Molecular Characterization of Antibiotic Resistant Genes In Psychrophilic Bacteria Isolated From Glaciers

By

Sabir Nawaz

Department of Microbiology

Faculty of Biological Sciences

Quaid-i-Azam University

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2023


بسم الله الرحمن الرحيم

My Humble Effort is

Dedicated to

My Father and Mother (Late),

And Sweet Brothers and Sisters

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Student Name: **Mr. Sabir Nawaz** Signature:_____

Examination Committee:

a) External Examiner 1: Signature:_____

Prof. Dr. Ghazala Kaukab Raja
Director University Institute of Biochemistry& Biotechnology
PMAS Arid Agriculture University, Murree Road, Rawalpindi

b) External Examiner 2: Signature:_____

Dr. Allah Nawaz
House No 667, Street No. 18,
Sector I-8/2, Islamabad

Supervisor Name: **Prof. Dr. Fariha Hasan** Signature:_____

Name of HOD: **Prof. Dr. Naeem Ali** Signature:_____

CONTENTS

List of Abbreviations

°C	Degree Centigrade
µg	Microgram
µL	Microliter
AMR	Antimicrobial Resistance
AO	Acridine Orange
ARB	Antibiotic-Resistant Bacteria
ARGs	Antibiotic-Resistant Genes
BLAST	Basic Local Alignment Search Tool
bp	Base Pairs
CAPs	Cold Acclimation Proteins
CARD	Comprehensive Antibiotic Resistant Database
CFU	Colony Forming Units
CIPs	Cold Induced Proteins
CLSI	Clinical Laboratory Standard Institutes
CSPs	Cold Shock Proteins
ct	Cycle Threshold
DNA	Deoxyribonucleic Acid
ESBLs	Extended Spectrum beta-Lactamases
HGT	Horizontal Gene Transfer
HKKH	Hindukush Karakorum and Himalayas
MAR	Multiple Antibiotic Resistance
MATE	Multidrug and Toxic Compounds Extrusion
MDR	Multi-drug Resistant
mg	Milli Gram
MGEs	Mobile Genetic Elements
MHA	Mueller Hinton Agar
MICs	Minimum Inhibitory Concentrations
mL	Milli Liter
mm	Milli Meter
MRSA	Methicillin-Resistant Staphylococcus aureus
NaCl	Sodium Chloride
NCBI	National Center for Biotechnology Information
NDM	New Delhi Metallo beta-Lactamase
ng	Nanogram
OTU	Operational Taxonomic Unit
PBPs	Penicillin Binding Proteins
pH	Power of Hydrogen
PUFA	Polyunsaturated Fatty Acids
rRNA	Ribosomal Ribonucleic Acid
RT-PCR	Real-time Polymerase Chain Reaction
β	Beta

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Summary

The cryosphere was once thought uninhabitable due to life-detrimental conditions but is found teeming with extremophiles of all life forms including viruses, bacteria, fungi, and Eukaryotes, and a decade ago scientists started to explore the polar cold regions for the occurrence of antibiotic resistance bacteria harbored with antibiotic-resistant determinants. Antibiotic production and resistance are prehistoric and currently, its spread is the preeminent global threat. The bacteria inhabiting the natural pristine cold environments are thought to be the potent reservoir of antibiotic-resistant genes. Besides the polar cold regions, the occurrence of the most congested non-polar glaciers in the region of Hindukush, Karakorum, and Himalayas, turned the attention to exploring the antibiotic-resistant bacteria in these areas since they are uncharted regarding the abundance of antibiotic-resistant genes. Therefore, the current study aims to explore the Passu glacier (Karakorum mountains range) for the presence of antibiotic-resistant culturable bacteria, the curing effect of acridine orange on glacier bacteria susceptibility to antibiotics, and the carriage of plasmid-mediated antibiotic resistance, the abundance of antibiotic-resistant genes, and mobile genetic elements like integron integrases, and their cloning efficacy in antibiotic-sensitive mesophilic bacteria. In this regard, we collected the glacial sediment, ice, and meltwater samples from the Passu glacier. Bacterial isolation was performed by evaluating the colony morphology and identified through *16S rRNA* amplicon sequencing. Kirby Bauer agar disc diffusion method was utilized to determine the bacterial *in vivo* antibiotic susceptibility profile. The molecular detection of antibiotic-resistant genes and the abundance of integron integrase genes were evaluated through conventional and real-time PCR amplification and quantification, respectively. To characterize the antibiotic-resistant genes, Sanger sequencing was performed and amino acids-based alignment with the counter reference antibiotic-resistant genes was performed to call the amino acids coverage and consensus variation, followed by tracing their evolutionary history through the Neighbor-joining method on Mega X software. The acridine orange in different concentrations, as a curing agent, was used to check the MICs of tested bacteria plus its plasmid elimination effect and to make a correlation between the plasmid-containing and plasmid-deprived bacteria, while evaluating their pre and post-curing antibiotic susceptibility profiling. The glacier's bacteria (psychrophiles) harbored antibiotic-resistant genes and were cloned in a pCR 2.1 vector utilizing TOP TA cloning kit and an antibiotic-sensitive competent *E. coli* (mesophile) was used to uptake the vector embedded with the desired antibiotic-resistant genes of interest, followed by the blue-white screening of transformed colonies. The Plasmid-PCR and Sanger

sequencing of each vector was performed, utilizing the M13 Forward, and reverse primers plus each ARG primer, to analyze the transformants, and SnapGene Software was utilized to map and annotate each plasmid vector construct.

From the Passu glacier, a total of 65 culturable bacterial isolates were retrieved. Predominantly identified bacteria were gram-negative 43 (66.15%) whereas, gram-positive isolates were 22 (33.84%). Among gram-negative bacteria, *Gammaproteobacteria* were dominant (62.79%), followed by *Betaproteobacteria* (18.60%) and *Alphaproteobacteria* (9.30%), and among gram-positive bacteria, *Actinobacteria* (50%) and *Firmicutes* (40.90%) were predominant. The glacial bacteria showed significant antibiotic resistance against a panel of 29 antibiotic groups and PCR amplification showed phylum *Proteobacteria* predominantly detected with 21 antibiotic-resistant genes including $_{bla}$*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 antibiotic-resistant genes, whereas, all isolates were negative for $_{bla}$*KPC, qnrA, vanA, ermA, ermB, intl2,* and *intl3*. The multiple antibiotic resistance indexes were higher for gram-negative, compared to gram-positive. Alignment of protein homology sequences of antibiotic-resistant genes with counter reference genes revealed amino acids coverage and consensus variations for $_{bla}$*NDM-1*, $_{bla}$*OXA-1*, $_{bla}$*SHV, mecA, aac(6)-Ib3, tetA, tetB, sul2, qnrB, gyrA,* and *intI1*. When different concentrations (0 to 200 µg/mL) of acridine orange were subjected to bacterial growth, 17/43, (39.53%) gram-negative and 15/22 (68.18%) gram-positive bacteria retained their slight growth up to 75 µg/mL acridine orange concentration, whereas, compared to gram-negative bacteria, gram-positive yielded higher MICs values. While comparing the pre and post-curing verificatory antibiotic susceptibility assays, 21/43 (48.83%) gram-negative and 7/22 (31.81%) gram-positive bacteria revealed no change in susceptibility pattern. The plasmid-cured bacterial strains showed 100% susceptibility to levofloxacin, ciprofloxacin, piperacillin, and imipenem whereas a major MAR index decline was observed for *Staphylococcus equorum* (HP19), *Leucobacter aridicollis* (HP22) and *Arthrobacter psychrochitiniphilus* (LP2) (0.7 to 0.2 each), *Serratia marcescens* (HP50) (0.6 to 0.1), *Flavobacterium antarcticum* (HP20) (0.6 to 0.2), *Flavobacterium saliperosum* (HP8) (from 0.5 to 0.1) and *Brevundimonas diminuta* (HP21) (0.7 to 0.4). The real-time PCR assay for the screening of integron integrase class 1, 2, and 3, among 28 multi-drug resistant bacteria carrying disparate antibiotic-resistant genes, revealed 20 strains (71.4%) were positive for integron integrase class 1, 12 (42.8%) for integron integrase class 2 while all negative for integron integrase class 3. Overall, gram-negative bacteria were more ubiquitous with integron

integrase class 1 (14, 70%) and integron integrase class 2 (10, 83.3%) with the lowest Ct (cycle threshold) values. A total of 12 (60%) *IntI1* positive strains were detected with strong gene copy/µL (Ct value ≤ 29), while all *intI2* positive bacteria had Ct > 29. Among the total 12 (42.8%) bacteria, gram-negative 10 (35.7%) while gram-positive 2 (7.1%) harbored both *intI1* and *intI2* genes. Strains from the glacier's sediment sample were more copious in *intI1* (16, 80%) and *intI2* (9, 75%), followed by water and ice samples. The ARGs ($_{bla}$*CTXM-15*, $_{bla}$*NDM-1*, *aac(6)-lb3*, and *gyrA*), detected among the MDR psychrophilic bacteria *Brevundimonas diminuta*, *Rahnella inusitata*, *Staphylococcus equorum*, and *Alcaligenes faecalis* were PCR amplified and poly-adenylated amplicons were purified and quantified for vector insertion. The desired ARGs of interest were successfully cloned in a pCR2.1-Topo vector and transformed using competent *E. coli* (One-Shot Mach1-T1) cells with ampicillin drug selection. Through the alpha complementation system (blue-white screening), transformed *E. coli* cells were screened out. The extracted plasmid vectors containing the desired gene of inserts were PCR amplified using appropriate primers and exact amplicons were visualized on the gel electrophoresis. The nucleotide sequences of $_{bla}$*CTXM-15*, $_{bla}$*NDM-1*, *aac(6)-lb3*, and *gyrA* cloned plasmid vectors were processed through SnapGene Software, and mapping and annotation were performed. The complete sequence of each plasmid vector construct with $_{bla}$*CTXM-15*, $_{bla}$*NDM-1*, *aac(6')-lb3*, and *gyrA* was obtained with disruption of the *lac operon lacZ* gene.

The present study manifests the first comprehensive assessment of the presence of antibiotic-resistant bacteria harbored with antibiotic-resistant genes and class 1 and 2 integron integrase genes, inhabiting a non-polar Passu glacier. The attained results highlight the mandatory focus to evaluate other non-polar glaciers regarding the bacterial diversity carrying antibiotic-resistant genes and mobile genetic elements. The glaciers located in the HKKH region provide agriculture and domestic water to billions of people in various countries, moreover, due to global warming effects, the glacier meltwater intermixes with the rivers and rainwater which poses a serious threat to the community's health and requires quintessential approach.

Introduction

Extremophile bacteria have been discovered to be a swarming population on the planet Earth, which was formerly believed to be uninhabitable due to its harsh environmental circumstances (Gilichinsky *et al.,* 2007). The inhabitants of extremes include Archaea, Bacteria, and some Eukaryotes and extremes might be physical like; radiations, temperature, and pressure, or geochemical like; salinity, redox potential, pH, desiccation, and oxygen species (Rothschild and Mancinelli 2001). Extremophile was termed by MacElroy (1974), to describe organisms that survive in harsh environments (MacElroy 1974). According to the environmental requirements for bacteria to survive in extreme environments, extremophiles are classified as psychrophilic (0 °C to 20 °C), thermophilic (41 °C to 122 °C), hyperthermophilic (80 °C to > 122 °C), acidophilic (pH 1–5), alkaliphilic (pH 9), piezophilic (elevated hydrostatic pressure, up to 50 MPa), and halophilic (salt concentration (low availability of free water) (Gupta *et al.,* 2014). On Earth, there is an unequal distribution of temperature, with 26% of the terrestrial ecosystems and 90% of the oceans being subjected to cold temperatures which include the deep ocean, mesosphere, and stratosphere, as well as high mountains, the Arctic, and the Antarctic. The deep sea makes up the majority (90% of ocean volume with a temperature < 5 degrees), followed by the snow region (35%, land's surface), the permafrost region (24%), regions of sea ice (13%, Earth's exterior), and glaciers zones (10%, land's surface) (Margesin and Miteva 2011; Zhang *et al.,* 2009). Glacier habitats are noted for having the worst environments for life compared to other regions of the cryosphere because of the scarcity of water and nutrients, the lack of light, the high hydrostatic pressure, and the subfreezing temperatures that vary from -56 to 10 °C. Nearly, the 15,861,766 kilometer^2 and a substantial reservoir of microorganisms of about 9.61 10^{25} cells were maintained in historical deposits in huge polar and non-polar glaciers thousands of years ago (Priscu *et al.*, 2007). Active ecological units with varying thermal predominance in geothermal, hydrological, and physical properties can be found in both the innermost subglacial layers as well as the uppermost supraglacial layers of glaciers (Hodson *et al.,* 2008). Microorganisms having the ability to survive in freezing temperatures, such as bacteria, viruses, fungi, and microeukaryotes, are found in great abundance and diversity in glaciers (Boetius *et al.,* 2015). Their tolerance to low temperature is a key evolutionary factor in microbe diversity in low-temperature environments and a crucial tool to comprehend how they endure and diversify their species. The makeup of the microbial population in glaciers has been the subject of numerous investigations, previously (Liu *et al.,*

2017; Franzetti *et al.,* 2013). *Micrococcus*, *Yersinia*, *Acinetobacter*, *Brevibacterium*, *Chromobacterium*, *Cryobacerium*, *Serratia*, *Corynebacterium*, *Bacillus*, *Arthrobacter*, *Hymenobacter*, *Cytophaga*, *Denococcus*, *Flavobacterium*, *Cellulomonas*, *Listeria*, *Brochothrix*, *Streptococcus*, *Polaromonas*, *Nostoc*, *Clostridium*, *Sphingomonas*, and *Cyanobacteria* are the frequently reported species from different glaciers' environments (Anesio *et al.,* 2017; Reddy *et al.,* 2010; Vasut and Robeci 2009).

The psychrophiles must overcome some hurdles to their way of life to survive in the extreme cold environments of the cryosphere. Psychrophiles face difficulties in surviving at 0°C including the limited availability of liquid-state water, drastically reduced plasma membrane fluid due to the extremely low-temperature conditions, decreased availability of nutrients (oligotrophy), high salinity, increased hydrostatic pressure, hypoxia, and high doses of ultraviolet radiation. Surviving at 0°C also involves a two-fold reduction in biological activities compared to the physiological state of 37°C (nutrients, waste products). Additionally, because of the extremely low temperatures, all enzyme-added reactions, genomic replication, transcription, translation, protein folding, and supercoiling are severely downregulated. Intracellular ice formation also poses a threat to the cellular integrity of psychrophiles, and repeated freeze-thaw cycles have a detrimental impact on their cellular physiology (Sharma *et al.,* 2022). To withstand such difficulties, microorganisms use internal strategies, such as cell membrane alteration and increased exopolysaccharide and unsaturated fatty acid production, transcription and translation of cold-active enzymes (enzymes alteration), ice-nucleating, antifreeze proteins, and cold-induced proteins (cold-shock and cold acclimation proteins). Moreover, psychrophiles produce compatible solutes including trehalose, sorbitol, glycerol, glycine, betaine, and mannitol which decreases the freezing temperature of cytoplasm's aqueous phase, and macromolecules, particularly the enzyme, are stable. (Pathania *et al.,* 2022; Sharma *et al.,* 2022; Goyal *et al.,* 2022; Lorv *et al.,* 2014).

Production of antibiotics and bacterial resistance to them are both natural phenomena with ancient roots. Undoubtedly, the most significant scientific achievement of the 20th century was the development of antibiotics, which not only saved lives from various microbiological illnesses but also played a fundamental part in the extension of current surgical techniques. At the moment, the increasing prevalence of antibiotic resistance among bacteria poses the greatest threat to human and animal health, and this phenomenon is not only restricted to medical facilities but has also been prominently noted in cold, pristine environments (Van

Goethem *et al.,* 2018; D'Costa *et al.,* 2011). Scientists are looking for antibiotic-resistant genes in natural habitats as a result of the upsurge in community-acquired infections instigated by drug-resistant bacteria (Gibson *et al.,* 2015). Environmental microorganisms found in soil, glaciers, and marine environments are likely to be a significant source of genes for antibiotic resistance (Van Goethem *et al.,* 2018; Perron *et al.,* 2015; Segawa *et al.,* 2013; Ushida *et al.,* 2010). The identification of natural repositories for antimicrobial-resistant determinants demonstrates the critical contribution of the natural environment to the spread of ARGs (Whitman *et al.,* 1998).

Antibiotic resistance genes can spread from cell to cell by conjugation, transformation, and transduction and are frequently found on mobile genetic elements including plasmids, integrons, and transposons (Heuer *et al.,* 2011). The main factor causing the rapid emergence of bacterial resistance to antibiotics is the existence of resistant genes in the environmental and clinical resistome (Jian *et al.,* 2021). Due to the long-term persistence of DNA-carrying ARGs in the environment under the protection of deoxynucleotide enzymes, the ARGs are crucial in the evolution of resistant traits among pathogenic strains of bacteria even after death (Dantas *et al.,* 2008). Since bacteria in the natural environment have evolved mechanisms of resistance against antimicrobial compounds for billions of years without anthropogenic effect and before the widespread application of antibiotics for therapeutic purposes, it is believed that ARGs massively circulating in clinical settings originated in the natural pristine environments (Blair *et al.,* 2015; Forsberg *et al.,* 2012). The natural environment is thought to be a potent reservoir of ARGs and the spread of resistant strains and ARGs between the environment, people, and animals causes antibiotic resistance to become a global health concern. ARGs and resistant bacterial strains are constrained from spreading by several barriers, but pathogens can acquire resistance from other species through a variety of mechanisms, increasing the risk of contracting resistant strains and lowering the ability to treat and prevent bacterial infections (Larsson and Flach 2022; D'Costa *et al.,* 2011). Environmental variables are more likely to be involved in the uptake of novel resistance components from the soil, water, and other environments with highly variable ecological niches than they are in the development of mutation-based resistance in pathogenic strains. Pathogenic bacteria can acquire a wide variety of genes from ecological niches. ARGs, plasmids, and transposons get frequent gene exchanges as a result of certain environmental factors (Rinke *et al.,* 2013; Shintani *et al.,* 2020). Various bacteria have distinct ARGs that cause them to become resistant to certain antibiotics, such as

beta-lactams (*bla*), tetracycline (*tet*), aminoglycosides (*acc*), colistin (*mcr*), fluoroquinolone (*qnr*), vancomycin (*van*), and macrolides (*erm*). The horizontal transmission of intracellular and extracellular ARGs in various habitats manifested that intracellular ARGs prevalence was higher in nutrient-rich environments whereas extracellular ARGs prevalence was higher in aquatic conditions (Zarei-Baygi and Smith 2021; He *et al.*, 2020).

There is a substantial reservoir of AGRs in the soil ecosystem, and these AGRs can move to other ecosystems like the water and forest and be ingested by bacteria that affect both humans and animals. Significant drivers of ARG proliferation in soil are anthropogenic activities that entail the transfer of antibiotics from human microbiomes into environments like soil and water. Bacillus species and the Actinomycetes genus Streptomyces, which can directly release antibiotics in the soil's habitats, are two natural antibiotic producers, abundantly found in soil (Heuer *et al.,* 2011; McManus *et al.,* 2002). Similarly, ARGs are becoming more prevalent in water all over the world, and the alteration of bacteria into superbugs is beginning to pose preeminent threats to the environment and public health. One of the most important reservoirs and means of transmission for ARGs is water and antibiotics enter water systems by the discharge of unused medications, human waste, animal waste, and urine from antibiotic production facilities. The aquatic environment is contaminated by the excretion of a substantial portion (90%) of antibiotics administered to mammals, as waste (urine, feces) thus accelerating the dissemination of antibiotic resistance (Larsson and Flach 2022; Amarasiri *et al.,* 2020; Wang *et al.,* 2018; Ma *et al.,* 2015). Besides other natural pristine environments, ecosystems comprising cold habitats like glaciers, and permafrost are a large reservoir of ARGs, and several studies documented the existence of ARGs in glaciers. ARGs have been reported from the Gulkana glacier in the United States, the Mackay glacier in Antarctica, Uganda, Nepal, and Bhutan, and Ürümqi in Africa (China) (Van Goethem *et al.,* 2018; Ushida *et al.,* 2010). A massive collection of ARGs from glaciers and other cold habitats including $_{bla}$*CTX-M*, $_{bla}$*SHV*, $_{bla}$*OXA*, $_{bla}$*GES*, $_{bla}$*TEM*, $_{bla}$*CMY*, $_{bla}$*VEB*, $_{bla}$*DHA*, *intI1*, $_{bla}$*IMP*, *vanA*, *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*, *tetO#1*, *tetO#2*, *tetS#1*, *tetS#2*, *tetX*, *tetW*, *cmlA*, *cmlV*, *cmrA*, *mefA*, *mefE*, and *cmx* have been reported by many researchers across the globe (Makowska *et al.,* 2020; Shen *et al.,* 2019; McCann *et al.,* 2019; Segawa *et al.,* 2013; Allen *et al.,* 2010; Ushida *et al.,* 2010) (Table 1).

Table 1. Worldwide detection of antibiotic-resistant genes from different cryosphere environments

Country/region	Location/glaciers	ARGs and MGEs	Reference
Greenland Norway	Greenland, Svalbard, and Caucasus glaciers	*blaCTX-M*, *blaSHV*, *blaOXA*, *blaGES*, *blaTEM*, *blaCMY*, *blaVEB*, *blaDHA*, and *intI1*	Makowska *et al.*, 2020
Norwegian territory	Spitsbergen (High Arctic Island)	*blaOXA*, *blaSHV*, *blaCTX-M*, *blaNDM-1*, *aph3'*, *cat1*, *catA4*, *catB4*, *catB5* and *catB6*	Makowska *et al.*, 2020
Canada	High Arctic glaciers	*blaCTX-M*, *blaNDM-1*, *tetA*, *tetB*, *tetC*, and *intI*1	McCann *et al.*, 2019
China	Urumqi glacier	*blaGES*, *blaTEM*, *blaCMY*, *blaVEB*, *blaIMP*, *blaOXA* and *intI1*	Shen *et al.*, 2019
Russia, Scandinavia	Pristine Antarctic soils	*blaCTX-M*, *blaCMY1*, *blaIMP*, *blaOXA*, *cat1*, *catA4*, *catB3msrA*, *msrB*, *tetA*, *tetB*, *tetC*, *tetD*, *tetG*, *tetL*, *tetM*, *tetO*, *tetS*, *tetS2*, *tetX*, *tetW*, *vanA*, *vanB*, *dfrA*, *intI1*, and *Efflux pump genes*	Van Goethem *et al.*, 2018
China, Tajikistan, Kyrgyzstan, Bhutan and Nepal Africa South America North America Greenland	Glaciers of Central Asia, Himalayas Uganda glacier Chile Alaska Antarctica, Arctic	*blaIMP*, *blaOXA*, *vanA*, *blaCMY1*, *blaCMY2*, *ampC2*, *strA*, *msrA*, *msrB*, *aacC1*, *aacC2*, *aac2'Ic*, *aac3*, *aac6'*, *ermA*, *ermC*, *ermM*, *ermML*, *ermTR*, *aadA*, *aadB*, *aadE*, *aadK*, *aph6*, *aph3'*, *cat1*, *catA4*, *catB3B4*, *catB5B8*, *catB7* *strA*, *msrA*, *msrB*, *catB5B8*, *catB7*, *tetD*, *tetG*, *tetL*, *tetM*, *tetO#1*, *tetO#2*, *tetS#1*, *tetS#2*, *tetX*, *tetW*, *cmlA*, *cmlV*, *cmrA*, *mefA*, *mefE*, and *cmx*	Segawa *et al.*, 2013
USA, Eastern Alaska China, Xinjiang	Gulkana glacier Ürümqi glacier	*blaCTX-M*, *ampC*, *cmrA*, *msrA*, *msrB*, *aacC1*, *aph6*, *tetD*, *tetE*, and *tetG*	Ushida *et al.*, 2010
Greenland Iceland	Arctic and sub-Arctic glaciers	*blaNDM-1*, *blaSHV-1*, *blaKPC* and *blaGES-1*	Allen *et al.*, 2010

ARGs are spread across clinical pathogens by horizontal gene transfer, which has contributed to the enormous expansion of antibiotic resistance from commensal and environmental bacteria to pathogenic ones. Genetic material transfer respects no ordered limitations, as opposed to

vertical legacy, which is the transmission of genetic material from parent to offspring. Instead, genes are transferred within the genus level while still among tangentially related ancestries that belong to various domains. Moreover, a few pathways for gene flow between environmentally disparate heredities have been discovered (Hassoun-Kheir *et al.*, 2020; Von Wintersdorff *et al.*, 2016). A bacterial cell that is "capable" or susceptible can modify its surroundings to take in new genetic material. Via a process known as transduction, a virus propagates genome contents among various donor and recipient bacteria. This method is dependent on the bacteriophage virus class, with subsequent bacterial infection and release of virion particles. During conjugation, one bacterium can exchange genetic contents with another bacterium through direct contact. Whole chromosomes, various mobile genetic elements, operons, superoperons that encode complicated biological pathways, and gene fragments are among the types of genetic information that can be transferred horizontally from the natural environment to the community (Schneider 2021; Villa *et al.*, 2019).

For an accurate analysis of antibiotic resistance in natural environments, it is essential to comprehend the function of antibiotics and the elements of their resistance in natural habitats. As many of the antibiotics used to treat illnesses are produced by soil microorganisms, it has been theorized that the function of these compounds in nature should be to limit the growth of the microbial competitors of antibiotic manufacturers. It has been proposed that some antibiotics may function as signaling agents at the low amounts that are undoubtedly present in the majority of natural habitats. Moreover, antibiotic detoxification in primary producers or the development of toxin resistance in plants or their accompanying microbiota is linked to the antibiotics, now prevalent in pathogenic bacteria (Bais *et al.*, 2005; Waksman and Woodruff 1940). It's believed that the penicillin-binding proteins needed to make peptidoglycan evolved into the plasmid-encoded beta-lactamases acquired by pathogenic bacteria through HGT and that their ability to break down beta-lactams was a consequence of their original function. In addition, although having a synthetic origin, quinolones are a favorite target of MDR efflux transporters, and environmental bacteria that were identified before quinolones were developed, may efflux these drugs, showing that the primary goal of those determinants is not to enhance resistance. (Meroueh *et al.*, 2003; Alonso *et al.*, 1999).

Surprisingly, many ancient bacteria have many molecular characteristics with contemporary bacteria. In some instances, the age of these species can be determined by geological data, while their modernity can be determined by DNA evidence. There exists an abundance of

frequent bacteria in the environment that are resistant to several antibiotics. Antibiotic resistance in microbes can occasionally be attributed to repeated antibiotic exposure, human diseases, as well as the transmission of resistance traits between and within microbes (Pareek *et al.,* 2015; Hershkovitz *et al.,* 2008). Environment-related microorganisms develop resistance to a range of native or modified antibiotics. Some ways naturally occurring ARGs in psychrophilic bacteria can spread to human diseases and vice versa. Many antibiotics, including kanamycin, tetracycline, chloramphenicol, beta-lactam antibiotics, and polymyxin B, have been rendered ineffective against certain psychrophilic bacteria (Martinez 2009; Martinez *et al.,* 2008). The geographical spread of ARGs and AR bacteria between the clinical and natural environments may also be aided by plausible means of transmission of these genes and mobile genetic elements through hydrological structures, winds, migrating birds, vertebrate feces, tourists, and airborne bacteria. Moreover, the blending of glacial melt and rainwater promotes bacterial dispersion and HGT, which aids in the global dissemination of a variety of ARGs (Makowska *et al.,* 2020; Literak *et al.,* 2014; Segawa *et al.,* 2013).

Several ARGs have been identified in bacterial populations living in extremely cold environments around the world, including the Gulkana glacier in Eastern Alaska and the Ürümqi glacier in China (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). Worldwide, polar cold habitats are thoroughly investigated for microbial diversity, antibiotic resistance, biogeochemical processes, and the impact of climatic changes. The non-polar cold habitats, on the other hand, have not received adequate attention. Scientists began investigating the Karakorum Mountains over a decade ago in search of microbial biodiversity and AR bacteria (Rafiq *et al.,* 2017; Shen *et al.,* 2012; Reddy *et al.,* 2010; Zhang *et al.,* 2010).

Considering the prevalence of antibiotic resistance among psychrophilic bacteria inhabiting the cryosphere including glaciers, the major goals of the current study were to screen out Pakistan's unexplored non-polar glacier for culturable bacterial diversity and antibiotic resistance genes. In this work, bacterial isolates from the Passu Glacier were retrieved and investigated for culturable diversity, antibiotic susceptibility, plasmid-mediated antibiotic resistance, and the abundance of ARGs plus different classes of integrons integrases. Furthermore, to examine the uptake efficiency of psychrophilic bacteria-originated ARGs by the mesophilic bacteria,

various ARGs were cloned in a competent mesophilic *E. coli* strain. The findings of this first comprehensive study will help in understanding the culturable bacterial community structure, and abundance of antibiotic-resistant genes and integron integrase genes in an anthropogenic-free psychrophilic bacteria, isolated from Passu glacier located in Northern Pakistan.

Aim and Objectives

Aim

Molecular characterization of antibiotic-resistant genes in psychrophilic bacteria isolated from glacier

Objectives

1. Culturable diversity of bacteria isolated from Passu glacier
2. To study the antibiotic-resistant profile of bacteria isolated from glacier
3. To detect and characterize antibiotic-resistant genes among bacteria isolated from glacier
4. Plasmid curing effect of acridine orange on glacier bacteria susceptibility to antibiotics
5. Quantification of integron integrase class 1, 2, and 3 abundances in glacier bacteria using Real-time quantitative PCR
6. Cloning of glacier bacteria harbored antibiotic-resistant genes in mesophilic *Escherichia coli* strain

Review of Literature

The continuous existence of life in all its forms depends on three fundamental components obtained from the environment: organic substances, free energy, and liquid water. On the planet Earth, all life forms necessitate several requisite organic materials in the form of nucleobases, amino acids, carbohydrates, and lipids for their ultimate survival and continuity (Aerts *et al.*, 2014). The biospheres' creatures extract these requisite pieces of machinery from the surrounding environments in favorable conditions and any imbalanced extremity in the environmental factors in the form of temperature, pH, pressure, salinity, liquid water availability, desiccation, radiation, and oligotrophy, drastically reduce the uptake of these essential components and challenges their existence. Moreover, extreme environmental factors negatively affect the organisms' normal cellular machinery, which is mandatory for survival and growth, and make them more vulnerable to environmental hostility. Such life-detrimental hostile environments are called "Extreme Environments" and the evolved group of organisms that flourish in these environments are termed "Extremophiles", coined in 1974 by MacElroy (MacElroy 1974).

1 Extreme Environments and Extremophiles

Extremophiles are a class of organisms that can grow well and flourish in harsh circumstances called extreme environments from the human beings' perspective (Figure 2.1). These organisms have successfully evolved the intracellular mechanisms to nurture their life in detrimental conditions (Kumar *et al.,* 2022). Prokaryotes have dominated the extremophilic group through stages of evolution and virtually have colonized all the hostile spots on Earth (Merino *et al.,* 2019). Based on the extreme environments the inhabiting organisms are categorized into different groups.

1.1 Thermophiles and hyperthermophiles (High-temperature environment)

The microorganisms that tend to survive and flourish in higher temperatures are called thermophiles and hyperthermophiles. Thermophiles exist in growth temperatures ranging from 45°C to 80°C whereas hyperthermophiles can grow beyond 80°C (Michael *et al.,* 2006). Such microorganisms are abundantly found in geothermally heated habitats on the earth like the volcano soils and hydrothermal vents (deep sea) (Nakagawa and Takai 2006). Most

thermophiles use oxygen and nitrate as electron receptors and react with ferrous ions, oxygen, and reduced sulfur with a chemoautotrophic mode of nutrition (Amend *et al.*, 2003).

1.2 Psychrophiles (Low-temperature environment)

Psychrophiles are cold-loving extremophiles that grow and thrive in the cold regions of the biosphere (cryosphere). Psychrophiles inhabiting habitats stretch from the stratosphere to the deep oceans. Based on cardinal growth temperatures, Psychrotrophs and Psychrophiles are two phenotypic distinctive overlapping groups of extremophilic bacteria thriving in cold environments (Morita, 1975). Generally, the minimal, optimal, and maximal growth temperature ranges are <0°C, 15°C, and 20°C (Psychrophiles) whereas >0°C, >20°C >30°C (Psychrotrophs) (Casanueva *et al.,* 2010). A major portion of the deep sea has 2°C while at the polar ice-caps, it goes down below 0°C and the saline sea water lowers the temperature beyond -1.8 °C. Moreover, upon freezing the seawater, the temperature drastically lowers to -20 °C. At such lower temperatures, the psychrophiles can perform their cellular metabolisms, and in such frozen areas, the flourishing psychrophiles are also halophilic (grow in elevated salt content) (Rodrigues *et al.,* 2009).

1.3 Acidophiles and Alkaliphiles (Acidic and alkaline environment)

The microorganisms that are termed “Acidophiles” optimally survive at pH 2.0 (Morozkina *et al.,* 2010). In the majority of cases, an acid environment is self-imposed by the residing microorganisms, and low pH results from their normal cellular metabolism, compared to other extreme habitats, and over time they develop mechanisms to cope with the low pH (Rampelotto 2010). In volcano and mine drainage areas, acidophilic microorganisms oxidize sulfidic minerals and elemental sulfur to obtain energy resulting in low pH values (Rohwerder and Sand 2007). On the contrary, the alkaliphile microorganisms grow in elevated pH values > 9.0 with optimal growth at pH 10 and little or no activity at < 8 pH (Horikoshi 1999). In alkaline environments, due to the silicate minerals' serpentinization, elevated concentrations of Ca^{+2} are generated, as seen in hyperalkaline water from springs (Grant 2006). In natural environments, alkaliphiles also exist and flourish in higher NaCl concentrations and are called “Haloalkaliphiles” (Gareeb and Setati 2009).

1.4 Halophiles (Saline or high salt environment)

The microorganisms that thrive in higher concentrations of salt are called halophiles, ranging from 10% sodium chloride to even growing in salt crystals (Yadav *et al.,* 2015). To balance the inter and intracellular osmotic pressure, these halophilic microorganisms produce some other solutes like potassium chloride in their cytoplasm (Oren 2002). Xerophilic are microorganisms that survive and grow in very low liquid water contents (Gupta *et al.,* 2014).

1.5 Piezophiles (High-pressure environment)

Piezophiles are the evolved group of microorganisms that grow and flourish in environments with elevated hydrostatic pressure. The earth's crust is found abundant with such microbes and is successfully isolated from a depth of > 10 Km of the oceans. Piezophiles are capable of tolerating pressure up to 110 Mpa. Piezophilic microorganisms' growth rate is very low, compared to others with low population densities (Rampelotto 2010).

1.6 Radiophile (High-radiation environment)

Radiophiles are microorganisms that survive in elevated doses of UV radiation. These microorganisms have adapted the mechanism to protect and compensate for DNA damage by UV radiation (Gupta *et al.,* 2014).

1.7 Metalophiles (High-metals environment)

Metalophiles are a group of microorganisms that grow and survive in higher concentrations of heavy metals. They can flourish in the occurrence of elevated concentrations of Mercury, Cadmium, Lead, and Chromium (Gupta *et al.,* 2014).

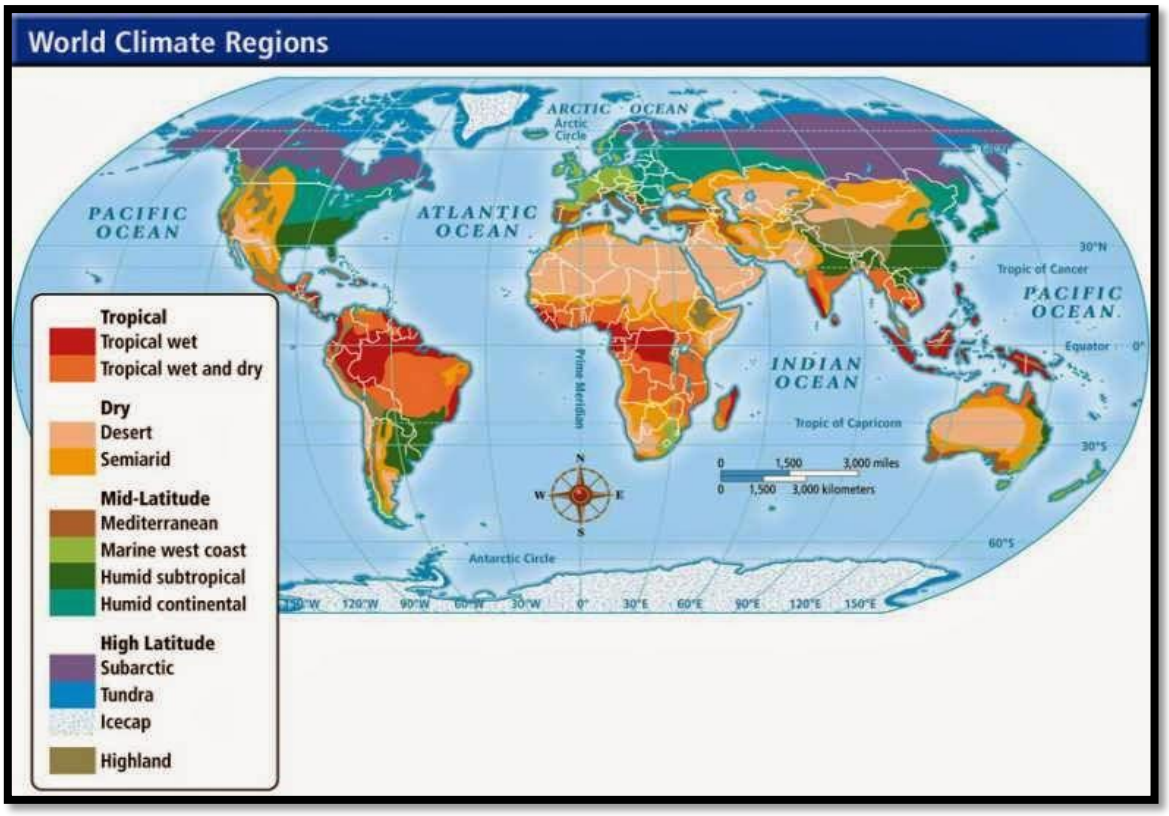

Figure 2.1. World climate map (world climate regions across the globe)
https://www.mapsofindia.com/world-map/climate.html

2 Cold environments on Earth

On the earth's surface, around 90% region is occupied by oceans and 26% by terrestrial ecosystems that are exposed to low temperatures (Zhang *et al.,* 2009), which span from the Arctic to the Antarctic, from high mountains (Himalayas, Alps, and Rocky mountains), to deep ocean, mesosphere, and stratosphere. The deep sea represents the major portion (90% of ocean volume with < 5°C), snow (35% of land surface), permafrost (24% of land surface), sea ice (13% of the earth's surface), and glaciers (10% of land surface) (Margesin and Miteva 2011; Cowan *et al.,* 2007). Other cold habitats include regions of cold soil, cold deserts, cold caves, and lakes (Margesin and Miteva 2011). This classifies the cold temperature habitat as the planet's Earth most ubiquitous "extreme" diversified ecosystem. The hostile cold ecosystems, present on the earth's surface, once considered uninhabitable regions, are teeming with microorganisms (Gilichinsky *et al*., 2007). Through the course of evolution, microorganisms have evolved to survive in hostile life-detrimental environments.

The cold habitats across the globe exist in two geographical forms.

2.1 Polar cold habitats

Polar cold regions include Arctic and Antarctic regions where exist massive collections of glaciers, permafrost, ice sheets, and cold deserts. Antarctica is a continent that occupies the South Pole of the Earth. It is fully covered by sheets that are thousands of meters thick and contains 90% of the world's fresh water. Antarctica receives extremely little precipitation in the form of annual snowfall, which causes the center of the continent to be incredibly dry and turns it into a desert. It is divided into three distinct zones: Maritime Antarctica, the Sub-Antarctic, and Continental Antarctica (Post *et al.,* 2019). The driest and coldest territory on Earth, Antarctica is defined as having 99% of its land covered by ice. The average temperature of Antarctica is 2°C, with extremes of -20°C and 10°C (Convey and Peck 2019).

The Arctic region is located at the North Pole at the top of the ecosystem. While the average winter temperature is -30 degrees Fahrenheit, the hottest month is more than 50 degrees. Included in it is the Arctic Ocean, which is completely covered in ice, the northernmost territories of Norway, Russia, Canada, Alaska, and regions of Greenland and Spitsbergen. The Arctic Circle (the sun never sets in the summer), and the northern border (the average temperature in July is 10°C) are their boundaries (Liang *et al.,* 2019). The Arctic islands are located in the latitude range 80-85°N and are classified into five bioclimatic subzones (A to E), with subzone A being the chilliest in the far north and subzone E being the warmest in the far south. Due to lower air and soil temperatures, organisms have harsher environments as latitude increases (Walker *et al.,* 2005; Billings 1992) (Figure 2.2)

Figure 2.2. World's North and South Poles. https://www.mapsofindia.com/north-south/poles.html

Permafrost is the term used to describe the frozen soils found at high altitudes, such as those existing in regions of the Arctic and sub-Arctic, which occupied a territory of 24% in the Northern Hemisphere. Climate changes may hasten the microbes-based decomposition of organic compounds with the liberation of CO2 and CH4 (greenhouse gases) into the environment. Permafrost melting causes erosion, landslides, ground subsidence, lake desertion, and changes in the composition of the vegetation at high altitudes (Schuur *et al.,* 2015; Dobinski 2011) (Figure 2.3).

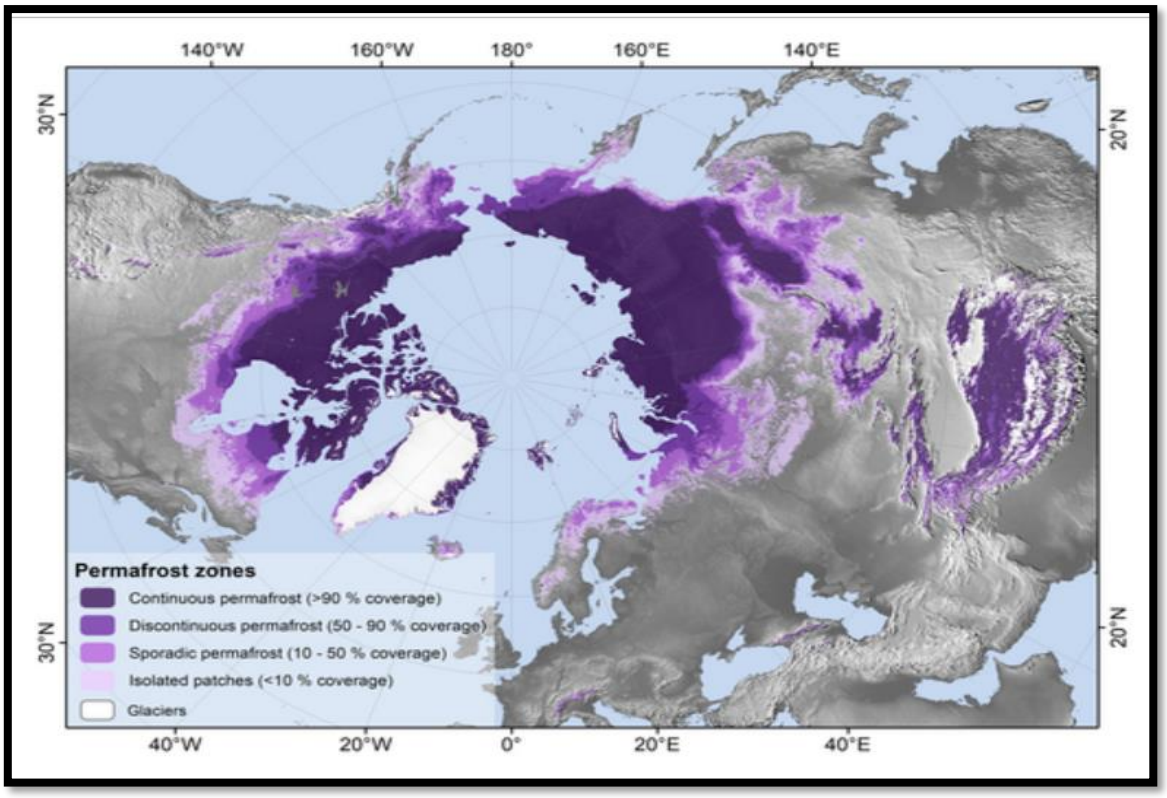

Figure 2.3. Permafrost zones across the world (adapted from Obu 2021)

Cold deserts have permeable soil that is heavily salted and slit, with heavy winter snowfall and high autumn rainfall of up to 260 mm (region dependent). In winter temperature lies in the range of -2 to 4° C, whereas in summer 21 to 26° C. Across the globe, permafrosts exist in the regions of Antarctica, Greenland, Atacama, Africa, China, Afghanistan, Iran, Pakistan, Russia, the Western United States of Great Britain, and North America (Nearctic area) (Snyder *et al.*, 2019).

2.2 Non-polar cold habitats

Non-polar cold regions are comprised of the Hindukush, Karakorum, and Himalayas (HKKH) region, the European Alpine, the height of mountains, and the depth of oceans. Due to the occurrence of largely congested glaciers in the region of HKKH, it is termed as "third pole" of the world and more than 54,252 glaciers occupied over an area of 60,000 km^2 are present in this region, the majority with <0°C annual temperature (Rafiq *et al.*, 2019).

The Alps, which are found in eight European Alpine countries, are the greatest mountain range in Europe. The European Alps mountain range, which stretches between roughly 44-48°N and

316.5°E, is about 200 km wide and 800 km long overall (Sternai *et al.,* 2012). The average elevation of Alps Mountain is about 2500 m, with the highest peaks being between 4400 and 4800 m. In the Mediterranean mountains that surround the valleys, the temperature lies in the range of -5°C to 8°C in Winter, while from 15°C to 24°C in the summer. The vast Eurasian landmass, the Atlantic environment, and the Mediterranean Sea all have an impact on the climate variability in the European Alps (Sternai *et al.,* 2012; Begert *et al.,* 2005).

Pakistan, which has a surface area of 796,095 km2 (307,374 sq m), is a country in South Asia. It is the 33rd largest nation in the globe and is home to the Himalayas, Karakoram, and Hindu Kush (HKKH) mountain ranges in its northern region (third pole of the world) because they form the largest glaciated mass outside of the polar zone. It included the world's highest mountains, with fourteen peaks all higher than 8000 meters, and 10 major rivers flowed from it (Schickhoff 2005). The HKKH experiences extensive glaciation due to its height and acts as a massive reservoir of water for many rivers. More than 1.4 billion people receive drinking water from HKKH glaciers in 9 countries as well as 60,000 km2 of agricultural land. The HKKH has occupied over 20,000 different glaciers, the Karakoram with 5000, and the Himalayas with 12000 (Khan 2015; Kääb *et al.,* 2012; Inman 2010; Schickhoff 2005).

In the HKKH mountains, Pakistan is home to one of the greatest glacier reserves in the world, and the important glaciers located in northern regions are Passu (Passu glacier), Batura (Batura glacier), Baltoro glacier, Siachen glacier, and Tirich Mir glacier. The 60 km-long Passu glacier, which is located in the Karakoram Mountains, is the fifth-longest in the world. The Batura Glacier and numerous other significant glaciers in the area are connected to the Passu Glacier in numerous ways. The peaks are substantially impacted by changes in the global atmosphere, and the Himalayan region glaciers are severely exposed to climate alterations (Hasson 2016; Armstrong 2010; Upadhyay 2009) (Figure 2.4).

The hostile deep-sea environments are categorized as nutrient deficient, dark (no sunlight), having elevated pressure, and low and extremely high temperatures, which also come in non-polar cold habitats. The deep water is a harsh habitat because of these circumstances. Oceans with > 200 m are thought to be thriving with all life forms, and that are trapped in a frozen aqua sphere (Marsh *et al.,* 2015; Nagano *et al.,* 2010).

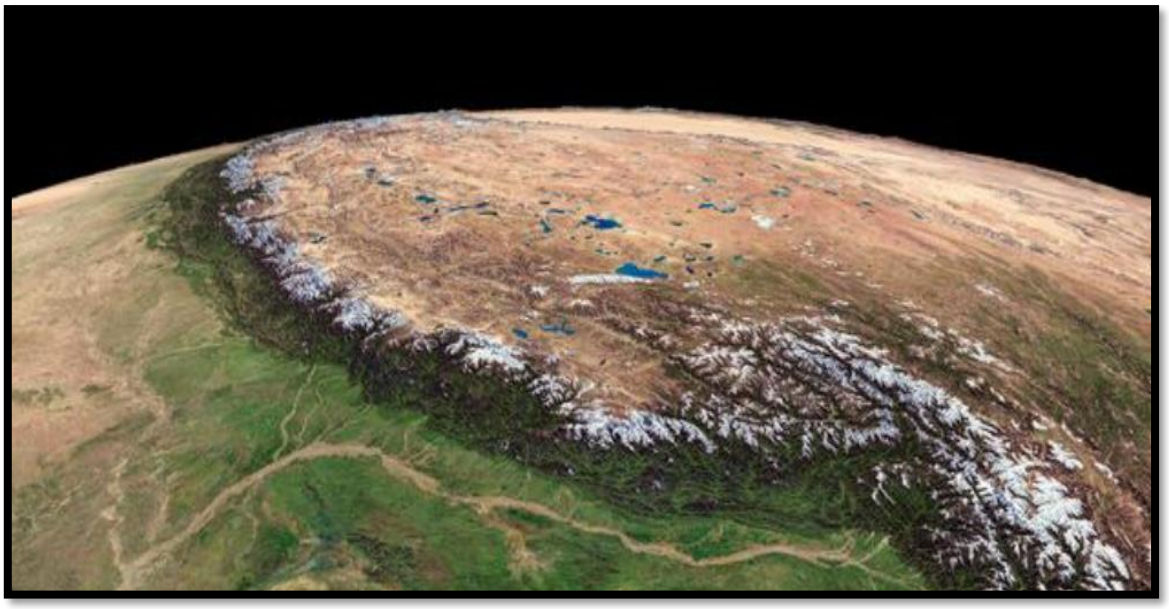

Figure 2.4. Hindukush Karakorum Himalayas (HKKH) region panoramic view. (https://www.mapsofindia.com/HKKH/image.html)

A glacier is a physique of extremely compressed and recrystallized ice that is under continuous gravitational pull due to its gigantic body mass. Only in locations where all of the wintertime snow does not melt and turn into ice grains in the summer can a glacier grow (Firn). Every year as more snow falls, it weighs down, squeezing out most of the air to solidify as glacier ice. Even while there were still tiny air bubbles, the pore spaces were no longer interconnected. Little earth sample-containing fissured air bubbles were buried and turned into ice. These air samples are important resources for scholars to understand past biosphere conditions. Gravity-driven glaciers deposit and move enormous quantities of sediment (Figure 2.5).

Landscapes created by glaciers are distinct from those created by streams. Glaciers exist in two geographical forms, one is Alpine (mountain glaciers) and other is Continental (Greenland and Antarctica glaciers) (Holderegger and Thiel-Egenter 2009; Takeuchi *et al.,* 2006). The valley glacier can be found in alpine regions and descends rather far from the valley's starting point. When many valley glaciers unite in a plain, level location near a mountain range, a Piedmont glacier is created. Alpine Glacier is both closer to and farther from the equator. Elevated peaks, close to the equator are located in the regions of Europe, Asia, South, and North America, and Africa. On the other hand, distant peaks from the equator are located in the Himalayas region (Asia), North America (Rocky Mountains region), and Europe’s Alps. Sierra Nevada

(California's high mountains region) faces extensive glaciations but exists with a small number of Alpine glaciers (Takeuchi *et al.,* 2006).

Figure 2.5. Passu glacier image (https://www.google/passuglacier/image/html)

3 Bacterial diversity in cold habitats

It was discovered more than a century ago that samples of snow and ice included microorganisms (McLean 1918). Afterward, across the globe from different cryosphere including glaciers, permafrost, cold soil, frozen lakes, subsoil, and cold caves, researchers have reported diverse microbial communities (Rafiq *et al.,* 2019, 2017; Anesio *et al.,* 2017; Anesio and Laybourn 2012; Margesin *et al.,* 2011; Amato *et al.,* 2007; Takeuchi *et al.,* 2006). Research on the richness of organisms inhabiting the Polar and Alpine cryoconite holes revealed the presence of yeasts, heterotrophic bacteria, viruses, algae, and cyanobacteria (Pittino *et al.,* 2018) including several novel cold-loving bacteria like *Spingomonas glacialis*, *Pedobacter*, and *Cryconitis* (Zhang *et al.,* 2010). Moreover, it has also been noted that cyanobacteria predominate among the communities thriving in lakes, streams, and ice of high Arctic regions (Velichko *et al.,* 2021), followed by permafrost inhabitants aerobic methanotrophic bacteria species like *Methylobacter tundripaludum* and *Methylobacter psychrophilus* (Altshuler *et al.,* 2022). Due to subzero temperatures, gamma radiation, freeze-thaw stress, and a wide variety

of antibiotics, permafrost soil is considered an extreme ecosystem for native microbial communities (Alcaraz 2019). From permafrost regions, more than 70 taxa have been reported, Actinobacteria and Firmicutes (gram-positive) were reported predominantly, followed by Xanthomonadaceae (gram-negative), respectively (Steven *et al.,* 2009; Steven *et al.,* 2008). Besides other cold habitats, the Arctic lakes have been explored regarding the bacterial diversity as well as the Alpine lakes, where α and β-proteobacteria were dominated (Gendron 2019; Adams *et al.,* 2010). On the other hand, from high mountains lakes, underlying water and surface layers β-proteobacteria and Actinobacteria have been frequently reported (Hörtnagl *et al.,* 2010). Six significant taxonomic groupings, including *Desulfobulbus*, *Desulfobacterium*, *Desulfosarcina*, *Desulfobacter*, *Desulfotomaculum*, and, *Desulfovibrio* were identified in the diversity of sulfate-reducing bacteria in Antarctica (Lake Fryxell) and the dominating phyla Bacteroidetes have been reported from the Mount Everest lakes (Karr *et al.,* 2006; Liu *et al.,* 2006). Considering the ancient permafrost sediments, methanotrophic, acetoclastic methanogens and some denitrifier bacteria have been explored that effectively metabolize methane to generate energy at lower temperatures including subzero temperature, followed by psychrophilic methanotrophs from Arctic soil (Gilichinsky *et al.,* 2008; Trotsenko and Khmelenina 2005; Rivkina *et al.,* 2004). Molecular techniques have also identified 73% abundant proteobacteria in the Himalayan Alps. Among these bacteria, gram-negative (51%) and gram-positive (48%) were in a viable state with the production of hydrolytic enzymes (Gangwar *et al.,* 2009).

From the Himalayas region, researchers explored different glaciers and have reported several 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.,* 2008, 2010), *Paenibacillus glacialis* (Kishore *et al.,* 2010), psychrotrophic proteolytic bacteria such as *Bacillus liceniformis*, *Bacillus subtilis* and *Pseudomonas aeruginosa* (Baghel *et al.,* 2005). The diversity of algae and fungi is less frequently reported in these cold environments (Lutz *et al.,* 2015; Margesin and Miteva 2011).

Both saltwater and freshwater glaciers can be found in the Antarctic, with rich and diversified microbial communities that include novel psychrophilic species (Säwström *et al.,* 2008). The distribution of microbial communities in these subglacial ecosystems is mostly under the direct

influence of glacier hydrology, lithology, and preglacial ecology, and the native microorganisms utilize iron and sulfur as reducing compounds for their growth and energy requirements (Perreault *et al.,* 2007). The microbial communities and phylogenetic diversity of the deep sea are immense, and the novel genera *Photobacterium*, *Moritella*, *Colwellia*, *Shewanella*, *Psychromonas*, and *Marinomonas* are among the most prevalent proteobacteria (Dang *et al.,* 2009).

3.1 Psychrophiles' survival challenges in cold habitats

While thriving in cold hostile environments, the psychrophiles have to face some life-detrimental challenges, to sustain their existence in the cryosphere. The featured obstacles that are faced by all psychrophiles are categorized as follows;

a. Limited availability of liquid-state water
b. A very decreased rate of normal cellular biological functions due to low-temperature conditions.
c. Decreased availability of nutrients (oligotrophy), high salinity, increased hydrostatic pressure, hypoxia, and high doses of ultraviolet radiation include additional survival challenges.
d. Surviving at 0°C minimizes the biological activities by two-fold, compared to the 37°C physiological state.
e. Drastically reduced plasma membrane fluidity results in the imbalance of membrane permeability and the very least effective cellular transport import mechanisms (nutrients, waste products).
f. Enzymatic activity is highly affected and all enzyme-added reactions are nearly halted.
g. The genomic replication, transcription, translation, and as well as protein folding, and supercoiling are highly downregulated.
h. Due to extremely low-temperature conditions, intracellular ice formation challenges the cellular integrity of psychrophiles.
i. Frequent freeze-thaw cycles negatively affect the psychrophiles' cellular physiology.

3.2 Psychrophiles' survival strategies/adaptations

To cope with the life-detrimental challenges in the cryosphere, psychrophiles have to adopt some requisite unique life-saving mechanisms, to thrive and flourish in the harshness of cold

and other hostile environmental conditions (Sharma *et al.,* 2022; D'Amico *et al.,* 2006). The generally adopted mechanisms are described as follows;

3.2.1 Cell membrane alteration

Lower temperature drastically reduces the plasma membrane's normal physiology and the gel state transition of the lipid contents is either halted or severely compromised, making it difficult to perform the transition of liquid-to-solid phase, required for cellular division and contents transportation, due to the unavailability of temperature energy for the phase-transition temperature phenomenon (transition midpoint or Tm), required for plasma membrane elasticity and fluidity (Pathania *et al.,* 2022). The psychrophiles produce higher contents of the tangled gel state to minimize the obstruction in the gel phase or avoid it (Rewald *et al.,* 2012) (Figure 2.6).

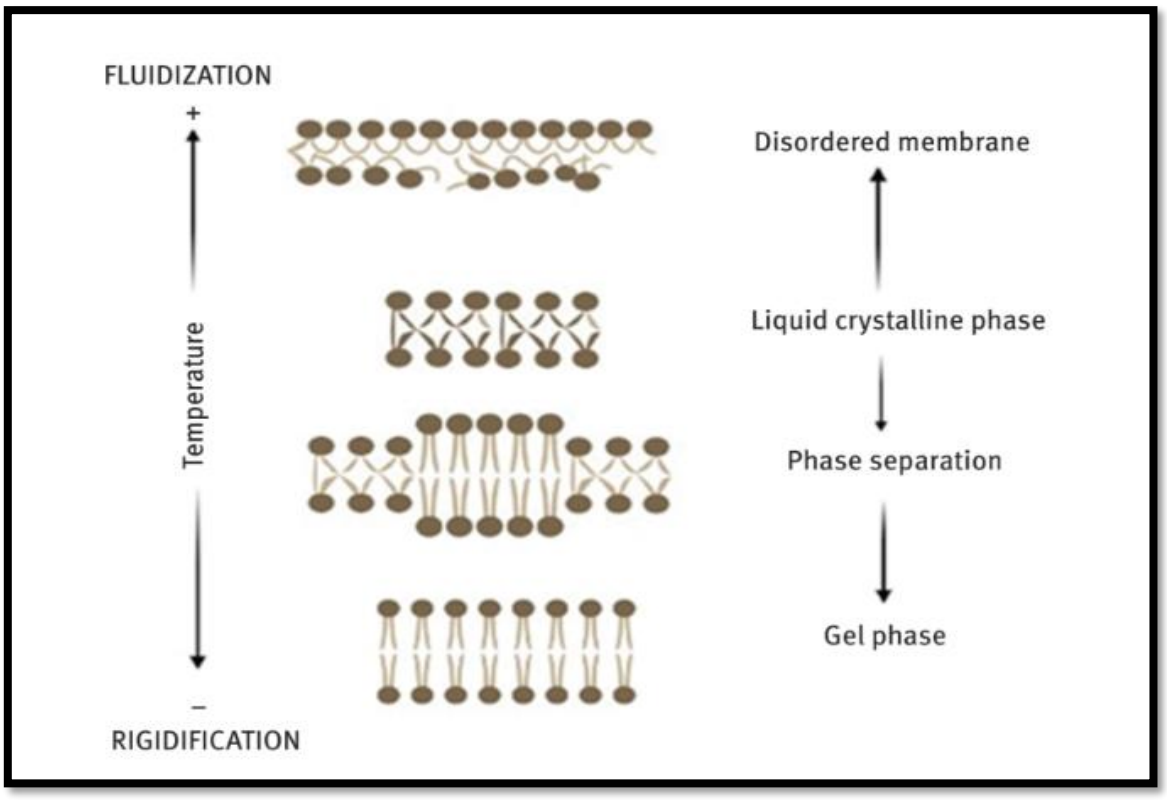

Figure 2.6. Diagrammatic representation of various alterations in membrane and the temperature-induced response of lipid bilayer (Adapted from Los and Zenchenko 2009)

The fluidity of the membrane is assumed to be influenced by the fatty acid contents, including the structure, saturation, and length of fatty acid chains in the membrane. One of the elements

that lower the membrane lipid's melting point and are thought to be crucial for boosting membrane fluidity is polyunsaturated fatty acid (PUFA). All these alterations are essential for the membrane to be structurally stable, elastic, and permeable, with enough fluidity, to perform normal membrane functions. The de novo elevated production of carotenoids, glycolipids, and polar lipids are the additional mechanisms adapted by psychrophiles to make their plasma membrane more functional (Pathania *et al.*, 2022; Velly *et al.*, 2015; Rewald *et al.*, 2012).

3.2.2 Antifreeze proteins and ice-nucleating proteins

Other particular mechanisms, like antifreeze proteins and ice nucleating proteins, are also available for preventing the fatal consequences of cytoplasmic freezing. Proteins that attach to ice and act as antifreeze agents stop the growth of recrystallization and the production of new ice crystals (Hudait *et al.*, 2019; Venketesh and Dayananda 2008). Many low-temperature microorganisms have produced ice-binding proteins, and antifreeze proteins have undergone thorough classification. Ice-nucleating proteins may be implicated in survival at lower temperatures by causing ice crystallization at temperatures closer to the melting point, which prevents supercooling of water. By lowering the freezing point to just 2°C, these proteins are very interested in the cryopreservation of biological material (Hudait *et al.*, 2019; Lorv *et al.*, 2014; Lee *et al.*, 2010; Wu *et al.*, 2009) (Figure 2.7).

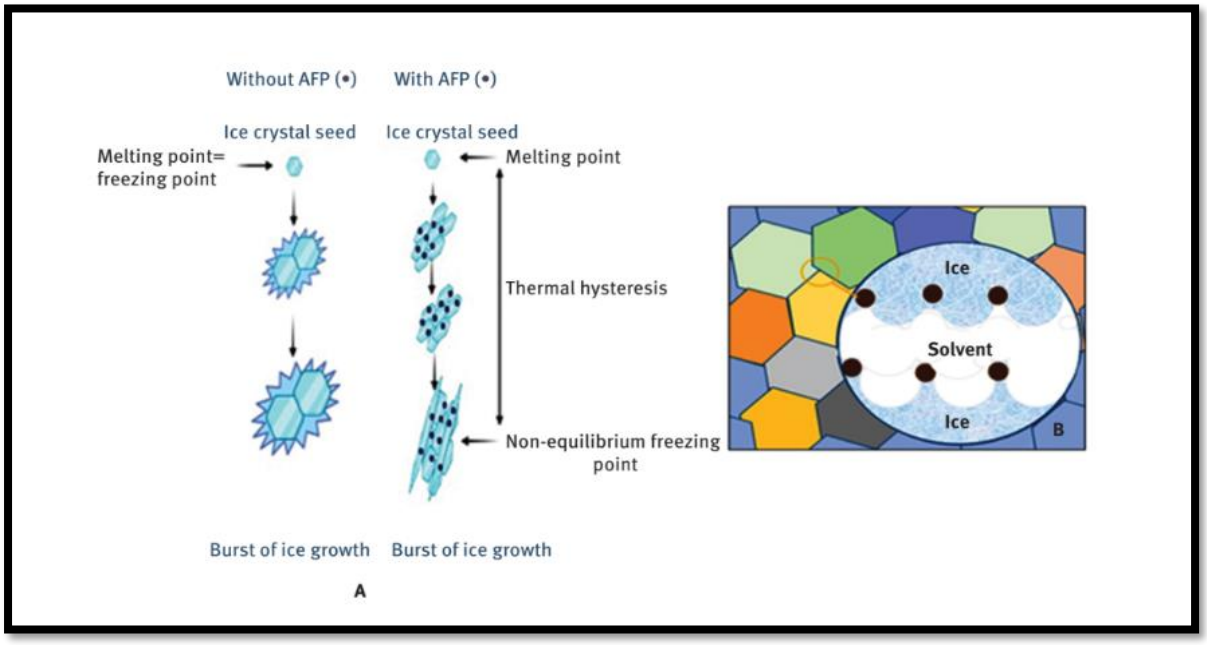

Figure 2.7. Graphical presentation of the workflow of anti-freeze proteins (A) inhibition of ice growth (B) inhibition of recrystallization process (adapted from Andrin 2015)

3.2.3 Cold-induced proteins (cold-shock and cold acclimation proteins)

A "cold shock reaction" is triggered by an unexpected drop in temperature in a variety of microorganisms, which results in the up or down-regulation of a large number of genes. Cold-induced proteins (CIPs) are the group of most important proteins that are essential for the survival of psychrophiles teeming in cold habitats. Based on their expression potential and kinetics, CIPs are categorized into two groups, cold-shock proteins (CSPs) and cold acclimation proteins (CAPs). In the cellular expression process, these groups sometimes overlap and are produced from a single promotor (Horn *et al.*, 2007). Cold shock proteins as small, conserved proteins link to single-stranded nucleic acids via cold shock domains (CSD) or nucleic acid binding motifs. Some cold RNA helicases that collaborate with other CSps in translation and replication are also affected by these protein reactions (Phadtare and Severinov 2010; El-Sharoud and Graumann 2007). The cold acclimation response in mesophiles is triggered by a set of genes, in response to the cold habitat, that are constitutive in psychrophiles and thus psychrophiles react differently to cold shock than mesophiles do. Both cold acclimation proteins and the vast majority of CSP homologs have been found and identified (Phadtare and Severinov 2010; Horn *et al.*, 2007) (Figure 2.8).

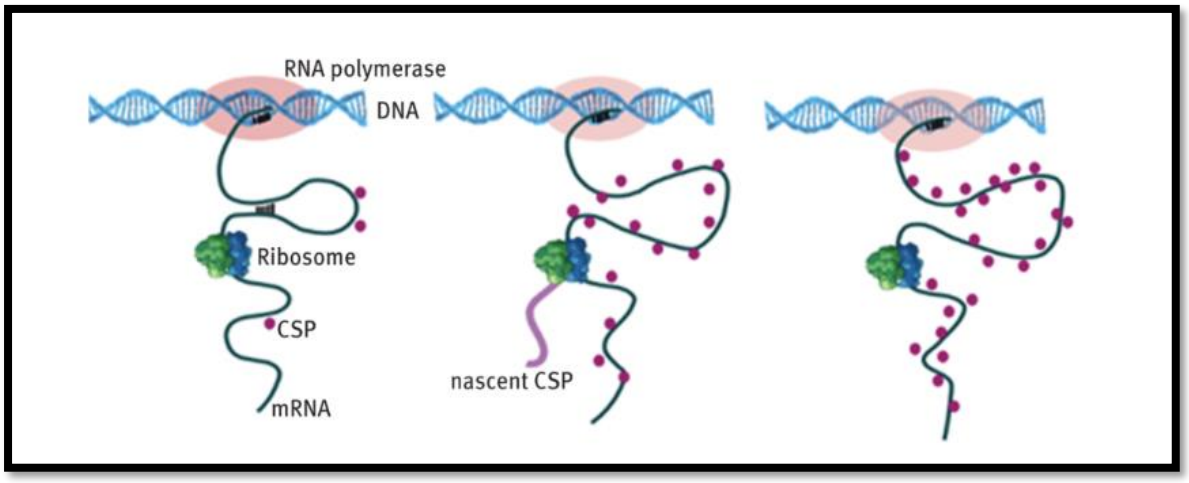

Figure 2.8. Schematic representation of cold-shock protein's (SCPs) role in the mRNA stabilization for normal cellular transcription (adapted from Horn *et al.*, 2007)

3.2.4 Enzymes alterations

Psychrophiles have the remarkable potential to produce several cold-adapted enzymes with higher specificity and functionality at extremely low temperatures. For several enzymes, the catalytic activity is at the maximum edge, compared to their mesophilic counterparts. Retaining enzyme

activity, stability, and flexibility is the mainstream strategy of psychrophiles to endure metabolism challenges in the cryosphere (Sharma *et al.*, 2022; Kuddus *et al.*, 2011). The reaction rate should be 10–60 times slower at 0 °C than it is at 30 °C, according to Arrhenius law. The physiology of microbes should not, however, be significantly hampered by this, and it is clear that enzymes from psychrophilic origins have been modified to function effectively at low temperatures (Duarte *et al.*, 2018; Casanueva *et al.*, 2010). There is a consistent pattern in psychrophilic enzymes: at lower temperatures, the low amount of structural stability permits larger stability rates. Because cold-active enzymes are more flexible, have a lower energy cost, and have a higher specific activity, the synergy between the substrate and active sites is increased. The polypeptide framework of the active site of the psychrophilic enzyme is thought by several scientists to be much more persistent than other regions of proteins (Sharma *et al.*, 2022; Siddiqui and Cavicchioli 2006). In general, small structural alterations, such as adjusting non-covalent connections in protein structure, resulted in either a decrease or an enhancement in the thermostabilities of low temperature-adapted enzymes. According to crystallographic research, low-temperature-suited enzymes have more readily available catalytic sites than mesophilic and thermophilic enzymes. This ability might be linked to structural adaptability and a reduction in the energy burden of induced fit mechanisms (Sharma *et al.*, 2022; Casanueva *et al.*, 2010; Marx *et al.*, 2007; Aghajari *et al.*, 2003).

3.2.5 Production of compatible solutes

In psychrophiles, osmotic tension, chilling, and desiccation are frequent occurrences. These species accumulate suitable solutes, including trehalose, sorbitol, glycerol, glycine, betaine, and mannitol, as one of their primary defenses against such stressful circumstances. The cytoplasm's aqua phase's freezing point drops as a result of the existence of such solubilized poly-hydroxylated chemicals, resulting in the structural and functional stability of macromolecules including the enzymes (Goyal *et al.*, 2022; Casanueva *et al.*, 2010; Borges *et al.*, 2002). The most crucial compatible solutes for cryoprotection under desiccation or freezing conditions are glycerol and mannitol. These chemicals can help sustain the turgor pressure even in the presence of decreased exterior water potential (Grant 2004). The primary soluble carbohydrates that aid in low-temperature adaption are acyclic sugar alcohols (polyols) that help in the regulation of coenzymes, and osmotic pressure and protect the cells from the freezing effects of intracellular fluids (Koštál *et al.*, 2007;). Another frequently found suitable solute in cold-loving extremophiles is trehalose, mainly found in psychrophilic fungi. Under

difficult and stressful circumstances (freezing temperatures), trehalose plays a crucial role in microorganism resistance development (Niu *et al.*, 2022).

4 Antibiotic resistance

Antibiotics are unarguably the most precious discovery of the 20th century, responsible for not only saving lives from different microbial infections but also playing a key role in modern medical procedures such as surgery. The introduction of chemotherapy for the treatment of diseases began in the early 20th century when Paul Ehrlich used Salvarsan for treating Syphilis and coined the term magic bullet for a drug that effectively treats the disease *(*Williams 2009*).* The modern era of drug discovery for chemotherapy started with the discovery of penicillin by Sir Alexander Fleming, which can treat many infectious diseases including *S. aureus* infections *(*Aminov 2010*).* Based on the mechanism of action, antimicrobial agents can be categorized into distinct classes. Agents that prevent the development of cell walls, cell membrane lysis, nucleic acid synthesis, protein synthesis, and metabolic processes are among the primary groups of these substances (Figure 2.9).

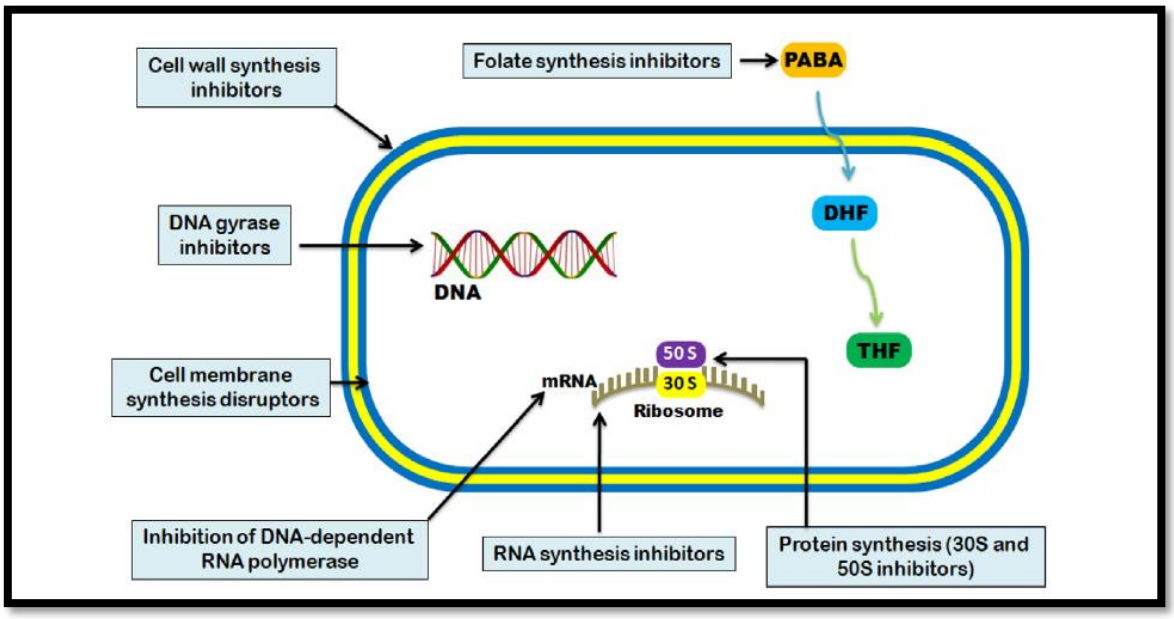

Figure 2.9. Antibiotics' different mechanisms of action (adapted from Uddin *et al.*, 2021)

Scientists and researchers discovered many notable antibiotics after the discovery of Penicillin to treat bacterial and other microbial infections. Nonetheless, due to the irrational use of antibiotics, the bacteria start evolving certain genes that impart resistance against the antibiotic

that was once sensitive to them. Antibiotic resistance is the ultimate capability of microorganisms to resist and hold on to any exposure to antimicrobial chemotherapeutics or medications (Frieri *et al.,* 2017). All classes of microorganisms can evolve resistance against any class of antibiotics. Bacteria evolve antibiotic resistance, viruses evolve antiviral resistance, protozoans evolve antiprotozoal resistance, and fungi evolve antifungal resistance via different mechanisms (Yousif *et al.,* 2022). Some of the microbes that become resistant to various drugs (extensively drug-resistant) or show complete resistance to all of the potential drugs (total drug-resistant) are known as superbugs (Coast *et al.,* 1996).

In the 1930s, the first case of resistance against sulfonamide was reported (Lobanovska and Pilla 2017). The resistance against Penicillin was reported soon after its introduction in chemotherapeutics in 1940 by Chain and Abraham in *E. coli* that produce β-lactamase to neutralize the effect of Penicillin (Abraham and Chain 1988). With the advent of time, more classes of antibiotics including aminoglycosides, quinolones, cephalosporin, and tetracycline, etc. were introduced but bacteria evolved resistance against such antibiotics. For example, *Staphylococcus aureus* became resistant to methicillin and the notorious Methicillin-resistant *Staphylococcus aureus* (MRSA) strains emerged (Chambers and DeLeo 2009). Similarly, *Mycobacteria tuberculosis* evolved resistance against multiple drugs including rifampicin, quinolones, and streptomycin resulting in MDR-TB (Müller *et al.,* 2013). With the emergence of new resistant clones and a lack of discoveries in finding new potential antimicrobial therapeutics, antibiotic resistance becomes a threat to the whole world. Antibiotic resistance among MDR bacteria poses a severe threat to public health with a higher mortality rate and nearly 4-95 million deaths are found to be associated with bacterial AMR (Murray *et al.,* 2002).

4.1 Mechanism of Antibiotic resistance

Antibiotic resistance is a natural process of overcoming the effects of antibiotics by microorganisms and is usually due to certain genetic changes in bacteria or due to the misuse of antibiotics which kill some bacterial populations and allow the remaining to flourish. The drugs need to reach the target site to exert their antimicrobial action, but bacteria have evolved several mechanisms to evade the effects of drugs imparting antibiotic resistance. The four kinds of resistant mechanisms are lowering the antimicrobial agent concentration, altering or inactivating the antibiotic, changing the target locations, and obtaining resistance from other related species or through mutations (Yousif *et al.,* 2022).

4.1.1 Lowering the intracellular concentration of antimicrobials

By actively eliminating the antibiotic or by blocking its entry, bacteria can lower the concentration of drugs inside their cells. The removal of drugs from bacteria can be actively achieved by the efflux pump present in bacteria. All of the bacterial genomes encode multiple different efflux pumps, which remove drugs from the bacterial cells. Efflux pumps can potentially determine the intrinsic susceptibility of microbial species to certain drugs and are clinically significant in imparting antibiotic resistance when overexpressed (Thakur *et al.,* 2021). Mutations in regulators and the acquiring of insertion nucleotide sequences that may function as potent promoters of efflux pump gene regulation can both contribute to the overexpression of the efflux pump in bacteria (Baylay *et al.,* 2019). In gram-negative and gram-positive bacteria, the efflux pump-mediated resistance is mainly due to the presence of resistance nodulation division (RND) and major facilitator superfamily (MFS) pump mechanisms (Poole 2005; Tikhonova and Zgurskaya 2004). Similarly, other efflux mechanisms including the MATE (multidrug and toxic compounds extrusion) like PmpA in *P. aeruginosa*, MepA in *Staphylococcus aureus*, and VcrM in *Vibrio cholera* can efflux benzalkonium chloride and fluoroquinolones (Kaatz *et al.,* 2005; He *et al.,* 2004; Huda *et al.,* 2003). Moreover, ATP-binding cassette transporter (ABC transporters) in bacteria also decrease the intracellular concentration of antimicrobials and results in antibiotic resistance like the PAtAB in *S. pneumonia* can efflux out the fluoroquinolones and MacAB can remove macrolides in *E. coli* (Greene *et al.,* 2018; Garvey *et al.,* 2011).

In addition, more antibiotic efflux mechanisms are usually acquired through an evolution where the evolving bacteria reduces the uptake of antimicrobials from the environment. Such an evolved mechanism is more frequently reported in gram-negative bacteria whose outer membranes serve as an effective permeable barrier plus the membrane-embedded porin protein channels that make the effective movement of solutes outside the cell. The membrane permeability can be reduced by either replacement of porins with much smaller channels or due to mutations in the porin gene in such a way that it affects the permeability (Baylay *et al.,* 2019) (Figure 2.10).

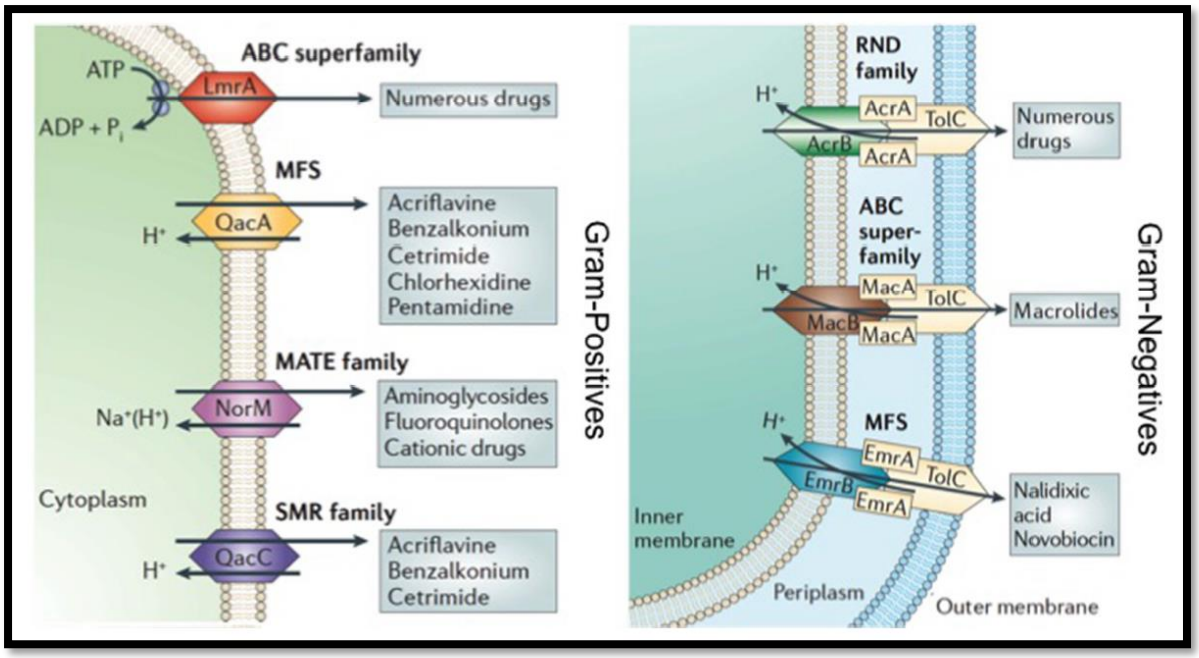

Figure 2.10. Illustration of different mechanisms of efflux pumps in Gram-negative and Gram-positive bacteria (adapted from Munita and Arias 2016)

4.1.2 Inactivation of Antibiotics

Antibiotic resistance can be mediated by the modification and degradation of antibiotic structure through hydrolysis, phosphorylation, etc (Kumar and Varela 2013). Enzyme-catalyzed degradation by hydrolysis is one of the major reasons for antibiotic resistance since the introduction of penicillin. Numerous antibiotic-modifying enzymes have been discovered that can potentially alter the structural integrity of antibiotics belonging to various classes like; β- lactam group, aminoglycosides, and macrolides. Hydrolytic enzymes including the β-lactamases target the intact structure and hydrolyze the β- lactam ring of β- lactam antibiotics and inactivate them. Moreover, they also hydrolyze other drugs like; cephalosporin (3rd and 4th generation), carbapenems, and penicillin by producing extended-spectrum β-lactamases (ESBLs) (Livermore 2008; Williams 1999). Many pathogenic agents having ESBLs are regularly being reported in clinical and environmental isolates. ESBLs in *P. aeruginosa*, *K. pneumonia*, and *Serratia marcescens* resulting in the emergence of highly resistant strains are consistently being reported worldwide in clinical samples (Ghafourian *et al.,* 2015; Rawat and Nair 2010). Similarly, macrolide esterase enzymes cause antibiotic degradation by hydrolyzing the structure of macrolide antibiotics (Wright 2005). The ester-bonded cyclic ring structure of macrolides is effectively hydrolyzed by the esterase enzymes. Enzyme-based resistance has

been reported in *E.coli, Providencia stuartii, Pseudomonas spp*., and *S. aureus* (Baylay *et al.*, 2019; Barthelemy *et al.*, 1984).

Antibiotic inactivation in bacteria can be due to the modification of drugs by the addition, replacement, or hydrolysis of functional groups attached to the antibiotics like, hydroxyls and amines. This modification led to alterations inside the chains that ultimately prevented the efficient binding of drugs to their target sites. The functional groups include acyl, phosphate, thoil, nucleotidyl, etc. (Wright 2005). *Phosphotransferase* and *acyltransferase* can impart resistance in aminoglycosides and chloramphenicol respectively (Ling 2019; Parulekar *et al.*, 2019) (Figure 2.11).

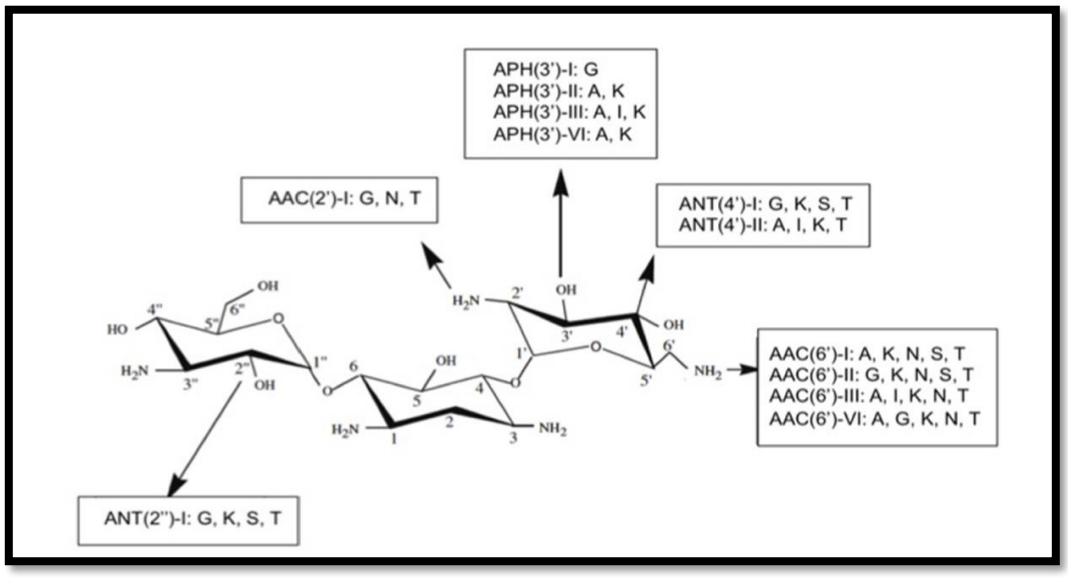

Figure 2.11. Nomenclature and illustrations of different aminoglycosides-modifying enzymes (adapted from Munita and Arias 2016)

4.1.3 Changing in the target site

Antibiotics' target site can be modified either due to mutations in the coding sequence or post-transcription modification by altering functional groups so that the drugs can no longer able to bind the target site. Some bacteria like *S. aureus* have acquired mechanisms to develop resistance against vancomycin drugs by modifying the chemical composition of the cell wall and the unavailability of target sites (Gardete and Tomasz 2014). It is also highly common for antibiotic resistance to develop as a result of gene mutations encoding target proteins. *Topoisomerase II* and *IV*, as well as *DNA gyrases*, are inhibited by fluoroquinolones. Most

bacteria's resistance to fluoroquinolones results from mutations in the *parC* and *gyrA* subunits of *topoisomerase*, and *DNA gyrase*, respectively (Everett *et al.*, 1996).

The target site can also be changed because it is frequently modified by methylation. *E. coli* can develop resistance to many antibiotics, such as streptogramins, macrolides, and lincosamides, by methylating an adenine molecule in *23S rRNA* (Baylay *et al.*, 2019). The alteration in target sites for linezolid is shown in Figure 2.12.

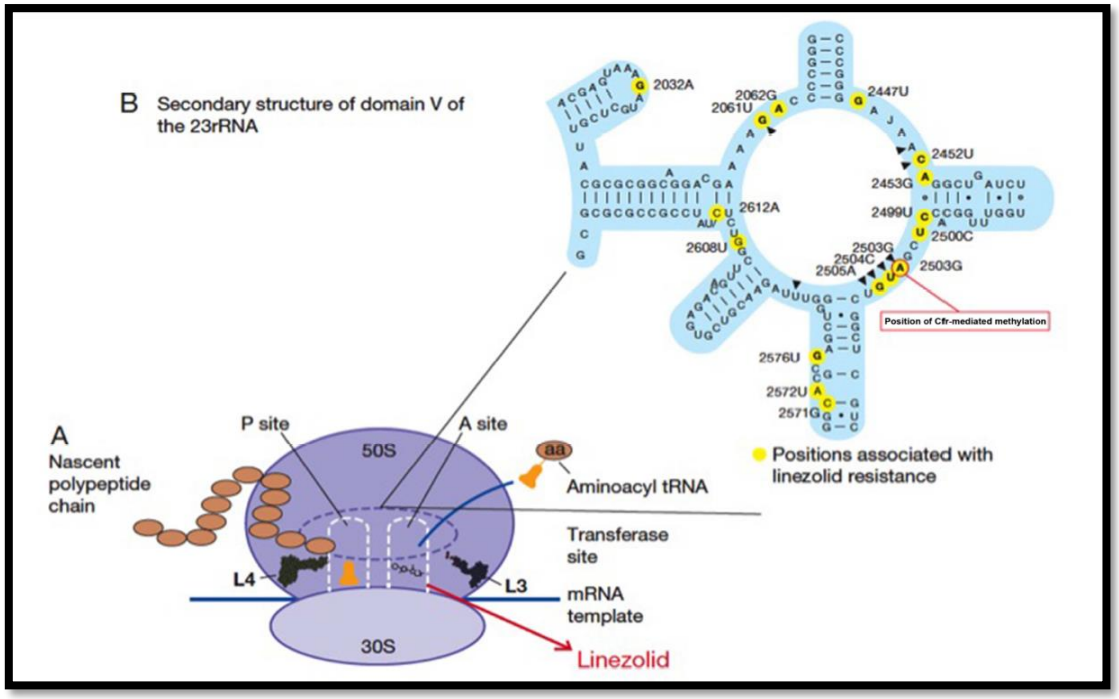

Figure 2.12. Alteration in the drug target sites (adapted from Munita and Arias 2016)

4.1.4 Acquired resistance

Bacteria have short generation times, produce large populations, and can acquire antibiotic resistance rapidly through de novo mutations. Mutations play a significant role in resistance, especially against synthetic drugs in which no reservoirs of antibiotic-resistant genes (ARGs) are present in natural pristine environments (Tenover 2006).

In addition to mutations, horizontal gene transfers play a significant role in the transmission of antibiotic resistance genes from related bacteria or the environment. Gene transfer from one bacterium to another can be achieved by transformation, transduction, and conjugation (Zhang and Feng 2016; Bennett 2008). Resistance usually involves genes, and mobile genetic elements

(MGEs), and antibiotic-resistant pathogens show a broad degree of tolerance and persistence against antibiotics (Bennett 2008).

5 Antibiotic resistance genes

Antibiotic resistance genes can spread from cell to cell by conjugation, transformation, and transduction and are frequently found on mobile genetic elements including plasmids, integrons, and transposons (Heuer *et al.,* 2011). ARGs hastened the microbial threat to public health in the last decade. Many genes have been found to confer resistance and significant elements resulting in the expression of ARGs in different strains of microbes through lateral transmission and desperately among the pathogens. The main factor causing the rapid emergence of bacterial resistance to antibiotics is the existence of genes for antibiotic resistance in the environment and clinical settings (Jian *et al.,* 2021). By exchanging plasmids at the gene level, pathogenic bacterial strains acquire ARGs and acquire resistance. The transfer of ARGs occurs through horizontal gene transfer (HGT) among strains carrying ARG-transposons, integrons, and plasmids to strains of the same or different species. Because DNA-carrying ARGs have been present in the environment for a longer period under the protection of the deoxynucleotide enzyme, they are crucial for the evolution of resistance in pathogenic strains of bacteria even after death (Dantas *et al.,* 2008; Bertolla *et al.,* 2000).

5.1 Antibiotic resistance and ARGs in a natural environment

Antibiotic resistance has become a global health concern due to the involvement of the transfer of resistant strains and ARGs between the environment, humans, and animals. Though multiple barriers restrict the movement of ARGs and resistant bacterial strains, pathogens are capable of acquiring resistance from other species through various means, thereby increasing the risk of getting infected by resistant strains and reducing the potential therapeutic options for bacterial infections (Larsson and Flach 2022). External environments are more likely to be involved in the acquisition of novel resistance determinants from the soil, water, and other surroundings with wildly heterogeneous ecological niches than they are in the development of mutation-based resistance in pathogenic strains (Rinke *et al.,* 2013; Forsberg *et al.,* 2012). The ecological niches provide a highly diverse gene pool that can be acquired by pathogenic bacteria. Some environmental conditions provide genetic elements like ARGs, plasmids, and

transposons with frequent gene exchanges (Flach *et al.,* 2017; Shintani *et al.,* 2020) (Figure 2.13).

Similar to human healthcare, the widespread application of antibiotics in animal husbandry places pressure on particular types of gut bacteria or other related internal systems to acquire and maintain ARGs, and when released in the environment results in their potential spread, increasing relatively abundant resistant bacterial strains. Different antibiotic-resistant genes of bacteria develop resistance against different types of antibiotics including β-lactams (*bla*), tetracycline (*tet*), aminoglycosides (*acc*), colistin (*mcr*), fluoroquinolone (*qnr*), vancomycin (*van*), and macrolides (*erm*) (He *et al.,* 2020). Zarei-Baygi and Smith (Zarei-Baygi and Smith 2021) describe the horizontal transferring routes of ARGs (both intracellular and extracellular) in different environments. In addition, they found that intracellular ARGs were copiously present in the nutritious environment while extracellular ARGs were more prevalent in the aquatic environments. The extracellular ARGs were more persistent compared to intracellular ARGS due to their ability to avoid *DNase* degradation and absorbance in soil particles. Zhuang *et al.* picturized the global persistence of ARGs in different environments and reported most common ARGs from farms, wastewater, and soil were tetracycline and sulfonamides while β-lactamide and glycopeptides (*bla*, *mecA*, *vanA*, and *vanB*) were more common in hospitals (Zhuang *et al.,* 2021).

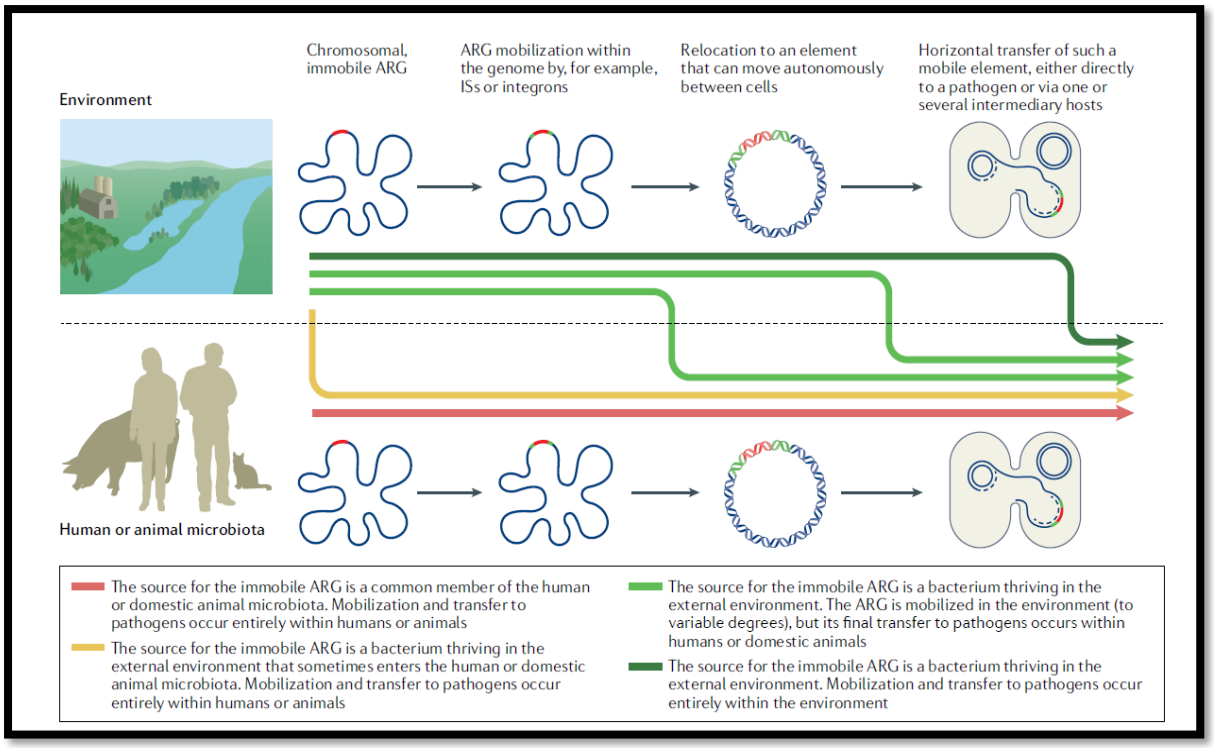

Figure 2.13. ARGs evolution to emergence: The originative driving force of the environment in the propagation of novel antibiotic-resistant genes (adapted from Larsson and Flach 2022)

5.1.1 Antibiotic-resistant genes in Soil

Soil ecosystem has a significant reservoir of AGRs which can potentially spread to other ecosystems such as water and forest and be acquired by pathogens that infect animals and humans. Anthropogenic activities that involve the emancipation of antibiotics of human microbiomes into an adjacent natural environment such as soil and water are significant sources of ARG proliferation in soil. The substantial use of antibiotics in plant agriculture and livestock husbandry may be one of the anthropogenic activities that greatly influence the establishment of ARGs in soil (Heuer *et al.*, 2011; McManus *et al.*, 2002). *Bacillus* species and the *Actinomycetes* genus *Streptomyces* are two naturally occurring antibiotic-producing species found in soil. Both species can directly discharge antibiotics into the soil environment, which can lead to antibiotic resistance in harmful bacterial species (Forsberg *et al.*, 2012).

According to Goethem *et al.* there are 177 naturally occurring ARGs in soil, with Gram-negative bacteria acquiring 71% of them whereas Gram-positive bacteria only take up 9%. The two common ARG families identified in the study were ARGs for efflux pumps/transporter systems and *undecaprenyl pyrophosphate phosphatase* which confer resistance to Bacitracin

(Van Goethem *et al.,* 2018). Osbiston *et al.* compared the agricultural soil with that of soil from urban areas and found higher levels of ARGs in agricultural soil using animal manure as fertilizer (Osbiston *et al.,* 2021). The use of antibiotics by animals, antibiotic residues in the soil, and the horizontal transmission of pathogenic gut microbiota from animals to humans all contribute to the accumulation of ARGs in agricultural soil. Similarly, Zheng *et al.* did a global analysis of 1088 soil samples by metagenomics and reported the detection of 558 ARGs in soil. The higher abundance was found in agricultural soil compared to non-agricultural soil while the most frequently detected ARG subtypes were *mexF*, *multidrug_ABC_transporter*, *macB*, *mdtB*, and *mdtC* (Zheng *et al.*, 2022).

5.1.2 Antibiotic-resistant genes in water

The improper use of antibiotics has sped up the emergence of bacteria that are resistant to drugs and genes that are resistant to antibiotics, raising severe issues for public health. Across the world, ARGs are becoming more prevalent in water, and the potential rise of superbugs and antibiotic-resistant bacteria is beginning to pose a severe threat to the environment and public health (Ma *et al.,* 2015). It is now well-accepted that one of the main reservoirs and transmission channels for ARGs is water (Amarasiri *et al.,* 2020). As residual drugs, urine, feces, and waste materials from antibiotic production facilities are disposed of, antibiotics end up in water bodies (Amaya *et al.,* 2012). According to estimates, more than 90% of antibiotics given to humans and animals end up in water streams as expelled waste (urine, feces), damaging the aquatic ecosystem (Wang *et al.,* 2018; Zhang and Li 2011). All of the antibiotics by somehow consistently ending up in the environment accelerate the pace of antimicrobial resistance and ARG development (Larsson and Flach 2022).

The World Health Organisation (WHO) has characterized the Southeast Asia (China and India) and Western Pacific regions as areas with significant antibiotic resistance and the presence of antibiotic resistance genes (ARGs) in their aquatic environments. These regions are recognized as major global hubs for antibiotic production and consumption. (Hanna *et al.,* 2023). Obayiuwana and Ibekwe reported the significant occurrence of ARGs in wastewater from pharmaceutical facilities in Nigeria. The study found the presence of *catA1* in 58.3% of isolates, *tet(E)* in 30%, *sulI* (31%), *aac(3)-IV* (28%), *ermC* (20%), and $_{bla}$*TEM*, and $_{bla}$*NDM-1* in 18.3% of the bacterial isolate. These ARGs encode resistance against chloramphenicol, tetracycline, sulfonamide, macrolide, and penicillin respectively (Obayiuwana and Ibekwe 2020). The

variety and abundance of ARGs were discovered in 79 wastewater treatment facilities by a multicentre metagenomics investigation that was carried out in 60 nations across Asia, Africa, North and South America, and Oceania. South America, Africa, and Asia have higher abundances of ARGs encoding resistance to phenolics and sulfonamides than Europe, North America, and Oceania, which have higher abundances of ARGs encoding macrolides (Hendriksen *et al.,* 2019). Moreover, Khan *et al.* documented the detection of the highest level of ARGs (*sulI* and *dfrA1*) in rivers and canals near drug manufacturing units in Pakistan (Khan *et al.,* 2013).

5.1.3 Antibiotic-resistant genes in cold habitats

Natural environments including cold habitats like glaciers, and permafrost are significant reservoirs of ARGs, and many studies reported the presence of ARGs and MGEs in cold environments including glaciers. Across the globe, many researchers have reported numerous classes of ARGs and MGEs from both polar and non-polar glaciers like; the Gulkana glacier (USA), Africa (Uganda), Himalayas (Nepal and Bhutan), Mackay glacier (Antarctica), Ürümqi (China), North Sikkim, Changme Khang and Khangpu (India) and HKKH (Pakistan) (Nawaz *et al.,* 2023; Sherpa *et al.,* 2020; Van Goethem *et al.,* 2018; Ushida *et al.,* 2010). The metagenomics analysis of North Sikkim glaciers (Changme Khang and Khangpu) samples detected the presence of bacitracin resistant gene (*bacA*) while metallic-resistant genes of copper (*cutA*, *cutC*, *cutE*, *cueB*, *copB*, and *copC*) and chromium (*yelf*, *nfsA*, and *chrR*) were explored in Changme Khangpu glacier. Whereas the metallic resistant genes of iron (*yefA*, *yefB*, *yefC*, and *yefD*) and cobalt (*dmef*, *corB*, *corC*, and *corD*) were disclosed in the Changme Khang glacier (Sherpa *et al.,* 2020; Segawa *et al.,* 2013). Following a similar study, a metagenomics approach was applied to detect the presence of ARGs and VFGs (virulence factor genes) in the Arctic permafrost region. The study reported 70 different types of antimicrobial drugs against 11 classes, 599 VFGs with 38 characteristic virulence factors, and 8 mobile genetic elements carrying ARGs (Kim *et al.,* 2022). Yuan *et al.* analyzed ARGs' presence in soil samples of Antarctica using metagenomics techniques and detected the presence of 79 ARG subtypes against 12 classes of antibiotics. Among these ARGs, 60% of them conferred antibiotic resistance through an efflux pump and approximately 16% were present on a plasmid (Yuan *et al.,* 2019).

6 Transference of ARGs from the natural environment to the community

Pathogenic bacteria's emergence and spread of antibiotic resistance have long been a significant problem for public health. It is becoming increasingly clear that ARGs are ubiquitously present in all pathogenic environmental bacteria, bacteriophages, and medicinal pathogens as well, forming what is known as a resistome (Zainab *et al.,* 2020). Via the transfer of resistance genes, pathogenic microorganisms can acquire resistance from this resistome, which is a reservoir of resistant genes. ARGs are transmitted in the clinical pathogen through horizontal gene transfer, which has become a source of dissemination of antibiotic resistance from commensal and environmental bacteria to pathogenic ones (Hassoun-Kheir *et al.,* 2020; Von Wintersdorff *et al.,* 2016). Moreover, through HGT the exchange of genetic material occurs between two organisms that do not have an antecedent-descendant link; it is a strategy that significantly speeds up the production of biotic innovation, which would otherwise take a long time to happen. Instead of vertical legacy, which is the transfer of genetic material from parent to offspring, genetic material transfer reveres no ordered limitations, with genes exchanged within the genus level while still among tangentially related ancestries that belong to other domains. Moreover, certain routes for gene flow between ecologically divergent heredities have been identified (Von Wintersdorff *et al.,* 2016).

The three methods that bacteria most usually utilize to diversify their DNA are transformation, conjugation, and transduction. Two of the three activities can be performed by each type of bacterial cell, but not all of them. A bacterial cell that is "capable" or susceptible can modify its surroundings to take in new genetic material. Via a process known as transduction, a virus spreads genetic material from one bacterium to another bacterium. This method is dependent on the bacteriophage virus class, which may infect bacterial cells and use them as hosts to produce further viruses. With direct touch, one bacterium can exchange genetic material with another bacterium through the process of conjugation (Schneider 2021). The quantity of genetic information that can be transported horizontally includes whole chromosomes, diverse mobile genetic elements, operons, superoperons that code complex biological pathways, and gene fragments. This fascinating technique unmistakably displays the special qualities of the microbial world. In the field of medicine, the ability of most microbes to acquire genetic information from other germs is of utmost significance. The issue of bacterial infections that are resistant to many conventional medications might be further worsened by the distribution

of ARGs in bacteria through HGT, mutation, and divergence of pathogenic organisms (Villa *et al.*, 2019; Andam and Gogarten 2011) (Figure 2.14).

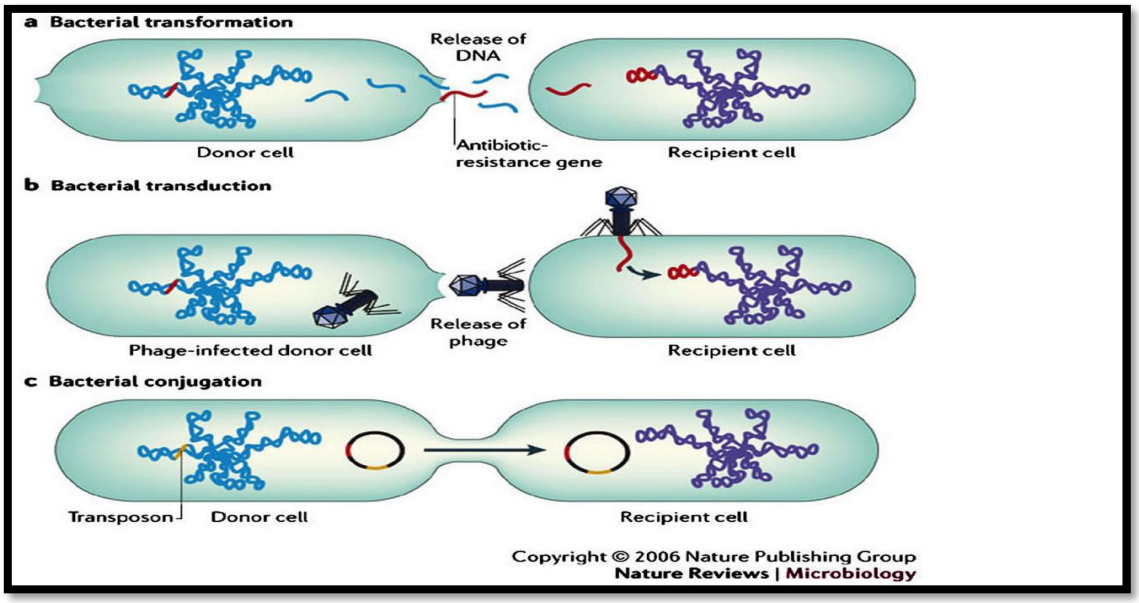

Figure 2.14. Genetic material flow among bacteria (adapted from Nature Reviews Microbiology)

7 Primitive role of ARGs in natural environments

Environmental microorganisms are not subject to the same strong antibiotic selective pressure as human infections, which are exposed to antibiotics during therapy, even if human activities can lead to antibiotic pollution of natural ecosystems. Understanding the role of antibiotics and the components of their resistance in these habitats is crucial for correctly analyzing antibiotic resistance in natural ecosystems. It has been hypothesized that the purpose of these substances in nature should be to restrict the growth of the microbial rivals of the antibiotic makers since several of the antibiotics used to treat illnesses are manufactured by soil microorganisms (Waksman and Woodruff 1940). In the low concentrations that are undoubtedly present in the majority of natural habitats, it has been suggested that some antibiotics may act as signaling agents. This idea is supported by experimental data demonstrating that low doses of antibiotics cause particular transcriptional alterations, independent of the broader microbial networks involved in stress response (Fajardo and Martínez 2008; Linares *et al.*, 2006).

In addition, the studies suggest that some antibiotics' principal function may be intercellular signaling in natural habitats, with bacterial growth inhibition occurring only at the high

concentrations required for therapeutic purposes. One aspect of antibiotic resistance genes that fit well with their function as barriers is that some of them, which are currently found in pathogenic bacteria, are associated with antibiotic detoxification in the primary producers or the resistance to toxicants created by plants or by their accompanying microbiota. Certain well-known antibiotic resistance drivers in natural ecosystems, however, do not always play a major role in preventing antibiotic activity (Bais *et al.,* 2005,2006; Pang *et al.,* 1994). Such as, it has been proposed that penicillin-binding proteins responsible for the production of peptidoglycan may have evolved into plasmid-encoded beta-lactamases, which are highly effective antibiotic resistance indicators procured by pathogenic bacteria via HGT, and that their activity against beta-lactams is a by-product of this original function (Meroueh *et al.,* 2003; Massova and Mobashery 1998). Moreover, the *Providencia stuartii* chromosomal *20-N-acetyltransferase*, an enzyme essential in the remodeling of the bacterial peptidoglycan, is thought to be an indicator of antibiotic resistance since it can render gentamycin inactive while having a different primary function (Macinga and Rather 1999). From these examples, it can be inferred that a factor that leads to the antibiotic resistance of human diseases can be involved in the primary metabolic functions of environmental bacteria in one‘s natural environment.

Likely, nature is also selected for other systems that do not necessitate antibiotic breakdown to provide a primarily functional purpose distinct from antibiotic resistance. The resistance against quinolones, a class of synthetic antibiotics introduced for use in therapy in the 1960s, serves as an illustration of this. Quinolones, while having a synthetic origin, are a preferred target of MDR efflux transporters, and environmental bacteria that were isolated before quinolones were created, can efflux these medications, demonstrating that those determinants' main purpose is not to promote resistance (Alonso *et al.,* 1999). The first quinolone resistance determinant encoded on a plasmid, *Qnr*, may experience a similar circumstance. It has been established that aquatic bacteria have chromosomally encoded *qnr* genes. Additionally, the idea that these determinants came from aquatic bacteria is supported by the high degree of synteny conservation in the regions around these genes and the absence of elements linked to transposition or insertion episodes in nearby environments where quinolone presence is not anticipated. (Sánchez *et al.,* 2008; Poirel *et al.,* 2005; Jacoby and Martinez 1998).

8 Assessing the ARGs link between the natural and clinical environment

Remarkably, many ancient bacteria have molecular similarities to modern bacteria. In certain situations, geological evidence shows how old these organisms are, whereas DNA evidence shows how modern they are. How germs can survive for such a long time is unknown. There are reports of bacteria from amber surviving for at least 40 million years despite the lack of a known mechanism (Hershkovitz *et al.,* 2008; Greenblatt *et al.,* 2004). The environment is frequently home to microbes that have developed resistance to several antibiotics. Sometimes regular antibiotic exposure, human pathogens, as well as the spread of resistance characteristics between and within microbes, are to blame for the development of antibacterial resistance in microbes (Pareek *et al.,* 2015; Kümmerer 2010, 2009).

Bacterial species naturally develop intrinsic resistance, not as a result of exposure to drugs. When species first respond favorably to an antibiotic but later develop resistance, this is known as acquired resistance. By trading genetic material or mutating, comparable and closely connected bacterial species may enable bacteria to become resistant to antibiotics. It's crucial to learn more about the potential for antibiotic resistance in bacteria from both natural and clinical situations to better comprehend the development of resistance (Martinez 2012; Martinez *et al.,* 2008). It is a natural occurrence for antibiotic-resistant strains of environmental and human pathogens to develop when bacteria are continuously exposed to sub-inhibitory concentrations of antibiotics. Hence, environmental microorganisms become resistant to a variety of antibiotics either native or adapted antimicrobials. Genes for antibiotic resistance that naturally exist in psychrophilic bacteria may be transmitted to human pathogens and vice versa through several mechanisms. Certain psychrophilic bacteria have developed resistance to many kinds of antibiotics, including kanamycin, tetracycline, chloramphenicol, beta-lactam antibiotics, and polymyxin B (Martinez 2009; Martinez *et al.,* 2008; Lo Giudice *et al.,* 2007).

Besides, reasonable means of transmission of antibiotic-resistant genes and mobile genetic elements all through the hydrological structures, winds, migrating birds, vertebrate feces, tourists, and airborne bacteria may encourage the geographical diffusion of ARGs and antibiotic-resistant bacteria between the clinical and natural environments (Makowska *et al.,* 2020; Literak *et al.,* 2014; Segawa *et al.,* 2013). Additionally, the mixing of glacier melt, and rainwater stimulates bacterial distribution and fosters HGT which contributes to diverse ARGs dissemination across the globe (Makowska *et al.,* 2020).

9 Nature's instabilities and impending evolution of antibiotic resistance

Since environmental bacteria are the potent source of ARGs, it is crucial to determine whether changes in natural ecosystems, which are mostly the result of human activity, might put the environment's microbiota under stress in a way that affects human pathogen resistance (Alonso and Martinez 2001). The flow of antibiotics that haven't been employed for treating or preventing human illnesses is still very high, even though the use of antibiotics for agricultural purposes has been outlawed in several nations. This has resulted in frequent antibiotic pollution at farms, rivers that collect wastewater, and areas getting antibiotic-contaminated manure, in addition to the antibiotics being used in clinics. This may hasten the evolution of resistance and raise the possibility that it will spread to human infections, which may also exist in these ecosystems (Baquero *et al.,* 2008; Cabello 2006). The integration of the plasmid-encoded *qnr* gene into a gene-transfer element in *Aeromonas spp*., which are chromosomally expressed in waterborne bacteria, may be a result of the application of quinolones in agriculture and could be a precursor to its transmission to human diseases. Interestingly, the same plasmid carrying the very same *qnr* gene has been discovered in geographically distinct areas, proving that once resistance genes for antibiotics are incorporated in gene-transfer components, they consequently have a better probability of dispersal and subsequently remaining in bacterial populations (Picao *et al.*, 2008).

The antibiotic-resistant genes ultimately make up a different sort of contamination that may be important for the resistance development in bacterial infections. Since the environment is the origin of resistant genes, the discovery of resistant species all over the world should not come as a surprise. The discovery of resistance determinants, which are already well-established in pathogenic bacteria, in settings without even a background of antibiotic contamination, on the other hand, is troublesome (Pallecchi *et al.,* 2008). Such as, resistant bacteria are present in distant human groups without a history of antibiotic exposure, and resistant strains are also well-established in populations of wild animals despite no antibiotic use (Bartoloni *et al.,* 2009; Livermore *et al.,* 2001). The same ARGs, linked to distinct genetic platforms (integrons, transposons, and plasmids), are found in both bacterial pathogens and uncontaminated environments with undetectable antibiotic concentrations, suggesting that there is a global distribution of ARGs conferring resistance. These ARGs are primed to be propagated by HGT with antibiotic selection because they are already prevalent in the environment and on genetic

platforms that are friendly to bacterial pathogens. This suggests that even in the absence of antibiotic selective pressure, some antibiotic-resistant genes can be challenging to eliminate. As a result, these genes may persist in the environment and affect the genetic diversity and ultimately the genetic drift of environmental microbes, because predefined resistance mechanisms cost the bacterium nothing and, in some circumstances, increase fitness and even for compensating mutations limiting bacterial load may be chosen even at pertinent fitness costs. (Balsalobre and de la Campa 2008; Löfmark *et al.,* 2008). Generally, the biocide use and heavy metal contamination may both favor resistance to antibiotics in natural habitats, additionally to the resistance of heavy metals and biocide resistance patterns linked to antibiotic resistance (Stepanauskas *et al.,* 2006).

Natural processes destroy antibiotics, however, the genetic components that include resistance genes are self-replicating and may be relatively persistent. In ecosystems where they have evolved without human interaction for thousands of years, a wide range of antibiotics and resistance genes share their environmental origins. As a result of this, in terms of evolution, human use of antibiotics, and genetic frameworks in communities of environmental bacteria are becoming more enriched with antibiotic-resistant genes, which is speeding up the dispersion and evolution of resistance among microbiotas.

10 Plasmid Curing of Bacteria

The process of extraction of plasmid from bacterial species is referred to as plasmid curing. It is considered an effective strategy for combating antibiotic resistance through the removal of ARGs from a bacterial population with negligible effect on the overall structure of the bacterial community (Buckner *et al.,* 2018).

The different strategies applied for plasmid curing employ phage therapy, CRISPR/Cas systems, phytocompounds, DNA intercalating agents, incompatible plasmids, detergents, and even psychotropic drugs. In addition, the interference with the normal plasmid conjugation process also facilitates the gradual elimination of plasmid-mediated resistance from the population over time (Buckner *et al.,* 2018).

The plasmid-curing agents work through a variety of mechanisms. These include disruption in replication of plasmid by intercalating with DNA (e.g., intercalating agents such as acridine orange and ethidium bromide), induction of breaks in DNA strands (ascorbic acid), interference

with supercoiling of plasmid (e.g., quinolones), prevention of conjugation by binding to *TraE* component of type IV secretion system and inhibition of its dimerization (e.g., *TraE* inhibitors) (Buckner *et al.*, 2018). However, the introduction of a smaller plasmid from the same incompatibility group but with a high copy number is an effective alternative to the chemical-based plasmid curing methods (Bringel *et al.*, 1989).

In addition, the application of phage as an anti-plasmid system is effective in producing the selection pressure with the resultant non-conjugate plasmid evolution or reduction of plasmid prevalence in the bacterial population. This strategy is utilized to select the antibiotic-sensitive population of bacteria (Chan *et al.*, 2016). The inhibition of the F-plasmid-based conjugation has been observed in *E. coli* in the presence of the g3p (coat protein) of M13 phage (Lin *et al.*, 2011).

The recent strategy involves the utilization of the bacterial adaptive immune system, referred to as the CRISPR/Cas system for plasmid curing. This involves either the targeting of the ARGs or the introduction of the double-stranded breaks ultimately reducing the plasmid stability or plasmid loss in some cases (Kim *et al.*, 2016; Lin *et al.*, 2016). However, the need for the exploration of other safe and more effective ways to cure ARGs containing plasmids still exists in this global crisis of AMR.

11 Bacterial Plasmid Cloning

The cloning of the PCR products for the generation of a recombinant plasmid through the utilization of a high-throughput compatibility system is crucial for the functional studies of biomolecules (Yao *et al.*, 2016). The plasmid cloning technology is classified into two major divisions that include ligase-based and ligase-free cloning. The ligase-independent method involves the Gateway (Hartley *et al.*, 2000), (Circular polymerase extension cloning) CPEC (Quan and Tian., 2009), Fast Cloning (Li *et al.*, 2011), and others. However, the robustness of ligase-dependent methods provides them an advantage over the ligase-independent methods (Yao *et al.*, 2016).

The ligase enzyme is required for the covalent binding of plasmid with DNA of interest in ligase-based cloning methods. These include cohesive-end ligation, blunt-end ligation, TA cloning, and TOPO cloning. The blunt-end-based ligation involves the ligation of DNA of interest with the linear vector but is considered inefficient for high-throughput technologies.

On the other hand, cohesive end-based ligation involves the highly efficient ligation of the cohesive ends of DNA insert and plasmid, but the internal site's presence makes it difficult to select appropriate digestion sites for restriction enzymes (Yao *et al.,* 2016).

TA cloning utilizes the Taq polymerase for adding adenosine to the 3' end of the PCR amplified products generating the 3'-A overhangs for them. The T-vectors have the 3'-T overhangs in their linear forms at both ends that promote their ligation (Bielefeldt-Ohmann and Fitzpatrick., 1997; Aranishi and Okimoto., 2004). It is a simple cloning method that can be utilized for the cloning of DNA insert with unknown sequences but for high throughput technologies, it's better to use an additional proofreading enzyme since Taq polymerase lacks the 3'-5' proofreading activity of exonuclease (Yao *et al.,* 2016).

The TOPO cloning method utilizes the DNA topoisomerase (TOPO) I with dual functionality as a ligase and restriction enzyme. It helps to achieve about 95% efficiency for cloning. The TOPO I identifies the pentameric sequence of 5'-(C/T)-CCTT-3' followed by the formation of a covalent bond with the 3'thymidine phosphate group. The cleavage of one DNA strand is then followed by DNA unwinding and religation of the cleaved strands. The linearized TOPO vector has the TOPO I covalently attached to its 3' end phosphate which then provides the efficient ligation for the compatible ends of the TOPO vector and DNA insert. It is one of the most versatile and efficient ligase-dependent cloning methods as it can be utilized for the i*n-vitro* transcription and functional expression in mammalian cells or competent Mach1™-T1^{R} or *E. coli* cells. In addition, the TOPO cloning method can be utilized to ensure expression through Gateway systems (Patel 2009).

The utilization of these cloning methods for the expression of antibiotic-resistant genes provides insight into the dissemination of these genes through HGT in natural environments. This is especially important in cold environments when psychrophilic bacteria from melting glaciers come in contact with the mesophilic bacteria and horizontally transfer their ARGs to them. However, the data on ARG transmission in psychrophilic bacteria of the non-polar glaciers is limited.

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Chapter 3. Bacterial diversity and antibiotic resistance

Research article 1.

Title:

Sabir Nawaz . Muhammad Rafiq . Ian L. Pepper . Walter Q. Betancourt . Aamer Ali Shah . Fariha Hasan. Prevalence and abundance of antibiotic-resistant genes in culturable bacteria inhabiting a non-polar Passu glacier, Karakorum Mountains range, Pakistan.

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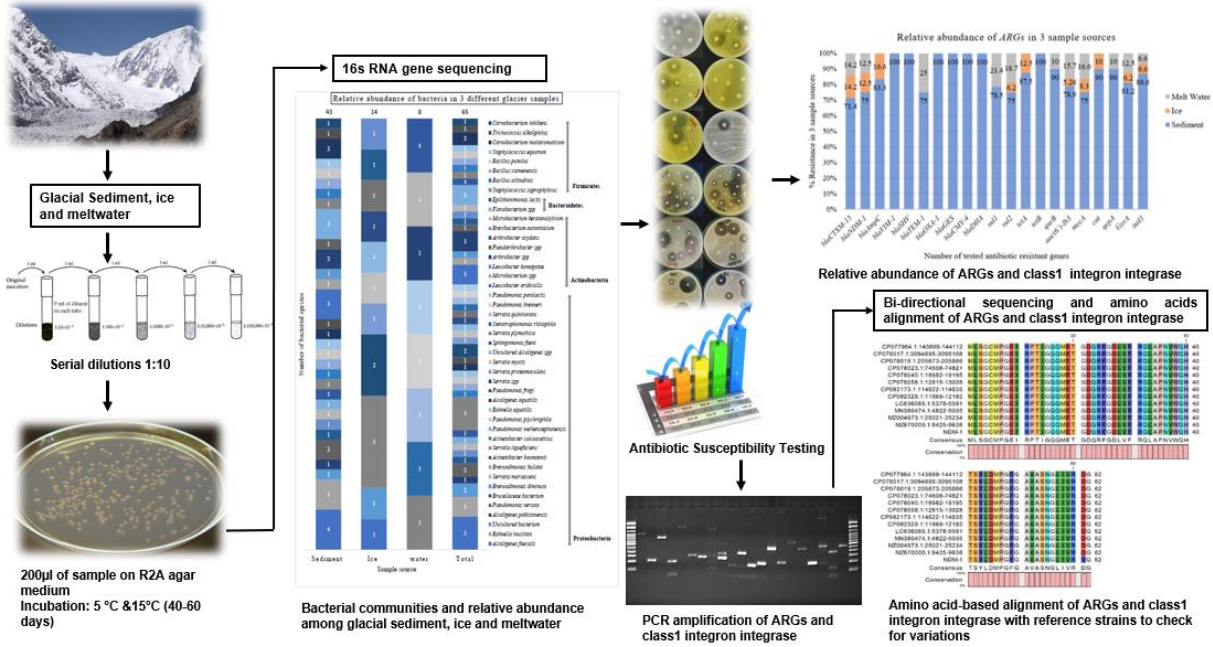
Prevalence and abundance of antibiotic-resistant genes in culturable bacteria inhabiting a non-polar Passu glacier, Karakorum Mountains range, Pakistan

Sabir Nawaz . Muhammad Rafiq . Ian L. Pepper . Walter Q. Betancourt . Aamer Ali Shah . Fariha Hasan

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, intl2,* and *intl3*. 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 *intI1*. 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.

Graphical abstract



Key words: Antibiotic resistance . antibiotic-resistant genes . glacial sediment . integron integrase . multiple antibiotic resistance index . ß-lactamase

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 °C to 20 °C), thermophilic (41 °C to 122 °C), hyperthermophilic (80°C to > 122 °C), acidophilic (pH 1-5), alkaliphilic (≥ pH 9), piezophilic (elevated hydrostatic pressure, up to 50 MPa), halophilic (salt concentration up to 5M), 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 km^2, 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 et al. 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. 2008, 2010), *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. 3.1).

Sample collection

The bacterial isolates were collected from glacial ice, meltwater, and sediment from three random distant locations (250 – 300 meters 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 -70ºC for subsequent uses.

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. Lious, 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 (1mL sample and 9 mL of sterile saline solution or 1g of sediment in 9 mL of sterile saline solution). Volumes of 200 µL were spread over R2A medium agar plates and incubated at 4°C and 15°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 µg, ciprofloxacin 5 µg, ofloxacin 5 µg, norfloxacin 10 µg and nalidixic acid 30 µg), Penicillins (amoxicillin 10 µg, penicillin G 10 µg, carbenicillin 100 µg, ticarcillin 75 µg, oxacillin 1 µg and piperacillin 100 µg), Cephalosporins (ceftriaxone 30 µg, cefotetan 30 µg, cephalexin 30 µg, cephalothin 30 µg, cefepime 30 µg, ceftazidime 30 µg, cephazolin 30 µg, cefpodoxime 10 µg and ceftaroline 30 µg), Carbapenems (meropenem 10 µg, imipenem 10 µg and ertapenem 10 µg), Monobactam (aztreonam 30 µg), Oxazolidinone (linezolid 30 µg), Macrolide (erythromycin 5 µg), Glycopeptide (vancomycin 30 µg) Glycycline (tigecycline 15 µg) and Nitroimidazole (metronidazole 5 µg).

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).

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 class1, 2 and 3 *integron integrase* genes were carried out in Eppendorf Gradient Thermocycler in a final volume of 30 µL reaction which contained 15 µL of Dream Taq green PCR 2x Master Mix (Thermo Scientific), followed by 1 µL of each oligonucleotide primer (1µM) (Integrated DNA Technologies), 8 µL of PCR grade nuclease-free water (Thermo Scientific) and 5 µL 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 (Table 3.1). 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* (27F, 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 searches through the Basic Local Alignment Search Tool (BLAST) program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for further evaluation. The gene sequences were submitted to the GeneBank Nucleotide Sequence Database, NBCI (National Center for Biotechnology Information), and the corresponding accession numbers are provided in (Table 3.2).

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 between 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°C and 15°C (Table 3.3). 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 (Table 3.4).

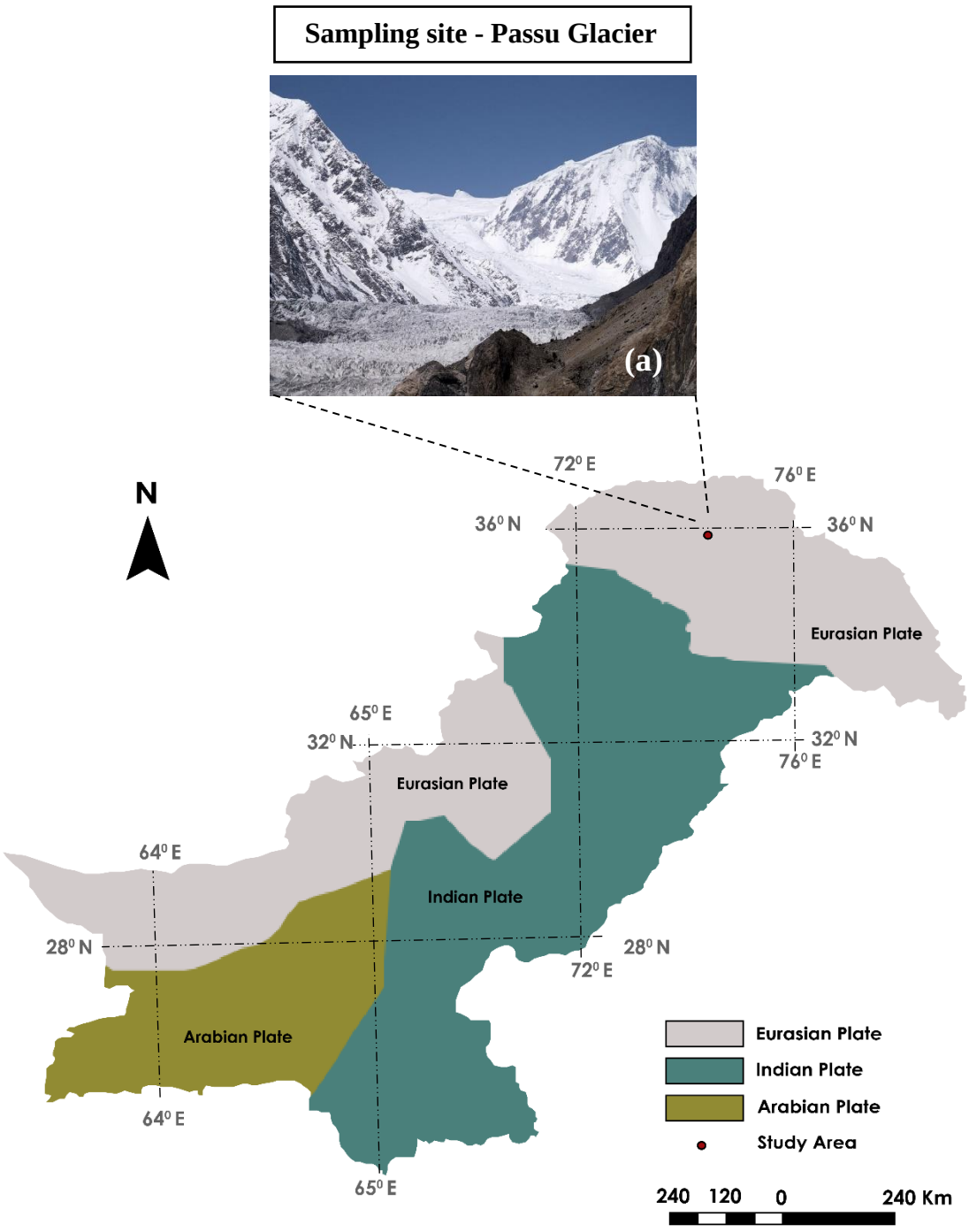


Fig. 3.1 Sampling site location: Passu glacier, Northern Pakistan, Karakorum Mountains Range (a) Photograph of Passu glacier from where samples were collected (Latitude 36° 27' 59.99" North and longitude 74° 53' 59.99" East)

Table 3.1 Target genes, primers sequences, amplicon sizes, and thermocycler conditions for PCR amplification

Target Genes	Primers sequences (5' – 3')	Product size (bp)	Thermocycle Profile					Reference
			Primary denaturation	Amplification (35 cycles)			Final Extension	
				Denaturation	Annealing	Extension		
16S rRNA	27F 5'-AGAGTTTGATCCTGGCTCAG-3' 1492R 5'-GGTTACCTTGTTACGACTT-3'	1350	95°C/2 min.	95°C/40 sec.	55°C/40 sec.	72°C/90 sec.	72°C/10 min.	Srinivasan *et al.*, 2015
*bla*CTXM	F: 5'-TTAATTCGTCTCTTCCAGA-3' R: 5'CAGCGCTTTTGCCGTCTAAG-3'	1000	95°C/5 min.	95°C/1 min.	50°C/45 sec.	72°C/90 sec.	72°C/10 min.	Baraniak *et al.*, 2002
*bla*NDM-1	F: 5'-CGCCATCCCTGACGATCAAA-3' R: 5'-CTGAGCACCGCATTAGCCG-3'	214	95°C/5 min.	95°C/30 sec.	59°C/45 sec.	72°C/50 sec.	72°C/10 min.	Tan *et al.*, 2018
*bla*KPC	F: 5'-GCTTCCCACTGTGCAGCTCATTC-3' R: 5'-CGCCCAACTCCTTCAGCAACAAATTG-3'	213	95°C/5 min.	95°C/30 sec.	66°C/45 sec.	72°C/50 sec.	72°C/10 min.	Tan *et al.*, 2018
*bla*AmpC	F: 5'-CCTCTTGCTCCACATTTGCT-3' R: 5'-ACAACGTTTGCTGTGTGACG-3'	189	95°C/5 min.	95°C/30 sec.	58°C/45 sec.	72°C/50 sec.	72°C/10 min.	Tan *et al.*, 2018
*bla*VIM-1	F: 5'-ACTGTCGGATACTCACCACTC-3' R: 5'-GTTATGGAGCAGCAACGATGT-3'	189	95°C/5 min.	95°C/30 sec.	57°C/45 sec.	72°C/50 sec.	72°C/10 min.	Tan *et al.*, 2018
*bla*SHV	F: 5'-ACTGAATGAGGCGCTTCC-3' R: 5'-ATCCCGCAGATAAATCACC-3'	300	95°C/5 min.	95°C/30 sec.	50°C/45 sec.	72°C/50 sec.	72°C/10 min.	Gniadkowski *et al.*, 1998a
*bla*TEM	F: 5'-ATTCTTGAAGACGAAAGGGC-3' R: 5'-ACGCTCAGTGGAACGAAAAC-3'	1150	95°C/5 min.	95°C/1min.	49°C/45 sec.	72°C/90 sec.	72°C/10 min.	Sáenz *et al.*, 2004
*bla*OXA	F: 5'-ACACAATACATATCAACTTCGC-3' R: 5'-AGTGTGTTTAGAATGGTGATC-3'	813	95°C/5 min.	95°C/30 sec.	49°C/45 sec.	72°C/50 sec.	72°C/10 min.	Sáenz *et al.*, 2004
*bla*GES	F: 5'-TTCCATCTCAAGGGATCACC-3' R: 5'-GCGTCAACTATTTGTCCGTG-3'	890	95°C/5 min.	95°C/30 sec.	50°C/45 sec.	72°C/50 sec.	72°C/10 min.	Mendonca *et al.*, 2009
*bla*CMY	F: 5'-CAATGTGTGAGAAGCAGTC-3' R: 5'-CGCATGGGATTTTCCTTGCTG-3'	1432	95°C/5 min.	95°C/1 min.	47°C/45 sec.	72°C/90 sec.	72°C/10 min.	Hanson *et al.*, 2002

$_{bla}$DHA	F: 5'-AACTTTCACAGGTGTGCTGGGT-3' R: 5'-CCGTACGCATACTGGCTTTGC-3'	404	95°C/5 min.	95°C/30 sec.	47°C/45 sec.	72°C/50 sec.	72°C/10 min.	Perez and Hanson 2002
sul1	F: 5' CGCACCGGAAACATCGCTGCAC 3' R: 5' TGAAGTTCCGCCGCAAGGCTCG 3'	163	95°C/5 min.	95°C/30 sec.	50°C/45 sec.	72°C/50 sec.	72°C/10 min.	Pei *et al.*, 2006
sul2	F: 5' TCCGGTGGAGGCCGGTATCTGG 3' R: 5' CGGGAATGCCATCTGCCTTGAG 3'	191	95°C/5 min.	95°C/30 sec.	50°C/45 sec.	72°C/50 sec.	72°C/10 min.	Pei *et al.*, 2006
tet(A)	F: 5' GCTACATCCTGCTTGCCTTC 3' R: 5' CATAGATCGCCGTGAAGAGG 3'	210	95°C/5 min.	95°C/30 sec.	55°C/45 sec.	72°C/50 sec.	72°C/10 min.	Li *et al.*, 2010
tet(B)	F: 5' TTGGTTAGGGGCAAGTTTTG 3' R: 5' GTAATGGGCCAATAACACCG 3'	659	95°C/5 min.	95°C/30 sec.	55°C/45 sec.	72°C/50 sec.	72°C/10 min.	Li *et al.*, 2010
qnrA	F: 5' ATTTCTCACGCCAGGATTTG 3' R: 5' GATCGGCAAAGGTTAGGTCA 3'	516	95°C/5 min.	95°C/30 sec.	53°C/45 sec.	72°C/50 sec.	72°C/10 min.	Kim *et al.*, 2008
qnrB	F: 5' GATCGTGAAAGCCAGAAAGG 3' R: 5' ATGAGCAACGATGCCTGGTA 3'	476	95°C/5 min.	95°C/30 sec.	53°C/45 sec.	72°C/50 sec.	72°C/10 min.	Kim *et al.*, 2008
vanA	F: 5' TCTGCAATAGAGATAGCCGC 3' R: 5' GGAGTAGCTATCCCAGCATT 3'	377	95°C/5 min.	95°C/30 sec.	47°C/45 sec.	72°C/50 sec.	72°C/10 min.	Zischka *et al.*, 2015
ermA	F: 5'-TATCTTATCGTTGAGAAGGGATT-3' F: 5'-CTACACTTGGCTTAGGATGAAA-3'	139	95°C/5 min.	95°C/30 sec.	62°C/45 sec.	72°C/50 sec.	72°C/10 min.	Moosavian *et al.*, 2014
ermB	F: 5'-CTATCTGATTGTTGAAGAAGGATT-3' R: 5'- TTTACTCTTGGTTTAGGATGAAA-3'	142	95°C/5 min.	95°C/30 sec.	59°C/45 sec.	72°C/50 sec.	72°C/10 min.	Moosavian *et al.*, 2014
aac(6')-Ib3	F: 5'-TTGCGATGCTCTATGAGTGGCTA-3' R: 5'-CTCGAATGCCTGGCGTGTTT-3'	482	95°C/5 min.	95°C/30 sec.	55°C/45 sec.	72°C/50 sec.	72°C/10 min.	Park *et al.*, 2006
mecA	F: 5'-GGGATCATAGCGTCATTATTC-3' R: 5'-AACGATTGTGACACGATAGCC-3'	527	95°C/5 min.	95°C/30 sec.	55°C/45 sec.	72°C/50 sec.	72°C/10 min.	Park *et al.*, 2006
cat	F: 5' ATGGCAATGAAAGACGGTGAGC 3' R: 5' TGCCGGAAATCGTCGTGGTATT 3'	122	95°C/5 min.	95°C/30 sec.	64°C/45 sec.	72°C/50 sec.	72°C/10 min.	Xi *et al.*, 2009
qepA	F: 5' AACTGCTTGAGCCCGTAGAT 3' R: 5' GTCTACGCCATGGACCTCAC 3'	596	95°C/5 min.	95°C/30 sec.	45°C/45 sec.	72°C/50 sec.	72°C/10 min.	Kim *et al.*, 2008
gyrA	F: 5'-AAATCTGCCCGTGTCGTTGGT- 3' R: 5'-GCCATACCTACGGCGATACC-3'	344	95°C/5 min.	95°C/30 sec.	58°C/45 sec.	72°C/50 sec.	72°C/10 min.	Park *et al.*, 2011

intI1	LC1-GCC TTG ATG TTA CCC GAG AG LC5-GAT CGG TCG AAT GCG TGT	196	95°C/5 min.	95°C/30 sec.	60°C/ 1 min.	72°C/50 sec.	72°C/10 min.	Pavelkovich *et al.*, 2014
intI2	LC2- TGC TTT TCC CAC CCT TAC C LC3- GAC GGC TAC CCT CTG TTA TCT C	195	95°C/5 min.	95°C/30 sec.	60°C/ 1 min.	72°C/50 sec.	72°C/10 min.	Pavelkovich *et al.*, 2014
intI3	LC1-GCC ACC ACT TGT TTG AGG A LC2-GGA TGT CTG TGC CTG CTT G	138	95°C/5 min.	95°C/30 sec.	60°C/ 1 min.	72°C/50 sec.	72°C/10 min.	Pavelkovich *et al.*, 2014

Table 3.2 List of GeneBank (NCBI) accession number and BLAST identities of study isolates retrieved from Passu glacier

Isolate Codes	Accession Numbers	Homologous Strains	Identity (%)	Querry Cover (%)
SUB10766215 HP1	OL721773	*Leucobacter aridicollis*	99.74	97
SUB10766215 HP2	OL721774	*Alcaligenes faecalis*	99.75	98
SUB10766215 HP3	OL721775	*Rahnella inusitata*	100	100
SUB10766215 HP4	OL721776	*Alcaligenes faecalis*	97.04	98
SUB10766215 HP5	OL721777	*Alcaligenes faecalis*	99.87	97
SUB10766215 HP6	OL721778	*Alcaligenes pakistanensis*	99.74	97
SUB10766215 HP7	OL721779	*Staphylococcus saprophyticus*	99.38	99
SUB10766215 HP8	OL721780	*Flavobacterium saliperosum*	96.04	97
SUB10766215 HP10	OL721781	*Pseudomonas versuta*	98.18	100
SUB10766215 HP11	OL721782	*Pseudomonas sp.*	97.62	99
SUB10766215 HP12	OL721783	*Flavobacterium suaedae*	97.37	99
SUB10766215 HP13	OL721784	*Bacillus altitudinis*	100	100
SUB10766215 HP14	OL721785	*Bacillus xiamenesis*	100	100
SUB10766215 HP15	OL721786	*Brucellaceae bacterium*	98.24	100
SUB10766215 HP16	OL721787	*Alcaligenes faecalis*	98.39	100
SUB10766215 HP17	OL721788	*Microbacterium profundi*	97.38	100
SUB10766215 HP18	OL721789	*Bacillus pumilus*	99.63	100
SUB10766215 HP19	OL721790	*Staphylococcus equorum*	99.75	100
SUB10766215 HP20	OL721791	*Flavobacterium antarcticum*	97.72	98
SUB10766215 HP21	OL721792	*Brevundimonas diminuta*	99.25	99
SUB10766215 HP22	OL721793	*Leucobacter aridicollis*	99.63	100
SUB10766215 HP23	OL721794	*Leucobacter sp.*	99.25	100
SUB10766215 HTP36	OL721795	*Carnobacterium maltaromaticum*	98.82	96
SUB10766215 HP50	OL721796	*Serratia marcescens*	99.50	99
SUB10766215 HP51	OL721797	*Leucobacter komagatae*	99.01	100
SUB10766215 HP52	OL721798	*Rahnella inusitata*	99.58	100
SUB10766215 HP53	OL721799	*Carnobacterium maltaromaticum*	98.64	100
SUB10766215 HP54	OL721800	*Brevundimonas bullata*	97.77	100
SUB10766215 HP55	OL721801	*Alcaligenes sp.*	99.71	100
SUB10766215 HP56	OL721802	*Rahnella sp.*	99.04	100
SUB10766215 HP57	OL721803	*Acinetobacter baunamii*	98.98	97
SUB10766215 HP58	OL721804	*Serratia liquefaciens*	100	100
SUB10766215 LP1	OL721805	*Arthrobacter oryzae*	99.13	100
SUB10766215 LP2	OL721806	*Arthrobacter psychrochitiniphilus*	98.89	100
SUB10766215 LP3	OL721807	*Trichococcus alkaliphilus*	99.75	100
SUB10766215 LP4	OL721808	*Pseudarthrobacter sp.*	99.25	100
SUB10766215 LP5	OL721809	*Acinetobacter calcoaceticus*	100	100
SUB10766215 LP6	OL721810	*Pseudomonas weihenstephanensis*	98.67	100
SUB10766215 LP7	OL721811	*Pseudomonas psychrophila*	98.43	99
SUB10766215 LP8	OL721812	*Carnobacterium inhibens*	96.89	99
SUB10766215 LP9	OL721813	*Rahnella aquatilis*	99.17	100
SUB10766215 LP10	OL721814	*Alcaligenes aquatilis*	99.54	100

SUB10766215 LP11	OL721815	*Pseudomonas fragi*	98.62	99
SUB10766215 HPI1	OL721816	*Serratia sp.*	98.75	99
SUB10766215 HPI2	OL721817	*Serratia liquefaciens*	98.42	100
SUB10766215 HPI3	OL721818	*Serratia proteamaculans*	98.53	99
SUB10766215 HPI4	OL721819	*Serratia sp.*	99.10	100
SUB10766215 HPI5	OL721820	*Serratia liquefaciens*	98.73	100
SUB10766215 HPI6	OL721821	*Serratia myotis*	98.86	100
SUB10766215 HPI7	OL721822	*Alcaligenes sp.*	98.26	100
SUB10766215 HPI8	OL721823	*Alcaligenes faecalis*	99.20	100
SUB10766215 LPI1	OL721824	*Serratia plymuthica*	97.87	100
SUB10766215 LPI2	OL721825	*Sphingomonas faeni*	100	100
SUB10766215 LPI4	OL721826	*Serratia plymuthica*	99.17	100
SUB10766215 LPI5	OL721827	*Serratia sp.*	98.43	100
SUB10766215 LPI6	OL721828	*Pseudarthrobacter oxydans*	99.88	96
SUB10766215 LPI7	OL721829	*Brevibacterium aurantiacum*	98.06	100
SUB10766215 LPW1	OL721830	*Serratia sp.*	98.78	99
SUB10766215 LPW2	OL721831	*Stenotrophomonas rhizophila*	99.42	100
SUB10766215 LPW3	OL721832	*Serratia quinivorans*	97.56	98
SUB10766215 LPW4	OL721833	*Epilithonimonas lactis*	98.50	100
SUB10766215 LPW5	OL721834	*Microbacterium keratanolyticum*	98.76	100
SUB10766215 LPW6	OL721835	*Pseudomonas brenneri*	100	100
SUB10766215 LPW7	OL721836	*Arthrobacter sp.*	97.33	99
SUB10766215 LPW8	OL721837	*Pseudomonas paralactis*	99.58	100

Table 3.3 Total viable count of retrieved isolates in terms of CFUmL^{-1} or g^{-1}

Sample/Source	**pH**	**Temp (°C)**	**CFUml^{-1} or g^{-1}**		**No. of colonies**
			4 °C	**15°C**	
Glacial Sediment	7	1	3.73 x 10^8	1.53 x 10^9	43
Glacial Ice	7	-2	9.92 × 10^5	3.65 x 10^6	14
Glacial meltwater	7	2	2.34 x 10^5	7.01 x 10^5	8

Table 3.4 Morphological and growth characteristics of isolates retrieved from glacier source

Isolate codes	Sample Source	Gram Reaction	Shape	Growth Temperature Range (C°)		
				4	15	37
HP1	Sediment	Gram positive	Rod shaped	✓	✓	✗
HP2	Sediment	Gram negative	Rod shaped	✓	✓	✗
HP3	Sediment	Gram negative	Rod shaped	✓	✓	✗
HP4	Sediment	Gram negative	Rod shaped	✗	✓	✗
HP5	Sediment	Gram positive	Rod shaped	✓	✓	✗
HP6	Sediment	Gram negative	Rod shaped	✓	✓	✗
HP7	Sediment	Gram positive	Cocci	✗	✓	✓
HP8	Sediment	Gram positive	Rod shaped	✗	✓	✗
HP10	Sediment	Gram negative	Rod shaped	✓	✓	✗
HP11	Sediment	Gram negative	Rod shaped	✓	✓	✗
HP12	Sediment	Gram negative	Rod shaped	✗	✓	✗
HP13	Sediment	Gram positive	Rod shaped	✓	✓	✗
HP14	Sediment	Gram positive	Rod shaped	✓	✓	✗
HP15	Sediment	Gram negative	Rod shaped	✓	✓	✗
HP16	Sediment	Gram negative	Rod shaped	✓	✓	✗
HP17	Sediment	Gram positive	Rod shaped	✓	✓	✗
HP18	Sediment	Gram positive	Rod shaped	✓	✓	✗
HP19	Sediment	Gram positive	Cocci	✗	✓	✓
HP20	Sediment	Gram negative	Rod shaped	✓	✓	✗
HP21	Sediment	Gram negative	Rod shaped	✗	✓	✓
HP22	Sediment	Gram positive	Rod shaped	✓	✓	✗
HP23	Sediment	Gram positive	Rod shaped	✓	✓	✗
HP36	Sediment	Gram positive	Rod shaped	✓	✓	✗
HP50	Sediment	Gram negative	Rod shaped	✗	✓	✓
HP51	Sediment	Gram positive	Rod shaped	✓	✓	✗
HP52	Sediment	Gram negative	Rod shaped	✓	✓	✗
HP53	Sediment	Gram positive	Rod shaped	✓	✓	✗
HP54	Sediment	Gram negative	Rod shaped	✗	✓	✓
HP55	Sediment	Gram negative	Rod shaped	✓	✓	✗
HP56	Sediment	Gram negative	Rod shaped	✓	✓	✗
HP57	Sediment	Gram negative	Rod shaped	✓	✓	✗
HP58	Sediment	Gram negative	Rod shaped	✗	✓	✓
LP1	Sediment	Gram positive	Rod shaped	✓	✗	✗
LP2	Sediment	Gram positive	Rod shaped	✓	✗	✗
LP3	Sediment	Gram positive	Cocci	✓	✗	✗
LP4	Sediment	Gram positive	Rod shaped	✓	✗	✗
LP5	Sediment	Gram negative	Rod shaped	✓	✓	✓

LP6	Sediment	Gram negative	Rod shaped	✓	✓	✓
LP7	Sediment	Gram negative	Rod shaped	✓	✗	✗
LP8	Sediment	Gram positive	Rod shaped	✓	✗	✗
LP9	Sediment	Gram negative	Rod shaped	✓	✗	✗
LP10	Sediment	Gram negative	Rod shaped	✓	✗	✗
LP11	Sediment	Gram negative	Rod shaped	✓	✓	✓
HPI1	Ice	Gram negative	Rod shaped	✓	✓	✗
HPI2	Ice	Gram negative	Rod shaped	✓	✓	✗
HPI3	Ice	Gram negative	Rod shaped	✓	✓	✗
HPI4	Ice	Gram negative	Rod shaped	✓	✓	✗
HPI5	Ice	Gram negative	Rod shaped	✓	✓	✗
HPI6	Ice	Gram negative	Rod shaped	✓	✓	✗
HPI7	Ice	Gram negative	Rod shaped	✓	✓	✗
HPI8	Ice	Gram negative	Rod shaped	✓	✓	✗
LPI1	Ice	Gram negative	Rod shaped	✓	✗	✗
LPI2	Ice	Gram negative	Rod shaped	✓	✗	✗
LPI4	Ice	Gram negative	Rod shaped	✓	✗	✗
LPI5	Ice	Gram negative	Rod shaped	✓	✗	✗
LPI6	Ice	Gram positive	Rod shaped	✓	✗	✗
LPI7	Ice	Gram positive	Rod shaped	✓	✗	✗
LPW1	Melt Water	Gram negative	Rod shaped	✓	✗	✗
LPW2	Melt Water	Gram negative	Rod shaped	✓	✗	✗
LPW3	Melt Water	Gram negative	Rod shaped	✓	✗	✗
LPW4	Melt Water	Gram negative	Rod shaped	✓	✗	✗
LPW5	Melt Water	Gram positive	Rod shaped	✓	✗	✗
LPW6	Melt Water	Gram negative	Rod shaped	✓	✗	✗
LPW7	Melt Water	Gram negative	Rod shaped	✓	✓	✓
LPW8	Melt Water	Gram negative	Rod shaped	✓	✓	✓

Key: **4°C only:** 20 (30.7%), **15°C only:** 3 (4.61%), **4°C + 15°C**: 31 (47.69%), **15°C + 37°C:** 6 (9.23%), **4°C + 15°C + 37°C:** 5 (7.69%) isolates

Abbreviations: **HP:** (High-temperature sediment Passu glacier), **LP:** (Low-temperature sediment Passu), **HPI:** (High-temperature ice Passu), **LPI:** (Low-temperature ice Passu), **LPW:** (Low-temperature water Passu)

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 effectiveness 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 letter 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, Glycycline, 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 3.5).

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% of 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* (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 3.6).

Table 3.5 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)
OXAZOLIDINONESs						
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

Table 3.6 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,FEP,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,EFP,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,FEP,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,FEP,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				
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 were 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

PCR amplification and sequencing of *16S rRNA*

The amplification of specific 1350 bp bands was visualized under the UV trans-illuminator (Fig. 3.2). 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. 3.3). Bacterial communities and their relative abundance, distribution plus structural community in glacial sediment, ice, and meltwater are shown in (Fig. 3.4).

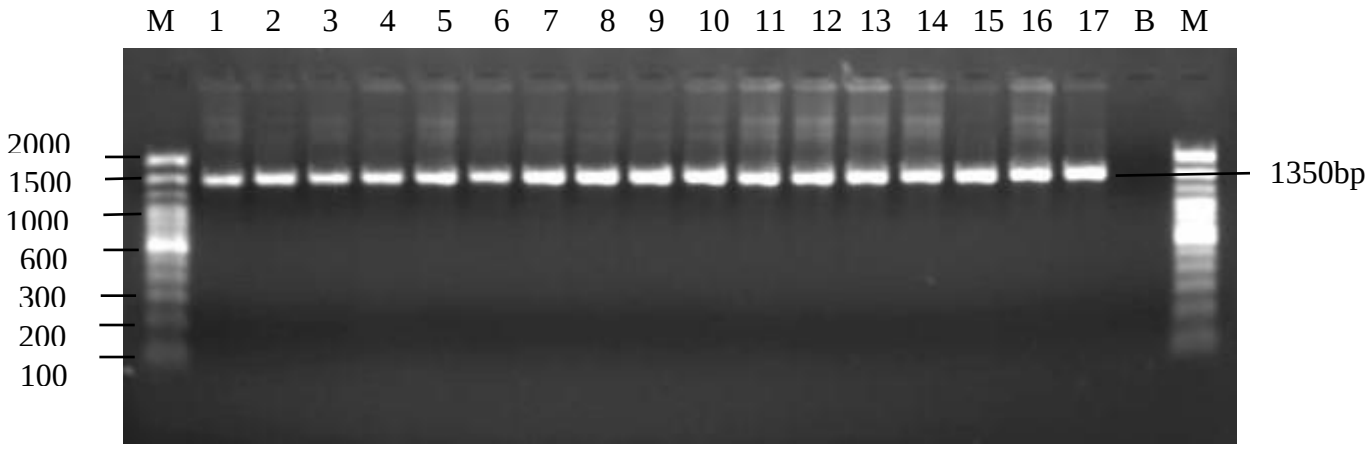


Fig. 3.2 PCR amplicons of *16S rRNA* gene on 1.5% agarose gel. Lane 1 to 17: 27F 1492R (1350bp), B: Blank, M: 100 bp DNA Ladder (Invitrogen)

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 $_{bla}$*CTXM-15* (21.53%), $_{bla}$*NDM-1* (24.61%), $_{bla}$*AmpC* (9.23%), $_{bla}$*VIM-1* (7.69%), $_{bla}$*SHV* (7.69%), $_{bla}$*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 *intl1* (24.6%), respectively. All tested strains were negative for $_{bla}$*KPC*, *qnrA*, *vanA*, *ermA*, *ermB*, *intl2*, and *intl3*. The representative amplified bands of desired genes are illustrated in (Fig. 3.5). 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.

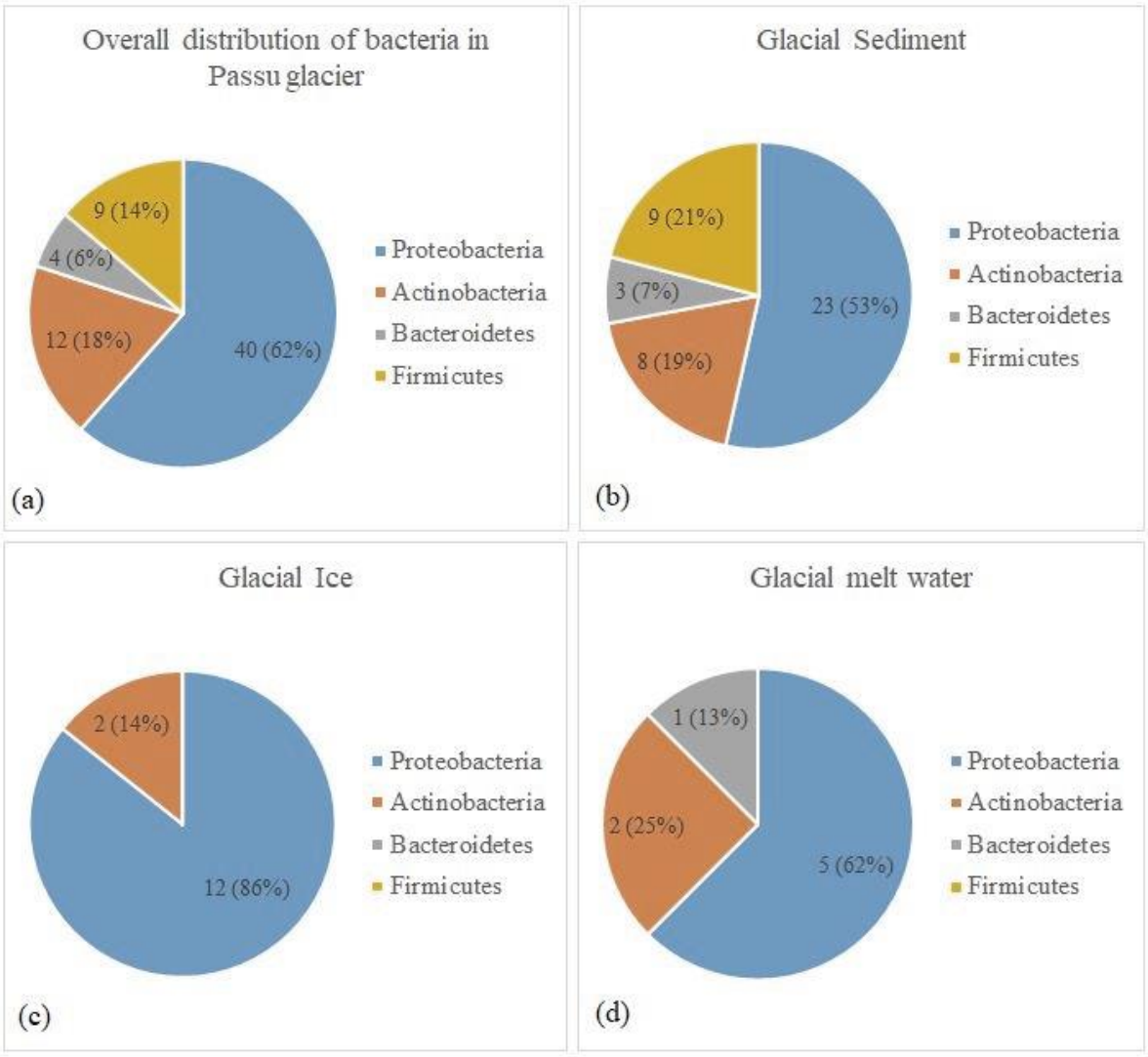


Fig. 3.3 Bacterial Phyla relative abundance in glacier samples (a) shows the whole bacteria abundance in glacier (b) bacterial phyla in glacial sediment (c) glacial ice and (d) glacial meltwater

Among the Gram-negative bacterial strains, *Brevundimonas diminuta* (HP21) and *Rahnella inusitata* (HP3) harbored *intl1* plus 19 and 17 ARGs, respectively. Thirteen ARGs were identified in *Alcaligenes faecalis* (HP55), however, *intl1 was* not associated with this bacterium. Meanwhile, *intl1* 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 *intl1*. The distribution of ARGs and *intI1* among the tested bacteria are categorized in (Table 3.7) and the relative abundance of ARGs in 3 different sample sources is summarized in (Fig. 3.6).

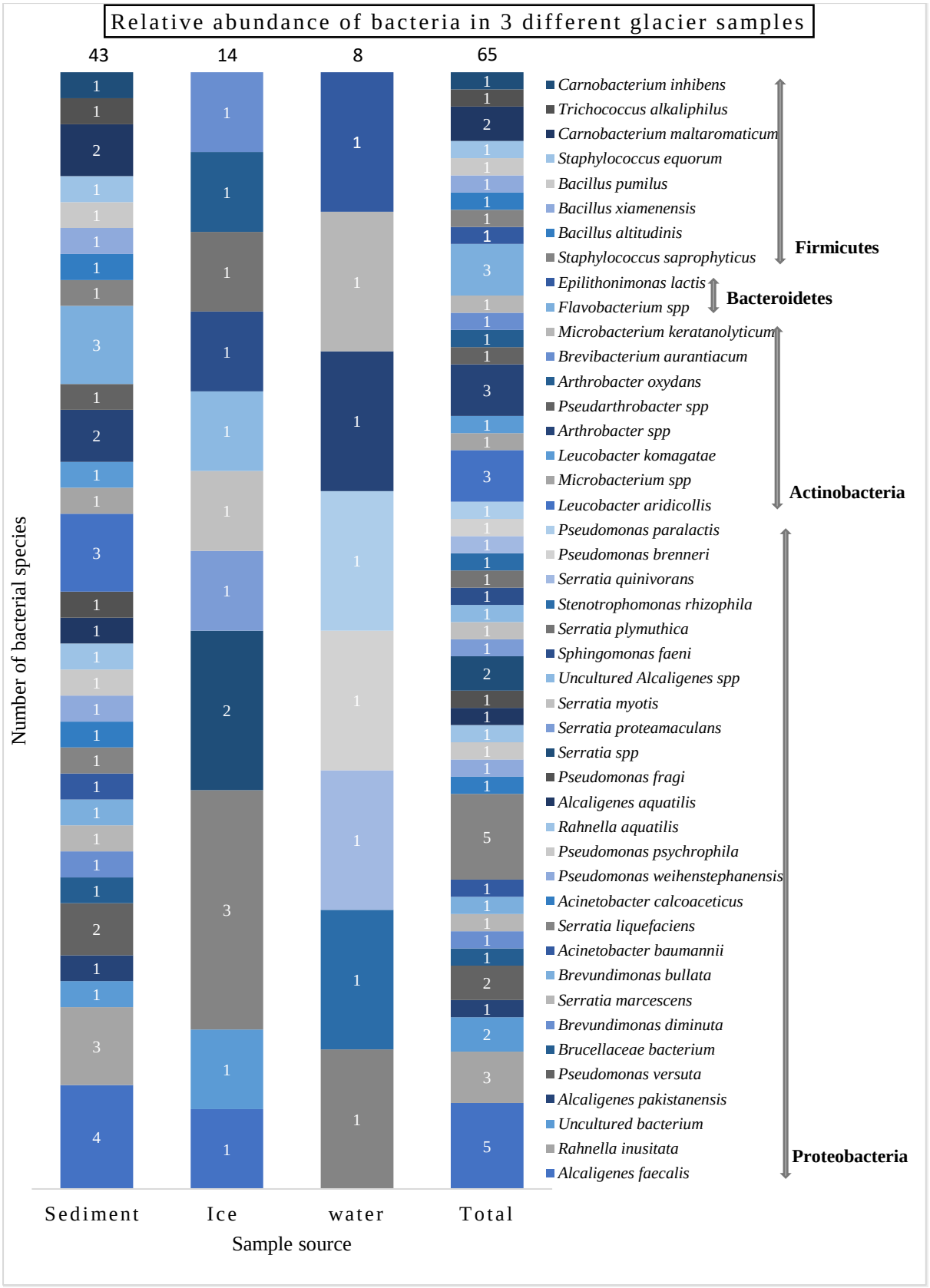


Fig. 3.4 Bacterial communities and their relative abundance, distribution plus structural community in glacial sediment, ice, and meltwater identified by amplicon sequencing of *16S rRNA* gene

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 3.8).

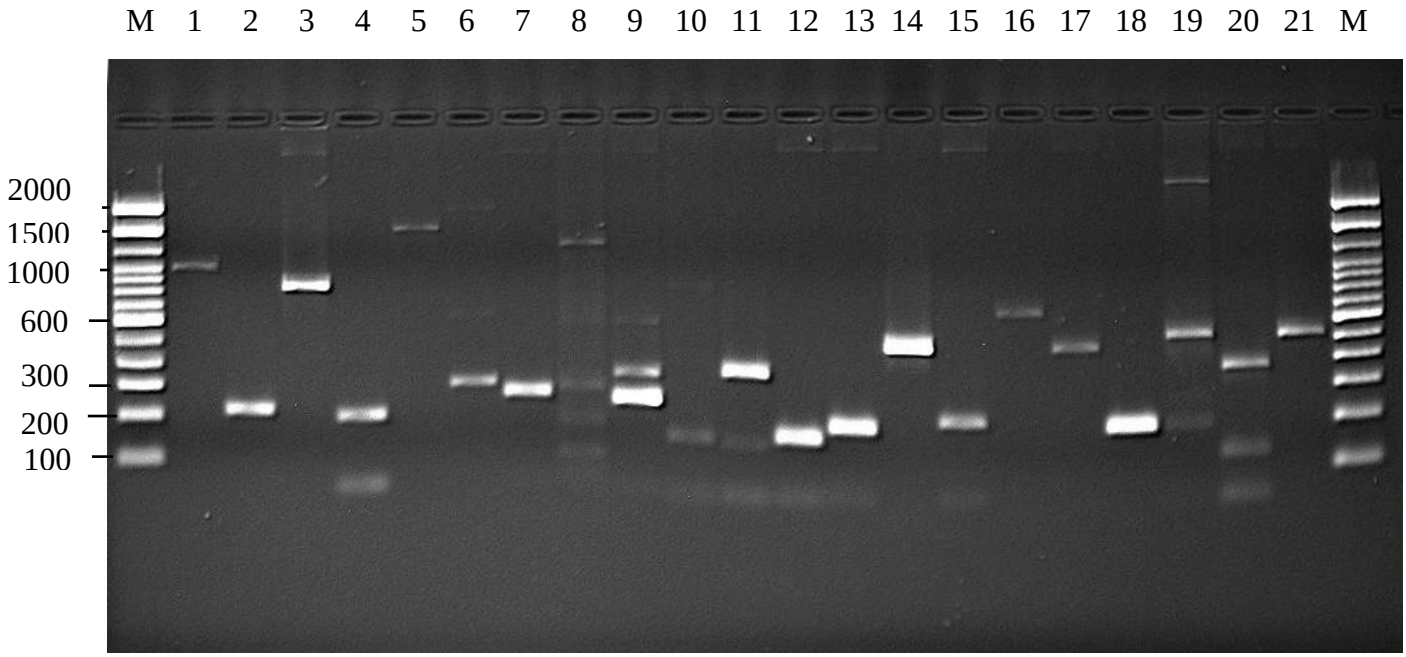


Fig. 3.5 PCR amplicons of ARGs and *intI1* on 1.5% agarose gel. **Lane 1:** *CTX-M* (1000bp), **Lane 2:** $_{bla}$*NDM-1* (214bp). **Lane 3:** $_{bla}$*OXA* (813bp), **Lane 4:** $_{bla}$*AmpC* (189bp). **Lane 5:** $_{bla}$*CMY* (1432bp), **Lane 6:** $_{bla}$*SHV* (300bp), **Lane 7:** $_{bla}$*VIM* (189bp), **Lane 8:** $_{bla}$*TEM* (1150bp), **Lane 9:** $_{bla}$*DHA* (404bp), **Lane 10:** $_{bla}$*GES* (890bp), **Lane 11:** *gyrA* (344bp), **Lane 12:** *sul1* (163bp), **Lane 13:** *sul2* (191bp), **Lane 14:** *aac(6')* (482bp), **Lane 15:** *tetA* (210bp), **Lane 16:** *tetB* (659bp), **Lane 17:** *qnrB* (476bp), **Lane 18:** *intl1* (196bp), **Lane 19:** *qepA* (596bp), **Lane 20:** *cat* (122bp), **Lane 21:** *mecA* (527bp), **M:** 100 bp DNA Ladder (Invitrogen)

Table 3.7 Distribution of antibiotic-resistant genes and class 1 *integron integrase* among Gram-negative and Gram-positive bacteria isolated from glacier

Isolate codes	ß-lactams ARGs										Non ß-lactams ARGs										Integron
	CTXM-15	*NDM-1*	*AmpC*	*VIM-1*	*SHV*	*TEM-1*	*OXA-1*	*GES*	*CMY-4*	*DHA*	*sul1*	*sul2*	*tetA*	*tetB*	*qnrB*	*aac(6')-Ib3*	*mecA*	*cat*	*qepA*	*gyrA*	*intI1*
HP2	-	-	-	-	-	+	-	-	-	-	+	+	-	-	+	+	+	-	+	+	-
HP3	+	+	+	+	+	+	-	+	+	+	+	+	-	-	+	+	-	+	+	+	+
HP7	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	-	-	+	+
HP18	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	+	+	-
HP19	+	+	-	-	-	-	-	-	-	-	+	+	-	-	+	+	+	+	+	+	+
HP20	+	+	-	-	-	+	-	+	-	-	+	+	-	-	+	+	-	+	-	+	+
HP21	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+
HP22	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+	+	-	+	+	+
HP23	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	+
HP50	+	+	+	+	+	+	-	-	-	+	+	+	+	-	-	+	-	+	-	-	+
HP51	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+	-	-	+
HP54	-	+	-	-	-	-	-	-	-	-	+	+	+	-	+	+	+	+	-	-	-
HP55	+	+	+	+	+	+	-	+	+	+	+	+	-	-	+	+	-	-	+	+	-
HP57	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
HP58	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+
LP1	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	+	-	-	-
LP2	+	+	-	-	-	-	-	-	-	-	+	+	-	-	-	+	+	-	-	+	-
LP5	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+	+	-	-	+	+
LP7	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-
LP10		-	-	-	-	+	-	-	-	-	-	+	-	-	-	+	+	-	-	+	+
LP11	-	+	-	-	-	+	-	-	-	-	+	+	-	-	-	+	+	+	+	-	-
HPI1	+	+	+	-	-	-	-	-	-	-	-	+	+	-	-	+	+	-	-	-	+
LPI7	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-
LPW6	+	+	-	-	-	+	-	-	-	-	+	+	-	-	-	+	-	-	-	-	+
LPW7	+	+	-	-	-	+	-	-	-	-	+	+	-	-	-	+	+	-	+	+	-
LPW8	-	-	-	-	-	+	-	-	-	-	+	+	-	-	+	+	+	-	-	+	-

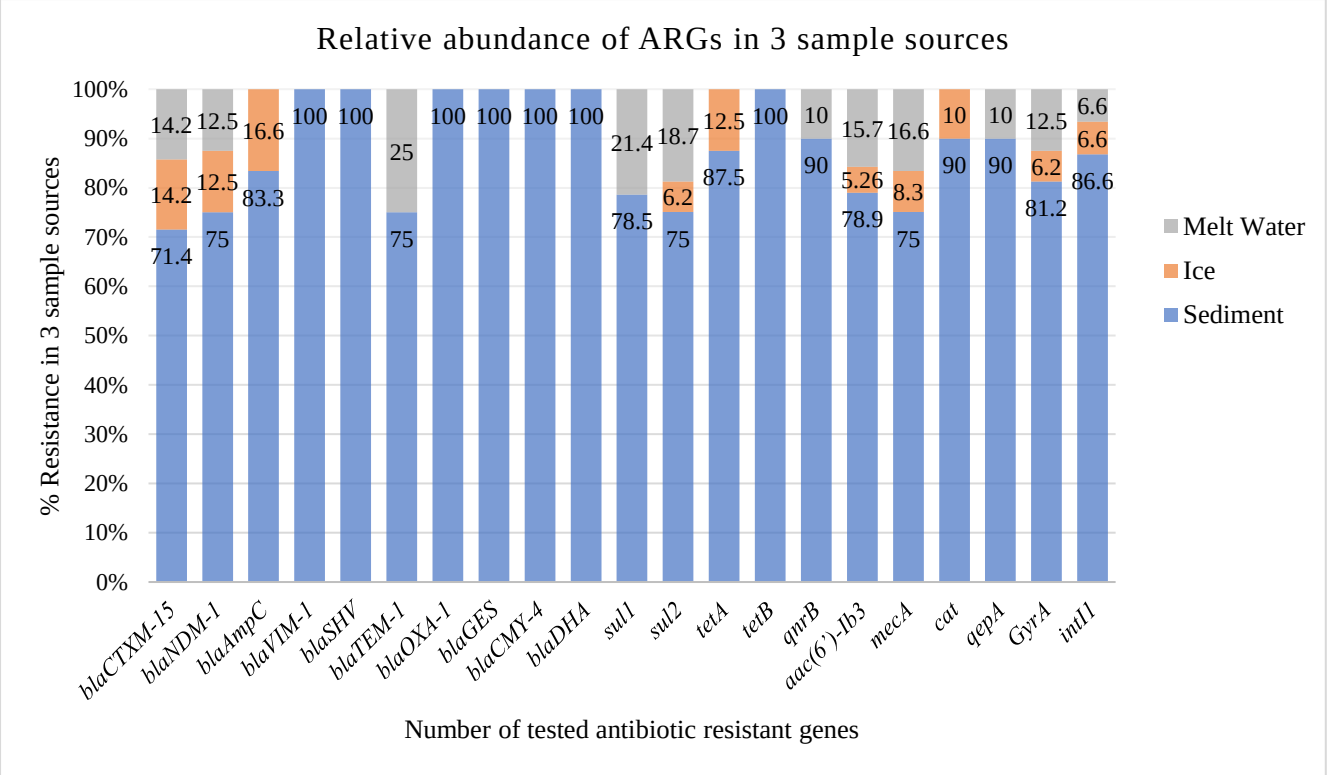


Fig. 3.6 Relative abundance of ARGs and class 1 *integron integrase* among glacier sediment, ice, and meltwater

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 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 (Fig. 3.7.1-7.15).

Table 3.8 Abundance of antibiotic-resistant genes and class 1 *integron integrase* among 4 bacterial phyla

ARGs	Proteobacteria n (%)	Bacteroidetes n (%)	Actinobacteria n (%)	Firmicutes n (%)	Total (n)
$_{bla}$*CTXM-15*	6 (42.85%)	1 (7.14%)	5 (35.71%)	2 (14.28%)	14
$_{bla}$*NDM-1*	10 (62.50%)	1 (6.25%)	4 (25%)	1 (6.25%)	16
$_{bla}$*KPC*	0	0	0	0	0
$_{bla}$*AmpC*	6 (100%)	0	0	0	6
$_{bla}$*VIM-1*	5 (100%)	0	0	0	5
$_{bla}$*SHV*	5 (100%)	0	0	0	5
$_{bla}$*TEM-1*	10 (83.33%)	1 (8.33%)	1 (8.33%)	0	12
$_{bla}$*OXA-1*	1 (100%)	0	0	0	1
$_{bla}$*GES*	3 (75%)	1 (25%)	0	0	4
$_{bla}$*CMY-4*	4 (100%)	0	0	0	4
$_{bla}$*DHA*	5 (100%)	0	0	0	5
sul1	9 (64.28%)	1 (7.14%)	3 (21.42%)	1 (7.14%)	14
sul2	11 (68.75%)	1 (6.25%)	3 (18.75%)	1 (6.25%)	16
tetA	4 (50%)	0	2 (25%)	2 (25%)	8
tetB	1 (20%)	0	2 (40%)	2 (40%)	5
qnrA	0	0	0	0	0
qnrB	6 (60%)	1 (10%)	1 (10%)	2 (20%)	10
vanA	0	0	0	0	0
ermA	0	0	0	0	0
ermB	0	0	0	0	0
aac(6)'-Ib3	13 (68.42%)	1 (5.26%)	4 (21.05%)	1 (5.26%)	19
mecA	7 (58.33%)	0	3 (25%)	2 (16.66%)	12
cat	5 (50%)	1 (10%)	3 (30%)	1 (10%)	10
qepA	5 (50%)	0	3 (30%)	2 (20%)	10
gyrA	7 (43.75%)	1 (6.25%)	5 (31.25%)	3 (18.75%)	16
intI1	9 (60%)	1 (6.66%)	3 (20%)	2 (13.33%)	15
intI2	0	0	0	0	0
intI3	0	0	0	0	0

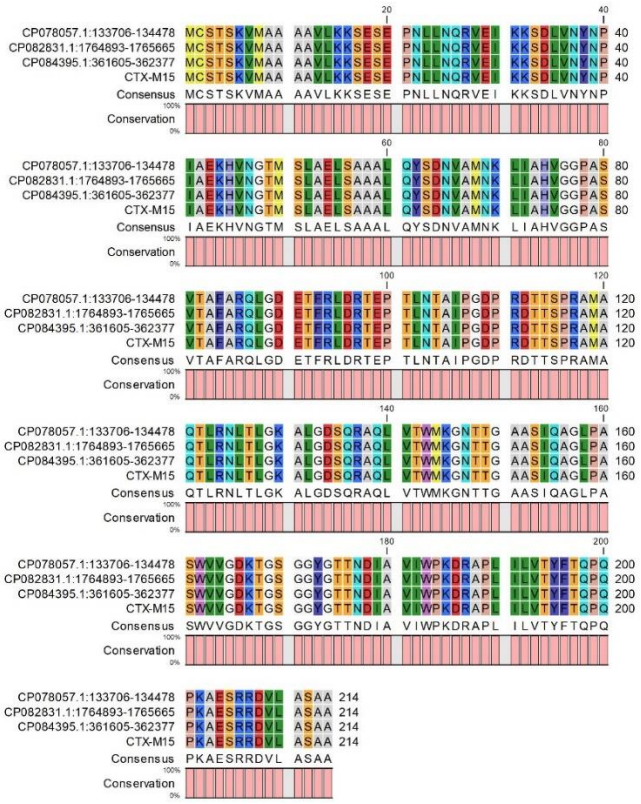


Fig. 3.7.1 Alignment of *CTXM-15* protein with the *CTXM-15* reference gene from 3 different strains (CP084395.1:361605-362377 *Klebsiella pneumoniae*, CP082831.1:1764893-1765665 *Escherichia coli* and CP078057.1:133706-134478 *Enterobacter hormaechei*) illustrating the amino acids coverage and consensus with no variation compared to references

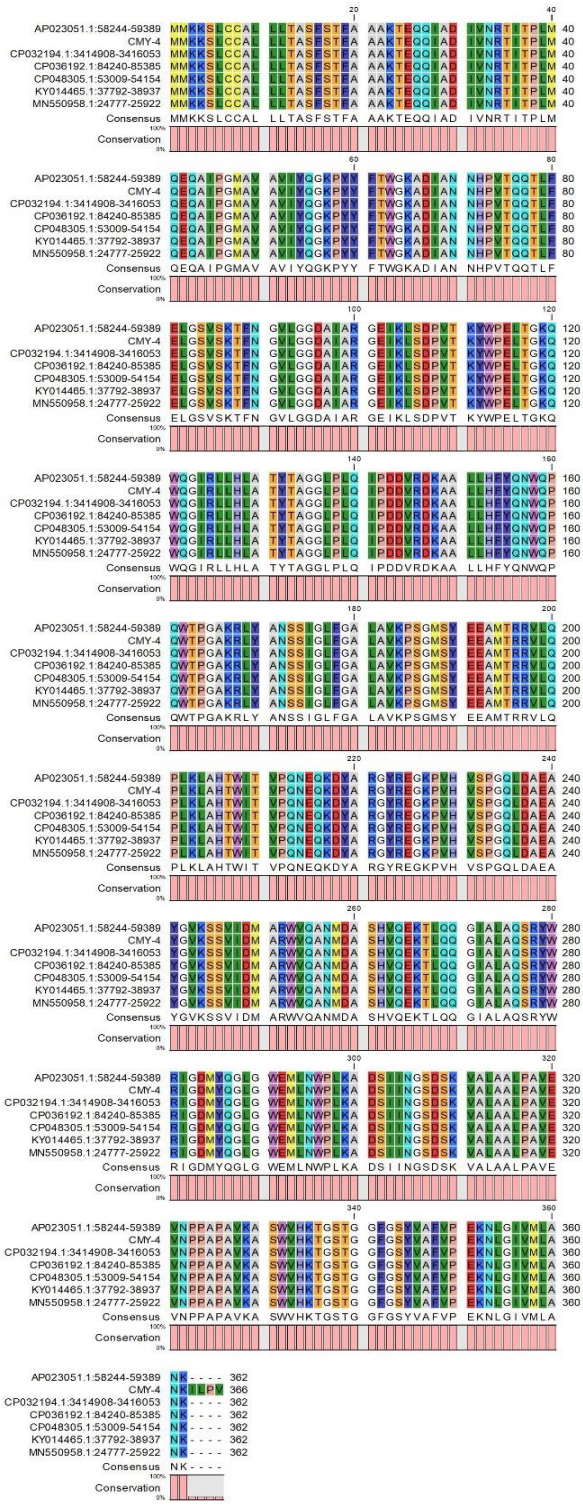


Fig. 3.7.2 Alignment of *CMY-4* protein with the *CMY-4* reference gene from 6 different strains (AP023051.1:58244-59389 *Citrobacter portucalensis*, CP048305.1:53009-54154 *Escherichia coli*, MN550958.1:24777-25922 *Proteus mirabilis*, CP036192.1:84240-85385 *Klebsiella pneumoniae*, CP032194.1:3414908-3416053 *Salmonella enterica* and KY014465.1:37792-38937 *Vibrio parahaemolyticus*) illustrating the amino acids coverage and consensus with no amino acids variation (*CMY-4* gene terminal amino acids variation are not considered here)

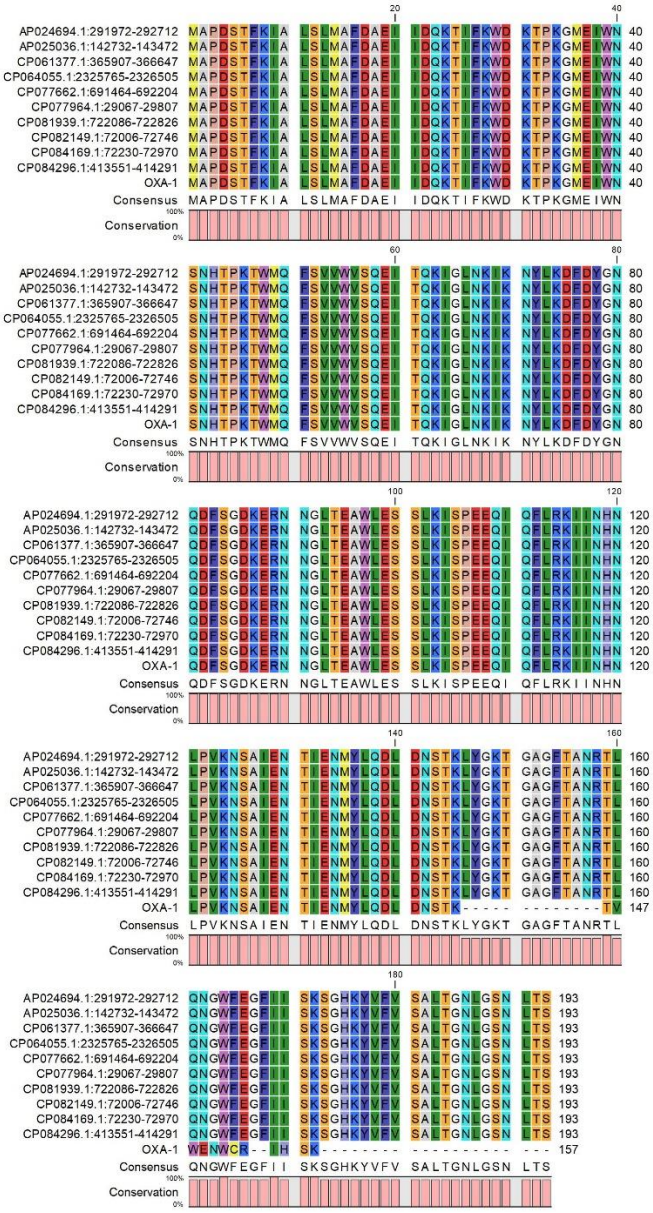


Fig. 3.7.3 Alignment of *OXA-1* protein with the *OXA-1* reference gene from 10 different strains (CP084296.1:413551-414291 *Providencia alcalifaciens*, CP084169.1:72230-72970 *Klebsiella pneumoniae*, CP077662.1:691464-692204 *Salmonella enterica*, CP082149.1:72006-72746 *Enterobacter cloacae*, AP024694.1:291972-292712 *Escherichia coli*, CP081939.1:722086-722826 *Avibacterium paragallinarum*, AP025036.1:142732-143472 *Enterobacter hormaechei*, CP061377.1:365907-366647 *Pseudomonas aeruginosa*, CP077964.1:29067-29807 *Proteus mirabilis* and CP064055.1:2325765-2326505 *Morganella morganii*) illustrating the amino acids variations (Leu160→ Val, 161Gln→ Trp, 162Asn→ Glu, 163Gly→ Asn, 165Phe→ Cys, 166Glu→ Arg and 170Ile→ His) at terminal end, compared to references

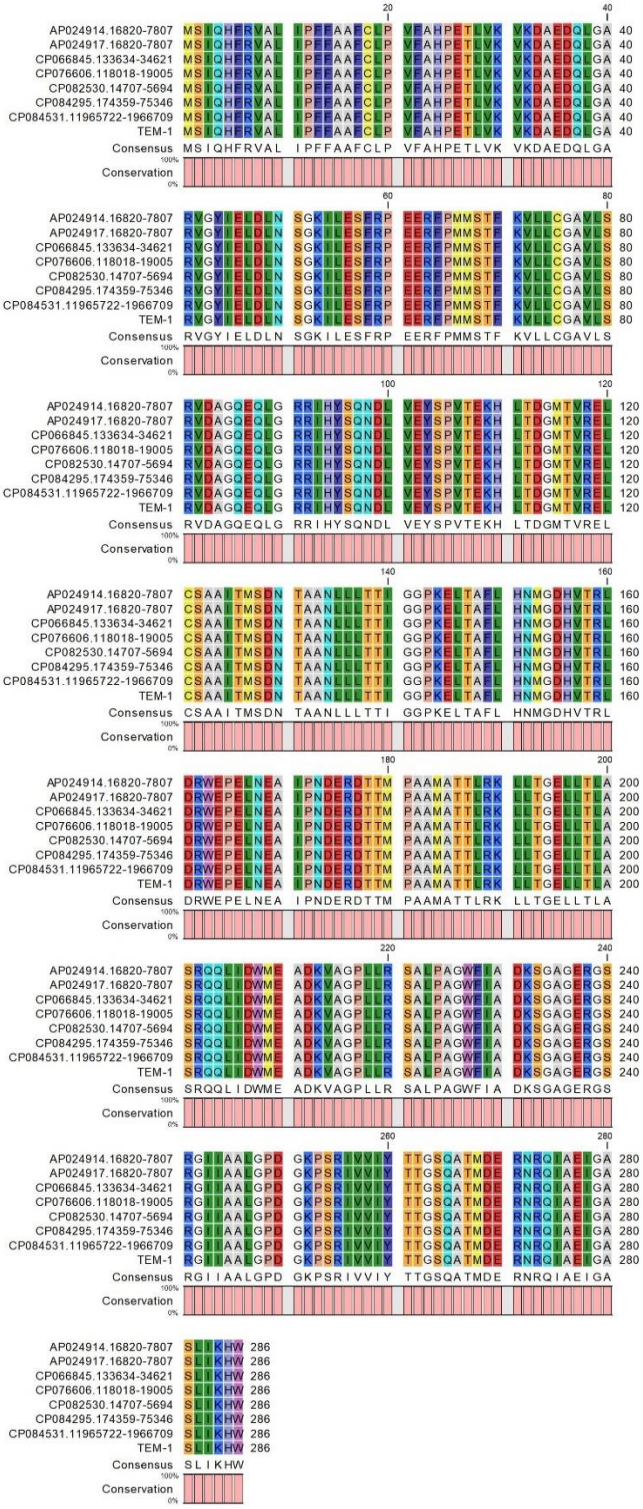


Fig. 3.7.4 Alignment of *TEM-1* protein with the *TEM-1* reference gene from 7 different strains (CP066845.1:33634-34621 *Escherichia coli*, CP084531.1:1965722-1966709 *Klebsiella pneumoniae*, CP084295.1:74359-75346 *Citrobacter werkmanii*, AP024917.1:6820-7807 *Serratia marcescens*, AP024914.1:6820-7807 *Enterobacter cloacae*, CP082530.1:4707-5694 *Salmonella enterica* and CP076606.1:18018-19005 *Raoultella ornithinolytica*) illustrating the amino acids coverage and consensus with no varaition compared to references

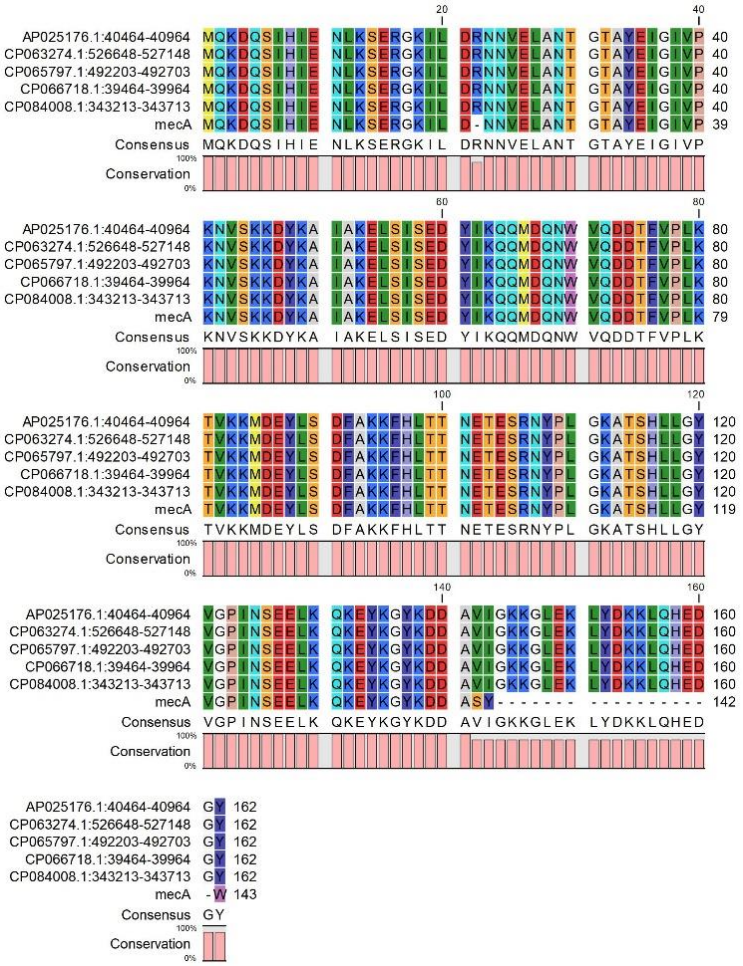


Fig. 3.7.5 Alignment of *mecA* protein with the *mecA* reference gene from 5 different strains (AP025176.1:40464-40964 *Staphylococcus aureus*, CP084008.1:343213-343713 *Staphylococcus epidermidis*, CP063274.1:526648-527148 *Staphylococcus haemolyticus*, CP066718.1:39464-39964 *Staphylococcus pseudintermedius* and CP065797.1:492203-492703 *Staphylococcus saprophyticus*) illustrating no amino acids variation (*mecA* gene terminal amino acids variation are not considered here)

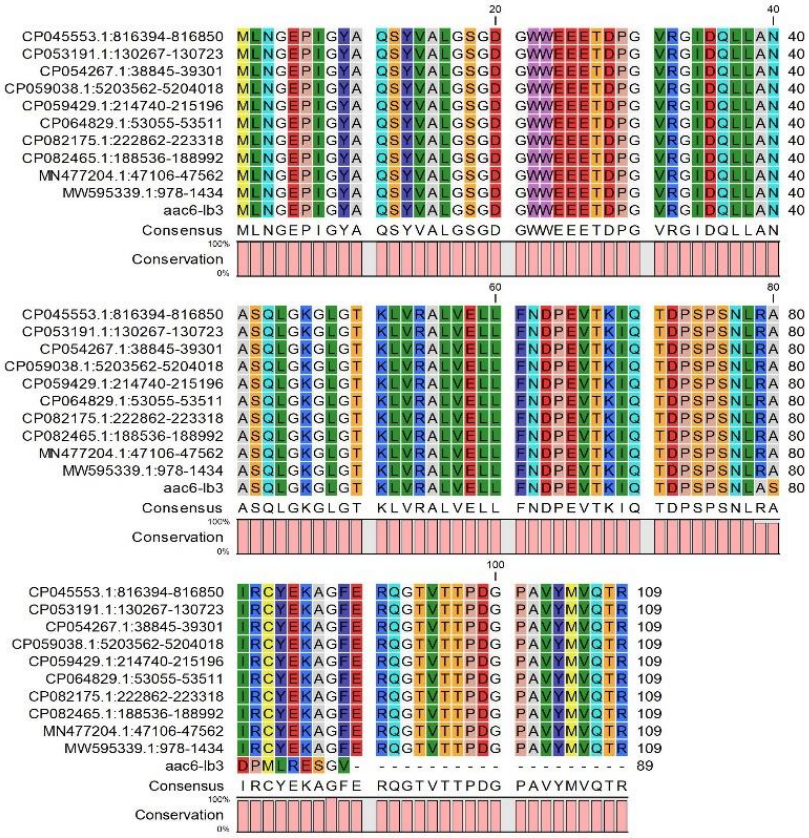


Fig. 3.7.6 Alignment of *aac6-Ib3* protein with the *aac6-lb3* reference gene from 10 different strains (CP045553.1:816394-816850 *Pseudomonas sp.*, CP054267.1:38845-39301 *Klebsiella pneumoniae*, MN477204.1:47106-47562 *Escherichia coli*, CP053191.1:130267-130723 *Enterobacter hormaechei*, CP082465.1:188536-188992 *Salmonella enterica*, CP082175.1:222862-223318 *Raoultella planticola*, MW595339.1:978-1434 *Pseudomonas aeruginosa*, CP064829.1:53055-53511 *Morganella morganii*, CP059429.1:214740-215196 *Citrobacter freundii* and CP059038.1:5203562-5204018 *Serratia marcescens*) illustrating the amino acids coverage and consensus with no variation (*aac6-lb3* gene terminal amino acids variation are not considered here)

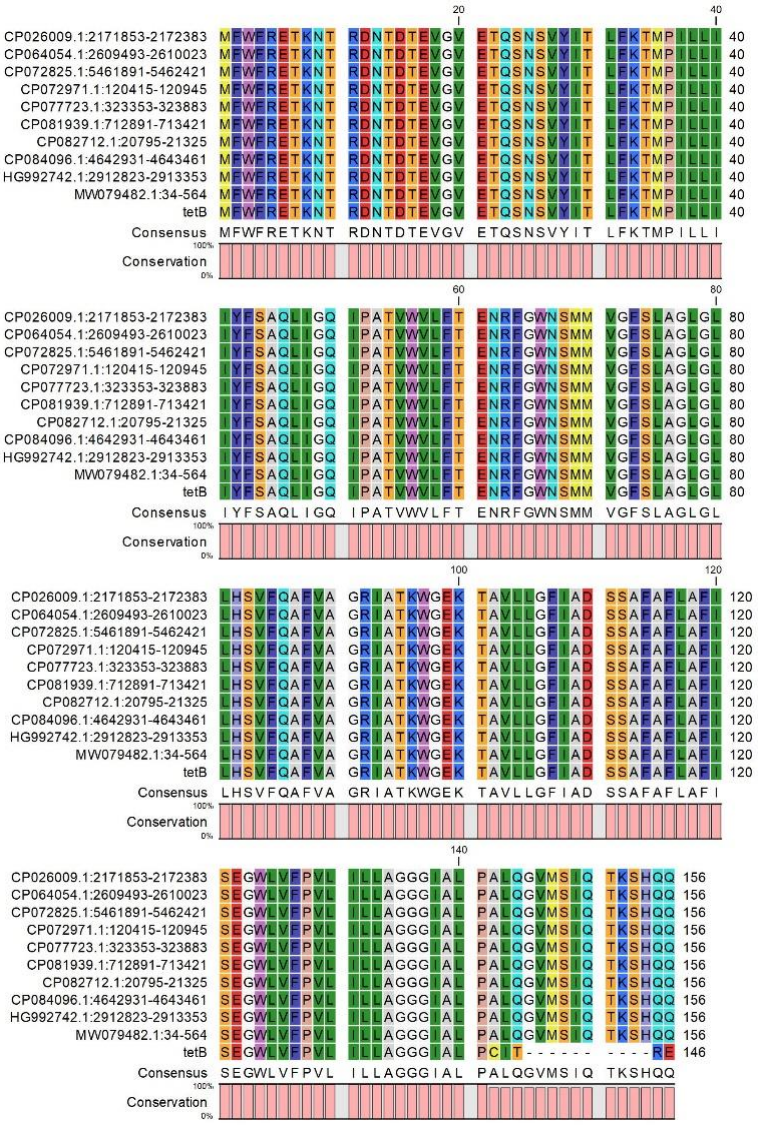


Fig. 3.7.7 Alignment of *tetB* protein with the *tetB* reference gene from 10 different strains (CP082712.1:20795-21325 *Salmonella enterica*, CP084096.1:4642931-4643461 *Escherichia coli*, CP081939.1:712891-713421 *Avibacterium paragallinarum*, CP077723.1:323353-323883 Pasteurella multocida, CP064054.1:2609493-2610023 *Morganella morganii*, CP072971.1:120415-120945 *Enterobacter sp.*, CP072825.1:5461891-5462421 *Klebsiella pneumoniae*, MW079482.1:34-564 *Uncultured bacterium*, HG992742.1:2912823-2913353 *Vibrio sp.* and CP026009.1:2171853-2172383 *Actinobacillus pleuropneumoniae*) illustrating the amino acids coverage and consensus with no amino acids variation (*tetB* gene terminal amino acids variation are not considered here)

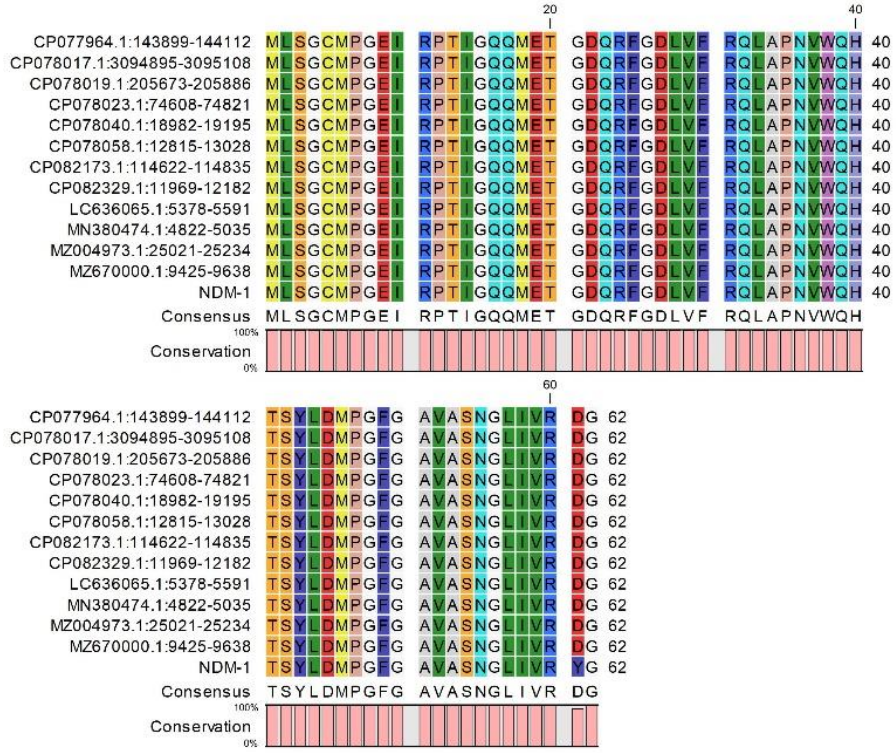


Fig. 3.7.8 Alignment of *NDM-1* protein with the *NDM-1* reference gene from 12 different strains (CP077964.1:143899-144112 *Proteus mirabilis*, CP078017.1:3094895-3095108 *Alcaligenes aquatilis*, CP078019.1:205673-205886 *Acinetobacter junii*, CP078023.1:74608-74821 *Citrobacter freundii*, CP078040.1:18982-19195 *Shewanella putrefaciens*, CP078058.1:12815-13028 *Enterobacter hormaechei*, CP082173.1:114622-114835 *Raoultella planticola*, CP082329.1:11969-12182 *Escherichia coli*, LC636065.1:5378-5591 *Pseudomonas aeruginosa*, MN380474.1:4822-5035 *Vibrio parahaemolyticus*, MZ004973.1:25021-25234 *Salmonella enterica* and MZ670000.1:9425-9638 *Providencia rettgeri*) illustrating the amino acids coverage and consensus with single amino acid variation (61Asp→ Tyr) compared to references

Fig. 3.7.9 Alignment of *SHV* protein with the *SHV* reference gene from 4 different strains (MW548273.1:559-832 *Klebsiella pneumoniae*, *CP079635.1:1030587-1030858 Klebsiella pneumoniae*, *CP079632.1:52754-53025 Klebsiella pneumoniae* and MZ700496.1:83-356 *Pseudomonas aeruginosa*) illustrating the amino acids coverage and consensus with 2 amino acids variation (80Val→ Leu and 84Arg→ Pro), compared to references

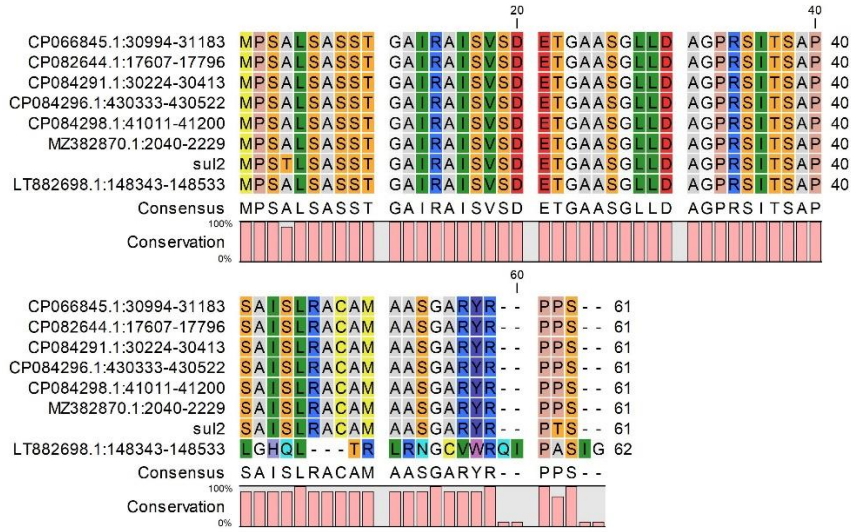


Fig. 3.7.10 Alignment of *sul2* protein with the *sul2* reference gene from 7 different strains (CP066845.1:30994-31183 *Escherichia coli*, CP082644.1:17607-17796 *Salmonella enterica*, CP084291.1:30224-30413 *Citrobacter werkmanii*, CP084296.1:430333-430522 *Providencia alcalifaciens*, CP084298.1:41011-41200 *Acinetobacter baumannii*, LT882698.1:148343-148533 *Klebsiella pneumoniae* and LT882698.1:148343-148533 *Enterobacter cloacae*) illustrating the amino acids coverage and consensus with 2 amino acids variation (4Ala→ Thr and 62Pro→ Thr), compared to references

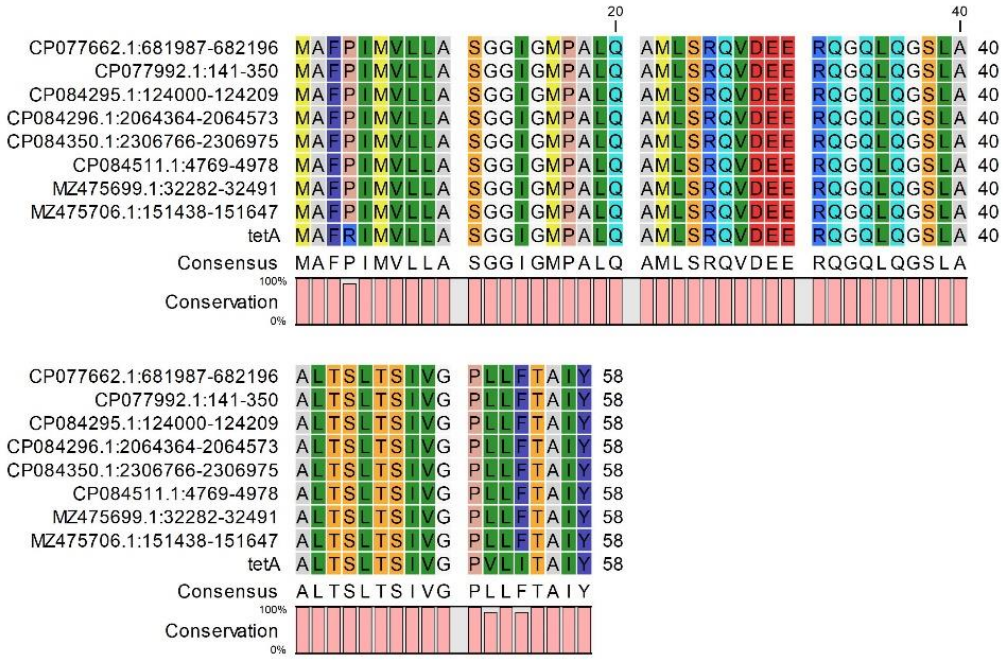


Fig. 3.7.11 Alignment of *tetA* protein with the *tetA* reference gene from 8 different strains (CP077662.1:681987-682196 *Salmonella enterica*, CP077992.1:141-350 *Pseudomonas aeruginosa*, CP084295.1:124000-124209 *Citrobacter werkmanii*, CP084296.1:2064364-2064573 *Providencia alcalifaciens*, CP084350.1:2306766-2306975 *Aeromonas caviae*, CP084511.1:4769-4978 *Edwardsiella tarda*, MZ475699.1:32282-32491 *Escherichia coli* and MZ475706.1:151438-151647 *Klebsiella pneumoniae*) illustrating the amino acids coverage and consensus with 3 amino acids variations (4Pro→ Arg, 52Leu→ Val and 54Phe→ Ile), compared to references

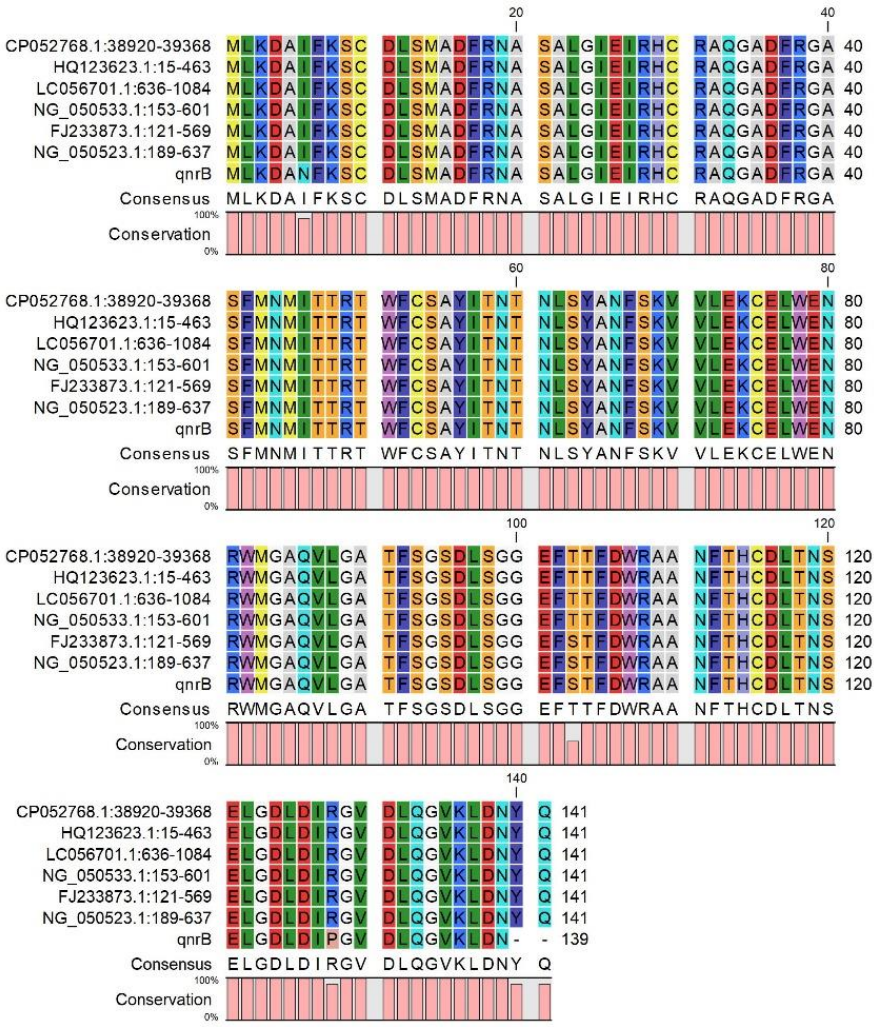


Fig. 3.7.12 Alignment of *qnrB* protein with the *qnrB* reference gene from 6 different strains (CP052768.1:38920-39368 *Salmonella enterica*, FJ233873.1:121-569 *Aeromonas veronii*, HQ123623.1:15-463 *Shigella flexneri*, LC056701.1:636-1084 *Escherichia coli*, NG_050523.1:189-637 *Pantoea agglomerans* and NG_050533.1:153-601 *Enterobacter cloacae*) illustrating the amino acids coverage and consensus with 3 amino acids variation (6Ile→ Asn, 103Thr→ Ser and 128Arg→ Pro), compared to references

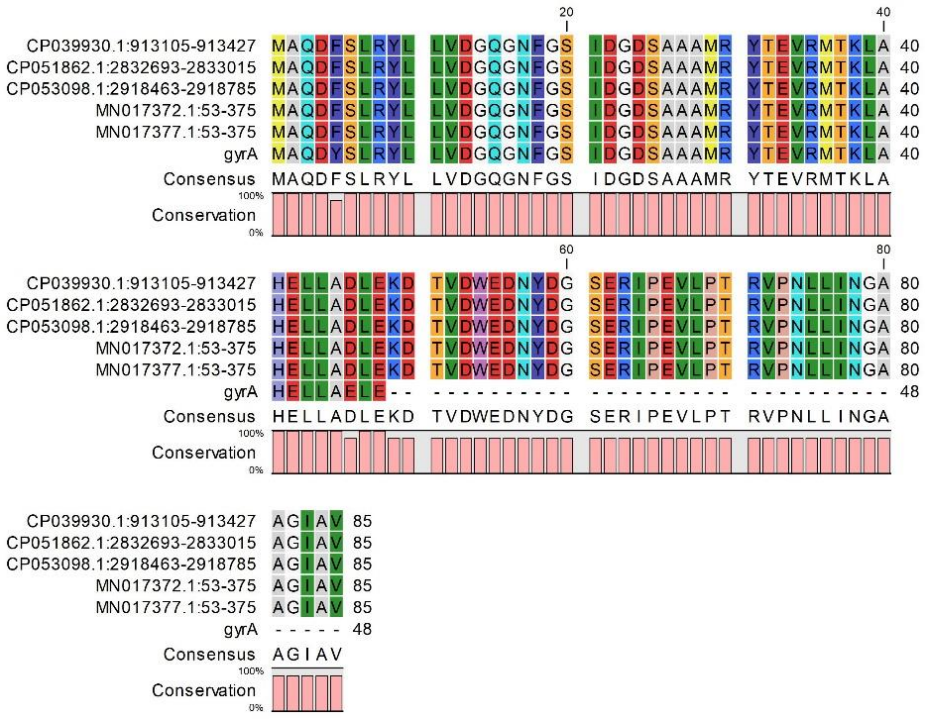


Fig. 3.7.13 Alignment of *gyrA* protein with the *gyrA* reference gene from 5 *Acinetobacter baumannii* different species (CP039930.1:913105-913427 *Acinetobacter baumannii*, CP051862.1:2832693-2833015 *Acinetobacter baumannii*, CP053098.1:2918463-2918785 *Acinetobacter baumannii*, MN017372.1:53-375 *Acinetobacter baumannii* and MN017377.1:53-375 *Acinetobacter baumannii*) illustrating the amino acids coverage and consensus with 2 amino acids variation (5Phe→ Tyr and 46Asp→ Glu), compared to references

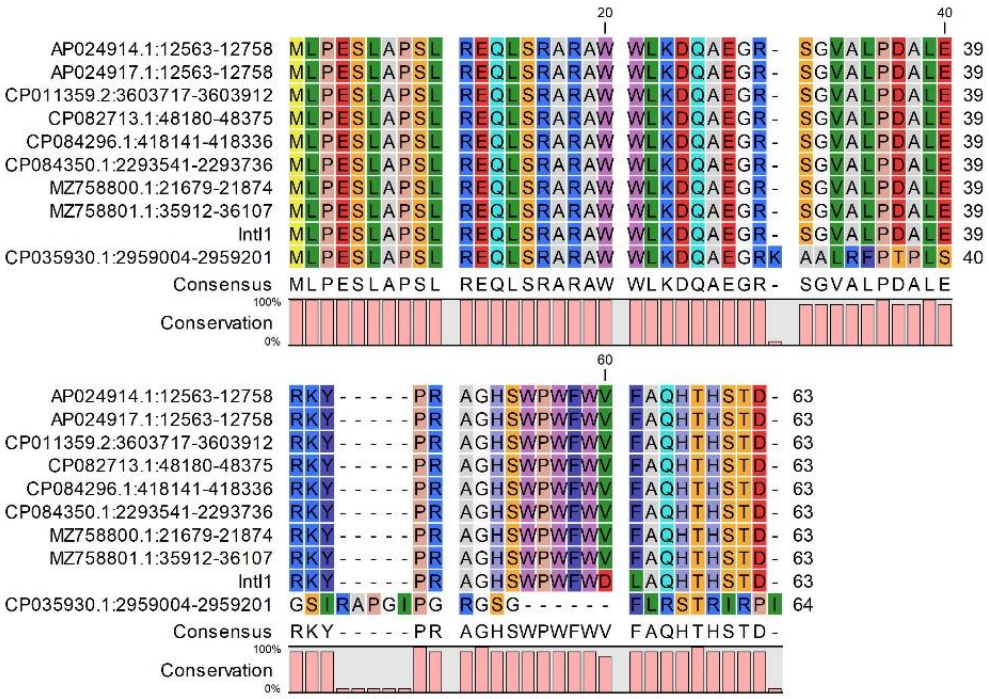
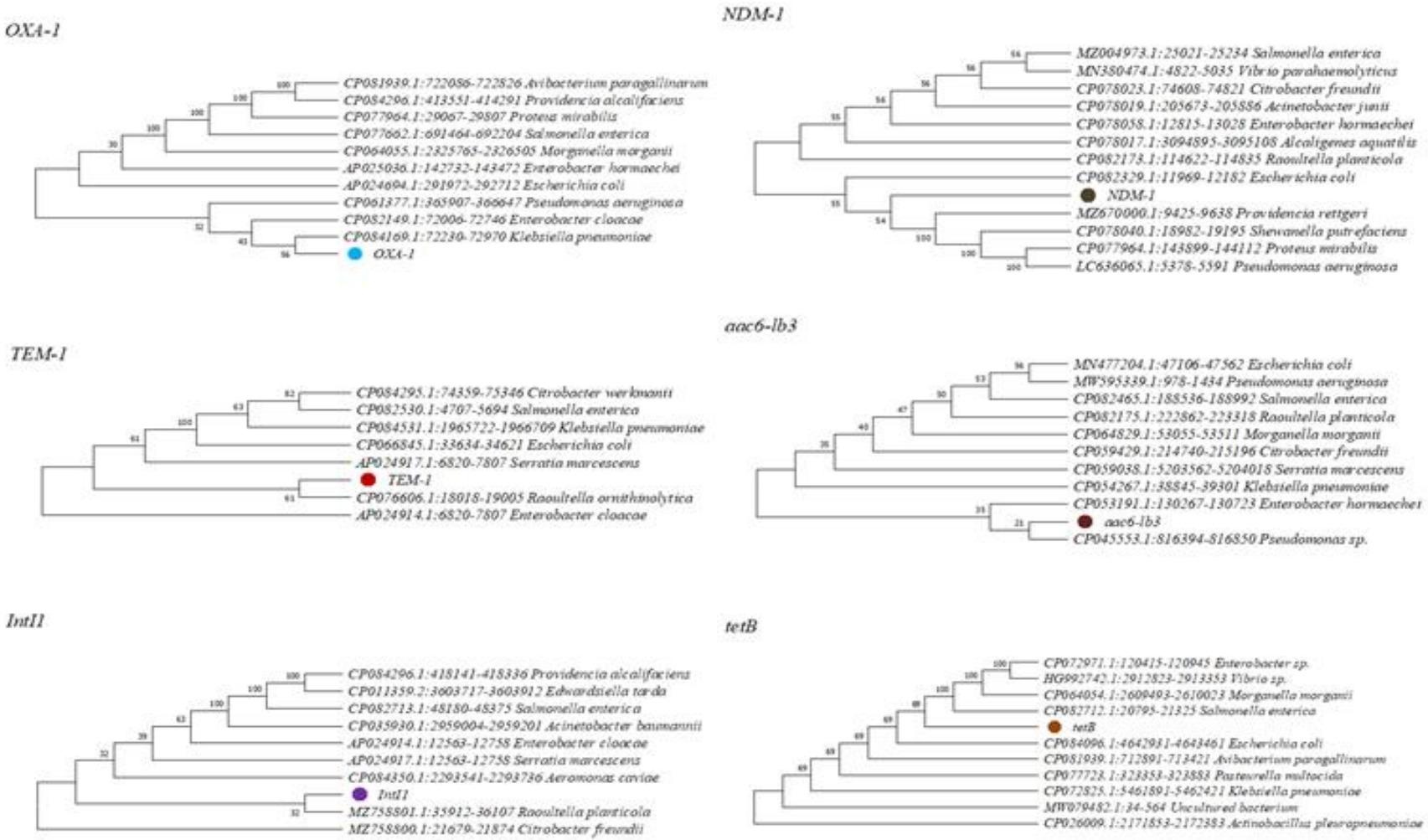


Fig. 3.7.14 Alignment of *IntI1* protein with the *IntI1* reference gene from 9 different strains (AP024914.1:12563-12758 *Enterobacter cloacae*, AP024917.1:12563-12758 *Serratia marcescens*, CP011359.2:3603717-3603912 *Edwardsiella*, CP035930.1:2959004-2959201 *Acinetobacter baumannii*, CP082713.1:48180-48375 *Salmonella enterica*, CP084296.1:418141-418336 *Providencia alcalifaciens*, CP084350.1:2293541-2293736 *Aeromonas caviae*, MZ758800.1:21679-21874 *Citrobacter freundii* and MZ758801.1:35912-36107 *Raoultella planticola*) illustrating the amino acids coverage and consensus with 2 amino acids variation (60Val→ Asp and 61Phe→ Leu), compared to references


OXA-1
CP081939.1:722086-722826 Avibacterium paragallinarum
CP084296.1:413551-414291 Providencia alcalifaciens
CP077964.1:29067-29807 Proteus mirabilis
CP077662.1:691464-692204 Salmonella enterica
CP064055.1:2325765-2326505 Morganella morganii
AP025036.1:142732-143472 Enterobacter hormaechei
AP024694.1:291972-292712 Escherichia coli
CP061377.1:365907-366647 Pseudomonas aeruginosa
CP082149.1:72006-72746 Enterobacter cloacae
CP084169.1:72230-72970 Klebsiella pneumoniae
OXA-1
NDM-1
MZ004973.1:25021-25234 Salmonella enterica
MN380474.1:4822-5035 Vibrio parahaemolyticus
CP078023.1:74608-74821 Citrobacter freundii
CP078019.1:205673-205886 Acinetobacter junii
CP078058.1:12815-13028 Enterobacter hormaechei
CP078017.1:3094895-3095108 Alcaligenes aquatilis
CP082173.1:114622-114835 Raoultella planticola
CP082329.1:11969-12182 Escherichia coli
NDM-1
MZ670000.1:9425-9638 Providencia rettgeri
CP078040.1:18982-19195 Shewanella putrefaciens
CP077964.1:143899-144112 Proteus mirabilis
LC636065.1:5378-5591 Pseudomonas aeruginosa
TEM-1
CP084295.1:74359-75346 Citrobacter werkmanii
CP082530.1:4707-5694 Salmonella enterica
CP084531.1:1965722-1966709 Klebsiella pneumoniae
CP066845.1:33634-34621 Escherichia coli
AP024917.1:6820-7807 Serratia marcescens
TEM-1
CP076606.1:18018-19005 Raoultella ornithinolytica
AP024914.1:6820-7807 Enterobacter cloacae
aac6-Ib3
MN477204.1:47106-47562 Escherichia coli
MW595339.1:978-1434 Pseudomonas aeruginosa
CP082465.1:188536-188992 Salmonella enterica
CP082175.1:222862-223318 Raoultella planticola
CP064829.1:53055-53511 Morganella morganii
CP059429.1:214740-215196 Citrobacter freundii
CP059038.1:5203562-5204018 Serratia marcescens
CP054267.1:38545-39301 Klebsiella pneumoniae
CP053191.1:130267-130723 Enterobacter hormaechei
aac6-Ib3
CP045553.1:816394-816850 Pseudomonas sp.
IntI1
CP084296.1:418141-418336 Providencia alcalifaciens
CP011359.2:3603717-3603912 Edwardsiella tarda
CP082713.1:48180-48375 Salmonella enterica
CP035930.1:2959004-2959201 Acinetobacter baumannii
AP024914.1:12563-12758 Enterobacter cloacae
AP024917.1:12563-12758 Serratia marcescens
CP084350.1:2293541-2293736 Aeromonas caviae
IntI1
MZ758801.1:35912-36107 Raoultella planticola
MZ758800.1:21679-21874 Citrobacter freundii
tetB
CP072971.1:120415-120945 Enterobacter sp.
HG992742.1:2912823-2913353 Vibrio sp.
CP064054.1:2609493-2610023 Morganella morganii
CP082712.1:20795-21325 Salmonella enterica
tetB
CP084096.1:4642931-4643461 Escherichia coli
CP081939.1:712891-713421 Avibacterium paragallinarum
CP077728.1:323353-323883 Pasteurella multocida
CP072825.1:5461891-5462421 Klebsiella pneumoniae
MW079482.1:34-564 Uncultured bacterium
CP026009.1:2171853-2172383 Actinobacillus pleuropneumoniae

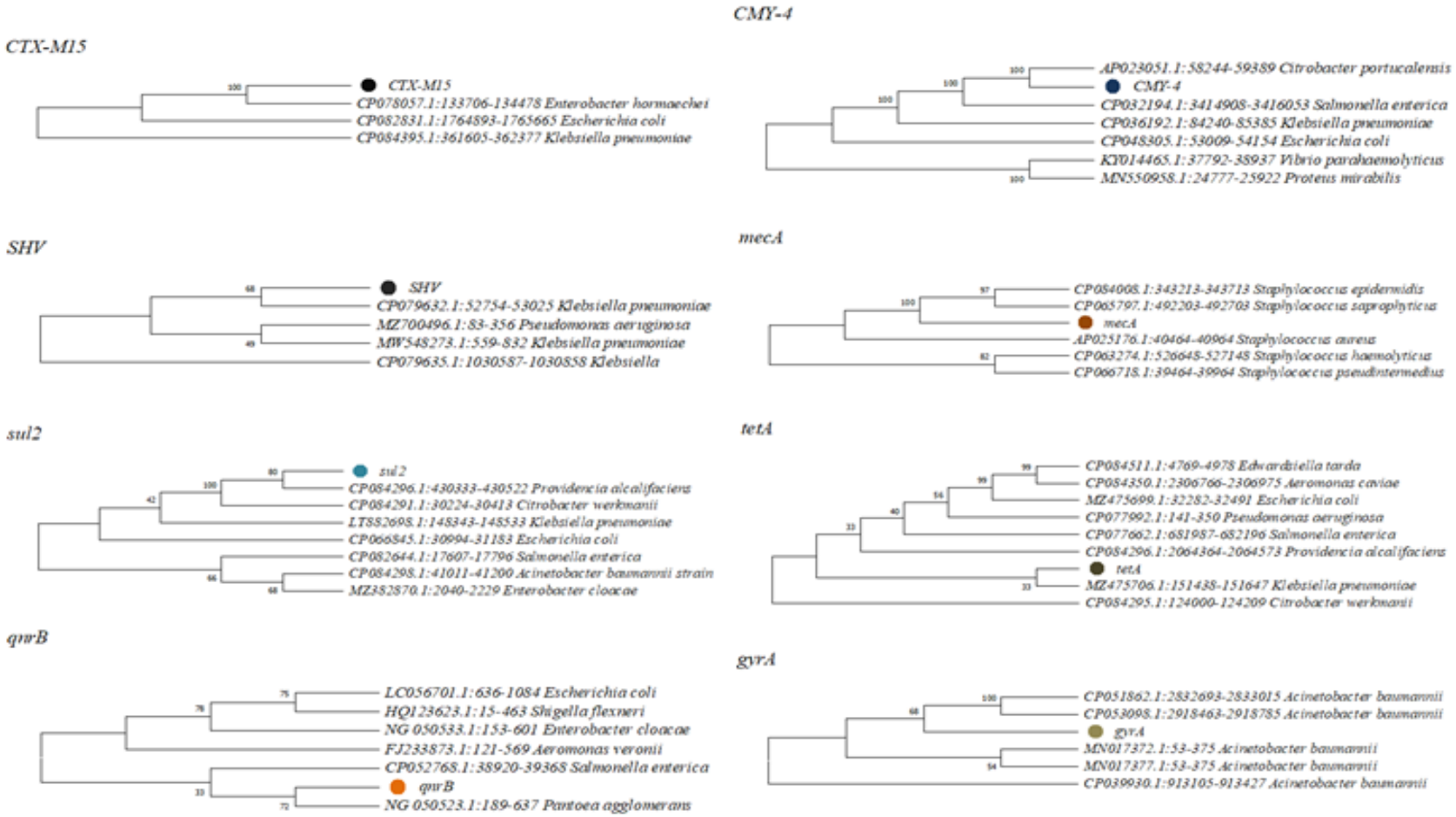


Fig. 3.7.15 The evolutionary history of ARGs with different clinical-based reference counterparts was deduced by Neighbor-Joining method and bootstrap consensus tree was derived from 500 replicates. Branches correlate with partitions rendered in <50% bootstrap replicates are eliminated. The replicate trees percentages in which the related taxa bunched together in bootstrap test are outlined next to branches. Using Poisson correction method, evolutionary spaces were calculated and are in unit of the number of amino acid substitution per site. All obscure locations were eliminated for each sequence pair. Evolutionary analyses were performed in MEGA X

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*, *Chlroflexi*, 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. 2017, 2019). 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 contrast with our findings. Seasonal variations in cold environments and geographical locations may contribute to bacterial diversity dominancy and variation because of the counter-selection of more adoptable bacteria (Zhang et al. 2007, 2010; 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 the 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, most frequently towards cephazolin (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 environments 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 was (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 (*ß lactam*, non- *ß 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 *ß-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 Ürümqi 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 *ß-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 *ß-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% *ß-lactamase* genes from high Arctic environments. Previous reports highlighted the presence of *ß-lactamase* genes and resistance against numerous *ß-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 *integrons* (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 *integrons* 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 *integrons* 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, *integrons* 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 (Zhang et al. 2018; Makowska et al. 2020).

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 baumanii*. 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 *integrons* 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.

Authors' contributions

SN, FH, and ILP conceived and designed the 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.

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Declarations

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.

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Chapter 4. Plasmid Curing

Research article 2.

Title:

Sabir Nawaz . Muhammad Rafiq . Shaista Andaleeb . Hafsa Kalsoom . Noor Hassan . Aamer Ali Shah . Fariha Hasan. Plasmid curing effect of acridine orange on a non-polar glacier bacteria susceptibility to antibiotics

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Plasmid curing effect of acridine orange on a non-polar glacier bacteria susceptibility to antibiotics

Sabir Nawaz . Muhammad Rafiq . Shaista Andaleeb . Hafsa Kalsoom . Noor Hassan . Aamer Ali Shah . Fariha Hasan

Abstract

Plasmid curing agents have plasmid obliterative potential, altering bacteria phenotypic characteristics, including antibiotic resistance. Bacteria with plasmid-encoded resistance are substantially decreased upon treatment with acridine orange. The study aims to determine the plasmid-borne antibiotic resistance by evaluating the plasmid-curing effect of acridine orange on glacier bacteria antibiotic susceptibility. A total of 65 bacterial strains were tested against 10 antibiotic classes and susceptibility patterns and multiple antibiotic resistance (MAR) index differences were evaluated pre and post-plasmid curing. To cure bacteria and investigate the MICs, various concentrations of acridine orange (0 to 200 µg/mL) were used. Gram-negative 17/43 (39.53%) and gram-positive 15/22 (68.18%) bacteria tolerated the highest concentration of 75 µg/mL acridine orange whereas gram-positive bacteria yielded a higher MICs value. Gram-negative bacteria 21/43 (48.83%) and gram-positive 7/22 (31.81%) revealed no change in susceptibility pattern. Cured bacteria showed 100% susceptibility to levofloxacin, ciprofloxacin, piperacillin, and imipenem whereas a major MAR index decline was observed for *Staphylococcus equorum* (HP19), *Leucobacter aridicollis* (HP22) (0.7 to 0.2 each) and *Serratia marcescens* (HP50) (0.6 to 0.1). Plasmid-borne antibiotic-resistant glaciated bacteria highlighted the horizontal transfer of antibiotic-resistant genes in natural environments and are startling which poses a major threat to mammal health and requires a quintessential approach.

Keywords. Acridine orange . antibiotic susceptibility . glacier bacteria . multiple antibiotic resistance index . plasmid curing agent

Introduction

Plasmids are small, additional chromosomal, double-stranded circular DNA fragments that exist independently inside the bacterial cell (Majumdar et al. 2006) and harbor genes for antibiotics and heavy metals resistance, catabolic pathways, and biosynthesis of certain antibiotics which bestow properties to host organisms (Kazeroon 2015; Ghosh et al. 2000; Datta and Hedges 1972). Plasmids perform an indispensable role in the accumulation and dissemination of antibiotic-resistant elements through horizontal gene transfer (HGT) and confer resistance to numerous antibiotics like; ß-lactams, quinolones, tetracyclines, aminoglycosides, macrolides, chloramphenicol, polymyxins and sulfonamides (Shintani et al. 2015). The induction of *ß-lactamases* occurs through several plasmids and resistance to *ß-lactamase* antibiotics has determined bacterial co-resistance to other antibiotics like aminoglycosides and quinolones (Amaya et al. 2012). Plasmids integrated with multi-drug resistant elements are mostly conjugative in natural environments and initiate the transfer of other plasmids beside themselves and possess the intrinsic potential to control their copy number inside host bacteria (Nordström 2006). Mobilizable plasmids are small and transfer DNA rather than self-transmission, with the aid of conjugative plasmids which occur both vertically and horizontally (Bennett 2008).

The phenomena of antibiotic resistance and propagation of resistant (R) plasmids among bacteria through HGT is a global threat to humans and the environment. A foremost approach to combat this phenomenon is to diminish the congregation and tenacity of antimicrobial-resistant plasmids among diverse bacteria populations (Vrancianu et al. 2020). By treating plasmid-containing bacteria with several techniques, conceivably bacteria can be deprived of plasmids to achieve plasmid-free derivatives (Mesas et al. 2004). Curing of a bacterial plasmid is a method used to vindicate and directly compare the relationship between plasmid-contained and plasmid-eliminated bacteria, regarding the carriage of specific genetic traits including the pivotal role of R plasmids in antibiotic resistance (Zaman et al. 2010; Trevors 1986). Several plasmids are impulsively prone to instantaneous remotion and sometimes deletion, nonetheless, a large number of plasmids are exceptionally stable and necessitate a curing agent supplemental approach for unanticipated deletion and segregation. To eliminate plasmids, various methods involving physical like; elevated growth temperatures, exposure to UV radiation, and chemical agents namely, intercalating dyes (acridine orange, acriflavine, ethidium bromide), sodium

dodecyl sulfate (SDS), crystal violet, novobiocin, mitomycin C and thymidine starvation have extensively used (Rezaee et al. 2007; Trevors 1986; Clowes 1972). Curing agents when exposed in sub-inhibitory concentration to tested bacterium cause the elimination of plasmid (Gohar et al. 2015).

Acridine orange is closely related to other members of the Xanthene class and used as a staining dye in biological procedures since 1929 and remains a very popular stain for its use in florescent microscopy and textile coloration (Rezaee et al. 2007). The adequacy of curing agents is sometimes uncertain in many bacteria as there exist no standard approaches and protocols to selectively eliminate or degrade all plasmids (Trevors 1986) and the effective quality of plasmid curing agents needs to be pinpointed. Generally, plasmid curing experiments are hit-and-miss based and rely on both options, the curing agent and culture conditions, as several curing agents work in an imprecise way (damage and stress out bacteria), whereas some act precisely (Zaman et al. 2010; Hohn and Korn 1969).

In the context of increased plasmid-mediated antibiotic resistance and dissemination through HGT, the current study was delineated to determine the plasmid-borne antibiotic resistance by evaluating the antibiotic susceptibility profile of glacier psychrophilic bacteria, pre, and post-treatment with acridine orange (a plasmid curing agent), isolated from Passu glacier's sediment, ice, and meltwater. Therefore, we put an exploratory effort into determining the curing efficacy of acridine orange on resistant bacteria plasmids to establish the association between R plasmid elimination and afterward, loss of antibiotic resistance, among psychrophilic bacteria.

Materials and Methods

Description of bacterial strains

This current study was carried out on bacterial strains, isolated from a non-polar Passu glacier, situated in the Karakorum mountains range in Pakistan. It covers an area of 72,971 square kilometers and lies at latitude 36° 27' 59.99" N and longitude 74° 53' 59.99" E. Due to the occurrence of congested glaciers, scientists sometimes term the Karakorum mountains as the third pole of the world.

Bacteria selection criteria for curing experiment

The bacterial strains were isolated and described previously (Nawaz et al. 2023) and their *16S rRNA* sequences were deposited to the GeneBank (NCBI) under the following accession numbers:

HP1 OL721773, HP2 OL721774, HP3 OL721775, HP4 OL721776, HP5 OL721777, HP6 OL721778, HP7 OL721779, HP8 OL721780, HP10 OL721781, HP11 OL721782, HP12 OL721783, HP13 OL721784, HP14 OL721785, HP15 OL721786, HP16 OL721787, HP17 OL721788, HP18 OL721789, HP19 OL721790, HP20 OL721791, HP21 OL721792, HP22 OL721793, HP23 OL721794, HTP36 OL721795, HP50 OL721796, HP51 OL721797, HP52 OL721798, HP53 OL721799, HP54 OL721800, HP55 OL721801, HP56 OL721802, HP57 OL721803, HP58 OL721804, LP1 OL721805, LP2 OL721806, LP3 OL721807, LP4 OL721808, LP5 OL721809, LP6 OL721810, LP7 OL721811, LP8 OL721812, LP9 OL721813, LP10 OL721814, LP11 OL721815, HPI1 OL721816, HPI2 OL721817, HPI3 OL721818, HPI4 OL721819, HPI5 OL721820, HPI6 OL721821, HPI7 OL721822, HPI8 OL721823, LPI1 OL721824, LPI2 OL721825, LPI4 OL721826, LPI5 OL721827, LPI6 OL721828, LPI7 OL721829, LPW1 OL721830, LPW2 OL721831, LPW3 OL721832, LPW4 OL721833, LPW5 OL721834, LPW6 OL721835, LPW7 OL721836, LPW8 OL721837.

Bacterial species were tested for antibiotic susceptibility evaluation to a panel of antibiotic classes and based on *in vivo* antimicrobial resistance to more than one antibiotic class (excluding intermediate resistant), a total of 65 bacterial strains, gram-positive (22 strains), and gram-negative (43 strains), were selected for plasmid curing analysis.

Plasmid curing experiment

Plasmid curing of resistant bacteria was performed following the methods previously described (Makia et al. 2013; Trevors 1986) with slight modifications, using acridine orange (OmniPur, Calbiochem) (curing agent, intercalating dye). In the experimental process, each bacterial strain was inoculated in Luria-Bertani broth and incubated. Among these, Psychrophilic bacterial strains were incubated at 4°C for 72 to 96 hours while Psychrotrophic bacteria were at 15°C for 24 to 48 hours, in an orbital shaker at 150 rpm. After the incubation period, 200µl of each tested bacterial culture (0.5 McFarland's standard) was taken and introduced into a set of 10 test tubes containing 5 mL fresh Luria-Bertani broth, respectively. Acridine orange (0.10 mg/mL) in different concentrations of 0, 10, 15, 25, 50, 75, 100, 125, 150, and 200 µg/mL was then added into all test tubes accordingly and incubated, to determine the sub-lethal concentrations of acridine orange. After an incubation period, 1mL aliquot from each test tube was inoculated onto fresh nutrient agar plates and incubated at the same conditions. Afterward, the plates were observed for growth, and isolated colonies were selected from the agar plate inoculated with the highest concentration of acridine orange (AO) that still allowed bacterial growth (Trevors 1986) and swabbed onto freshly prepared Mueller Henton Agar (MHA) plates for verificatory antibiotic susceptibility assay.

Pre and post-plasmid curing susceptibility testing

The bactetial strains were subjected to antibiotic sensitivity testing using Levofloxacin (5 µg), Ciprofloxacin (5 µg), Ofloxacin (5 µg), Norfloxacin (10 µg), Nalidixic acid (30 µg), Amoxicillin (10 µg), Penicillin G (10 µg), Carbenicillin (100 µg), Ticarcillin (75 µg), Oxacillin (1 µg), Piperacillin (100 µg), Ceftriaxone (30 µg), Cefotetan (30 µg), Cephalexin (30 µg), Cephalothin (30 µg), Cefepime (30 µg), Ceftazidime (30 µg), Cephazolin (30 µg), Cefpodoxime (10 µg), Ceftaroline (30 µg), Meropenem (10 µg), Imipenem (10 µg) Ertapenem (10 µg), Aztreonam (30 µg), Linezolid (30 µg), Erythromycin (5 µg), Vancomycin (30 µg), Tigecycline (15 µg) and Metronidazole (5 µg), by Kirby Bauer disc diffusion method (Bauer et al. 1996). Gram-positive bacteria were tested against 28 antibiotics while gram-negative bacteria were against 21 antibiotics.

The standardized inocula (0.5 McFarland's standard) of herein tested bacteria were swabbed onto fresh MHA plates and subsequent disc diffusion treatment with antibiotics of prior

resistance, to determine the antibiotic susceptibility post plasmid curing. After incubation, MHA plates were scrutinized and sizes of the zone of inhibition were juxtaposed and compared with those procured before plasmid curing. Particular bacterial strains with phenotypic resistance (no zone of inhibition) to specific antibiotic/antibiotics before AO treatment, if inspected with a zone of inhibition afterward, will be assumed as cured strains with possible plasmid elimination and encoded antibiotic resistance genes, against tested antibiotics.

Pre and post-plasmid curing MAR indexes

Multiple antibiotic resistance (MAR) index was calculated for all tested bacteria, pre and post-plasmid curing, by using the formula MAR index = a/b, designed by Krumperman, where "a" reflects the number of resistant antibiotics, and "b" displays a total number of antibiotics used for susceptibility testing. MAR index value ≥ 0.2 emerges from a hazardous source of contamination (Joseph et al. 2017; Krumperman 1983).

Statistical analysis

The generated data were subjected to descriptive statistics for analysis where applicable, using R software (version 3.3.3), chi-square, degree of freedom (*df*) and *p-value* were determined while the level of significance data was 0.05 (95%) and results were summarized in a suitable table for simplicity.

Results

Antibiotic resistance pattern of bacteria

All glaciated bacteria showed significant antibiotic resistance against a set of 29 antibiotics from 10 different classes and both gram-positive (22 strains, against 28 antibiotics) and gram-negative (43 strains, against 21 antibiotics) bacteria were subjected to plasmid curing testing. Among gram-positive bacteria, maximum resistance was showed by *Staphylococcus equorum* (HP19) to 22 antibiotics, followed by *Leucobacter aridicollis* (HP22) and *Arthrobacter sp.* (LP2) to 21 each, *Leucobacter komagatae* (HP51) to 20 and *Bacillus pumilus* (HP18) to 19 antibiotics, while minimum resistance was exhibited by *Carnobacterium maltaromaticum* (HP36) to 6 antibiotics. On the contrary, gram-negative bacteria in comparison were more resistant with *Brevundimonas diminuta* (HP21) resistance to a maximum of 16 antibiotics, followed by *Flavobacterium antarcticum* (HP20) and *Serratia marcescens* (HP50) to 14 each, *Alcaligenes faecalis* (HP2) and *Acinetobacter calcoaceticus* (LP5) each to 11 antibiotics, whereas minimum resistance pattern was shown by *Acinetobacter baumannii* (HP57) to 6 antibiotics. Antibiotic resistance patterns of glaciated bacteria selected for plasmid curing analysis are shown in (Table 4.1).

Table 4.1 Antibiotic resistance pattern and MAR indexes of bacteria pre and post-acridine orange treatment

Bacteria code	Pre-plasmid Curing			Post-plasmid Curing		
	Antibiotic resistance pattern	No. of resistant antibiotics (%)	MAR Index	Antibiotic resistance pattern	No. of resistant antibiotics (%)	MAR Index
Gram-negative						
HP2	OFX,NOR,NA,CTT,CL,KF,KZ,CPD, E,TGC,MET	11 (52.3)	0.5	CTT,CL,KF,KZ,CPD,E,TGC,MET	8 (38)	0.3
HP3	LEV,CTT,CL,KF,FEP,KZ,CPT,E, TGC,MET	10 (47.6)	0.4	CTT,CL,KF,FEP,KZ,CPT,E,TGC, MET	9 (42.8)	0.4
HP4	CTT,CL,KF,CAZ,KZ,ATM,E,TGC,M ET	9 (42.8)	0.4	CTT,CL,KF,CAZ,KZ,ATM,TGC, MET	8 (38)	0.3
HP6	NA,CL,KF,KZ,CPD,E,TGC,MET	8 (38)	0.3	NA,CL,KF,KZ,CPD,TGC,MET	7 (33.3)	0.3
HP10	NOR,CL,KF,KZ,CPD,E,MET	7 (33.3)	0.3	NOR,CL,KF,KZ,CPD,E,MET	7 (33.3)	0.3
HP11	NA,CL,KF,EFP,KZ,CPD,E,MET	8 (38)	0.3	E,MET	2 (9.5)	0.09
HP12	CIP,NOR,NA,CTT,CL,KF,KZ,CPD,E ,MET	10 (47.6)	0.4	CIP,NOR,NA,CTT,CL,KF,KZ,CP D,E,MET	10 (47.6)	0.4
HP15	CTT,CL,KF,KZ,CPD,ATM,E,MET	8 (38)	0.3	CTT,CL,KF,KZ,CPD,ATM,E,ME T	8 (38)	0.3

HP16	NOR,NA,CTT,CL,KF,KZ,CPD,E, MET	9 (42.8)	0.4	NOR,NA,CTT,CL,KF,KZ,CPD,E, MET	8 (38)	0.3
HP20	OFX,NOR,CRO,CL,KF,FEP,KZ,CPD ,CPT,IMP,ATM,E,TGC,MET	14 (66.6)	0.6	OFX,NOR,ATM,TGC,MET	5 (23.8)	0.2
HP21	LEV,CIP,OFX,NOR,NA,CRO,CTT,C L,KF,KZ,CPD,ETP,ATM,E,TGC,ME T	16 (76.1	0.7	CRO,CTT,CL,KF,KZ,CPD,ATM, TGC,MET	9 (42.8)	0.4
HP50	CRO,CTT,CL,KF,FEP,CAZ,KZ,CPD, CPT,MRP,ETP,ATM,E,MET	14 (66.6)	0.6	ATM,E,MET	3 (14.2)	0.1
HP52	NA,CRO,CTT,CL,KF,FEP,CAZ,KZ, CPD,CPT,MRP,ETP,ATM,E,MET	15 (71.4)	0.7	NA,CRO,CTT,CL,KF,FEP,CAZ,K Z,CPD,CPT,MRP,ETP,ATM,E,M ET	15 (71.4)	0.7
HP54	NOR,CTT,CL,KF,FEP,KZ,CPD,MRP ,ETP,MET	10 (47.6)	0.4	CTT,CL,KF,FEP,KZ,CPD,MRP,E TP,MET	9 (42.8)	0.4
HP55	NOR,CRO,CTT,CL,KF,FEP,CAZ,KZ ,CPD,CPT,MRP,ETP,ATM,E,MET	15 (71.4)	0.7	CRO,CTT,CL,KF,FEP,CAZ,KZ,C PD,CPT,MRP,ETP,ATM,E,MET	14 (66.6)	0.6
HP56	KF,MRP,MET	3 (14.2)	0.1	KF,MRP,MET	3 (14.2)	0.1
HP57	CL,KF,CAZ,KZ,MRP,MET	6 (28.5)	0.28	CL,KF,CAZ,KZ,MET	5 (23.8)	0.23
HP58	CL,KF,KZ,MRP,ETP,MET	6 (28.5)	0.2	MET	1 (4.7)	0.04
LP5	CIP,CL,KF,FEP,CAZ,KZ,CPD,ETP, ATM,E,MET	11 (52.3)	0.5	CL,KF,FEP,CAZ,KZ,CPD,ETP,A TM,E,MET	10 (47.6)	0.4
LP6	CTT,CL,KF,FEP,KZ,CPD,E,MET	8 (38)	0.3	CTT,CL,KF,FEP,KZ,CPD,E,MET	8 (38)	0.3
LP7	CTT,CL,KF,FEP,KZ,CPD,ATM,E,M ET	9 (42.8)	0.4	CTT,CL,KF,FEP,KZ,CPD,ATM, MET	8 (38)	0.3
LP9	CTT,CL,KF,FEP,KZ,CPD,ATM,E,M ET	9 (42.8)	0.4	CTT,CL,KF,FEP,KZ,CPD,ATM, MET	8 (38)	0.3
LP10	CIP,OFX,NOR,CAZ,CPD,CPT,MRP, ETP,ATM,E,TGC,MET	12 (57.1)	0.5	CAZ,CPD,CPT,MRP,ETP,ATM,E, TGC,MET	9 (42.8)	0.4
LP11	LEV,CL,KF,FEP,CAZ,KZ,CPD,MRP ,ATM,E,MET	11 (52.3)	0.5	CL,KF,FEP,CAZ,KZ,CPD,MRP,A TM,E,MET	10 (47.6)	0.4
HPI1	CRO,CTT,CL,KF,CAZ,KZ,CPD,CPT ,MRP,ETP,ATM,E,MET	13 (61.9)	0.6	CRO,CTT,CL,KF,CAZ,KZ,CPD,C PT,MRP,ETP,ATM,E,MET	13 (61.9)	0.6
HPI2	CL,KF,KZ,MRP,ETP,MET	6 (28.5)	0.2	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	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,MR P,ETP,ATM,E,MET	12 (57.1)	0.5	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	CL,KF,KZ,MRP,ETP,MET	6 (28.5)	0.2
HPI6	CL,KF,KZ,MRP,ETP,E,MET	7 (33.3)	0.3	CL,KF,KZ,MRP,ETP,E,MET	7 (33.3)	0.3
HPI7	CTT,KF,FEP,CAZ,KZ,CPD,CPT,AT M,MET	9 (42.8)	0.4	CTT,KF,FEP,CAZ,KZ,CPD,CPT, ATM,MET	9 (42.8)	0.4
HPI8	CTT,KF,CAZ,KZ,CPD,CPT,IMP,AT M,E,MET	10 (47.6)	0.4	CTT,KF,CAZ,KZ,CPD,CPT,IMP, ATM,E,MET	10 (47.6)	0.4
LPI1	CRO,CL,KF,CAZ,KZ,IMP,ATM,E,M ET	9 (42.8)	0.4	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	E,MET	2 (9.5)	0.09
LPI4	CL,KF,KZ,MRP,E,MET	6 (28.5)	0.3	CL,KF,KZ,MRP,E,MET	6 (28.5)	0.3
LPI5	CL,KF,KZ,MRP,IMP,ETP,E,MET	8 (38)	0.3	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	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	CL,KF,KZ,E,MET	5 (23.8)	0.2
LPW3	CL,KF,KZ,E,MET	5 (23.8)	0.2	CL,KF,KZ,E,MET	5 (23.8)	0.2
LPW4	KF,FEP,KZ,ETP,ATM,E,MET	7 (33.3)	0.3	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	ATM,MET	2 (9.5)	0.09
LPW7	NOR,CRO,CTT,FEP,CAZ,CPD,MRP,IMP,ATM,MET	10 (47.6)	0.4	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	CL,KF,CAZ,KZ,CPD,ATM,E,MET	8 (38)	0.3
Gram-positive						
HP1	NOR,AML,P,CAR,TIC,OX,CTT,KF,FEP,KZ,CPT,LZD,E,VA,TGC,MET	16 (57.1)	0.5	AML,P,CAR,TIC,OX,CTT,KF,FEP,KZ,CPT,LZD,E,VA,TGC,MET	15 (53.5)	0.5
HP5	NA,AML,P,CAR,OX.CTT,CL,KF,KZ,CPD,LZD,E,VA,MET	14 (50)	0.5	NA,LZD,VA,MET	4 (14.2)	0.1
HP7	LEV,AML,P,CAR,TIC,OX,CTT,CL,KF,KZ,CPD,LZD,E,VA,MET	15 (53.5)	0.5	AML,P,CAR,TIC,OX,CTT,CL,KF,KZ,CPD,LZD,E,VA,MET	14 (50)	0.5
HP8	AML,P,OX,PRL,CTT,KF,KZ,LZD,E,VA,MET	11 (39.2)	0.3	LZD,E,VA,MET	4 (14.2)	0.1
HP13	P,CTT,FEP,CAZ,CPD,CPT,MRP,ETP,MET	9 (32.1)	0.3	P,CTT,FEP,CAZ,CPD,CPT,MRP,ETP,MET	9 (32.1)	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	LZD,E,VA,MET	4 (14.2)	0.1
HP17	NOR,NA,AML,P,CAR,TIC,OX,CTT,CL,KF,KZ,CPD,LZD,E,VA,MET	16 (57.1)	0.5	NOR,NA,AML,P,CAR,TIC,OX,CTT,CL,KF,KZ,CPD,LZD,E,VA,MET	16 (57.1)	0.5
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	AML,P,CAR,TIC,OX,CRO,CTT,CL,KF,KZ,CPD,LZD,E,VA,MET	15 (53.5)	0.5
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	LZD,E,VA,TGC,MET	5 (17.8)	0.1
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	AML,P,CAR,TIC,OX,LZD,E,MET	8 (28.5)	0.2
HP23	CIP,OFX,NOR,NA,AML,P,CAR,OX,CL,KF,KZ,CPD,ETP,LZD,E,VA,MET	17 (60.7)	0.6	AML,P,CAR,OX,CL,KF,KZ,CPD,LZD,E,VA,MET	12 (42.8)	0.4
HP36	FEP,CPD,CPT,MRP,ETP,MET	6 (21.4)	0.2	FEP,CPD,CPT,MRP,ETP,MET	6 (21.4)	0.2
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	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
HP53	AML,P,CAR,TIC,OX,CTT,CL,KF,FEP,KZ,CPD,MRP,ETP,LZD,E,VA,MET	17 (60.7)	0.6	AML,P,CAR,TIC,OX,CTT,CL,KF,FEP,KZ,CPD,MRP,ETP,LZD,E,VA,MET	17 (60.7)	0.6

LP1	AML,P,CAR,TIC,OX,CTT,CL,KF,FEP,CAZ,KZ,CPD,LZD,E,VA,MET	16 (57.1)	0.5	AML,P,CAR,TIC,OX,CTT,CL,KF,FEP,CAZ,KZ,CPD,LZD,E,VA,MET	16 (57.1)	0.5
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	OFX,AML,P,TIC,OX,LZD,TGC,MET	8 (28.5)	0.2
LP3	AML,P,TIC,OX,CL,KF,FEP,KZ,CPD,LZD,E,VA,MET	13 (46.4)	0.4	AML,P,TIC,OX,CL,KF,FEP,KZ,CPD,LZD,E,VA,MET	13 (46.4)	0.4
LP4	AML,P,OX,CL,KF,FEP,CAZ,KZ,ETP,LZD,E,VA,MET	13 (46.4)	0.4	AML,P,OX,CL,KF,FEP,CAZ,KZ,LZD,MET	10 (35.7)	0.3
LP8	CIP,NOR,AML,P,OX,CPD,CPT,MRP,ETP,LZD,VA,MET	12 (42.8)	0.4	CPD,CPT,MRP,ETP,LZD,VA,MET	7 (25)	0.2
LPI6	NOR,NA,AML,CRO,CAZ,CPD,ETP	7 (25)	0.2	AML,CRO,CAZ,CPD	4 (14.2)	0.1
LPI7	NA,AML,P,OX,CRO,CTT,KF,FEP,CAZ,KZ,CPD,CPT,MRP,IMP,VA,MET	16 (57.1)	0.5	AML,P,OX,CRO,CTT,KF,FEP,CAZ,KZ,CPD,CPT,VA,MET	13 (46.4)	0.4
LPW5	LEV,CIP,OFX,NOR,AML,OX,CAZ,KZ,MRP,IMP,MET	11 (39.2)	0.3	AML,OX,CAZ,KZ,MET	5 (17.8)	0.1

Key: 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.

Effect of treatment with acridine orange on bacteria growth

Plasmid curing was performed by treatment with various concentrations of AO and determining the minimum inhibitory concentration (MICs). Glaciated bacteria exhibited various degrees of growth when treated with AO concentrations (0 to 200 µg/mL). All tested bacteria showed heavy growth at 0 to 25 µg/mL, except *Staphylococcus equorum* (HP 19), *Arthrobacter oryzae* (LP1), *Trichococcus alkaliphilus* (LP3) among gram-positive and *Alcaligenes faecalis* (HP2), *Pseudomonas versuta* (HP10), *Flavobacterium suaedae* (HP12), *Brucellaceae bacterium* (HP15), *Flavobacterium antarcticum* (HP20), *Brevundimonas bullata* (HP54), *Acinetobacter baumannii* (HP57) and *Serratia liquefaciens* (HP58) among gram-negative, which exhibited moderate growth. Furthermore, at 50 µg/mL, *Flavobacterium saliperosum* (HP8), *Staphylococcus equorum* (HP19), *Arthrobacter oryzae* (LP1), *Trichococcus alkaliphilus* (LP3), *Alcaligenes faecalis* (HP2), *Flavobacterium suaedae* (HP12), *Brucellaceae bacterium* (HP15), *Flavobacterium antarcticum* (HP20) and *Acinetobacter baumannii* (HP57) showed slight growth while rest of bacteria exhibited moderate growth.

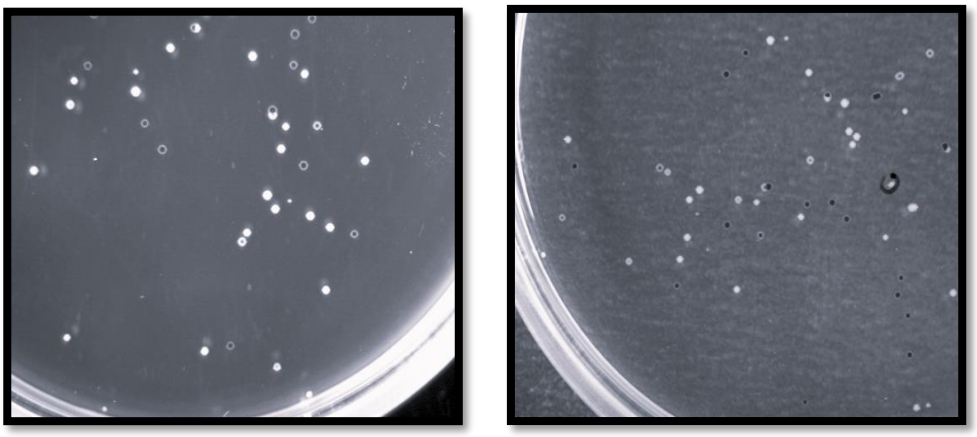
Meanwhile, at 75 µg/mL of AO, all bacteria exhibited slight growth except *Flavobacterium saliperosum* (HP8), *Staphylococcus equorum* (HP19), *Arthrobacter oryzae* (LP1), *Trichococcus alkaliphilus* (LP3), *Alcaligenes faecalis* (HP2), *Flavobacterium suaedae* (HP12), *Brucellaceae bacterium* (HP15), *Flavobacterium antarcticum* (HP20), *Brevundimonas bullata* (HP54), *Acinetobacter baumannii* (HP57) and *Serratia liquefaciens* (HP58) which showed no growth. Consistently at 100 to 200 µg/mL, none of the tested bacteria showed any visible growth. AO concentrations and MIC values were slightly higher for gram-positive bacteria in comparison to gram-negative, as illustrated in (Table 4.2). The effect of AO at concentrations of 75 µg/ µL on *Brevundimonas diminuta* (HP21) growth (isolated cured colonies) is shown in Fig. 4.1.

Table 4.2 Effect of different concentrations of acridine orange (curing agent) on bacteria growth

Bacteria code	**Different concentrations of acridine orange (µg/ml)**										**MICs (µg/ml)**
	0	10	15	25	50	75	100	125	150	200	
Gram-negative											
HP2	++	++	++	+	±	-	-	-	-	-	70
HP3	++	++	++	++	+	±	-	-	-	-	90
HP4	++	++	++	+	±	-	-	-	-	-	70
HP6	++	++	++	+	±	-	-	-	-	-	60
HP10	++	++	++	+	±	-	-	-	-	-	70
HP11	++	++	++	+	±	-	-	-	-	-	70
HP12	++	++	++	+	±	-	-	-	-	-	60
HP15	++	++	++	+	±	-	-	-	-	-	70
HP16	++	++	++	++	+	±	-	-	-	-	90
HP20	++	++	++	+	±	-	-	-	-	-	70
HP21	++	++	++	++	+	±	-	-	-	-	90
HP50	++	++	++	++	+	±	-	-	-	-	90
HP52	++	++	++	+	±	-	-	-	-	-	60
HP54	++	++	++	+	±	-	-	-	-	-	60
HP55	++	++	++	++	+	±	-	-	-	-	90
HP56	++	++	++	+	±	-	-	-	-	-	60
HP57	++	++	++	+	±	-	-	-	-	-	70
HP58	++	++	++	+	±	-	-	-	-	-	70
LTP5	++	++	++	++	+	±	-	-	-	-	80
LTP6	++	++	++	++	+	±	-	-	-	-	90
LTP7	++	++	++	+	±	-	-	-	-	-	70
LTP9	++	++	++	+	±	-	-	-	-	-	70
LTP10	++	++	++	++	+	±	-	-	-	-	90
LTP11	++	++	++	++	+	±	-	-	-	-	80
HPI1	++	++	++	++	+	±	-	-	-	-	80
HPI2	++	++	++	+	±	-	-	-	-	-	60

HPI3	++	++	++	++	+	±	-	-	-	-	80
HPI4	++	++	++	++	+	±	-	-	-	-	80
HPI5	++	++	++	+	±	-	-	-	-	-	70
HPI6	++	++	++	+	±	-	-	-	-	-	60
HPI7	++	++	++	++	+	±	-	-	-	-	90
HPI8	++	++	++	++	+	±	-	-	-	-	90
LPI1	++	++	++	++	+	±	-	-	-	-	90
LPI2	++	++	++	+	±	-	-	-	-	-	70
LPI4	++	++	++	+	±	-	-	-	-	-	70
LPI5	++	++	++	+	±	-	-	-	-	-	60
LPW1	++	++	++	++	+	±	-	-	-	-	80
LPW2	++	++	++	+	±	-	-	-	-	-	70
LPW3	++	++	++	+	±	-	-	-	-	-	60
LPW4	++	++	++	++	+	±	-	-	-	-	90
LPW6	++	++	++	++	±	-	-	-	-	-	70
LPW7	++	++	++	++	±	-	-	-	-	-	60
LPW8	++	++	++	++	±	-	-	-	-	-	70
Gram-positive											
HP1	++	++	++	++	+	±	-	-	-	-	80
HP5	++	++	++	++	+	±	-	-	-	-	90
HP7	++	++	++	++	+	±	-	-	-	-	90
HP8	++	++	++	++	±	-	-	-	-	-	60
HP13	++	++	++	+	±	-	-	-	-	-	70
HP14	++	++	++	++	+	±	-	-	-	-	80
HP17	++	++	++	++	+	±	-	-	-	-	80
HP18	++	++	++	++	+	±	-	-	-	-	80
HP19	++	++	++	+	±	-	-	-	-	-	70
HP22	++	++	++	++	+	±	-	-	-	-	90
HP23	++	++	++	++	+	±	-	-	-	-	90
HP36	++	++	++	+	±	-	-	-	-	-	70
HP51	++	++	++	++	+	±	-	-	-	-	90
HP53	++	++	++	++	+	±	-	-	-	-	80
LP1	++	++	++	+	±	-	-	-	-	-	70
LP2	++	++	++	++	+	±	-	-	-	-	80
LP3	++	++	++	+	±	-	-	-	-	-	70
LP4	++	++	++	++	+	±	-	-	-	-	90
LP8	++	++	++	++	+	±	-	-	-	-	80
LPI6	++	++	++	+	±	-	-	-	-	-	70
LPI7	++	++	++	++	+	±	-	-	-	-	90
LPW5	++	++	++	++	+	±	-	-	-	-	70

Key: ++ Heavy growth (60 - 100%), + moderate growth (30 – 50%), ± slight growth (15 - 20%), - no growth (0%)



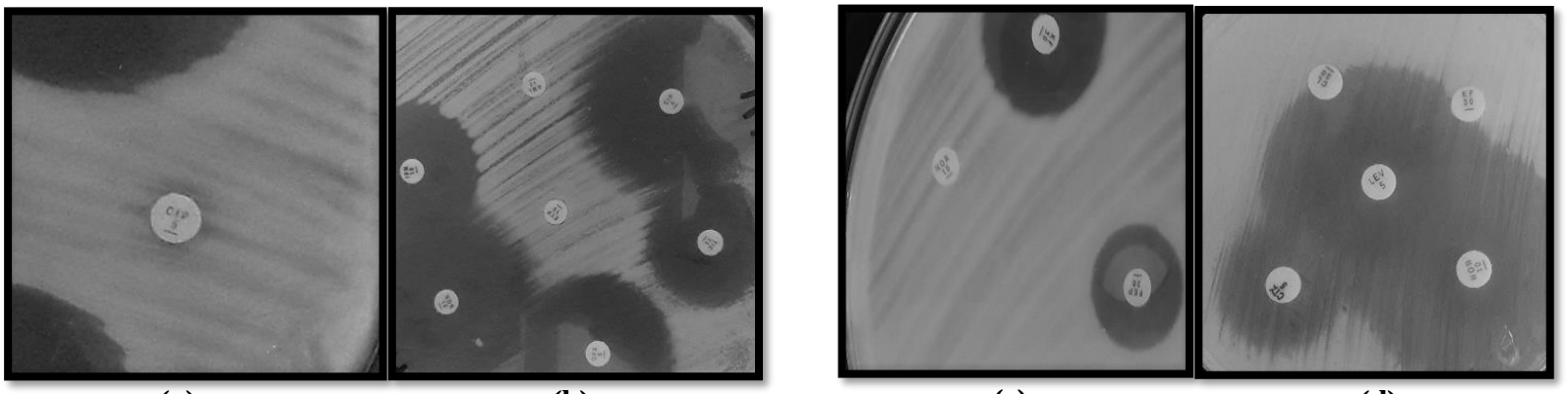
(a) **(b)**

Fig. 4.1 Effect of acridine orange on bacterial growth. (a) *Brevundimonas diminuta* (HP21) growth at Acridine Orange 75 µg/ µL concentration (b) Cured bacterial colonies utilized for subsequent MHA lawn formation for antibiotic susceptibility

Pre and post-plasmid curing susceptibility testing and MAR indexes

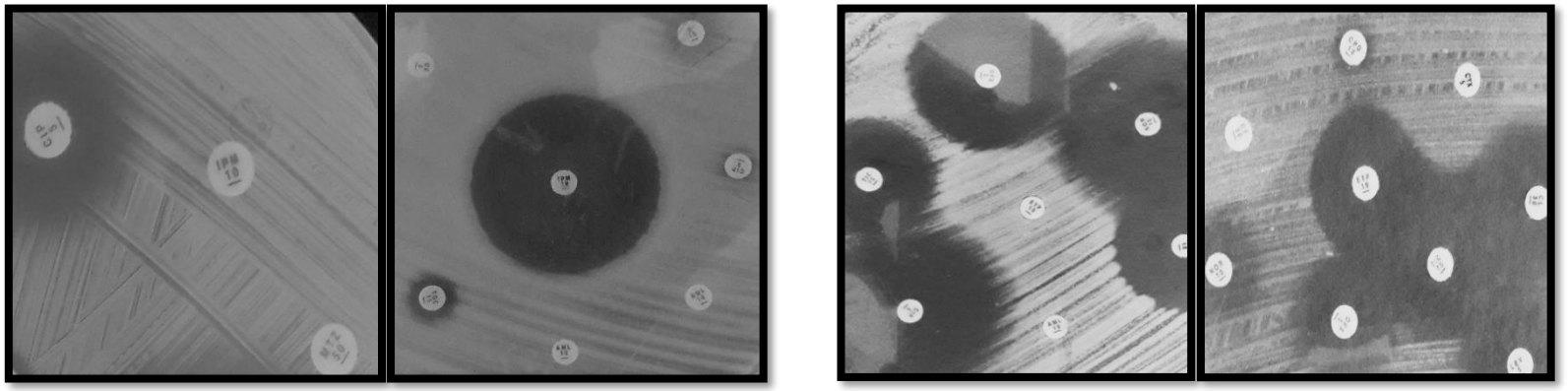
The antibiotic susceptibility profile was evaluated post-plasmid curing to find out changes in susceptibility patterns and to inspect for cured bacteria. Based on pre and post-antibiotic susceptibilities pattern differences, our findings showed that among gram-negative bacteria 21/43 (48.83%) whereas, 7/22 (31.81%) gram-positive were found with no change in susceptibility patterns. Post-plasmid treatment with AO, the investigated bacteria showed increased susceptibility to all prior resistant antibiotics except to aztreonam, linezolid, tigecycline, and metronidazole whose susceptibility patterns remained the same pre and post-curing. Effects of AO treatment on bacteria with the formation of zones of inhibition on MHA plates against antibiotics are shown in Fig. 4.2.1-2.5.

Among Fluoroquinolones, plasmid AO pre-treatment, susceptibilities range from 55% to 78.94% but post-treatment ranges from 89.47% to 100% for gram-positive. Gram-positive bacteria gained 100% susceptibility to levofloxacin and ciprofloxacin after AO treatment. For gram-negative pre and post susceptibilities ranges were 73.17% to 92.85% and 87.80% to 100%, respectively where 100% susceptibility was achieved against levofloxacin after curing. Penicillins were susceptible to glacier bacteria AO pre-treatment (4.76% to 95.23%) and post (28.57% to 100%) where the piperacillin susceptibility was 100% post-treatment. Cephalosporins against gram-positive bacteria were 18.18% to 63.63% susceptible initially but subsequently achieved 38.09% to 80% susceptibilities. Meanwhile, for gram-negative bacteria sensitivity values were 4.65% to 70.73% (AO pre-treatment) and 18.60% to 78.04% (AO post-treatment). Carbapenems were highly efficient against the tested bacteria. Gram-positive bacteria susceptibility ranged from 45.45% to 90.90% before plasmid curing but increased from 77.27% to 100% afterward. Against gram-negative bacteria increased susceptibilities patterns were observed from (51.21% to 88.09%) to (60.97% to 90.47%). Only imipenem among gram-positive bacteria achieved 100% susceptibility post-treatment. Similarly, erythromycin and vancomycin also showed increased susceptibility percentages after bacteria AO treatment. Antibiotic susceptibility profiles of bacteria pre and post-AO treatment are shown in (Table 4.3).



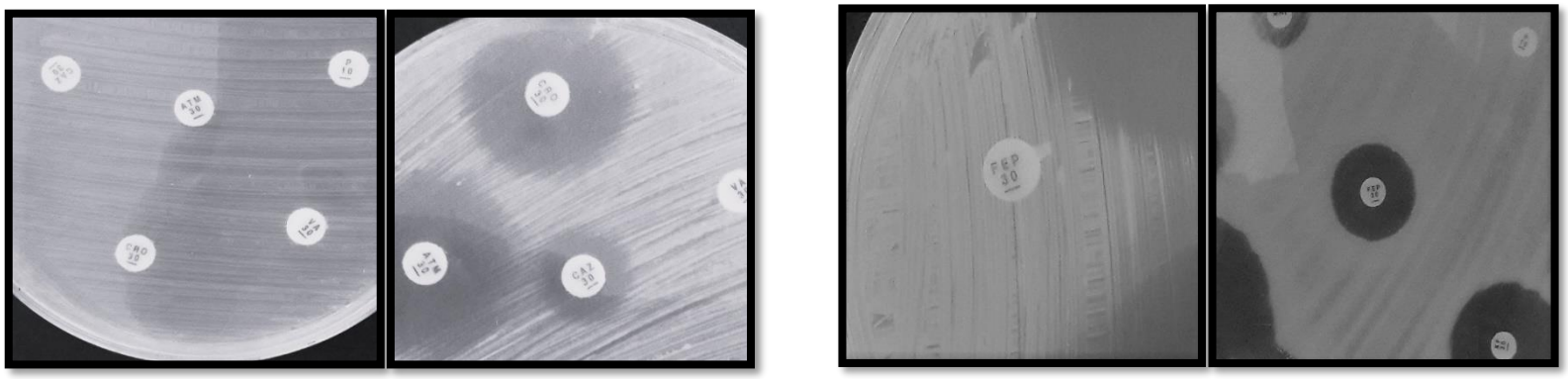
(a) (b) (c) (d)

Fig. 4.2.1 Effect of acridine orange on bacteria with the formation of zones of inhibition against Fluoroquinolones. (a, b) CIP (Ciprofloxacin), (c, d) NOR (Norfloxacin)



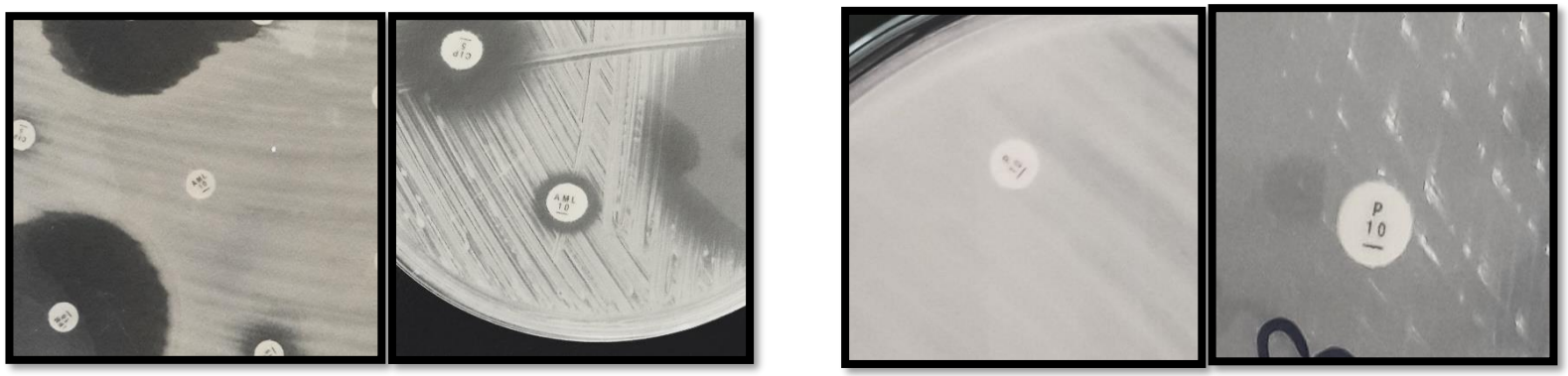
(a) (b) (c) (d)

Fig. 4.2.2 Effect of acridine orange on bacteria with the formation of zones of inhibition against Carbapenems. (a, b) IMP (Imipenem), (c, d) ETP (Ertapenem)



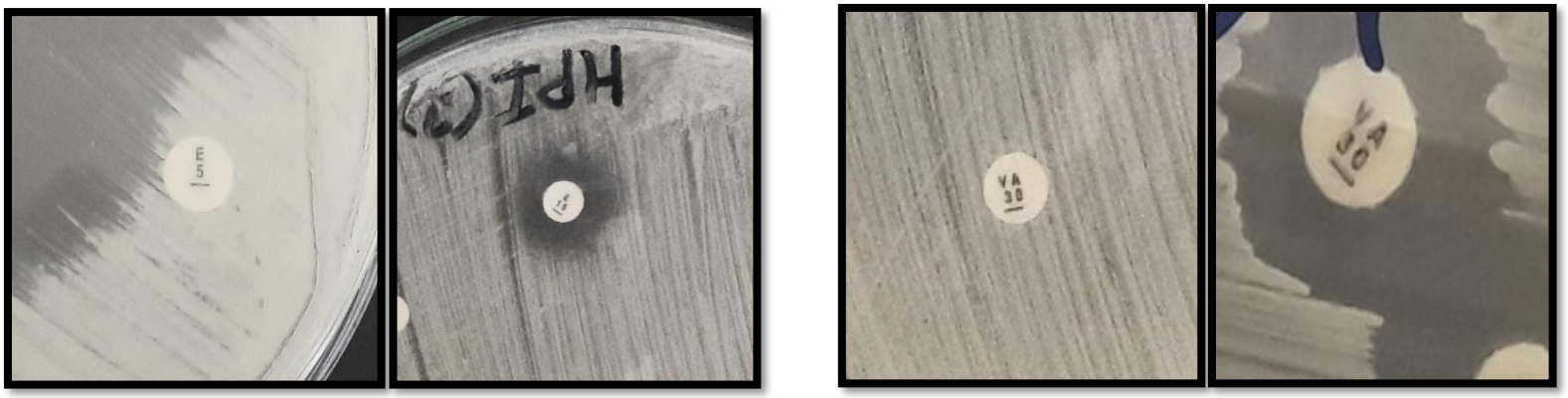
(a) (b) (c) (d)

Fig. 4.2.3 Effect of acridine orange on bacteria with the formation of zones of inhibition against Cephalosporins. (a, b) CRO (Ceftriaxone), (c, d) FEP (Cefepime)



(a) (b) (c) (d)

Fig. 4.2.4 Effect of acridine orange on bacteria with the formation of zones of inhibition against Penicillin. (a, b) AML (Amoxicillin), (c, d) P (Penicillin G)



(a) **(b)** **(c)** **(d)**

Fig. 4.2.5 Effect of acridine orange on bacteria with the formation of zones of inhibition against Macrolide, Glycopeptide. (a, b) E (Erythromycin), (c, d) V (Vancomycin)

Table 4.3 Antibiotic susceptibility profile of bacteria pre and post-acridine orange (curing agent) treatment

Antibiotics	Gram-positive				Gram-negative			
	Pre-plasmid curing		Post-plasmid curing		Pre-plasmid curing		Post-plasmid curing	
	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)
Fluoroquinolones								
Levofloxacin	15 (78.94)	4 (21.05)	19 (100)	0 (0)	39 (92.85)	3 (7.14)	42 (100)	0 (0)
Ciprofloxacin	15 (75)	5 (25)	20 (100)	0 (0)	35 (89.74)	4 (10.25)	38 (97.43)	1 (2.56)
Ofloxacin	14 (70)	6 (30)	19 (95)	1 (5)	33 (89.18)	4 (10.81)	36 (97.29)	1 (2.70)
Norfloxacin	11 (55)	9 (45)	19 (95)	1 (5)	30 (73.17)	11 (26.82)	36 (87.80)	5 (12.19)
Nalidixic acid	11 (57.89)	8 (42.10)	17 (89.47)	2 (10.52)	30 (78.94)	8 (21.05)	34 (89.47)	4 (10.52)
Penicillins								
Amoxicillin	1 (4.76)	20 (95.23)	6 (28.57)	15 (71.42)	NA	NA	NA	NA
Penicillin G	3 (13.63)	19 (86.36)	8 (36.36)	14 (63.63)	NA	NA	NA	NA
Carbenicillin	9 (42.85)	12 (57.14)	12 (57.14)	9 (42.85)	NA	NA	NA	NA
Ticarcillin	7 (36.84)	12 (63.15)	9 (47.36)	10 (52.63)	NA	NA	NA	NA
Oxacillin	3 (13.63)	19 (86.36)	8 (36.36)	14 (63.63)	NA	NA	NA	NA
Piperacillin	20 (95.23)	1 (4.76)	21 (100)	0 (0)	NA	NA	NA	NA
Cephalosporins								
Ceftriaxone	13 (65)	7 (35)	16 (80)	4 (20)	29 (70.73)	12 (29.26)	32 (78.04)	9 (21.95)
Cefotetan	7 (31.81)	15 (68.18)	13 (59.09)	9 (40.90)	21 (50)	21 (50)	23 (54.76)	19 (45.23)
Cephalexin	7 (33.33)	14 (66.66)	12 (57.14)	9 (42.85)	5 (11.90)	37 (88.09)	11 (26.19)	31 (73.80)
Cephalothin	5 (22.72)	17 (77.27)	11 (50)	11 (50)	2 (4.65)	41 (95.34)	8 (18.60)	35 (81.39)
Cefepime	11 (50)	11 (50)	13 (59.09)	9 (40.90)	23 (60.52)	15 (39.47)	26 (68.42)	12 (31.57)
Ceftazidime	13 (59.09)	9 (40.90)	15 (68.18)	7 (31.81)	23 (56.09)	18 (34.90)	25 (60.97)	16 (39.02)
Cephazolin	4 (18.18)	18 (81.81)	10 (45.45)	12 (54.54)	3 (6.97)	40 (93.02)	9 (20.93)	34 (79.06)
Cefpodoxime	4 (19.04)	17 (80.95)	8 (38.09)	13 (61.90)	15 (35.71)	27 (64.28)	19 (45.23)	23 (54.76)
Ceftaroline	14 (63.63)	8 (36.36)	16 (72.72)	6 (27.27)	29 (70.73)	12 (29.26)	32 (78.04)	9 (21.95)
Carbapenems								
Meropenem	13 (59.09)	9 (40.90)	17 (77.27)	5 (22.72)	21 (51.21)	20 (48.78)	25 (60.97)	16 (39.02)
Imipenem	20 (90.90)	2 (9.09)	22 (100)	0 (0)	37 (88.09)	5 (11.90)	38 (90.47)	4 (9.52)
Ertapenem	10 (45.45)	12 (54.54)	17 (77.27)	5 (22.72)	22 (53.65)	19 (46.34)	27 (65.85)	14 (34.14)
Monobactam								
Aztreonam	NA	NA	NA	NA	19 (45.23)	23 (54.76)	19 (45.23)	23 (54.76)
Oxazolidinones								
Linezolid	5 (22.72)	17 (77.27)	5 (22.72)	17 (77.27)	NA	NA	NA	NA
Macrolide								
Erythromycin	5 (23.80)	16 (76.19)	8 (38.09)	13 (61.90)	6 (14.28)	36 (85.71)	14 (33.33)	28 (66.66)
Glycopeptide								
Vancomycin	4 (18.18)	18 (81.81)	7 (31.81)	15 (68.18)	NA	NA	NA	NA
Glycycline								
Tigecycline	10 (76.92)	3 (23.07)	10 (76.92)	3 (23.07)	25 (78.12)	7 (21.87)	25 (78.12)	7 (21.87)
Nitroimidazole								
Metronidazole	0 (0)	21 (100)	0 (0)	21 (100)	0 (0)	43 (100)	0 (0)	43 (100)

Key: S; Sensitive, I; Intermediate, R; Resistant, NA; not applicable

In addition, after AO treatment, there was observed a significant decrease in MAR indexes from 87.69% to 65.31% bacteria with >0.2 values. The massive decline was observed for *Staphylococcus equorum* (HP19), *Leucobacter aridicollis* (HP22) and *Arthrobacter psychrochitiniphilus* (LP2) (0.7 to 0.2 each), *Serratia marcescens* (HP50) (0.6 to 0.1), *Flavobacterium antarcticum* (HP20) (0.6 to 0.2), *Flavobacterium saliperosum* (HP8) (from 0.5 to 0.1) and *Brevundimonas diminuta* (HP21) (0.7 to 0.4). MAR indexes pre and post-AO treatment are recorded in (Table 4.1).

Results obtained stipulated that plasmid-cured bacteria exhibited lower resistances and MAR indexes, due to plasmid elimination by AO treatment, which designated that several bacterial species herein studied, harbored plasmid-encoded antibiotic-resistant genes (ARGs) and loss of plasmid resulted in subsequent loss of phenotypic antibiotic resistances.

Statistical analysis

To verify the significant association between resistant antibiotic classes, tested bacteria and pre-pro plasmid curing antibiotic susceptibilities ranges, data were scrutinized using R software (version 3.3.3), and *chi-square*, *df*, and *p-values* were determined. A *Chi-square* value less than the *p-value* was considered significant data. For gram-positive only oxazolidinones had a *p-value* greater than *chi-square* with significant data, whereas Monobactam, Macrolide, and Glycycline yielded significant data with a *p-value* greater than *chi-square*, among gram-negative isolates (Table 4.4).

Table 4.4 Glacier bacteria antibiotic susceptibility data statistical analysis (*Chi-square, degree of freedom,* and *p-value*)

Antibiotic Class	**Gram-positive**						**Gram-negative**					
	Pre-plasmid curing			**Post-plasmid curing**			**Pre-plasmid curing**			**Post-plasmid curing**		
	Chi-square	df	p-value	Chi-square	df	p-value	Chi-square	df	p-value	Chi-square	df	p-value
Fluoroquinolones	4.5343	4	0.3385	20.911	4	0.0003298	4.7678	4	0.312	5.266	4	0.2611
Penicillins	51.136	5	8.11e-10	24.66	5	0.0001621	NA	NA	NA	NA	NA	NA
Cephalosporins	36.303	8	1.546e-05	17.023	8	0.02988	45.186	8	3.392e-07	37.903	8	7.846e-06
Carbapenems	9.5143	2	0.00859	2.1951	2	0.3337	1.2222	2	0.5427	2.0625	2	0.3566
Monobactam	NA	NA	NA	NA	NA	NA	0.090909	1	0.763	0.090909	1	0.763
Oxazolidinones	0.090909	1	0.763	15	1	0.0001075	NA	NA	NA	NA	NA	NA
Macrolide	11.267	1	0.000789	8.3333	1	0.003892	7.3636	1	0.006656	0.090909	1	0.763
Glycopeptide	15	1	0.000107	5.4	1	0.02014	NA	NA	NA	NA	NA	NA
Glycycline	5.4	1	0.02014	5.4	1	0.02014	0.090909	1	0.763	0.090909	1	0.763
Nitroimidazole	15	1	0.000107	15	1	0.000107	11	1	0.000911	11	1	0.000911

Key: df; degree of freedom, NA; not applicable

Discussion

For functional analysis of bacterial plasmids, the elimination of plasmids with the aid of a curing agent is an essential step. To eliminate plasmids, several physical methods and chemical agents like; acridine orange, acriflavine, and ethidium bromide are widely used (Rezaee et al. 2007; Trevors 1986; Clowes 1972). Acridine orange acts on plasmid by preferential inhibition of the DNA gyrase activity responsible for plasmid replication (Spengler et al. 2006). The efficacy of curing agents mostly depends on host bacteria and the size/type of plasmids because only a few perform better with desirable outcomes (Imre et al. 2006). The tested glaciated bacteria were subjected to different concentrations of AO and exhibited different growth patterns. AO concentration <75 µg/mL was non-lethal for the majority of tested bacteria whereas gram-negative (17/43, 39.53%) and gram-positive (15/22, 68.11%) were able to retain their slight growth (15 to 20%) at 75 µg/mL. At higher concentrations (100 to 200µg/mL), no bacteria were inspected with any visible growth. Lack of growth at higher concentrations may be due to the toxic effect of AO on bacteria growth by inhibiting replication (Spengler et al. 2006). The highest MIC values were recorded for gram-positive bacteria in comparison to gram-negative. *Leucobacter aridicollis* (HP1), *Staphylococcus saprophyticus* (HP7), *Bacillus xiamenesis* (HP14), *Alcaligenes faecalis* (HP16), *Brevundimonas diminuta* (HP21) and *Acinetobacter calcoaceticus* (LP5) were among the strains which tolerated the highest (75 µg/mL) of acridine orange. The recorded MIC values in the current study were in comparison to those reported by Rezaee et al. (2007) for two curing agents, 80 to 90 µg/mL for AO and 70 to 90 µg/mL for acriflavine while curing *E. coli* strains. Moreover, Zaman et al. (2010) cured bacteria plasmids at an AO concentration of 75 µg/mL with a low frequency of cured strains in comparison to our findings.

Ethidium bromide as a plasmid-curing agent inhibits plasmid replication and is effectively used for curing bacterial plasmids. Mbim et al. (2016) achieved cured bacterial strains at 50 to 300 µl of ethidium bromide concentration where *Pseudomonas aeruginosa*, *E. coli,* and *Proteus sp.* retained their growth at 250 µl and only *Pseudomonas aeruginosa* were able to grow at 300 µl concentration of curing agent. In this case, the highest concentrations of curing agent might be due to the testing of clinical strains for plasmid elimination as clinical bacteria is more adapted to antibiotics and curing agents, but this seems to be not true in all cases. Dasmeh et al. (2015) reported lower MIC values for clinical *Acinetobacter baumanii* strains, treated with AO, in

comparison to our results. Furthermore, Zaman et al. (2010) compared the curing efficacies of AO, sodium dodecyl sulfate, and ethidium bromide against clinical *E. coli* plasmids and reported the comparable AO MIC values to our findings and further evaluated ethidium bromide as a more effective curing agent to eliminate plasmids among different bacteria. It is generally considered that plasmid curing experiments work on a trial and error basis and usually depend on culture conditions and curing agent. Moreover, non-specificity and selectivity for different strains mainly contribute to screening out an ideal curing agent (Zaman et al. 2010; Hohn and Korn 1969).

After AO treatment, bacteria were subjected to antibiotic susceptibility testing to evaluate post plasmid curing susceptibility profile. Our findings showed that both gram-positive and negative bacteria showed a decrease in susceptibility profiles AO post-treatment. When antibiotic susceptibilities differences were compared, among gram-negative 21/43 (48.83%), whereas, 7/22 (31.81%) gram-positive bacteria had no change in antibiotic susceptibility pattern. Moreover, for linezolid, tigecycline, and metronidazole there were no differences in susceptibility profile, pre- and post-AO treatment, suggesting that either plasmid is not eliminated or resistant genes are located on bacteria chromosomes. It is frequently reported that most bacteria have intrinsic resistant mechanisms where ARGs are chromosomally encoded which enable bacteria to confer resistance against antibiotics (Raghada et al. 2013; Zaman et al. 2010; Spengler et al. 2006).

On the contrary, in our study, a major susceptibility decrease was reported against norfloxacin, nalidixic acid (Fluoroquinolones), and ertapenem (Carbapenems). Gram-positive bacteria achieved 100% susceptibilities against levofloxacin, ciprofloxacin, piperacillin, and imipenem after AO treatment, whereas gram-negative bacteria were found 100% susceptible to levofloxacin. In support of our findings, Dasmeh et al. (2015) successively cured all bacteria, treated with AO, where ceftazidime achieved (100%) susceptibility and major resistance decline was reported against meropenem (78.2%) and cefotaxime (82.6%). Similarly, higher antibiotic susceptibility values for unasyn, streptomycin, metronidazole, ciprofloxacin, pefloxacin, ceftriaxone, cefotaxime, and cefuroxime have been reported by Orhue et al. (2017) after bacteria AO treatment except for ampicillin, ampicillin-clavulanate, nitrofurantoin, and erythromycin. Moreover, Onyeadi and Agbagwa (2019) also reported the successful AO curing of *E. coli* plasmids and antibiotic resistances were reduced drastically but overall results were

in contradiction to our study. The susceptibilities decrease after post-plasmid treatment with AO might be due to the elimination effect of the curing agent on bacteria plasmids, as described by Raghada et al. (2013).

Extrachromosomal, double-stranded plasmid exist independently inside bacteria (Majumdar et al. 2006) and harbor antibiotic-resistant markers, which confer resistant traits to host organism and other counterparts via HGT (Kazeroon 2015; Montefour et al. 2008; Ghosh et al. 2000; Datta and Hedges 1972). Plasmids induce the production of *ß-lactamases* which confer co-resistance to ß-lactamase and other antibiotics like; aminoglycosides and quinolones (Amaya et al. 2012) that make bacteria multi-resistant. This describes the correlation that exists between the plasmid and multiple resistances. Decrease in susceptibility degrees, post-plasmid curing, manifest that some ARGs were located on plasmids (Subbiah et al. 2017; Raj 2012). While comparing the post-antibiotic susceptibility patterns of the current study, gram-positive bacteria were more prone to plasmid loss in comparison to gram-negative. Bacteria treated with AO can be deprived of plasmids with small sizes while disrupting the structural integrity of larger plasmids in some cases (Zaman et al. 2010). Considering the susceptibilities differences pre and post-plasmid curing, it may be concluded that either several bacterial plasmids were not cured or some bacteria contained no plasmid with encoded ARGs.

MAR index values of plasmid-cured bacteria decreased drastically. AO pre-treatment, 87.69% of bacteria had >0.2 MAR indexes whereas 65.31% had MAR indexes >0.2, afterward. A decrease in MAR indexes might be due to the loss of plasmids, harbored with antibiotic-resistant elements. Plasmids are possessed with numerous genetic elements that code for antibiotics and heavy metal resistance, antimicrobial biosynthesis, and metabolic pathways (Kazeroon 2015). Associated characteristics are highly transferrable through HGT among diverse bacteria and the elimination of such plasmids deprives bacteria of the aforementioned properties (Ghosh et al. 2000; Datta and Hedges 1972). In our study, a drastic decrease in MAR index values was observed for *Staphylococcus equorum* (HP19) (0.7 to 0.1), *Flavobacterium saliperosum* (HP8) (from 0.5 to 0.1), *Leucobacter aridicollis* (HP22) and *Arthrobacter psychrochitiniphilus* (LP2) (0.7 to 0.2 each), *Flavobacterium antarcticum* (HP20) (0.6 to 0.2), *Serratia marcescens* (HP50) (0.6 to 0.1) and *Brevundimonas diminuta* (HP21) (0.7 to 0.4), suggesting that these bacteria contained plasmids, harbored with several ARGs for more than one antibiotic class, to which the bacteria were resistant before AO treatment.

Conclusion

The environmental psychrophilic bacteria isolated from the glacier were found to possess plasmid-mediated antibiotic resistances, based on post-AO plasmid curing antibiotic susceptibility verificatory assay. A significant decrease was observed in antibiotic susceptibility patterns and MAR indexes after plasmid treatment with AO. The presence of plasmid-borne antibiotic resistance and the promising donor behavior of environmental bacteria through HGT pose a global threat to public health and the environment and need to be addressed properly in pristine environments primarily in melting glaciers.

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Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

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

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Chapter 5. Integron integrases

Research article 3.

Title:

Sabir Nawaz . Muhammad Rafiq . Ian L. Pepper . Walter Q. Betancourt . Aamer Ali Shah . Fariha Hasan. Quantification of class 1 and 2 integron integrases abundance in bacteria isolated from glacier using real-time PCR

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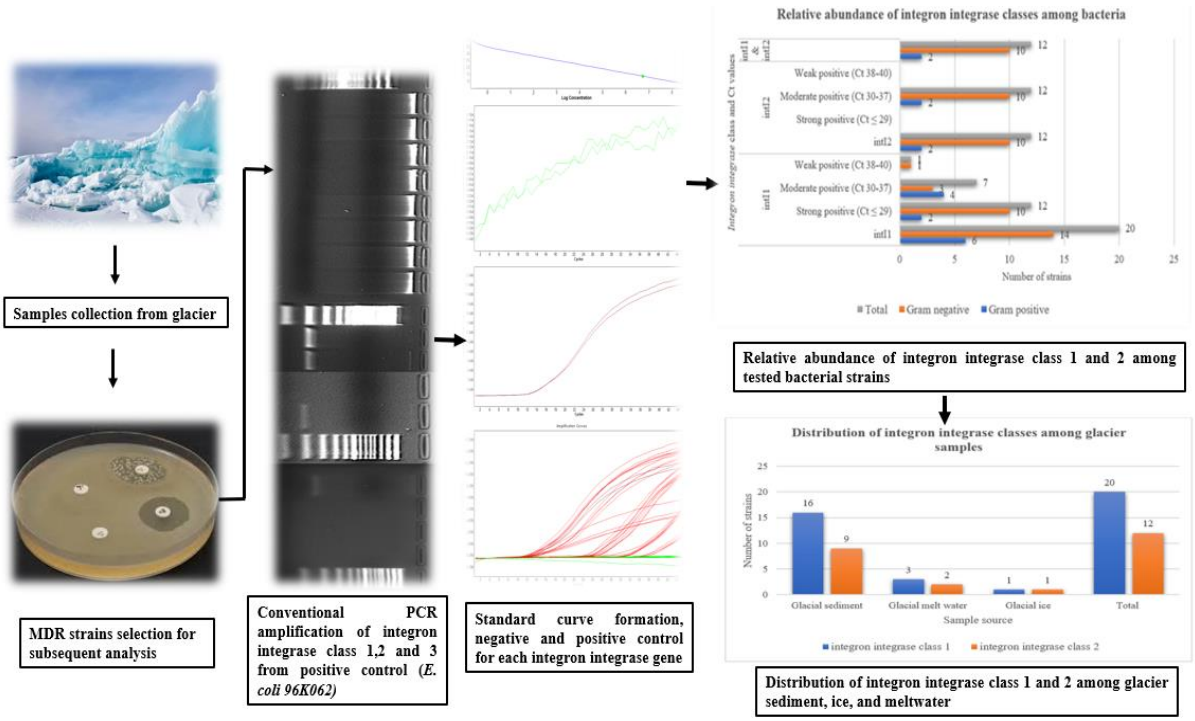
Quantification of class 1 and 2 integron integrases abundance in bacteria isolated from glacier using real-time PCR

Sabir Nawaz . Muhammad Rafiq . Ian L. Pepper . Walter Q. Betancourt . Aamer Ali Shah . Fariha Hasan

Abstract

Integrons are genetic contents that facilitate the acquisition and dissemination of antibiotic-resistant elements in bacteria, thereby enhancing their potential for adaptation. The study objective was to detect and quantify different classes of integron integrase genes through real-time PCR assay, among 28 multidrug-resistant bacteria, isolated from glacier sediment, ice, and meltwater. Conventional PCR amplicons of integron integrase class 1, 2, and 3 from positive control strain *E. coli 96K062* were serially diluted, and standard curves were prepared and subsequently used as positive controls for real-time PCR quantification. Fluorescence threshold values for probes were 2000 for FAM fluorophore, 1000 for Texas Red, and 500 for Cy5. Overall, 20 strains (71.4%) were positive for integron integrase class 1, and 12 (42.8%) for integron integrase class 2, whereas all were negative for integron integrase class 3. Gram-negative bacteria were more prevalent with integron integrase class 1 (14, 70%) and integron integrase class 2 (10, 83.3%) with the lowest Ct (cycle threshold) values. *IntI1*-positive bacterial strains with strong gene copy/µL (Ct value ≤ 29) were 12 (60%), while all *intI2*-positive bacteria had Ct > 29. Among the total 12 (42.8%) bacterial strains, gram-negative 10 (35.7%) while gram-positive 2 (7.1%) harbored both *intI1* and *intI2* genes. Glacial sediment bacteria were more abundant in *intI1* (16, 80%) and *intI2* (9, 75%), followed by water and ice. The higher prevalence of *intI1* and *intI2* among psychrophilic bacteria manifests their alarmable ubiquitous presence in anthropogenic-free glacier environments and requires featured scrutiny.

Graphical abstract



Keywords: Glacier environment . integron integrase gene . multidrug-resistant bacteria . real-time PCR

Introduction

Predominately, antibiotic resistance originates in natural environments under stressful conditions and is an extremely ancient phenomenon as massive antibiotic-resistant genes (ARGs) from 30,000 old permafrost sediments have been reported (D'Costa et al. 2011; Allen et al. 2009). Microbial antibiotic production is a life-sustaining strategy to compete for nutrients and other resources in oligotrophic environments (Zhang et al. 2018). A diverse range of cold-adapted bacteria and fungi have been found flourishing in cold challenging environments including glaciers (Zawierucha et al. 2018; Cook et al. 2016). Bacteria inhabiting natural pristine cold environments are the potent reservoir of antibiotic-resistant elements (Van Goethem et al. 2018; Segawa et al. 2013; Ushida et al. 2010). On the contrary, the

anthropogenic effect majorly contributes to the expeditious evolution and dissemination of antibiotic-resistant elements in natural ecosystems (Hawkey 2008). In natural environments, the rapid ongoing antibiotic resistance evolution and dissemination among a diverse set of bacteria is mainly due to horizontal gene transfer (HGT) phenomena and a complex process that involves various mechanisms like; mutations, transfer of ARGs via exchangeable mobile genetic elements (MGEs) such as conjugative plasmids and transposons, embedded with insertion sequences and integrons, which greatly enhance their mobility and play an essential role in the emergence of multidrug resistance (Abraham et al. 2014; Barlow et al. 2004).

Integrons, reported in the 1980s, are conserved powerful mobile genetic sequences that can capture and successfully integrate gene cassettes, coding for antibiotic resistances, to plasmids, chromosomes, and pathogenicity islands through site-specific recombination. Besides antibiotic resistance, gene cassettes also code for transposases, phosphatases, esterases, and transporter proteins (Stalder et al. 2012). Gene cassettes are DNA features that encode resistant elements with recombination site *attC* (59 base elements), recognized by the *integrase* (Domingues et al. 2012). Initially, integrons were confined to clinical strains, although they have been recently reported in other non-clinical environments like; wastewater, river water, arctic soil, permafrost, and glaciers, yet their ultimate spread and abundance in frozen alpine environments are still to be evaluated (Makowska et al. 2020; McCann et al. 2019; Domingues et al. 2012; Peymani et al. 2012; Zhang et al. 2009; Bennett 2008). Intensive misuse of antibiotics in human medicine, agriculture, and farming greatly enhances bacteria antibiotic selective pressure resulting in a swift spread of ARGs and integrons through HGT and declared as biotic pollution and an alarm ecological problem. The contamination of natural ecosystems with ARGs and integrons harboring bacteria poses a potential threat related to public health (Segawa et al. 2013). Arguably, migrating birds, aerosol bacteria, and anthropogenic activities are most likely the key vehicles for ARGs and integrons delivery to the remote sites where they disseminate through HGT among the diverse bacterial communities (Tan et al. 2018; Segawa et al. 2013).

Integrons are composed of main three core segments. The first element *intI* gene encodes an enzyme *integrase* (*intI*) that belongs to the tyrosine recombinase family and catalyzes the site-specific recombination of exogenous gene cassettes (Messier and Roy 2001). The second core element is *attI*, an integron-associated recombination site, recognized by the *integrase*. The

third essential element, *integron-associated promotor (Pc)*, transcribes and expresses the recombined gene cassettes inside the *integron* region. The integrons have been classified into five classes, based on the variations of amino acid sequences within the enzyme *integrase* (Cambray et al. 2010). Integron integrase class 1 was the first widely described integron (Stokes and Hall 1989) and is found most prevalent with resistance gene cassettes and carries a diverse array of antibiotic resistance elements, which confer resistance against a group of antibiotics like β-lactams, aminoglycosides, fluoroquinolones, macrolide, chloramphenicol, erythromycin and majorly contribute to the emergence of multi-drug resistant bacteria (Deng et al. 2015; Gillings 2014; Cambray et al. 2010). Integron integrase class 1 is considered a marker to check the level of antibiotic resistance and proxy for anthropogenic contamination in a natural environment (Gillings 2017). Integron integrase class 1 comprised two conserved segments (3' and 5' conserved segments), including internal *gene cassettes*, encoded with exogenous ARGs (Lima et al. 2014). Integron integrase class 2 is found to exist abundantly with transposon family *Tn7* and encodes for *integrase 2* (*intI2*), recombination site (*aatI 2*), and *gene cassette* at 3' terminal. The integron integrase class 3 has a similar structure to class 2 integron and exists rarely in *Serratia marcescens, Pseudomonas aeruginosa*, *Klebsiella pneumonia*, and *Vibrio cholera* (Deng et al. 2015; Hansson et al. 2002).

The prevalence of integron integrase genes has been reported in pervasive distributions among bacteria thriving in cold polar regions (Makowska et al. 2020; McCann et al. 2019; Van Goethem et al. 2018; Segawa et al. 2013; Peymani et al. 2012; Ushida et al. 2010), but on the flip side, study on the occurrence of integrons among bacteria inhabiting non-polar cold regions is limited. Therefore, regarding the significance and involvement of integrons in the dissemination of ARGs, the current study aimed to screen out multidrug-resistant bacteria for the occurrence and abundance of integron integrase genes, isolated from non-polar Passu glacier.

Materials and methods

Bacterial strains

The current study was conducted on 28 environmental multidrug-resistant (MDR) bacterial strains isolated from different sources of a non-polar Passu glacier (glacial sediment, ice, and meltwater), in Pakistan. Bacterial strains with resistance to ≥ 3 different antibiotic classes and carriage of multiple ARGs were selected and screened out for occurrence and abundance of different classes of integron integrase genes through real-time PCR assay. The details of bacterial species isolation, identification, and antibiotic susceptibilities were described previously (Nawaz et al. 2023) and GeneBank accession number and BLAST similarities of MDR bacteria are shown in Table 5.1.

Bacterial DNA extraction

Genomic DNA Extraction (Gene JET Ko721, Thermo Scientific) was used to extract the PCR-competent genomic DNA. In the extraction procedure, 200 µl bacterial suspension with *Proteinase K* and *Lysozyme* (20 µl each), followed by 200 µl of Lysis solution, were thawed at 56°C for 30 minutes in a shaking water bath. In the next step, 400 µl of 50 % ethanol was properly mixed with the lysate solution by vertexing, and wash buffers were used to wash the samples. To elute the genomic DNA, 200 µl of elution buffer was used. To confirm and visualize the genomic DNA, 1.5% horizontal agarose gel electrophoresis with TAE buffer and 1g/ml ethidium bromide at 100 V and 40 minutes was performed (Meyers et al. 1976).

Conventional PCR for positive control

The *E. coli 96K062* strain was used as standard positive for integron integrase class 1, 2, and 3 genes. To amplify each integron fragment from positive control, genomic DNA along with specific primers were subjected to conventional PCR (Eppendorf Thermocycler). A final 30 µl PCR reaction mixture included 1 µL of each primer (Integrated DNA Technologies), 8 µl of nuclease-free water, 15 µl PCR 2x Master Mix (Dream Taq, Thermo Scientific) and 5 µl of template DNA. PCR amplification was performed under conditions: 1 cycle (95°C for 5 minutes), 35 cycles (95°C for 30 seconds, 60°C for 1 minute, 72°C for 50 seconds), and 1 cycle (72°C for 10 minutes). The primer sequences and PCR amplicon sizes are described in (Table 5.2). Desired PCR amplified products of integron integrase class 1, 2, and 3 genes were purified

using PCR Purification Kit (Gene JET, Thermo Scientific) and quantified with Qubit 3 Fluorometer and dsDNA HS Assay Kit (Invitrogen), for subsequent uses.

Standard curve formation

The standard curves for each integron integrase class were generated, to define the association between the input genomic concentration and cycle threshold (Ct) value. Ct value reflects the total number of fluorescent signal cycles required to exceed the background threshold level and is inversely proportional to the target nucleic acid concentration. For standard curve formation, standards of known genome copy numbers were prepared by serial dilution of appropriate respective purified PCR amplicons of each integron class. A 10 µl of each PCR product was added to 90 µl of Tris EDTA buffer in an Eppendorf tube and further serially diluted (10^{10} to 10^{0}). Real-time PCR for each standard curve was performed on Light Cycler 480 using a reaction mixture of 25 µL which included 12.5 µl reaction mixture, 2 µl forward and reverse primers, 1 µl probe, and 4.5 µl nuclease-free water. From each dilution, 5 µl was added to make the final volume of the reaction 25 µl.

Real-time quantitative PCR assay

For integron integrase class 1, 2, and 3, a real-time PCR assay was performed to find the desired genes occurrence and concentrations, in a total reaction volume of 25 µl, containing 12.5 µl light cycler reaction mixture, followed by 1 µl each forward and reverse primers, 1 µl each probe, 4.5 µl nuclease-free water and 5 µl of template DNA, respectively. real-time PCR program was run at 95°C for 10 minutes in an initial step, followed by 45 cycles at 95°C for 30 seconds and annealing temperature at 60°C for 1 minute with a run time of 1 hour and 45 minutes, for each integron integrase class. Fluorescence threshold values were 2000 for the *FAM fluorophore* (*intI1* probe), *1000 for Texas Red* (*intI2* probe) 500 for *Cy5* (*intI3* probe) (Table 5.2). All reactions were performed in duplicates, containing no template control. A sequence detection system (software v1.9.1, Applied Biosystems) was operated to analyze the results. The threshold fluorescence and baseline levels were adjusted to appropriate magnitudes. To assess the product specificity, melt curve analysis was performed (Dissociation curves v 1.0, Applied Biosystems).

Results

Multidrug-resistant bacterial species

Bacterial species were isolated from the Passu glacier previously and sequences were submitted to NCBI (GeneBank) to obtain accession numbers. A total of 28 bacteria were selected for the screening of different classes of integron integrase gene, based on multidrug resistivity, with phenotypic resistance to ≥ 3 disparate antibiotic classes and carriage of multiple ARGs. Antibiotic-resistant profiles and the abundance of numerous ARGs among glacier psychrophilic bacteria (used in this current study) were described previously (Nawaz et al. 2023). Among tested species 20 (71.4%) were gram-negative and 8 (28.5%) were gram-positive. The distribution of selected MDR bacterial strains among glacial samples was like this, 23 species (82.1%) were from glacial sediment, 1 (3.5%) from ice and 4 (14.2%) were from meltwater, respectively.

Table 5.1 List of GeneBank (NCBI) accession numbers and BLAST identities of multidrug-resistant glaciated bacteria

Sr.No.	Isolate Code	Accession Number	Homologous Species/Strains	Identity %	Querry Cover (%)
1	SUB10766215 HP2	OL721774	*Alcaligenes faecalis*	99.7	98%
2	SUB10766215 HP3	OL721775	*Rahnella inusitata*	100	100
3	SUB10766215 HP7	OL721779	*Staphylococcus saprophyticus*	99.3	99
4	SUB10766215 HP12	OL721783	*Flavobacterium suaedae*	90.3	100
5	SUB10766215 HP14	OL721785	*Bacillus xiamenesis*	100	100
6	SUB10766215 HP18	OL721789	*Bacillus pumilus*	99.6	100
7	SUB10766215 HP19	OL721790	*Staphylococcus equorum*	99.7	100
8	SUB10766215 HP20	OL721791	*Flavobacterium antarcticum*	93.7	99
9	SUB10766215 HP21	OL721792	*Brevundimonas diminuta*	99.2	99
10	SUB10766215 HP22	OL721793	*Leucobacter aridicollis*	99.6	100
11	SUB10766215 HP23	OL721794	*Leucobacter sp.*	99.2	100
12	SUB10766215 HP50	OL721796	*Serratia marcescens*	99.5	99
13	SUB10766215 HP51	OL721797	*Leucobacter komagatae*	99	100
14	SUB10766215 HP52	OL721798	*Rahnella inusitata*	99.5	100
15	SUB10766215 HP54	OL721800	*Brevundimonas bullata*	97.7	100
16	SUB10766215 HP55	OL721801	*Alcaligenes sp.*	99.7	100
17	SUB10766215 HP57	OL721803	*Acinetobacter baunamii*	98.9	97
18	SUB10766215 HP58	OL721804	*Serratia liquefaciens*	100	100
19	SUB10766215 LP2	OL721806	*Arthrobacter psychrochitiniphilus*	98.8	100
20	SUB10766215 LP5	OL721809	*Acinetobacter calcoaceticus*	100	100
21	SUB10766215 LP7	OL721811	*Pseudomonas psychrophila*	98.4	99
22	SUB10766215 LP10	OL721814	*Alcaligenes aquatilis*	99.5	100
23	SUB10766215 LP11	OL721815	*Pseudomonas fragi*	98.6	99

24	SUB10766215 HPI1	OL721816	*Serratia sp.*	98.7	99
25	SUB10766215 LPW4	OL721833	*Epilithonimonas lactis*	98.5	100
26	SUB10766215 LPW6	OL721835	*Pseudomonas brenneri*	100	100
27	SUB10766215 LPW7	OL721836	*Arthrobacter sp.*	96.3	99
28	SUB10766215 LPW8	OL721837	*Pseudomonas paralactis*	99.5	100

Table 5.2 Primers/Probes sequences for integron integrase class 1, 2, and 3 for Real-time PCR assay

Primers/ Probes	**Primer/Probe Sequences (5′ - 3′)**	**Annealing Temperature**	**Product Size**	**Location**	**Reference**
Primers					
intI1-LC1 *intI1-LC5*	GCC TTG ATG TTA CCC GAG AG GAT CGG TCG AAT GCG TGT	60°C	196	*intI1*	Barraud *et al.*, 2010
intI2-LC2 *intI2-LC3*	TGC TTT TCC CAC CCT TAC C GAC GGC TAC CCT CTG TTA TCT C	60°C	195	*intI2*	
intI3-LC1 *intI3-LC2*	GCC ACC ACT TGT TTG AGG A GGA TGT CTG TGC CTG CTT G	60°C	138	*intI3*	
Probes					
intI1-probe	(6-FAM) ATT CCT GGC CGT GGT TCT GGG TTT T (BHQ1)	-	-	*intI1*	Pavelkovich *et al.*, 2014
intI2-probe	(Texas Red) TGG ATA CTC GCA ACC AAG TTA TTT TTA CGC TG (BHQ2)	-	-	*intI2*	
intI3-probe	(Cy5) CGC CAC TCA TTC GCC ACC CA (BHQ3)	-	-	*intI3*	

Table 5.3 Antibiotic resistance profile of multidrug-resistant glaciated bacteria for Real-time qPCR quantification of class 1 and 2 *integron integrases*

Test Bacteria	**Antibiotic resistance profile**	**Resistant antibiotics (n,%)**
HP2 Alcaligenes faecalis	OFX,NOR,NA,CTT,CL,KF,KZ,CPD,E,TGC,MET	11/21 (52.3)
HP3 Rahnella inusitata	LEV,CTT,CL,KF,FEP,KZ,CPT,E,TGC,MET	10/21 (47.6)
HP7 Staphylococcus saprophyticus	LEV,AML,P,CAR,TIC,OX,CTT,CL,KF,KZ,CPD,LZD,E,VA,MET	15/28 (53.5)
HP12 Flavobacterium sp.	CIP,NOR,NA,CTT,CL,KF,KZ,CPD,E,MET	10/21 (47.6)
HP14 Bacillus xiamenensis	LEV,AML,P,CAR,TIC,OX,CTT,CL,KF,FEP,KZ,MRP,ETP,LZD,E,VA, MET	17/28 (60.7)
HP18 Bacillus pumilus	CIP,OFX,NOR,NA,AML,P,CAR,TIC,OX,CRO,CTT,CL,KF,KZ,CPD,LZD, E,VA,MET	19/28 (67.8)
HP19 Staphylococcus equorum	CIP,OFX,NOR,NA,AML,P,CAR,TIC,OX,CRO,CTT,CL,KF,CAZ,KZ,CPD, ETP,LZD,E,VA,TGC,MET	22/28 (78.5)
HP20 Flavobacterium antarcticum	OFX,NOR,CRO,CL,KF,FEP,KZ,CPD,CPT,IMP,ATM,E,TGC,MET	14/21 (66.6)
HP21 Brevundimonas diminuta	LEV,CIP,OFX,NOR,NA,CRO,CTT,CL,KF,KZ,CPD,ETP,ATM,E,TGC, MET	16/21 (76.1)
HP22 Leucobacter aridicollis	LEV,OFX,NOR,NA,AML,P,CAR,TIC,OX,CRO,CTT,CL,KF,KZ,CPD,	21/28 (75)

	CPT,ETP,LZD,E,VA,MET	
HP23 Leucobacter sp.	CIP,OFX,NOR,NA,AML,P,CAR,OX,CL,KF,KZ,CPD,ETP,LZD,E,VA, MET	17/28 (60.7)
HP50 Serratia marcescens	CRO,CTT,CL,KF,FEP,CAZ,KZ,CPD,CPT,MRP,ETP,ATM,E,MET	14/21 (66.6)
HP51 Leucobacter komagatae	AML,P,CAR,TIC,OX,CRO,CTT,CL,KF,FEP,CAZ,KZ,CPD,CPT,MRP, ETP,LZD,E,VA,MET	20/28 (71.4)
HP52 Rahnella inusitata	NA,CRO,CTT,CL,KF,FEP,CAZ,KZ,CPD,CPT,MRP,ETP,ATM,E,MET	15/21 (71.4)
HP54 Brevundimonas bullata	NA,CRO,CTT,CL,KF,FEP,CAZ,KZ,CPD,CPT,MRP,ETP,ATM,E,MET	15/21 (71.4)
HP55 Alcaligenes faecalis	NOR,CRO,CTT,CL,KF,FEP,CAZ,KZ,CPD,CPT,MRP,ETP,ATM,E,MET	15/21 (71.4)
HP57 Acinetobacter baumannii	CL,KF,CAZ,KZ,MRP,MET	6/21 (28.5)
HP58 Serratia liquefaciens	CL,KF,KZ,MRP,ETP,MET	6/21 (28.5)
LP2 Arthrobacter psychrochitiniphilus	OFX,AML,P,TIC,OX,CRO,CTT,CL,KF,FEP,CAZ,KZ,CPD,CPT,MRP, ETP,LZD,E,VA,TGC,MET	21/28 (75)
LP5 Acinetobacter calcoaceticus	CIP,CL,KF,FEP,CAZ,KZ,CPD,ETP,ATM,E,MET	11/21 (52.3)
LP7 Pseudomonas psychrophila	CTT,CL,KF,FEP,KZ,CPD,ATM,E,MET	9/21 (42.8)
LP10 Alcaligenes aquatilis	CIP,OFX,NOR,CAZ,CPD,CPT,MRP,ETP,ATM,E,TGC,MET	12/21 (57.1)
LP11 Pseudomonas fragi	LEV,CL,KF,FEP,CAZ,KZ,CPD,MRP,ATM,E,MET	11/21 (52.3)
HPI1 Serratia sp.	CRO,CTT,CL,KF,CAZ,KZ,CPD,CPT,MRP,ETP,ATM,E,MET	13/21 (61.9)
LPW4 Epilithonimonas lactis	KF,FEP,KZ,ETP,ATM,E,MET	7/21 (33.3)
LPW6 Pseudomonas brenneri	CRO,CTT,CL,KF,CAZ,KZ,CPD,CPT,MRP,ETP,ATM,E,MET	13/21 (61.9)
LPW7 Arthrobacter sp.	NOR,CRO,CTT,FEP,CAZ,CPD,MRP,IMP,ATM,MET	10/21 (47.6)
LPW8 Pseudomonas paralactis	NOR,NA,CL,KF,CAZ,KZ,CPD,ATM,E,MET	10/21 (47.6)

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.

Positive control and standard curve formation

The conventional PCR amplified desired fragments of integron integrase class 1, 2, and 3 genes were obtained and further utilized for real-time PCR assay as positive control and are shown in (Fig. 5.1). The purified product quantities recorded from Qubit Fluorometer were integron integrase class 1 (25.5 ng/µL), integron integrase class 2 (21.6 ng/µL) and integron integrase class 3 (17.3 ng/µL), respectively. Each positive control product was serially diluted (10^{10} to 10^{0}) and subjected to Light cycler amplification. The standard curves for integron integrase classes 1, 2, and 3 were formed and are recorded in (Fig. 5.2.1-2.3). The standard of 5.45E + 06 genome copy/µl was used as real-time PCR positive control for integron integrase class 1, 2, and 3 quantifications.

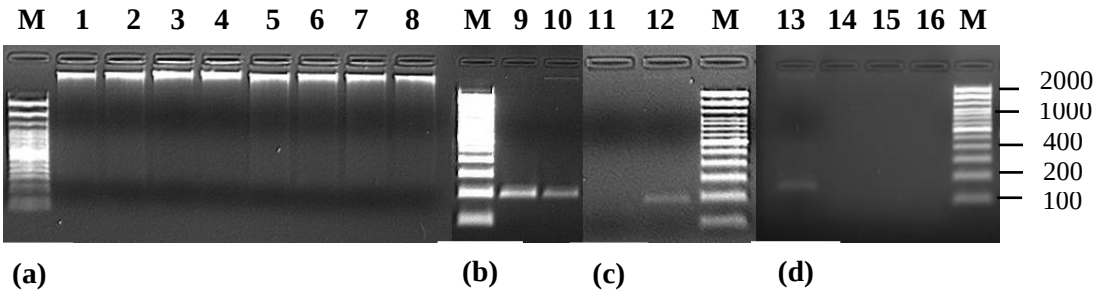


Fig. 5.1 (a) Genomic DNA (Lane 1 to 8) **(b)** PCR amplified bands of integron integrase class 1 (196bp) (Lane 9,10) **(c)** integron integrase class 2 (195bp) (Lane 12) **(d)** integron integrase class 3 (138bp) (Lane 13) and M (100 bp DNA Ladder, Invitrogen)

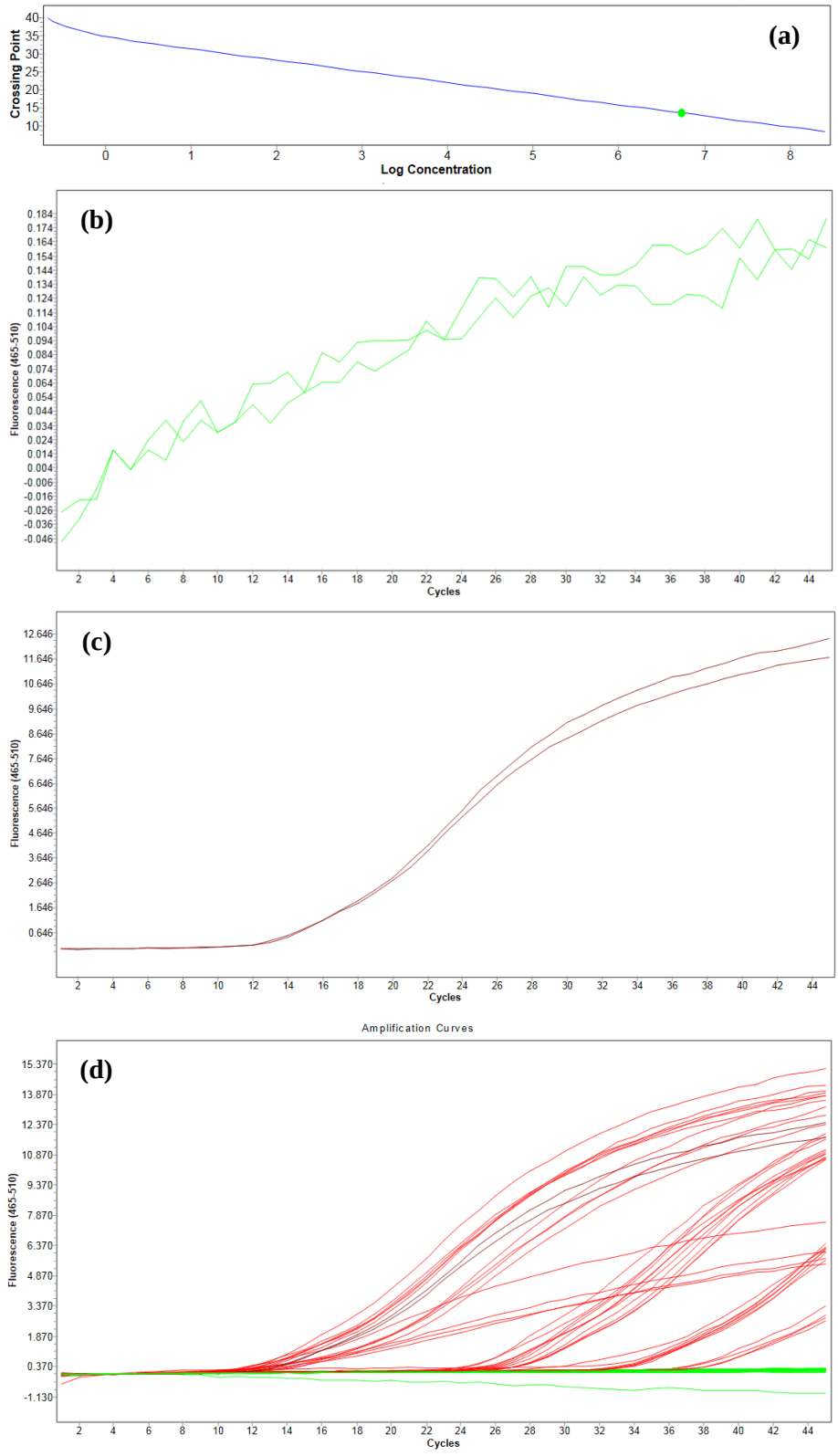


Fig. 5.2.1 Real-time PCR integron integrase class 1 assay **(a)** Standard curve **(b)** Negative control **(c)** Positive control **(d)** Amplification curves for positive strains

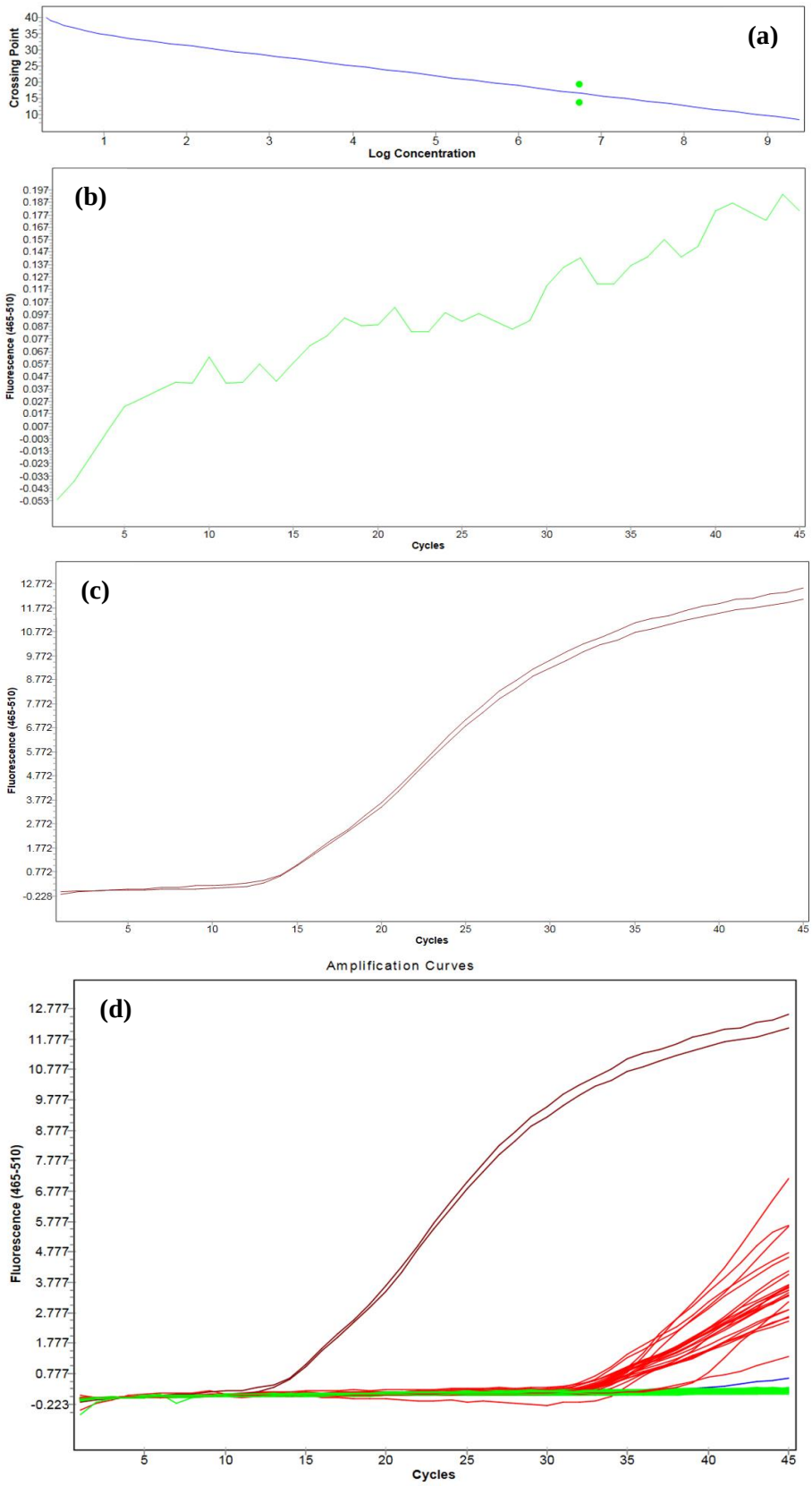


Fig. 5.2.2 Real-time PCR integron integrase class 2 assay **(a)** Standard curve **(b)** Negative control **(c)** Positive control **(d)** Amplification curves for positive strains

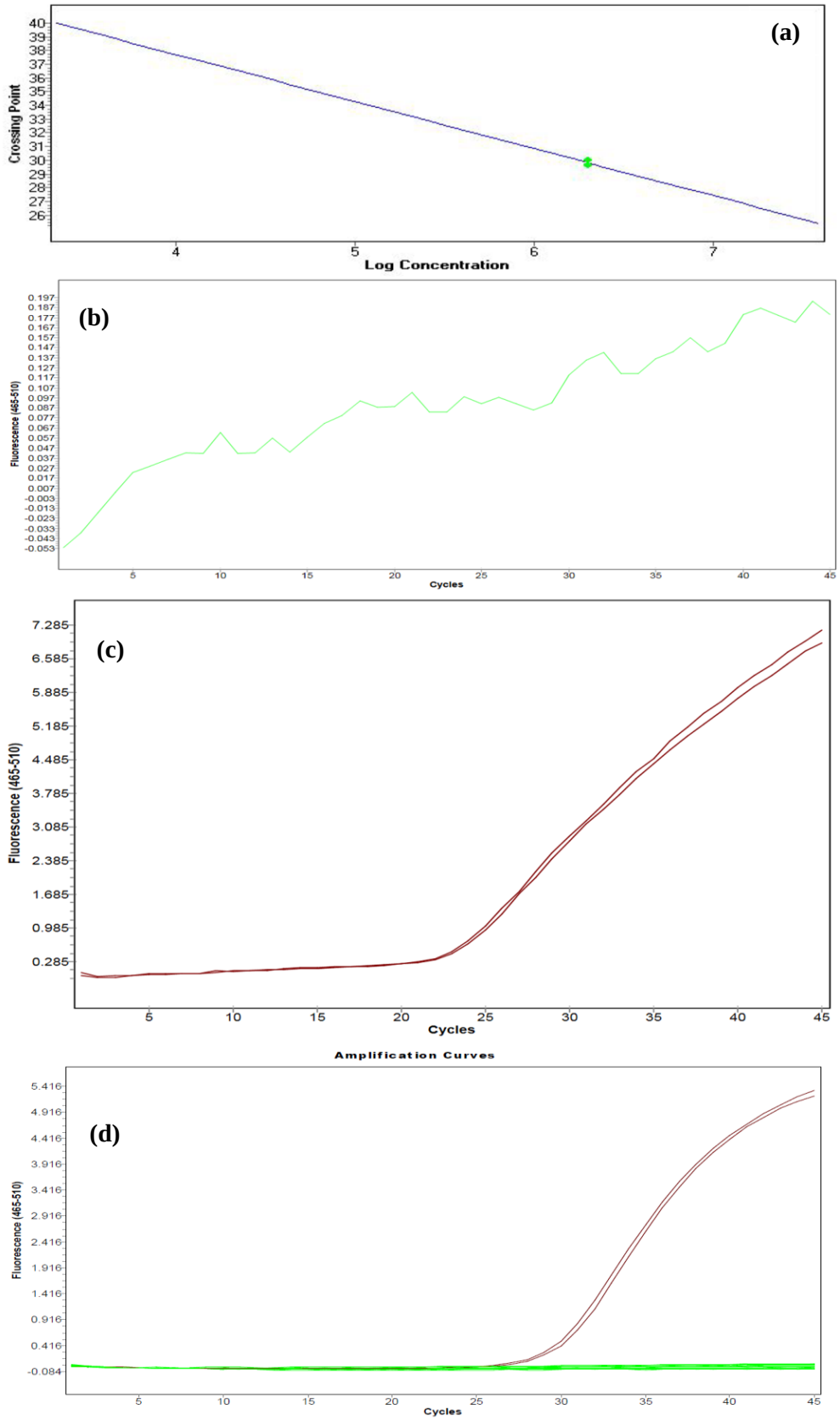


Fig. 5.2.3 Real-time PCR integron integrase class 3 assay **(a)** Standard curve **(b)** Negative control **(c)** Positive control **(d)** Amplification curves for the standard only (all strains were negative)

Real-time PCR assay for *integrons integrase* classes

Among tested bacteria, 20 (71.4%) were positive for integron integrase class 1, 12 (42.8%) for integron integrase class 2 whereas all strains were negative for integron integrase class 3. In comparison, gram-negative bacteria harbored *intI1* (14, 70%), and *intI2* (10, 83.3%), whereas gram-positive bacteria were detected with *intI 1* (06, 30%) and *intI2* (02, 16.6%). For *intI1*, strong positives with Ct values (Ct ≤ 29) were recorded for strains 12, 60% (gram negative 10 (50%), gram-positive 2 (10%)), while no strain was found with Ct ≤ 29 for *intI2*. All *intI2* positive strains had Ct > 29, indicating the moderate or minimal target nucleic acid in the samples. Relative abundance of integron integrase class 1 and 2, based on Ct values among tested strains are shown in (Fig. 5.3).

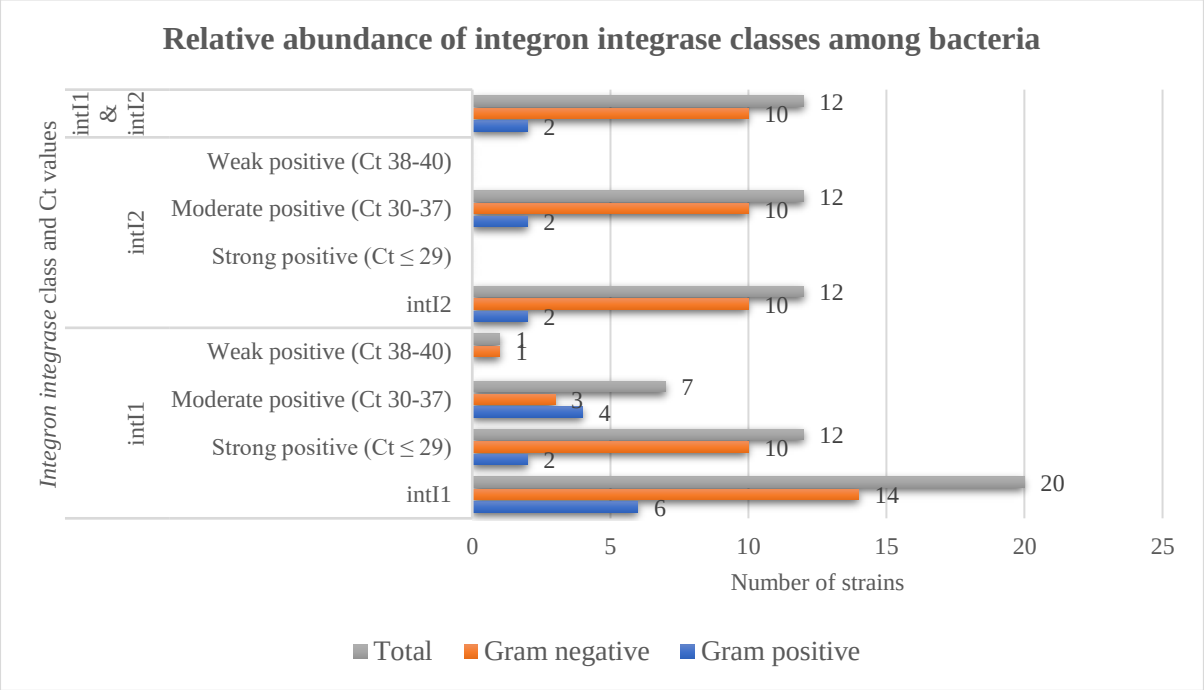


Fig. 5.3 Relative abundance of integron integrase class 1 and 2 among tested bacterial strains (based on Ct values)

Among gram-negative, samples with abundant *intI1* target nucleic acids were HP3 *Rahnella inusitata* (2.78E + 08, Ct = 11.2), followed by HP21 *Brevundimonas diminuta* (2.02E + 08, Ct = 11.6), HP50 *Serratia marcescens* (3.88E + 06, Ct = 16.9), HP57 *Acinetobacter baumannii* (3.22E + 05, Ct = 20.9) and LP5 *Acinetobacter calcoaceticus* (1.02E + 07, Ct = 13.1), respectively, whereas strain HP58 *Serratia liquefaciens* (2.02E + 00, Ct = 40) was found with minimal amount of *intI1* nucleic acid. Gram-positive bacteria with lowest Ct values for *intI1* were HP22 *Leucobacter aridicollis* (9.13E + 07, Ct = 12.7) and HP51 *Leucobacter komagatae* (1.73E + 05, Ct = 21.1) while with highest values were HP19 *Staphylococcus equorum* (1.01E + 00, Ct = 35.7) and HP14 *Bacillus xiamenensis* (1.26E + 01, Ct = 34.3). Moreover, for *intI2* lowest Ct values were found among HP3 *Rahnella inusitata* (6.37E + 00, Ct = 31.91), followed by HP50 *Serratia marcescens* (6.54E + 00, Ct = 31.8) and LP10 *Alcaligenes aquatilis* (7.90E + 00, Ct = 31.7) among gram-negative while strain HP22 *Leucobacter aridicollis* (3.93E + 00, Ct = 32.5) among gram-positive, respectively. Strains 12 (42.8%) harbored both *intI1* and *intI2* genes among which 10 (83.3%) were from gram-negative bacteria and 2 (16.6%) were from gram-positive. These strains included HP3 *Rahnella inusitata*, HP20 *Flavobacterium antarcticum*, HP21 *Brevundimonas diminuta*, HP22 *Leucobacter aridicollis***,** HP23 *Leucobacter sp.***,** HP50 *Serratia marcescens***,** HP52 *Rahnella inusitata*, LP5 *Acinetobacter calcoaceticus***,** LP10 *Alcaligenes aquatilis***,** HPI1 *Serratia sp.***,** LPW6 *Pseudomonas brenneri*, and LPW7 *Arthrobacter specie*. The quantitative real-time PCR Ct values of *intI1* and *intI2* genes for corresponding strains are recorded in (Table 5.4). Considering the distribution of integron integrase class 1 and 2 among sample sources, the glacial sediment strains were found more abundant in *intI1* (16, 80%) and *intI2* (9, 75%), followed by meltwater *intI1* (3, 15%) and *intI2* (2, 16.6%) and ice *intI1* (1, 5%) and *intI2* (1, 8.3%), respectively and are illustrated in (Fig. 5.4).

Table 5.4 Real-time PCR values of integron integrase class 1 and 2 for tested bacterial strains

Test strains	***intI1***		***intI2***		***intI3***
	Gene copy number/µl	**Ct value**	**Gene copy number/µl**	**Ct value**	
HP2 Alcaligenes faecalis	Negative	-	Negative	-	Negative
HP3 Rahnella inusitata	2.78E + 08	11.2	6.37E + 00	31.9	Negative
HP7 Staphylococcus saprophyticus	1.52E + 01	31.6	Negative	-	Negative
HP12 Flavobacterium sp.	Negative	-	Negative	-	Negative
HP14 Bacillus xiamenensis	1.26E + 01	34.3	Negative	-	Negative
HP18 Bacillus pumilus	Negative	-	Negative	-	Negative
HP19 Staphylococcus equorum	1.01E + 00	35.7	Negative	-	Negative
HP20 Flavobacterium antarcticum	2.93E + 03	26.6	4.67E + 00	32.3	Negative
HP21 Brevundimonas diminuta	2.02E + 08	11.6	5.51E + 00	32.1	Negative
HP22 Leucobacter aridicollis	9.13E + 07	12.7	3.93E + 00	32.5	Negative
HP23 Leucobacter sp.	4.37E + 01	29.8	2.88E + 00	33	Negative
HP50 Serratia marcescens	3.88E + 06	16.9	6.54E + 00	31.8	Negative
HP51 Leucobacter komagatae	1.73E + 05	21.1	Negative	-	Negative
HP52 Rahnella inusitata	7.05E + 01	29.2	3.06E + 00	32.9	Negative
HP54 Brevundimonas bullata	Negative	-	Negative	-	Negative
HP55 Alcaligenes faecalis	1.47E + 01	34	Negative	-	Negative
HP57 Acinetobacter baumannii	3.22E + 05	20.9	Negative	-	Negative
HP58 Serratia liquefaciens	2.02E + 00	40	Negative	-	Negative
LP2 Arthrobacter psychrochitiniphilus	Negative	-	Negative	-	Negative
LP5 Acinetobacter calcoaceticus	1.02E + 07	13.1	4.98E + 00	32.2	Negative
LP7 Pseudomonas psychrophila	Negative	-	Negative	-	Negative
LP10 Alcaligenes aquatilis	4.72E + 03	26	7.90E + 00	31.7	Negative
LP11 Pseudomonas fragi	Negative	-	Negative	-	Negative
HPI1 Serratia sp.	3.53E + 03	23.1	5.30E + 00	32.1	Negative
LPW4 Epilithonimonas lactis	1.16E + 00	35.4	Negative	-	Negative
LPW6 Pseudomonas brenneri	1.16E + 04	24.8	1.98E + 00	33.6	Negative
LPW7 Arthrobacter sp.	1.98E + 00	33.6	1.34E + 00	34.3	Negative
LPW8 Pseudomonas paralactis	Negative	-	Negative	-	Negative

Key: Ct value; The number of genomic cycles required to exceed the fluorescent signal across the threshold (background). Ct ≤ 29- strong positive, Ct 30 to 37- moderate positive, Ct 38 to 40- weak positive

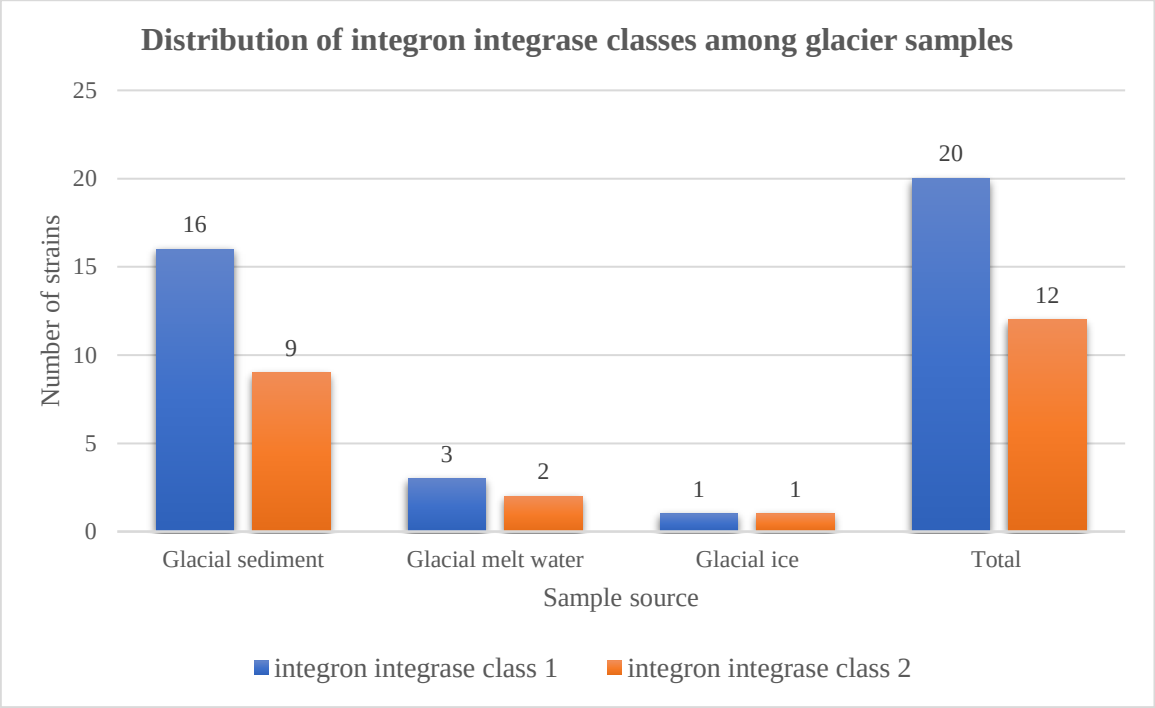


Fig. 5.4 Distribution of integron integrase class 1 and 2 among glacier sediment, ice, and meltwater bacteria

Discussion

Antibiotic resistance is ancient, originates in natural environments (D'Costa et al. 2011) and environmental bacteria are the potent reservoir of antibiotic-resistant elements (Van Goethem et al. 2018) and dissemination of ARGs is mainly due to MGEs like *integrons* (Abraham et al. 2014). The current study illustrated the occurrence of *integrons integrase* class 1 and 2 genes, among the environmental bacteria isolated from the glacier's habitat, that were resistant to ≥ 3 unique antibiotic classes (MDR). The real-time PCR results showed a higher prevalence of *intI1* with strong genome copies per microliter, followed by *intI2* with a moderate or weak number of genome copies per microliter, while all strains were negative for *intI3*. Moreover, our study showed the highest prevalence of *intI1*, n= 20 (71.4%), and *intI2*, n= 12 (42.8%) among MDR bacteria isolated from the glacier's environment. The glacier environment as a terrestrial ecosystem is considered a favorable habitat from the evolutionary point of view to study the intricate process of antibiotic resistance, dissemination, and evolution (Van Goethem et al. 2018). In a natural environment, among bacterial genera, the shared antibiotic-resistant elements are greatly ascribed to the horizontal gene transfer of MGEs (Hu et al. 2016).

ARGs and MGEs are extensively distributed in pristine environments with a vast sequence diversity, including temperate and extreme habitats like; riverine sediments, forest soils, glacier sediment, hot springs, and cold soils (Van Goethem et al. 2018; Elsaied et al. 2007; Ushida et al. 2010), moreover, have been found in association with *integron* genes circulating in clinical settings. The integron integrase class 1 and 2 are known as extensive resistance acquisitive and disseminative MGEs, circulation among clinical bacteria in the clinical environments (Deng et al. 2015; Essen et al. 2007). On the other hand, *integrons* with a remarkable ability to recombine and express a variety of *gene cassettes* in a natural environment, are sometimes assigned to adoptive functions rather than antibiotic resistance, while considering their ubiquitous presence (Hardwick et al. 2008). The abundance of *integrons* in natural habitats might be due to anthropogenic effects and can be used as an indicator of human activities in a natural environment (Koczura et al. 2016). In a glacier extreme environment, MGEs might be actively involved in the acquisition of antibiotic resistance (Hu et al. 2017). Considering the ARGs and MGEs in natural environments like glacier and permafrost sediments and soils, real-time PCR detection is more sensitive and reliable compared to traditional PCR detection, where some ARGs and MGEs are not detected might be due to low target genome copy number (Zhang et

al. 2015; Ushida et al. 2010). In our study, the *intI1* positive strains with Ct < 29 (strong nucleic acid contents) were positive for conventional PCR, whereas the strains with Ct > 29 (moderate or weak nucleic acid contents) and all tested bacteria for *intI2* (Ct value > 32) were remained negative (Nawaz et al. 2023). Therefore, *intI1* and all *intI2* negative strains on conventional PCR were found with very low genome copy numbers through real-time PCR detection (Table 2). The increased percentages highlight the higher sensitivity and capability of real-time PCR to detect the very minute amount of target nucleic acid, in comparison to the conventional PCR (Zhang et al. 2015; Ushida et al. 2010).

The glacial sediment contains nutrient-rich resources with slightly higher temperatures compared to the glacial ice and meltwater and possesses diverse bacterial genera (Parnell and McMahon 2016). Zhang et al. (2018) identified a massive number of intI1 from sediment, permafrost, and glacial soil whereas, *intI2* and *intI3* were not detected. The *integrons* were mostly detected among the genera *Pseudomonas*, *Acinetobacter*, *Bacillus*, *Citrobacter*, and *Sphingopyxis*. Our finding showed that glacial sediment harbored more abundant *intI1* positive bacteria (16, 80%) and *intI2* (9, 75%), followed by glacial melt water *intI1* (3, 15%) and *intI2* (2, 16.6%), whereas glacial ice contained *intI1* (1, 5%) and *intI2* (1, 8.3%) positive bacteria and mostly detected among *Pseudomonas, Acinetobacter, Leucobacter, Alcaligenes, Bacillus, Staphylococcus, Serratia* and *Flavobacterium*. Shen et al. (2019) detected 48.8% multidrug resistance genes from Urumqi Glacier (China), among which the *intI1* gene with 80–100% of the relative abundance of overall MGEs (11.3%), was found, which exceeds over current percentages of detection. Moreover, Hardwick et al. (2008) real-time PCR study reported a higher prevalence (96.1%) of *intI1* among Cowan Creek sediment samples (Sydney). The significantly higher abundance of *intI1* in sediment might have resulted from the diverse bacterial genera and long continuance of ARGs and MGEs in sediment, sustaining the antibiotic-resistant elements reservoir. Furthermore, the genome contents and plasmid DNA are comparatively sturdier in sediment than in water (Ma et al. 2013). In addition, contrasted to our findings, Koczura et al. (2016) reported a higher prevalence of *intI1* among bacteria isolated from river water compared to sediment bacteria. The study also elaborated on the lower prevalence of *intI2* whereas isolates were negative for *intI3*. A study from Pearl River (China) by Chen et al. (2015) reported nearly comparable values for *intI1*, ranging from 1 E + 05 to 2 E + 06 when analyzing the concentrations. Another study on the surface urban sea and river water reported a 26% (5/19) prevalence of *intI1* among antibiotic-resistant *Vibrio spp.* (Taviani

et al. 2008). From municipal water treatment plants, Ferreira et al. (2007) reported 9% of *intI1*, among *Enterobacteria*. The lake water is considered stable in comparison to the running river water. The pristine lakes are thought to be possessed with ARGs and MGEs. Berglund et al. (2014) study on a frozen lake Nydala, reported the presence of *intI1* in all sediment and water samples with a quantified value of 3.86E + 06.

The super-resistant bacteria among diverse soil genera, usually contain MGEs, more precisely the integron integrase (class 1 and 2) as a mandatory tool for the propagation of resistant determinants. In pristine habitats, considering MGEs, the *intI1* gene acts as a marker for the HGT potential among bacteria. The occurrence of *intI1* is more abundantly reported in soil-inhibiting bacteria with a key role in the HGT transfer of ARGs and MGEs (Gundogdu et al. 2013). A study on the diverse soil bacteria by Zhang et al. (2015) reported the presence of *intI1* in all soil samples while less prevalence of *intI2* supported the ubiquitous distribution of *intI1* and its role in the dissemination of AR elements among soil microbiota. The seasonal variations might play a crucial role in the abundance of MGEs and ARGs among bacteria thriving in a pristine environment. It is illustrated that environmental bacteria are found in abundance with MGEs and ARGs in winter and spring compared to other seasons (Koczura et al. 2016). *Acinetobacter species* have a remarkable ability to acquire intrinsic resistance to antibiotics and are known for the acquisition of foreign genes conferring antibiotic resistance (Bonomo and Szabo 2006). In our current study, *Acinetobacter baumannii* (HP57), *Acinetobacter calcoaceticus* (LP5), *Serratia marcescens* (HP50), *Brevundimonas diminuta* (HP51), and *Rahnella inusitata* (HP3) were detected with *integrons* with lowest Ct values (11.2 to 20.9) (Table 2).

There exists a close association between ARGs and MGEs which play a mandatory role in the propagation of multi-antibiotic resistances among bacteria (Frost et al. 2005) but such dissemination is unable to provide enough means to create a link between the relative contribution of known channels while considering the heterogeneity and complexity of the natural environment (Bellanger et al. 2014; Van et al. 2002). The *integrons*-positive bacteria are mostly found with a broad range of antibiotic resistance to several drugs and among gram-negative bacteria, there is an intense association between the occurrence of multidrug resistance and the presence of *integrons* (mostly *intI1*) (Li et al. *2013*; Wu et al. 2012). This might be the reason that in our study the prevalence of integron integrase genes was much higher as only

MDR bacteria were subjected to screen out integron integrase genes and detected with *intI1* 20 (71.4%) and *intI2* 12 (42.8%). Furthermore, both *intI1* and *intI2* positive strains were also found in association with several ARGs like; *blaCTX-M, blaNDM-1*, *blaKPC*, *blaAmpC, blaVIM-1, blaSHV*, *blaTEM, blaOXA, blaGES*, *blaCMY*, *blaDHA*, *sul1*, *sul2*, *tetA, tetB, qnrB, aac(6')-Ib3*, *mecA, cat*, *qepA* and *gyrA* (Nawaz et al. 2023), supporting the previous reports that dissemination of ARGs and MGEs are closely linked in the respective environment (Shen et al. 2019).

Integron class 1 possesses a conserved portion within the gene cassette at downstream direction integrated with *sul1*, *qacE*Δ1, and *orf5* genes, that encode dihydropteroate synthetase (sulfonamide resistance, *sul1*), an exporter protein conferring resistance to quaternary ammonium compound (*qacE*) and hypothetical peptide with undisclosed function (Paulsen et al. 1993). In our findings, the majority of *intI1*-positive bacteria were also found positive for the *sul1* gene (Nawaz et al. 2023). A similar association has been delineated by various studies (Makowska et al. 2016; Chen et al. 2015). The *intI1* with no association with *sul1* gene has been reported for species like; *Stenotrophomonas maltophilia* (Chang et al. 2007) and *Salmonella spp*. (Chen et al. 2004). The continuous and prolonged flow of wastewater from municipal areas actively plays a major role in the dissemination of *integrons* and existing genes (*aadA, aac6, qacE*) in natural environments. From wastewater isolated *Methicillin-resistant Staphylococcus aureus* (MRSA) was found to coexist with *intI1* and other ARGs on the same *integron gene cassettes*. Moreover, the potential widespread association of *intI1* and *qacE* in soil and associated ecosystems has been reported in several studies (Wan and Chou 2015; Jechalke et al. 2014), supporting the diverse distribution of *integrons* and associated ARGs. The distribution of ARGs and MGEs among sites close to human activities is found in more abundance and potential association with *intI1*, *sul1*, *aac6*, and *dfrA* (Khan et al. 2013). Zhang et al. (2009) reported the co-existence of *intI1* (33%) in activated sludge samples of five sewage treatment plants in Hong Kong, with *sul1*, *qacE*, *dfrA*, and *aadA* genes. Borruso et al. (2016) reported the highest prevalence of *intI1*-containing *gene cassettes* with sequences similar to the 3' conserved segment of *intI1*, from agriculture wastewater and highlighted the coexistence of *sul1*, *qacE* and aminoglycoside resistance genes (*aadA* and *aac6*) within the integron integrase class 1 *gene cassettes*. In our study, there might be some association between *intI1* and *aac6*, as a significant number of tested *intI1*-positive strains were positive for the *aac6* gene (Nawaz et al. 2023).

As an alternative to selective antibiotic pressure in a clinical environment, *integrons* in harsh environments confer selection due to the challenges thrown by the environment. The discovery of novel integron integrase class 1 with non-resistive *gene cassettes* conferring no resistance to antibiotics, supported this phenomenon and the ubiquitous distribution of *integrons*. The high prevalence of *intI1* and *intI2*, in our study, can be correlated with environmental stress, which triggers the selective pressure on the acquisition and dissemination of ARGs and MGEs in remote pristine environments, among the upset bacterial community thriving in the glacier environment. The clinical *intI1* has evolved from *Betaproteobacteria* but the ubiquitous distribution of *integrons* especially among the natural environments is still to be evaluated as *intI1* comprised of only one type among 100 *integron* classes (Stokes et al. 2006). The high prevalence of integron integrase class 1 and 2 among the glaciated MDR bacteria is a vital challenging question to be answered as the occurrence of *integron* other discrete classes among environmental bacteria has not been explored and such abundance may not reflect the ultimate distribution of *integrons* in pristine environments.

Conclusion

In summary, the MDR bacteria isolated from the glacier environment were found abundantly possessed with integron integrase class 1 and 2, predominantly detected among gram-negative and glacial sediment bacteria. The prevalence of *intI1* was higher than *intI2*, with the presence of strong nucleic acid concentrations. The majority of gram-negative bacteria with maximal *intI1* genome copy per microliter remained positive for the *intI2* gene. The presence of MGEs like integron integrase class 1 and 2, among the glacier bacteria, highlights the HGT acquisition and dissemination of ARGs and MGEs in a natural environment. The exploration of pristine cold habitats for the occurrence of MGEs, especially of melting glaciers is limited and requires continued surveillance to evaluate the diversification of antibiotic resistance in the natural environment.

Authors' contributions

SN, FH, and ILP conceived and designed the 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.

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Declarations

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.

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Chapter 6. Cloning

Title: Cloning of glacier bacteria harbored antibiotic-resistant genes in mesophilic *Escherichia coli* strain

Literature review

The word clone has been derived from the "klon" (a Greek word) which means "twig" which demonstrates the recreation process of a new plant from the twig. In more precise scientific linguistics, the word "clone" means any genetic copy of a human being, plant, cell, molecule, or genes. Gene cloning can be defined as the identical replication process of particular DNA (nucleotide) sequences by utilizing advanced molecular biology techniques. The extraction of any nucleotide sequences from one biological system, followed by subsequent incorporation into the destination vector for furthermore proliferation into another species devoid of any alteration to the native DNA sequences is called molecular cloning. Molecular cloning generates many copies of the desired nucleotide fragments and desired expression characteristics are obtained in vitro (Ashwini *et al.,* 2016). The amazing role of the restriction enzymes in the form of desired DNA sequences cutting and ligation is among the exceptional discoveries in the 20th century, followed by various other techniques including PCR added, used for more precise and site-directed alteration of DNA sequences (Backman and Ptashne 1978; Cohen *et al.,* 1973). To perform the cloning procedure, a few fundamental approaches are utilized; restriction endonucleases-based cleavage of DNA fragments, ligation of the desired nucleotides to the specific genetic vehicle (vector), the transformation of the recipient organism, and selection/screening of the transformants (Ashwini *et al.,* 2016).

In the 21st century, highly advanced cloning techniques have come out where the genomic sequences and DNA coding efficiencies of different organisms can be resolved, opening many ways to analyze and determine the functions of various unknown genes and their respective genetic characteristics. Synthetic biology, as a more advanced field, provided the featured opportunities to analyze the complex diversity of heritable genetic contents. These astonishing advancements are ultimately possible by utilizing the extremely knowledgeable and very loftier cloning procedures including the plasmid Fusion System, Golden Gate, Gateway, and the Independent DNA Sequence and Ligation Cloning System (Siegel *et al.,* 2004; Walhout *et al.,* 2000; Nebert *et al.,* 2000; Hartley *et al.,* 2000; Liu *et al.,* 1998; Bethke and Sauer 1997).

Advanced Cloning Approaches

1. Conventional Cloning

Conventional cloning follows the basic and simple workflow procedure where the desired DNA fragments are extracted or obtained through PCR, followed by the insert ligation into a suitable plasmid cloning vector, and for propagation, the recombinant plasmid vector uptaking and transformation into an appropriate recipient host organism, and finally the screening of the transformants (host containing desired recombinant vector). To increase the cloning efficiency and increased percentages of transformants, many unique and specific enzymes are used which enhance the overall productivity of molecular cloning (Figure 6.1).

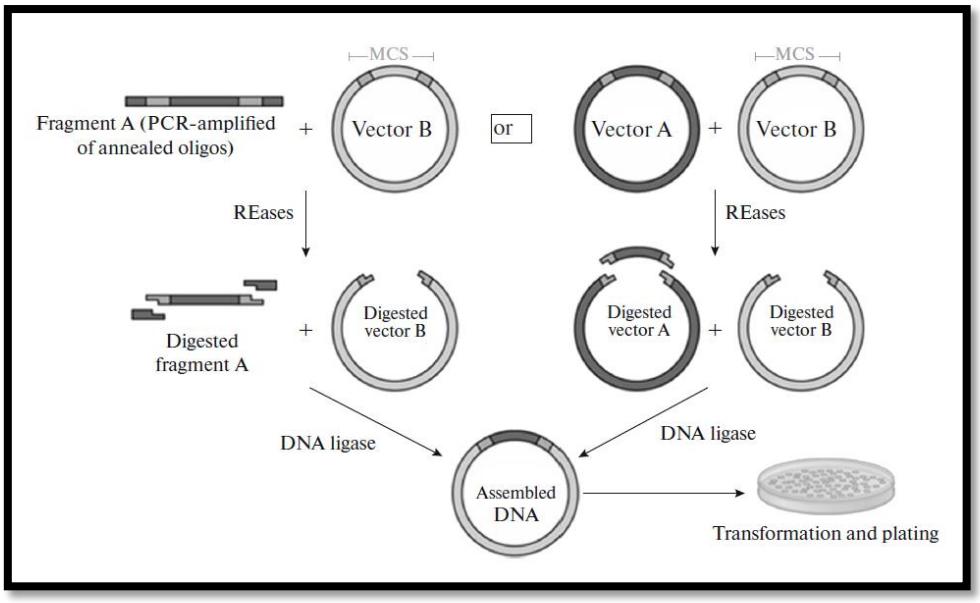

Figure 6.1. The mechanism of Conventional Cloning based on restriction and ligation enzymes (https://www.neb.com/products/restriction-endonucleases/restriction)

1.1.TOPO/TA Cloning

Some cloning procedures don't require the activity of ligases (Ashwini *et al.*, 2016). The Topo/TA Cloning is a ligation ligation-independent technique and only requires the activity of Taq polymerase. TOPO Cloning is a very rapid cloning technique without the use of restriction enzymes. To perform the TOPO cloning procedure mostly the Vector pCR 2.1-TOPO is used and the detailed map of sequences is illustrated in Figure 6.2.

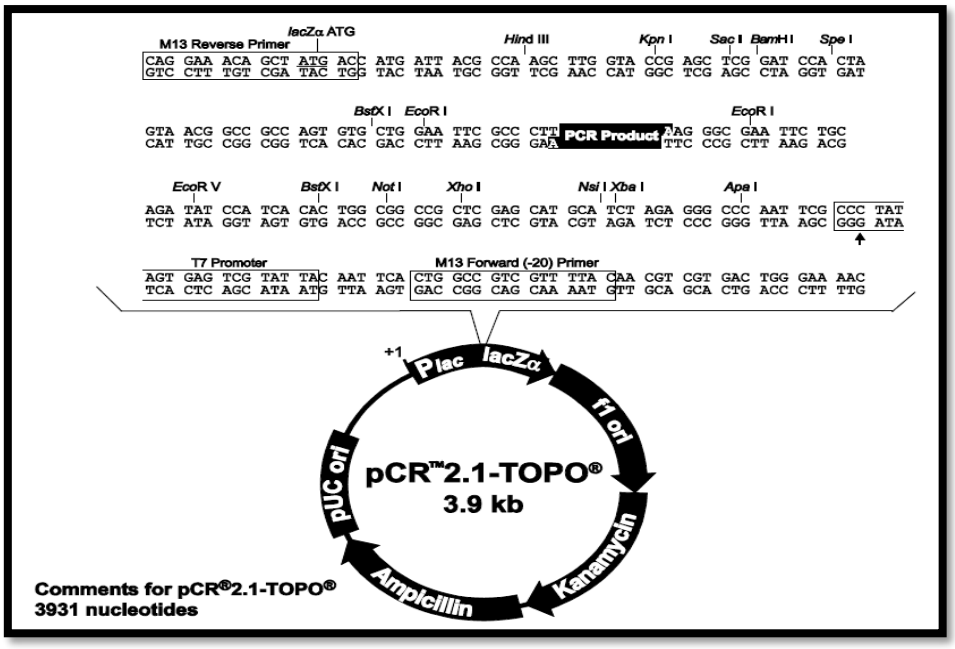

Figure 6.2. The complete map and features of the TOPO Cloning Vector pCR 2.1 (TOP TA Cloning Kit (Invitrogen)

In the experimental process, *Taq polymerase* aided, 3' end polyadenylated PCR products are produced, of the desired DNA insert, and the adenine overhang PCR products are ligated to the linearized vector with Thymine overhangs and the adenine and thymine base pairing occurs, that's why it's also known as "TA" cloning. TA cloning uses the T4 DNA polymerases which create the single-strand desired DNA overhangs (Figure 6.3).

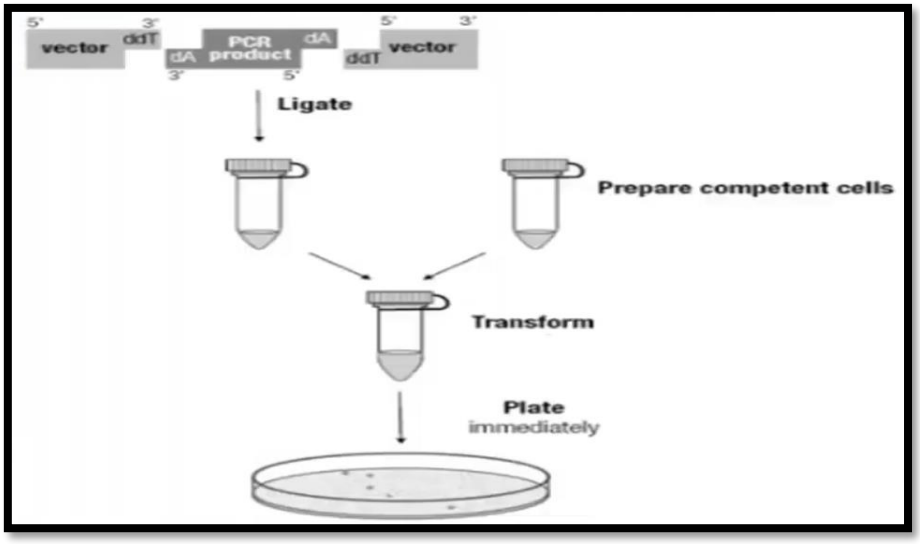

Figure 6.3. TOPO/TA Cloning working principle, adenine and thymine overhangs are shown which results in base pairing (TOP TA Cloning Kit (Invitrogen)

To analyze the TA cloning transformants (desired clones), the Alpha complementation system (blue-white screening) is used. In this screening system, a substrate (X- Gal, 5-bromo-4-chloro-3-indolyl ß-D-glactopyranoside) is used to detect the normal function of *ß- galactosidase*. The *ß- galactosidase* is the ultimate product of the *lac operon* lacZ gene and oxides the colorless X- Gal (5-bromo-4-chloro-3-indolyl ß-D-glactopyranoside) to a blue-color product (5-bromo-4-chloro-indoxyl and galactose). The host contains the lacZ gene with deficient amino acid sequences (11-41) making it non-functional. On the contrary, the plasmid vector contains the alpha peptide (deficient amino acid sequences) to complement the defect and make the enzyme active. Within the vector, among the alpha peptide region, multiple cloning sites (MLS) are present, and if the gene of the insert is integrated here, the fully active enzyme is not synthesized, and the activity is halted, resulting in white/off-white color colonies. Whereas, if the DNA insert is not present on the vector, the active enzyme will act on the substrate and blue-color colonies will be produced (Figure 6.4) (Wu *et al.*, 2010; Top TA Cloning Kit, Invitrogen). Recently, like TA cloning, GC cloning has also been introduced, where the DNA polymerase *Tth*, *TfI*, and *Taq* (non-proofreading enzymes), incorporate 3'G to each blunt DNA fragment through a G tailing reaction or PCR. Afterward, the 3'G overhang (DNA insert) is bas paired with and ligated to the 3'C overhang vector (GC Cloning Kit, Invitrogen).

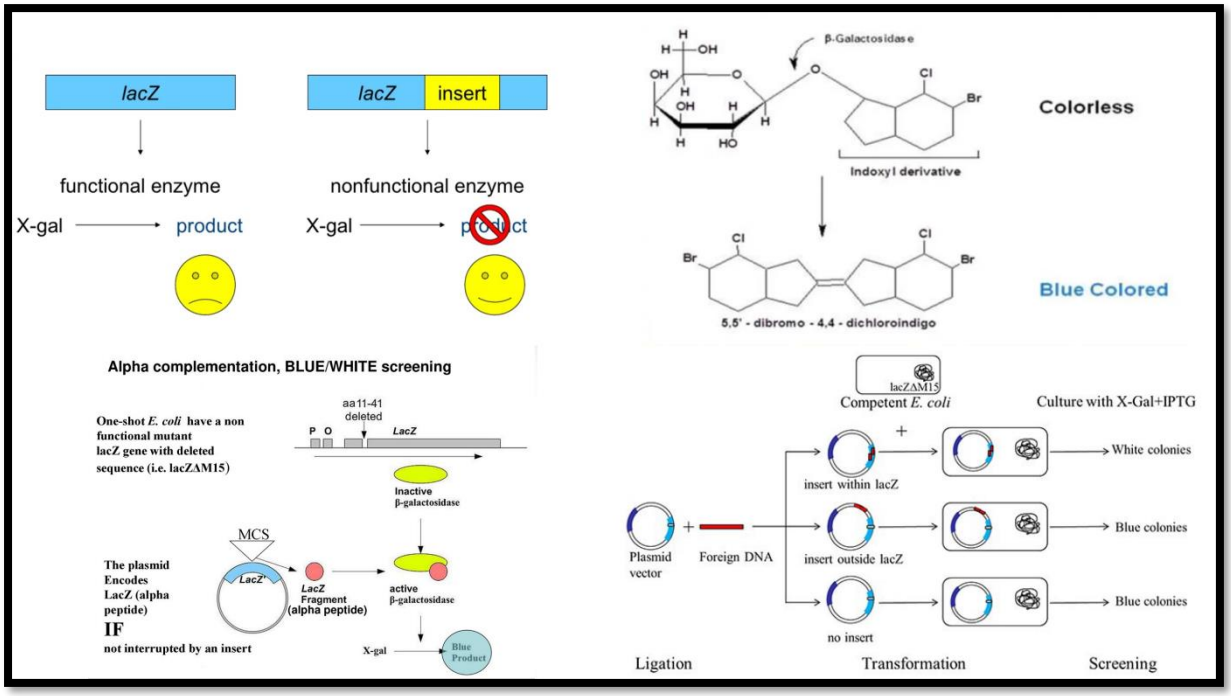

Figure 6.4. TOPO Cloning overall mechanism, X-Gal conversion, insertional inactivation, and alpha complementation screening of transformed cells (TOP TA Cloning Kit (Invitrogen)

2. Gateway Cloning

The gateway cloning system works on the principle of site-specific recombination. In the experimental process, the λ phage self-integration and excision up and downstream of the bacterial chromosome are performed (Katzen, 2007). To catalyze the LR and BP reactions, two different requisite mandatory enzymes; LR clonase and BP clonase are used, resulting in the shuffling of genomic contents of different plasmids with flanked *att* recombination sites. The mixture of BP clonase enzymes contains phage integrase. The desired DNA fragment, flanked by *attB* sites, is integrated into a suitable donor vector that contains two *attP* sites. Afterward, both the requisite sites (*attB* and *attP*) are recombined, the desired DNA content is incorporated into the specific donor vector, and the formed access clone is fringed by L sites. In addition, the enzymatic reaction mixture of the enzyme LR clonase (containing integrases, host integration factor, plus an enzyme phage excisionase) catalyzes the overall LR reaction, utilizing the entry clones as the main substrate. The *attL* flanked, desired DNA fragments are transferred into the recipient vector that contains various R sites (Figure 6.5). The second recombination takes place between the sites *attL* and *attR* which generates the attB-flanked novel expression clone (Ashwini *et al.,* 2016). Moreover, the entry clones' uptake and assemblage can also be acquired through the use of a restriction-ligation system where the desired DNA content is ligated into a suitable vector. Where the multiple cloning sites (MCSs) are generated by the *attL* region (Cheo *et al.*, 2004). The gateway cloning system also allows the insertion and integration of desired DNA multi-fragments into a single vector, synchronously. In this system, several genes are expressed at the same time, whose induction is controlled by a tissue-specific or an active inducible promotor (Karimi *et al.,* 2007). The multi-site expressive gateway system is successfully used for protein-protein interactions, gene stalking, genomic content recombination, and gene silencing (Karimi *et al.,* 2007; Alvarez *et al.,* 2006; Schwab *et al.,* 2006; Chen *et al.,* 2006; Burch-Smith *et al.,* 2004; Robertson, 2004; Puig *et al.,* 2001).

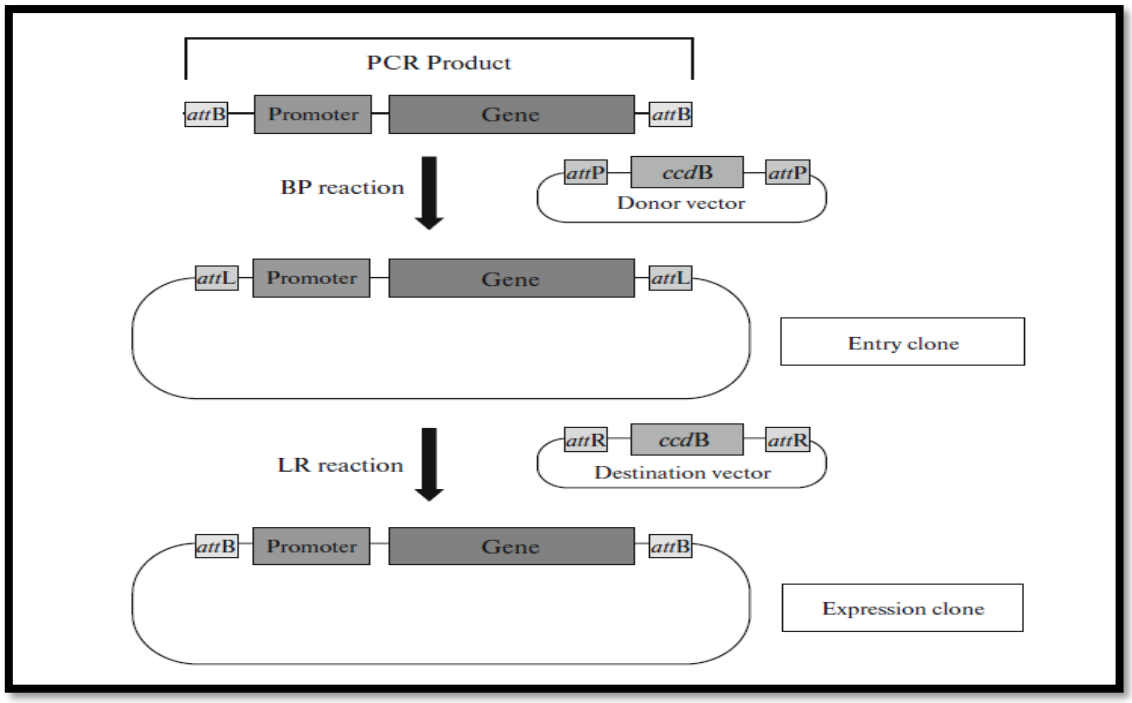

Figure 6.5. The overall mechanism of Gateway Cloning, the LR and BP reactions (www.invitrogen.com)

3. Golden Gate Cloning

The Golden Gate Cloning is another advanced synthetic biology procedure, which involves the assembly of various desired DNA contents through the utilization of specific restriction enzymes called IIS. These enzymes are highly effective and multi-featured which provides several advanced approaches to uplift the assembly standards (Figure 6.6) (Engler *et al.*, 2008). Considering the DNA assembly procedure, it's more productive, and time-saving, and minimizes the imposing stages of molecular cloning like the PCR amplification of desired DNA fragments, gel extraction, and purification, and designing of specific target primers. In the experimental process, this technique only requires the four base pairs' intersections (fusion sites) that are utilized to connect the adjoining modules. These modules are highly adjustable to generate high throughput assembly (Liu *et al.*, 1998). This technique also neglects the overlapped flanked sequences and recombination sites that are required for the efficient recombination process. The BbsI, BsmbI, and BsaI are among the most effective commonly used restriction enzymes, used for this process (Ashwini *et al.*, 2016).

In a single-step Golden Gate Cloning reaction, various transcriptional units are prepared and collected from modular parts used as a standard. Moreover, the advanced order assembly, to

construct multi-genomic fragments, can be achieved through similar approaches (Werner *et al.*, 2012; Weber *et al.*, 2011; Sarrion-Perdigones *et al.*, 2011). Golden gate cloning, in contrast to traditional cloning, restricts itself to those sequences that are less sequence-independent, specifically to the chosen type IIS identification spot should be absent from the innermost segments of the respective DNA contents that must be put together. In this process, the domestication phenomena take place which is the removal of the interior manifestations of the distinguishing structures for the type IIS enzyme utilized for the construction of all beginning modules. The very end result is the organized, flawless construction of DNA fragments in a separate step. Nevertheless, the Golden Gate Assembly is a powerful technique that, aside from its use in the creation of specifically tailored TALENS (Transcription Activator-like Effector Nucleases) with a mainstream role in in-vivo gene editing procedure, generates several site-directed alterations and assembles numerous DNA fragments (Ashwini *et al.*, 2016).

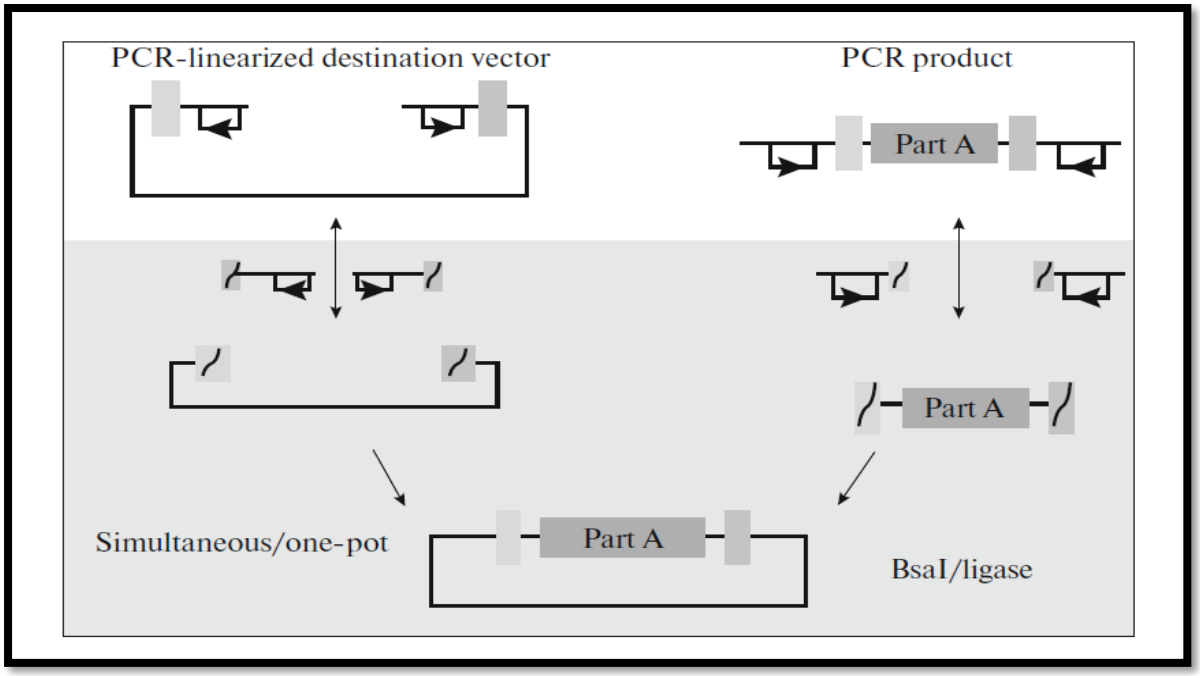

Figure 6.6. Mechanism of Golden Gate Cloning (Ashwini *et al.*, 2016)

4. Sequence and Ligation Independent Cloning

SLIC refers to the in-vitro technique that adheres to the RecA-independent pathway for homologous recombination. It uses a PCR-generated insert (12 base pairs) and single-stranded corresponding overhangs produced by an enzyme (T4 DNA polymerase) within the desired

vector. In the reaction mixture, one dNTP is sufficient to balance the activity of the 3' to 5' and the 5' to 3' polymerase at the location of this nucleotide's initial occurrence (Li and Elledge 2007). A segment of the DNA sequence required to be replicated into a particular desired vector is linearized either by PCR amplification or restriction digestion, followed by PCR amplification using oligos with 5' termini that have roughly the sequence homology of 25 bp to the terminal base pairs of the destination vector. While missing the requisite dNTPs, the enzyme T4 DNA polymerase is used to remedy the linearized target vector, and amplicons are generated by the PCR, which contains the target genes separately. When the corresponding single-stranded 5' overhangs at the ends of the linearized target vector and the PCR amplicons are sufficiently subjected, dCTP is attached to stop the bite-down the enzymatic reaction of T4 DNA polymerase, which holds 3' to 5' exonuclease action. The PCR product and the bite-down linearized destination vector are combined, and then they are annealed to one another. Due to the absence of ligase in the procedure, a specific plasmid containing four single-stranded spaces or cuts is produced (Figure 6.7). Without the need for the precise sequences necessary for the ligation and recombination, recombinant DNA assembled by SLIC achieved a faultless reassignment of intrinsic components. This method can be used for any gene, making it easy to forecast the combinatorial assembly of the desired gene with the vector while saving the researcher time, effort, and money (Ashwini *et al.*, 2016; Werner *et al.*, 2012).

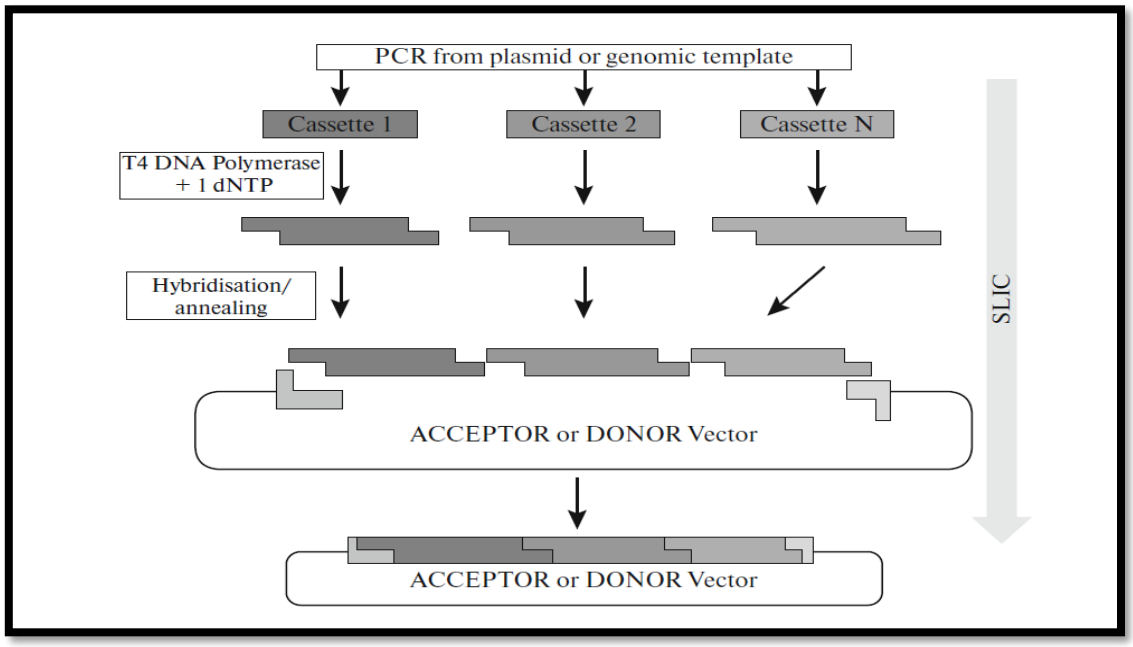

Figure 6.7. Working principles of Sequence and Ligation Independent Cloning (SLIC) (Ashwini *et al.*, 2016)

The use of these cloning techniques for the production of antibiotic-resistant genes sheds light on how HGT spreads these genes in the environment. This is crucial in cold environments as mesophilic bacteria may come into contact with psychrophilic bacteria from melting glaciers and horizontally transfer their ARGs to them. Therefore, considering the fact that glaciers and other cold habitats native bacteria are integrated with numerous antibiotic-resistant genes (ARGs) and mobile genetic elements (MGEs) (Van Goethem *et al.,* 2018; Segawa *et al.,* 2013; Ushida *et al.,* 2010) which disseminates through the horizontal gene transfer (HGT) among the diverse bacterial population and their ultimate flow to the community and hospital's inhabiting pathogenic bacteria, poses a major threat to the public health in the form of emerging antibiotic resistance and least therapeutic options. Therefore, the current study was designed to assess the uptake of psychrophilic bacteria-born ARGs by a mesophilic competent *E. coli* strain through the cloning process.

Materials and Methods

1. Selection of Inserts (ARGs) for Cloning

A total of four antibiotic-resistant genes (ARGs) were selected to be cloned in a competent mesophilic *E. coli*. strain. The ARGs have been detected among different Psychrophilic bacterial strains including *Brevundimonas diminuta*, *Rahnella inusitata*, *Staphylococcus equorum*, and *Alcaligenes faecalis*. Among four ARGS, two belonged to the ß-lactam group antibiotics ($_{bla}$*CTXM-15* and $_{bla}$*NDM-1*) while two to non- ß-lactam antibiotics (*aac(6)-lb3* and *gyrA*).

2. TOPO/TA Cloning

To clone the desired ARG inserts into a suitable vector, TOPO Cloning was performed by utilizing the TOP TA Cloning Kit (Invitrogen), following the manufacturer's guidelines. TOPO cloning is a restriction enzyme-free, rapid cloning technique where the Adenine-overhang-PCR-product is ligated to the linearized Thymine-overhang-vector and to analyze the transformants, the Alpha complementation system (blue-white screening) is used. The vector used in this study was pCR2.1-Topo Vector and supplied by Invitrogen.

3. PCR amplicons production

PCR amplification of each ARG of interest ($_{bla}$*CTXM-15*, $_{bla}$*NDM-1*, *aac(6)-lb3* and *gyrA*) was performed in Eppendorf Gradient Thermocycler in a final reaction volume of 50 µL while following the manufacturer's protocols;

DNA Template	→ 10 – 100 ng
10X PCR Buffer	→ 5 µL
50 mM dNTPs	→ 0.5 µL
Primers (100–200 ng each)	→ 1 µM each
Water (add to final volume)	→ 49 µL
Taq Polymerase (1 unit/ µL)	→ 1 µL
Total Volume	→ 50 µL

The forward and reverse primers sequences of *$_{bla}$CTXM-15*, *$_{bla}$NDM-1*, *aac(6)-lb3*, *gyrA*, M13 forward, M13 reverse, and the expected amplicon size are summarized in Table 6. A final extension of 7–30 minutes at 72°C after the last cycle was added to each PCR run, to ensure that all PCR products are full-length and 3´ adenylated. 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).

Table 6. ARGs of interest (DNA fragments), primer sequences, and amplicon sizes

Target Genes	Primers sequences (5' – 3')	Product size (bp)	Reference
$_{bla}$CTXM	F: 5'-TTA ATT CGT CTC TTC CAG A-3' R: 5'CAG CGC TTT TGC CGT CTA AG-3'	1000	Baraniak *et al.*, 2002
$_{bla}$NDM-1	F: 5'-CGC CAT CCC TGA CGA TCA AA-3' R: 5'-CTG AGC ACC GCA TTA GCC G-3'	214	Tan *et al.*, 2018
aac(6')-Ib3	F: 5'-TTG CGA TGC TCT ATG AGT GGC TA-3' R: 5'-CTC GAA TGC CTG GCG TGT TT-3'	482	Park *et al.*, 2011
gyrA	F: 5'-AAA TCT GCC CGT GTC GTT GGT- 3' R: 5'-GCC ATA CCT ACG GCG ATA CC-3'	344	Park *et al.*, 2011
M13	F: 5'-CTG GCC GTC GTT TTA C R: 3'-CAG CAA ACA GCT ATG AC	-	TOP TA Cloning Kit (Invitrogen)

4. TOPO Cloning Reaction

To clone the desired ARG of interest into the pCR2.1-Topo Vector, a TOPO Cloning reaction mixture was prepared, utilizing the following manufacturer's protocol;

Fresh PCR product = 0.5-4µl

Salt solution (200mM NaCl, 10mM MgCl2) = 1µl

Water = 5µl

pCR2.1-TOPO vector = 1µl

Final volume = 6µl

Afterward, the reaction mixture was incubated at room temperature at 22 to 25°C. After incubation, the reaction mixture was placed on ice to perform the subsequent one-shot transformation.

5. Transformation of Competent Cells (One-Shot Mach1-T1)

The process of transformation was carried out utilizing the manufacturer's supplied chemically competent *E. coli* strain with ampicillin selection, as an antibiotic marker. In the transformation experimental process, 2 µL of TOPO Cloning reaction mixture was added to one vial of chemically competent *E. coli* (One-Shot Mach1-T1) and mixed gently. The reaction vial was placed on ice for about 5 to 30 minutes for incubation. Afterward, the cells were heat-shocked at 42°C for 30 seconds without shaking, followed by their immediate transfer on the ice. A volume of about 250 µL of S.O.C medium (Room temperature) was added to the tubes and kept for incubation at 37°C for 1 hour in a horizontal shaker (200 rpm). After incubation, a volume of 10 to 50 µL from each transformation tube was spread over a prewarmed selective media plate contained with ampicillin drug, and the plates were incubated overnight at 37 °C.

6. Analysis of Transformants

Post-incubation, the plates were inspected for white/off-white colonies for each transformant. About ~10 colonies from each transformed plate were selected and used for subsequent transformant analysis. To analyze the cloned vector construct, the following two approaches were utilized.

6.1. PCR Amplification of Plasmid (vector) ARG inserts

About 2 to 6 white/off-white colonies were picked and incubated overnight in an LB medium containing 50 µg/mL ampicillin antibiotic. The isolated colonies from each culture plate were collected and subjected to plasmid DNA extraction using PureLink Quick Plasmid Miniprep Kit (Invitrogen), following the manufacturer's guidelines. Afterward, Plasmid DNA as a template for PCR amplification of each cloned ARG was performed in Eppendorf Gradient Thermocycler, by using the respective forward and reverse primers, and the desired amplicon bands were visualized on the gel documentation system.

6.2. Sanger Sequencing of Plasmid Vector constructs

In the second approach, the extracted plasmid vector constructs of each ARG of interest, accompanied by the respective ARGs primers and vector primers (M13 Forward and M13 Reverse) were sent to the University of Arizona Genetic Core (https://uagc.arl.arizona.edu/services/services/dna-sequencing) for sanger sequencing. Sequences read of 600 bases per reading in one direction was performed on Applied Biosystems 3730XL DNA Analyzer. For mapping and annotation of each plasmid vector construct, SnapGene Software was used.

Results

1. PCR amplicons production

The PCR amplicons of 4 ARGs were obtained using the respective primers and used as DNA inserts for the cloning experiment, subsequently. The presence of the amplified bands was revealed for *$_{bla}$CTXM-15* (1000bp), *$_{bla}$NDM-1* (214bp), *aac(6)-lb3* (482bp), and *gyrA* (344bp), on 1.5% agarose gel and visualized through Gel documentation system. The ARG amplicons were full-length and 3' adenylated and are shown in Figure 6.8.

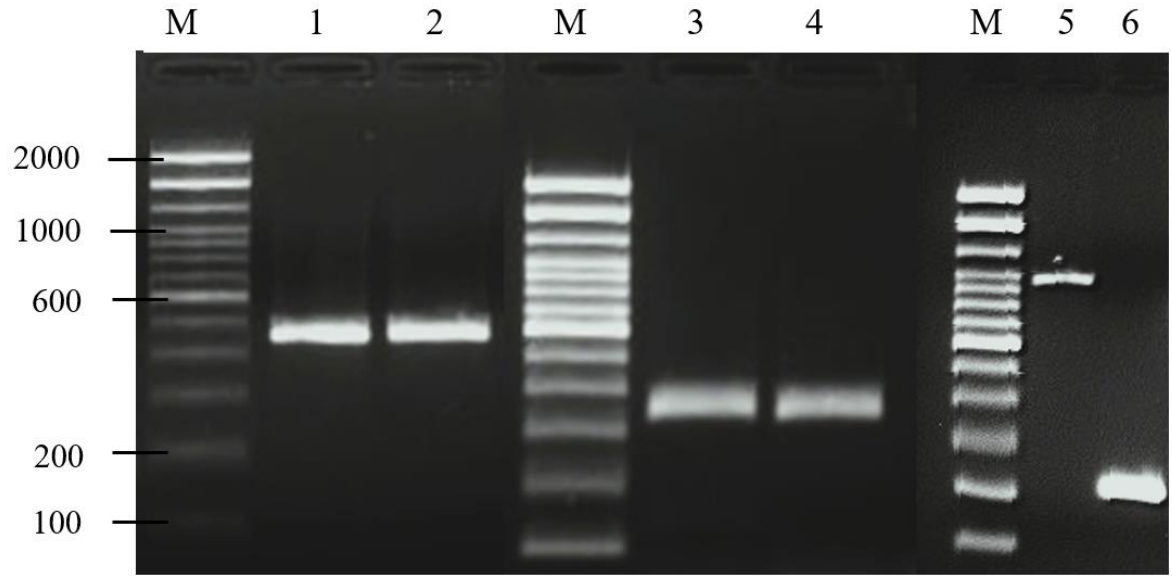

Figure 6.8. PCR amplified product bands on 1.5% agarose gel. Lane 1, 2 *aac(6')-Ib3* (482bp); Lane 3, 4 *GyrA* (344bp); Lane 5 *CTX-M15* (1000bp); Lane 6 *NDM-1* (214bp); M (100 bp DNA Ladder, Invitrogen)

2. Transformation of *E. coli* with antibiotic-resistant genes of interest

To screen out the transformed *E. coli*, the Alpha complementation system (blue-white screening) was used. A chromogenic substrate was used to detect the activity of *ß-galactosidase* enzyme. The host contains the non-functional lacZ gene and the plasmid vector contains the alpha peptide to complement the defect and make the enzyme active. If the foreign DNA (ARG of interest) is inserted within the *lacZ* gene fragments of the vector, the enzyme activity is halted and white/off-white color colonies will be produced. On the contrary, if the

DNA fragment is inserted outside the *lacZ* gene, leaving it active, the colonies will be blue-color. If there is no insert at all then also blue-color colonies will be produced. When the culture plates of each ARG-transformed *E. coli* were observed, both blue and white/off-white color colonies were inspected, indicating both transformed and non-transformed bacteria. Considering the $_{bla}$*CTXM-15*, $_{bla}$*NDM-1*, *aac(6)-lb3*, and *gyrA* inserts, the transformed (white/off-white) and non-transformed (blue) *E. coli* colonies are shown in Figures 6.9.1-9.4.

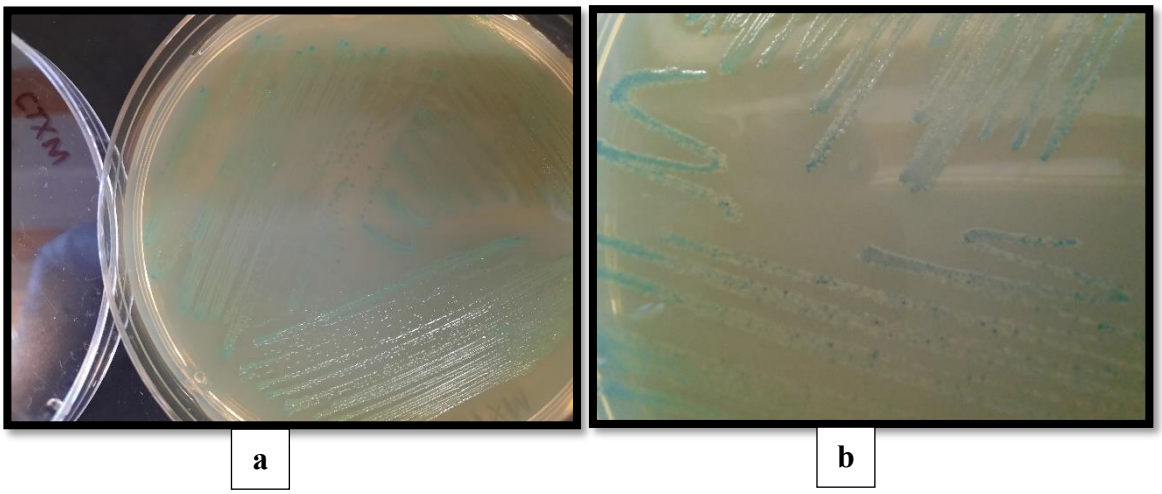

Figure 6.9.1 (a,b) White/off-white colonies are the transformed bacteria with $_{bla}$*CTX-M* insert. Blue colonies are non-transformed lacking $_{bla}$*CTXM15* insert

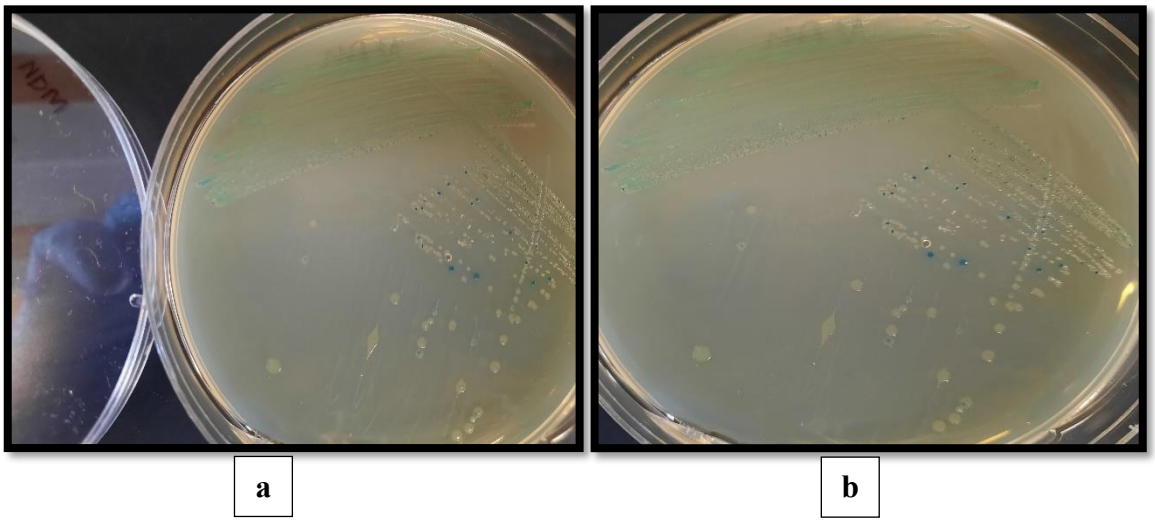

Figure 6.9.2 (a,b) White/off-white colonies are the transformed bacteria with $_{bla}$*NDM-1* insert. Blue colonies are non-transformed lacking $_{bla}$*NDM-1* insert

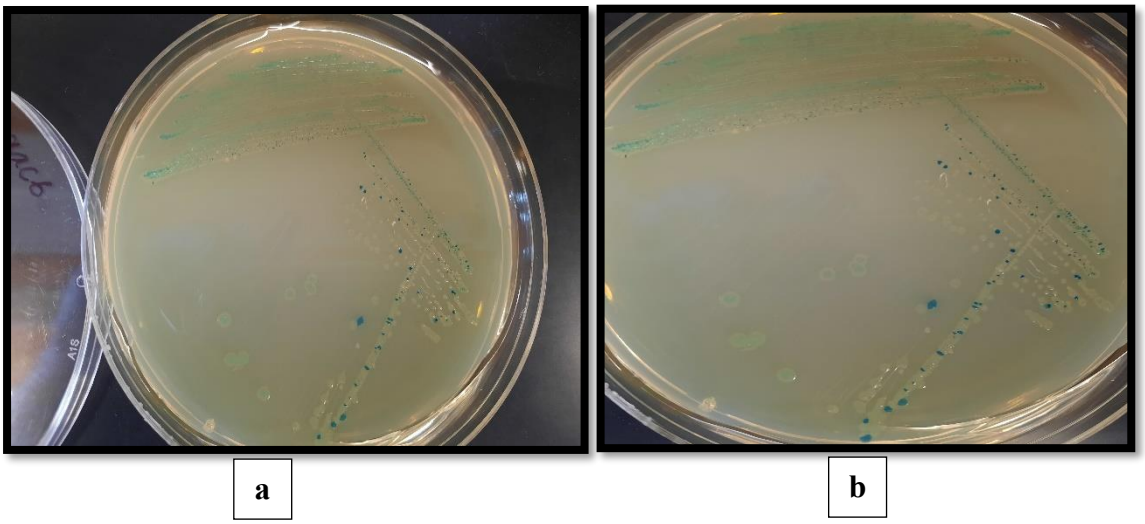

Figure 6.9.3 (a,b) White/off-white colonies are the transformed bacteria with *aac6-lb3* insert. Blue colonies are non-transformed lacking *aac6-lb3* insert

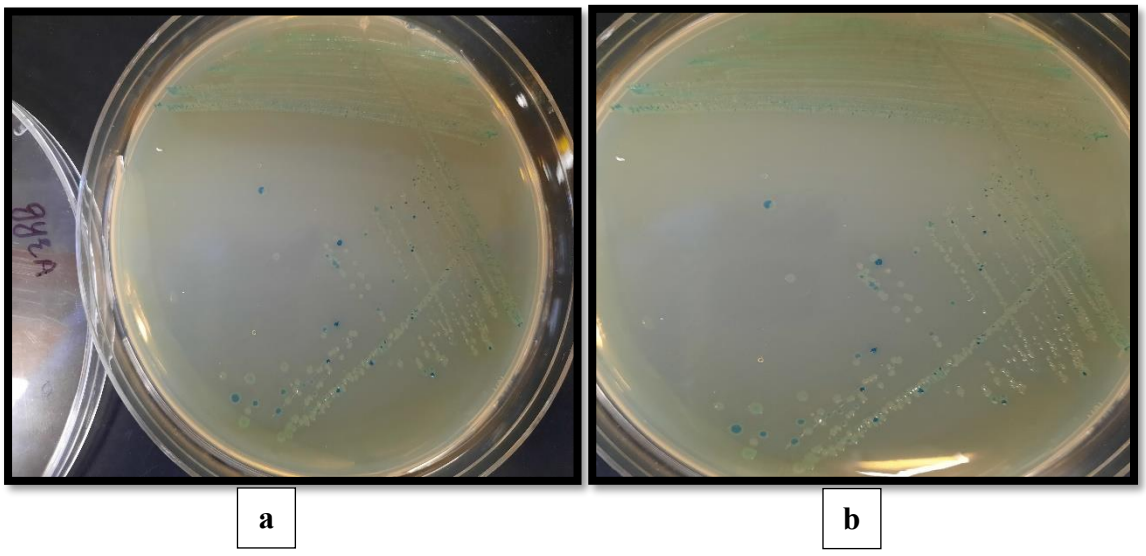

Figure 6.9.4 (a,b) White/off-white colonies are the transformed bacteria with *gyrA* insert. Blue colonies are non-transformed lacking *gyrA* insert

3. PCR amplified Plasmid (vector) ARG inserts

To analyze the transformants, the plasmid DNA (vector construct) of each cloned ARG was extracted and subjected to PCR amplification, utilizing the forwards and reverse primers of each ARG insert. When the PCR amplicons were analyzed and visualized on agarose gel, exact product bands were found for the respective cloned DNA fragments (ARGs). The PCR amplicons of cloned $_{bla}$*CTXM-15*, $_{bla}$*NDM-1*, *aac(6)-lb3*, and *gyrA* genes are illustrated in Figure 6.10.

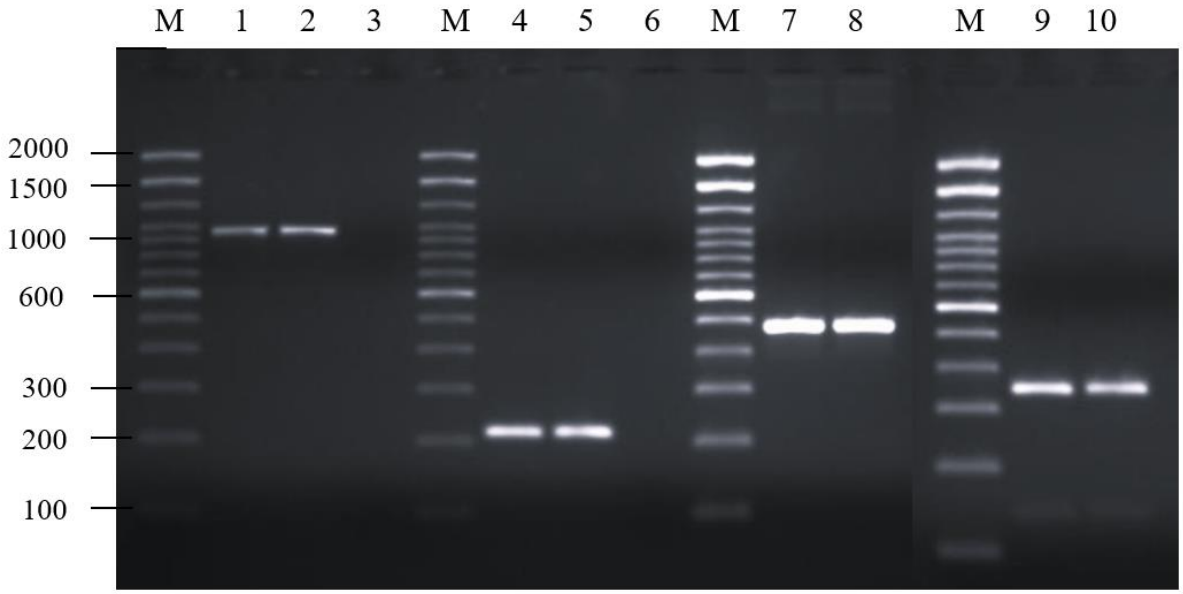

Figure 6.10. Plasmid vector PCR amplified bands of ARGs of insert on 1.5% agarose gel. Lane 1,2 $_{bla}$*CTXM-15* (1000bp), 3 blank; Lane 4,5 $_{bla}$*NDM-1* (214bp), 6 blank; Lane 7,8 *aac(6')-lb3* (482bp); Lane 9, 10 *gyrA* (344bp); M (100 bp DNA Ladder, Invitrogen)

4. Sanger Sequencing of Plasmid Vector constructs

After analyzing the ARGs transformed *E. coli* cells by plasmid vector PCR amplification, the nucleotide sequences of each extracted plasmid vector construct were obtained by the Sanger Sequencing method. The sequences for $_{bla}$*CTXM-15*, $_{bla}$*NDM-1*, *aac(6)-lb3*, and *gyrA* cloned plasmid vectors were uploaded to the SnapGene Software and analyzed for mapping and annotation. The complete sequence of plasmid vector construct with $_{bla}$*CTXM-15* insert is

shown in Figure 6.11, *blaNDM-1* in Figure 6.12, *aac(6')-lb3* in Figure 6.13, and *gyrA* in Figure 6.14.

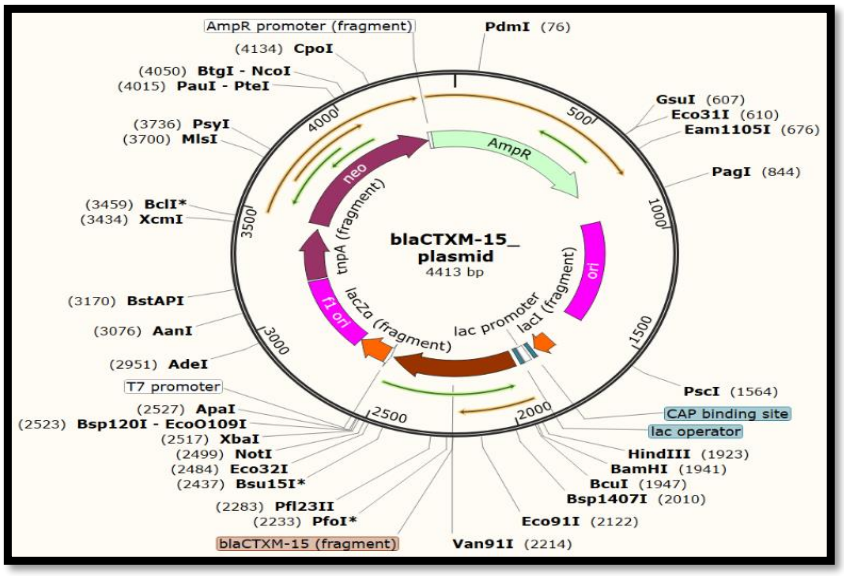

Figure 6.11. The complete sequence of plasmid vector construct with *blaCTXM-15* insert

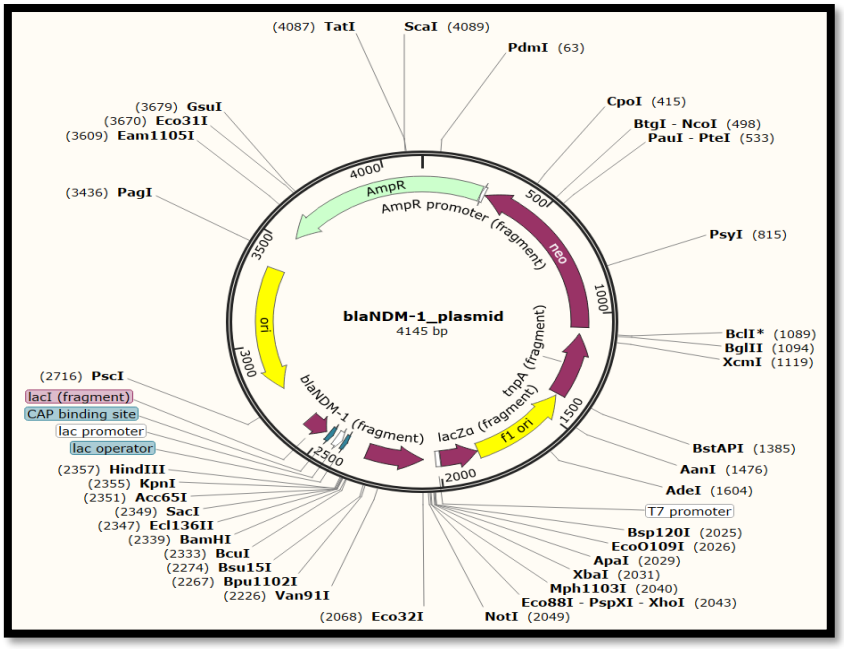

Figure 6.12. The complete sequence of plasmid vector construct with *blaNDM-1* insert

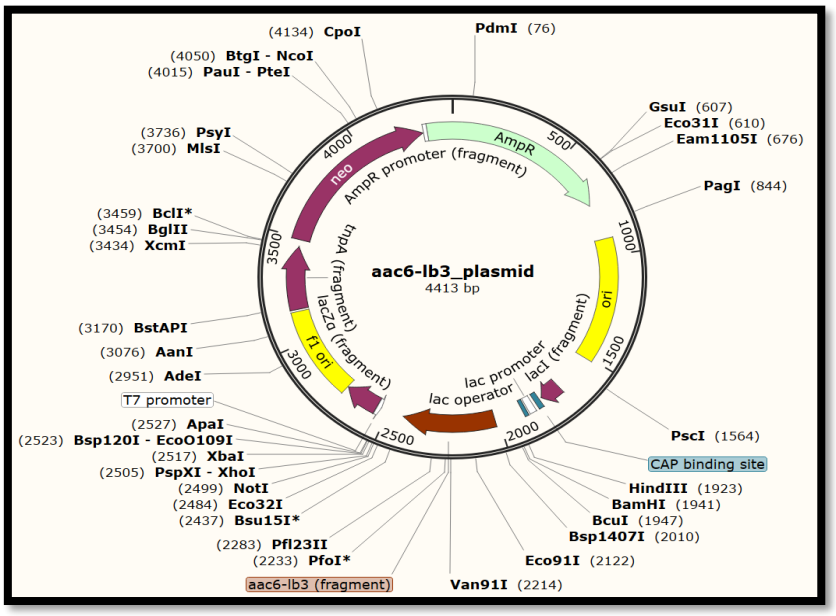

Figure 6.13. The complete sequence of plasmid vector construct with *aac6-lb3* insert

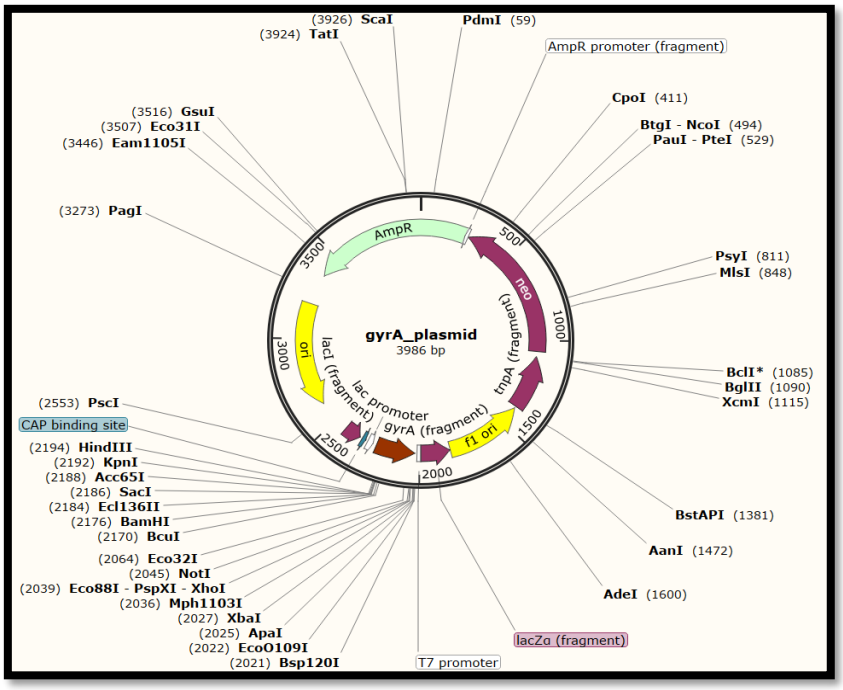

Figure 6.14. The complete sequence of plasmid vector construct with *gyrA* insert

Discussion

The pristine cold environments across the globe are declared to be teeming with Bacteria, Archaea, Viruses, and some Eukaryotes (Rothschild and Mancinelli 2001) and are copiously found with numerous ARGs and MGEs. As a result of global warming, the rapid melting of glaciers and other cold habitats emancipate these sleeping threatful entities which ultimately contaminate the drinking and domestic water. Upon interaction with the antibiotic-sensitive bacteria, these emancipated AR bacteria transfer the antibiotic-resistant determinants through HGT and make them adapt to the respective traits. Moreover, the implemented resistant traits (Plasmids, transposons, integrons) are further propagated to the other antibiotic-sensitive communities making the scenario more alarming. In this current study, the ARGs detected among the Psychrophilic bacteria isolated from a non-polar Passu glacier were deliberately introduced into a vector, and afterward their uptake by antibiotic-sensitive mesophilic bacteria (*E. coli*) through the functional cloning process. ARGs showing resistance to Penicillins, Cephalosporins, Carbapenems, Aminoglycoside, and Fluoroquinolones were successfully cloned and uptaken by mesophilic bacteria, emphasizing the role of natural pristine environments in the dissemination of ARGS and MGEs. Previously, the study of AR bacteria was confined to the infectious pathogenic bacteria that cause numerous infectious diseases in humans and animals, but recently due to the emergence of infectious diseases in the community and the involvement of non-clinical AR bacteria, provoked the researchers' interest to the environment and pristine habitats including the cryosphere (Gibson *et al.,* 2015).

Glaciers are very important to the biosphere regarding pandemics and endemics of various infectious diseases caused by Viruses and Bacteria (Rogers *et al.,* 2004). The glaciers were once thought to be the massive reservoir of fresh water on planet Earth and used for domestic and drinking water purposes but in the last 10 years, the literature gained from the extensive research on the glaciers and other cold environments, regarding the presence of AR bacteria has declared these water reservoirs as highly polluted and preeminently threatened (Van Goethem *et al.,* 2018). Besides AR bacteria, numerous bacterial species with stringent pathogenicity potential have been detected, inhabiting the glaciers' environment (Qi *et al.,* 2022). These bacterial communities effectively exchange different habitats through seasonal variations, hydrothermal vents, migrating birds, wind, airborne bacteria, and other temporal gene flow mechanisms making the mixing of AR bacteria more prominent and diverse

(Makowska *et al.,* 2020; Literak *et al.,* 2014). On the flip side, the natural environments are highly affected by anthropogenic activities in the form of careless use of antibiotics for both therapeutic and agricultural purposes which ultimately adds to the dissemination of antibiotic resistance in natural environments (Hawkey 2008). Moreover, the flourishing of bacteria in antibiotic-rich habitats (agricultural soil, animals' gut) also provides additional channels for antibiotic emergence and elevated infiltration of pollutant antibiotics to the adjacent environments (Malik *et al.,* 2008; Gibbs *et al.,* 2006). The emergence of antibiotic resistance occurs through mutual interaction between bacteria possessing antibiotic-resistant determinants and those lacking them. Horizontal gene transfer (HGT) serves as the primary mechanism for the effective transportation of antibiotic resistance genes (ARGs) between geographically diverse bacterial communities.

In this current study, the ARGs (*$_{bla}$CTXM-15*, *$_{bla}$NDM-1*, *aac(6)-lb3*, and *gyrA*), detected among the MDR Psychrophilic bacteria *Brevundimonas diminuta, Rahnella inusitata, Staphylococcus equorum*, and *Alcaligenes faecalis* were PCR amplified and poly-adenylated amplicons were purified and quantified for vector insertion. The desired ARGs of interest were successfully cloned in a pCR2.1-Topo Vector (Provided with TOPO Cloning Kit) and transformed using competent *E. coli* (One-Shot Mach1-T1) cells with ampicillin drug selection. To screen out the transformed *E. coli* cells, the blue-white screening method (Alpha complementation system) was utilized. The extracted plasmid vectors were subjected to PCR amplification for the desired inserts, using the appropriate primers for ARGs plus M13 Forward, and reverse and subsequently for sanger sequencing the plasmid vectors to confirm the presence of each ARG insert within the plasmid vector. Our current study results manifest the ultimate key role of environmental bacteria in the acquisition and dissemination of various ARGs and MGEs among different bacterial communities as well as the link between the clinical and environmental settings. The acquisition of glacier bacteria harbored ARGs by mesophilic antibiotic-sensitive bacteria also demonstrates the ecological fitness of environmentally derived genetic contents (ARGs, MGEs) in the distant bacterial species and are reported in several studies (Nielsen *et al.,* 2022; Clemente *et al.,* 2015). The common ancestral origin of distant habitat-inhabiting bacteria usually demonstrates the least fitness cost, considering the heterogeneity of the genetic contents and environment (Baltrus 2013). The cost of fitness and cloning/expression of ARGs and other resistant traits are correlated variably in different bacterial species.

Utilizing the cloning approach, the successful integration of ARGs antibiotic-sensitive bacteria and making the resistant one is obvious from various studies. In a study conducted by Chen *et al.* (2003), nine different types of ARGs, retrieved from *Serratia marcescens* were effectively cloned into an antibiotic-hyper-sensitive *E. coli* strain, followed by *Pseudomonas aeruginosa*, which gained the resistant mechanisms against different drugs including; Erythromycin, Tetracycline, Norfloxacine, and intercalating agent Ethidium bromide which supported our results, whereas in the current study, we successfully cloned ARGs in antibiotic-sensitive *E. coli* strain; showing resistance to Penicillins, Cephalosporins, Carbapenems, Aminoglycoside, and Fluoroquinolones. In some cases, cloning a single ARG results in the acquisition of multiple resistant traits including the efflux pump mechanisms that are mostly involved in MDR bacteria (Chen *et al.,* 2003). Besides cloning of specific ARGs in antibiotic-sensitive bacteria, to acquire antibiotic resistance, cloning of other cellular genes also alters the phenotypic characteristics of the transformed bacteria, as reported by Bharathi *et al.* (2011) which cloned the genes responsible for membrane fusion protein in *Acinetobacter baumanii* and making it antimicrobial-resistant which is supported by other studies (Ghanem 2011). In addition, Ito *et al.* (1999) designated the methicillin-sensitive *Staphylococcus aureus* as a methicillin-resistant strain by cloning the entire *mecA* DNA fragment (51,699 bp) from a Japanese *S. aureus* strain. Among bacterial antibiotic resistance, the efflux pump mechanism either acquired or innate nature is of significant importance and majorly contributes to the multi-drug resistant (MDR) phenotypes among diverse bacterial communities (Blair *et al.,* 2015). The successful integration of genes responsible for the efflux pump system in a recipient bacterium, through the molecular cloning process, alters the bacterial characteristics into the MDR traits. A study conducted by Sekiya *et al.* (2003) added the antibiotic efflux pump characteristics to the lacking counterpart by cloning the MexH1-OmpD from *Pseudomonas aeruginosa*, in a vector (pUCT20T) and subsequently successful integration. Moreover, such multi-drug efflux pump acquisition has also been shown in other studies; against the *EmeA* from *Enterococcus faecalis* (Lee *et al.,* 2003) and against NorM (*Neisseria gonorrhea*) and YdhE (*E. coli*) (Long *et al.,* 2008).

Conclusion

The ARGs detected among psychrophilic bacteria isolated from a non-polar Passu glacier were successfully integrated into pCR2.1-Topo Vector and the subsequent transformation of

mesophilic *E. coli* manifest the starring role of pristine environmental bacteria in the acquisition and dissemination of ARGs and MGEs through HGT phenomena among environmental as well as clinically relevant bacteria. The liberated bacteria from melting glacier when interacting with community bacteria, transfer of ARGs occurs which immensely enhance antibiotic resistance and pose the foremost threat to the community's health.

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Conclusions

Culturable bacterial diversity, antibiotic resistance, and abundance of ARGs

- A Total of 65 bacterial isolates were collected from Passu glacier with the glacial sediment as the bacterial richest source 43 (66.1%), followed by ice 14 (21.5%) and meltwater 8 (12.3%), respectively.
- The majority of isolates, 31 (47.69%) showed growth at 4°C + 15°C.
- Gram-negative bacteria were predominant 43 (66.15%), compared to gram-positive 22 (33.84%).
- Most gram-negative bacteria belonged to *Proteobacteria* 39 (90.6%) whereas gram-positive to *Actinobacteria* 11 (50%).
- Out of the total of 65 bacteria, 34 (52.4%) were MDR whereas 9 (13.8%) were XDR, followed by their abundance among sediment and gram-negative bacteria.
- HP19 (*Staphylococcus equorum)* (gram-positive) was the most resistant strain to (22/29, 75.86%) antibiotics, while HP21 (*Brevundimonas diminuta)* (gram-negative) to (16/21, 76.19%) antibiotics.
- Overall resistance was least against Fluoroquinolones, Carbapenems, and Glycycline, while substantial against Nitroimidazole, Oxazolidinones, and Glycopeptide.
- Among gram-positive bacteria, 86.04% had > 0.2 MAR index value, whereas gram-negative had 90.9%.
- HP19 (*Staphylococcus equorum)* (gram-positive) and HP55 (*Alcaligenes faecalis)* from gram-negative showed the highest MAR index value of 0.7.
- ARGs and *IntI1* gene were abundantly detected among gram-negative bacteria and glacial sediment bacteria harbored maximum ARGs, followed by meltwater and ice, respectively.
- Proteobacteria was found abundant in ARGs and *IntI1*, followed by Actinobacteria, Firmicutes, and Bacteroidetes, respectively.
- The ARGs *$_{bla}$CTXM-15*, *$_{bla}$NDM-1*, *aac(6')-Ib3*, *sul1*, *sul2*, *gyrA*, and *intI1* were frequently detected among glacier bacteria, while a single isolate harbored *$_{bla}$OXA-1*.
- HP21 (*Brevundimonas diminuta)* and HP3 (*Rahnella inusitata)* (gram-negative) were detected with most ARGs (19,17) plus *intl1*, respectively.
- HP19 (*Staphylococcus equorum)* and HP22 (*Leucobacter aridicollis)* (gram-positive) were found positive for maximum ARGs (11 and 8) plus *intl1*, respectively.

- The ARGs and *IntI1* amino acids alignment with the clinical-based counterparts were found with several variations against $_{bla}$*OXA-1*, $_{bla}$*NDM-1*, $_{bla}$*SHV*, *sul2*, *tetA*, *qnrB*, *gyrA*, and *IntI1*.

Plasmid-mediated antibiotic resistance (plasmid curing analysis)

- Gram-positive bacteria tolerated higher acridine orange concentrations and were more prone to plasmid loss, compared to gram-negative.
- Gram-negative (21/43, 48.83%), whereas gram-positive (7/22, 31.81%) were found with no change in antibiotic susceptibility pattern pre and post-treatment with acridine orange.
- Post acridine orange curing, bacteria exhibited lower resistance to antibiotics except to Aztreonam, Linezolid, Tigecycline, and Metronidazole.
- Levofloxacin, Ciprofloxacin, Piperacillin, Meropenem, and Imipenem achieved 100% susceptibility post-plasmid curing with acridine orange.
- A massive decrease in MAR index values from 87.6% to 65.3% isolates with > 0.2 MAR index values was observed post-curing.

Real-time PCR quantification of integron integrase genes

- Glacial sediment bacteria were predominantly possessed with *IntI1* and *IntI2* compared to meltwater and ice with a higher prevalence of *IntI1*.
- Ct values were lowest for *IntI1*, indicating the strong nucleic acid contents, compared to *IntI2*.
- Real-time PCR positive *IntI2* (all isolates) and *IntI1* (Ct value >31) were negative on conventional PCR.
- Gram-negative isolates were more prevalent with *IntI1* and *IntI2* with the lowest Ct values, compared to gram-positive.
- *IntI1* strong nucleic acid content was detected in *HP21 Brevundimonas diminuta* (Ct = 11.63) (gram-negative) and *HP22 Leucobacter aridicollis* (Ct = 12.7) (gram-positive), respectively.

Cloning of antibiotic-resistant genes in *E. coli*

- Antibiotic-resistant genes ($_{bla}$*CTXM-15*, $_{bla}$*NDM-1*, *aac6-Ib3*, and *gyrA*), detected among psychrophilic bacteria were successively cloned in a pCR 2.1 vector.

- Glacier bacteria harbored antibiotic-resistant genes were successfully taken up by the mesophilic antibiotic-sensitive *E. coli* strain.
- Glacier bacteria harbored ARGs can be easily disseminated through horizontal gene transfer into other habitats.
- This also emphasizes the role of pristine environments in the acquisition and dissemination of antibiotic-resistant genes.

Advances made in this study

- A first comprehensive description of the presence of ARGs and class 1 and 2 *integron integrases* among Pakistani glacier native bacteria.
- Explored the non-polar glacier (Passu) as a contaminated source habitat with a higher risk to public health.
- Variations among amino acid sequences in $_{bla}$*OXA-1*.
- Variations among amino acid sequences in $_{bla}$*NDM-1*.
- Variations among amino acid sequences in $_{bla}$*SHV*.
- Amino acids variations in *sul2*, *tetA*, *qnrB*, *gyrA*, and class 1 *integron integrase*.
- Non-polar glacier bacteria were found with significant plasmid harbored ARGs.

Future Prospects

- Next-generation sequencing of massively ARGs-possessed strains (HP19 *Staphylococcus equorum*, HP21 *Brevundimonas diminuta*, HP55 *Alcaligenes faecalis*) to determine other MGEs, Insertion sequences, and gene cassettes in the entire genome.
- Stability and cost of glacier bacteria harbored MDR plasmids and to check their transfer to indigenous soil bacteria (HGT potential).
- Sequence alignment data (amino acids variation, altered 3D protein structure) might be useful for molecular docking and structure-based novel drug targets.

Appendices

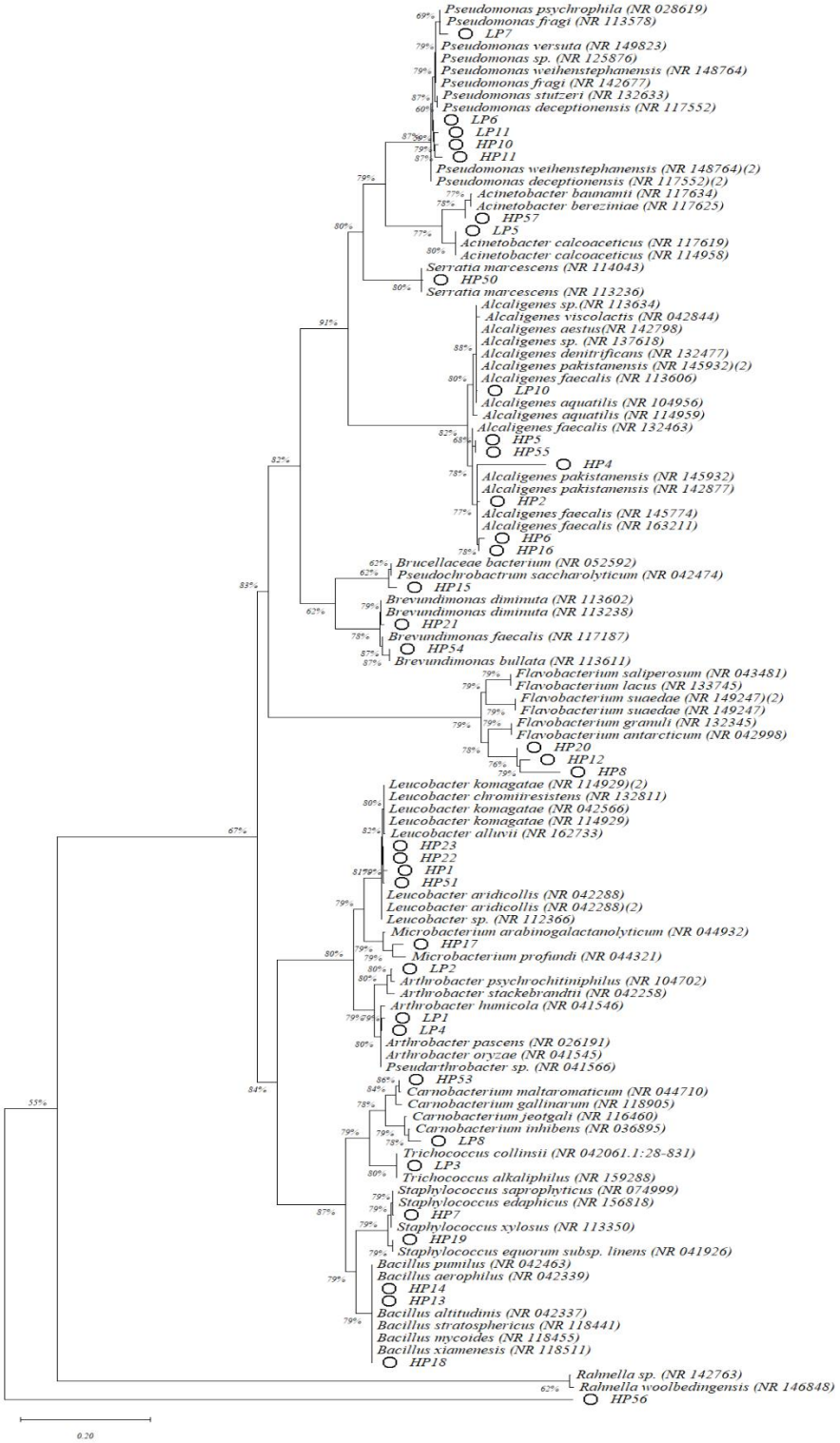


Figure A1. Phylogenetic tree of isolates (27F) retrieved from Passu glacier's sediment by Maximum Likelihood method constructed in MEGA X software

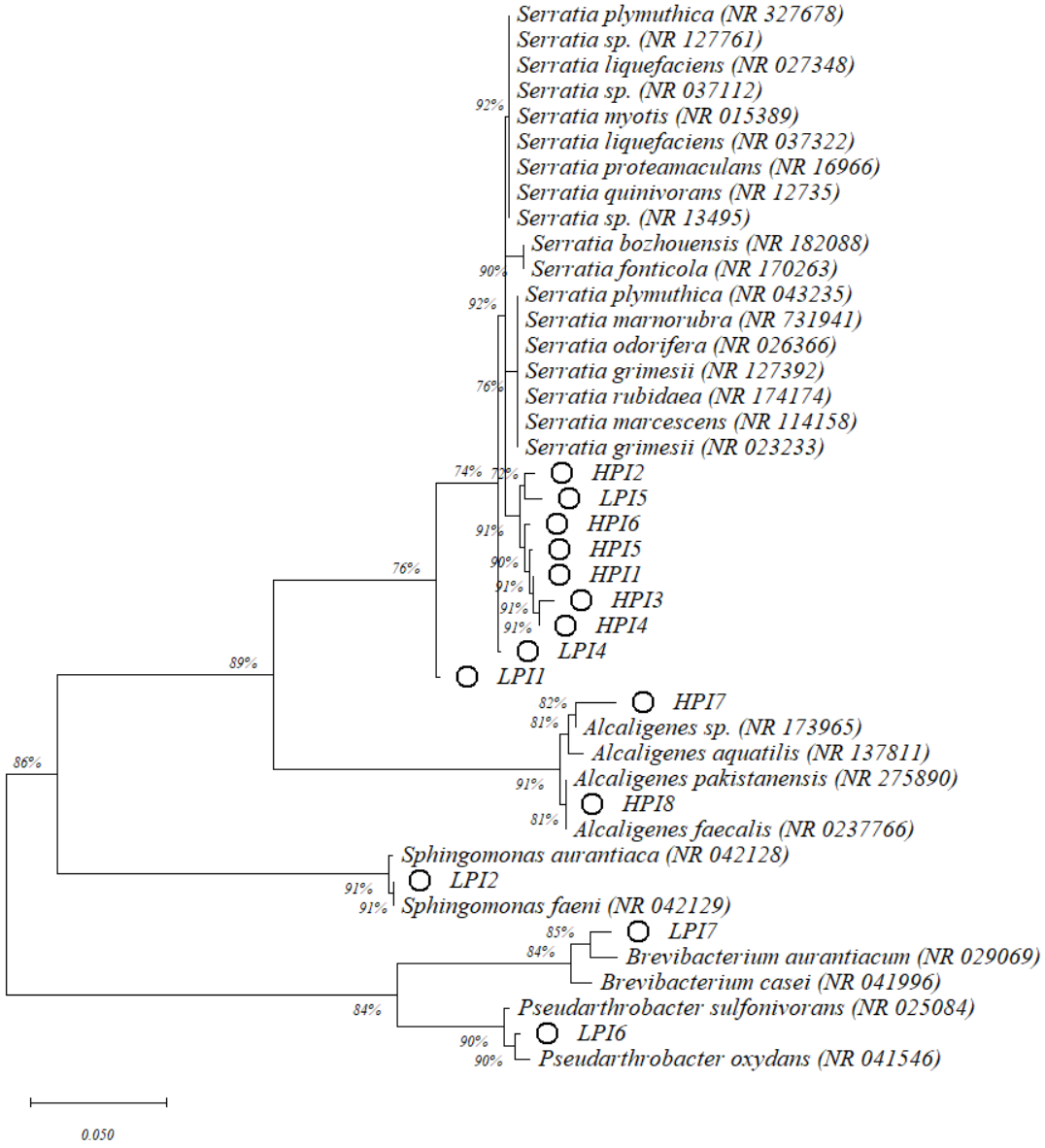


Figure A2. Phylogenetic tree of isolates (27F) retrieved from Passu glacier's ice by Maximum Likelihood method constructed in MEGA X software

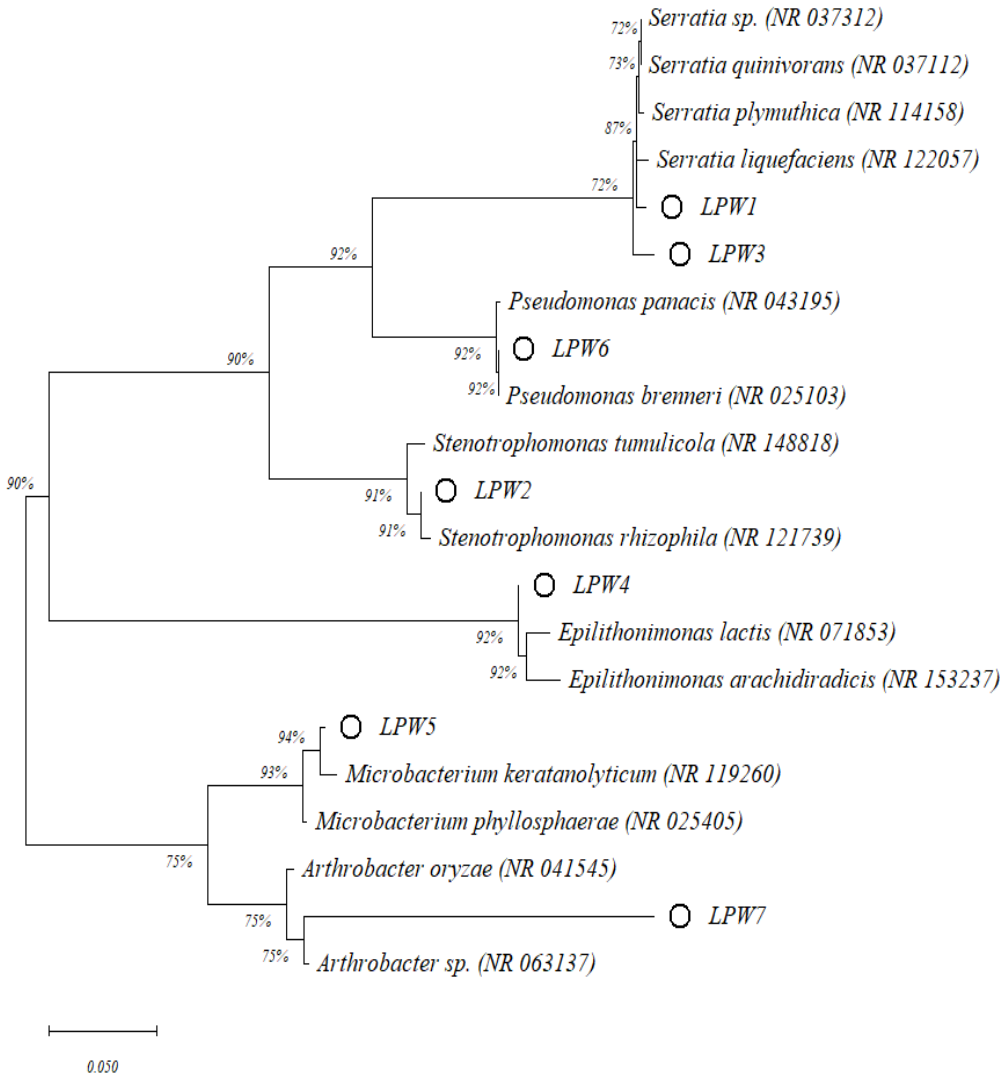


Figure A3. Phylogenetic tree of isolates (27F) retrieved from Passu glacier's meltwater by Maximum Likelihood method constructed in MEGA X software

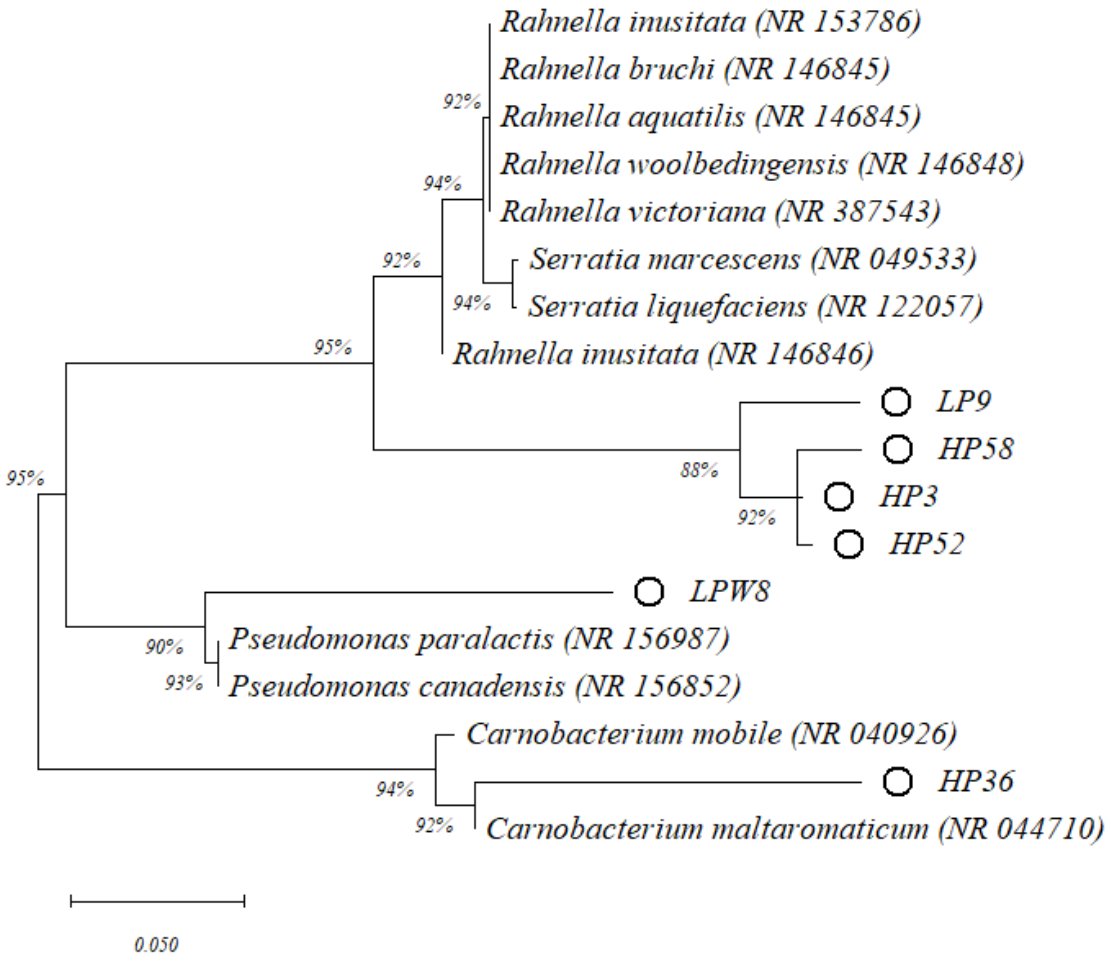
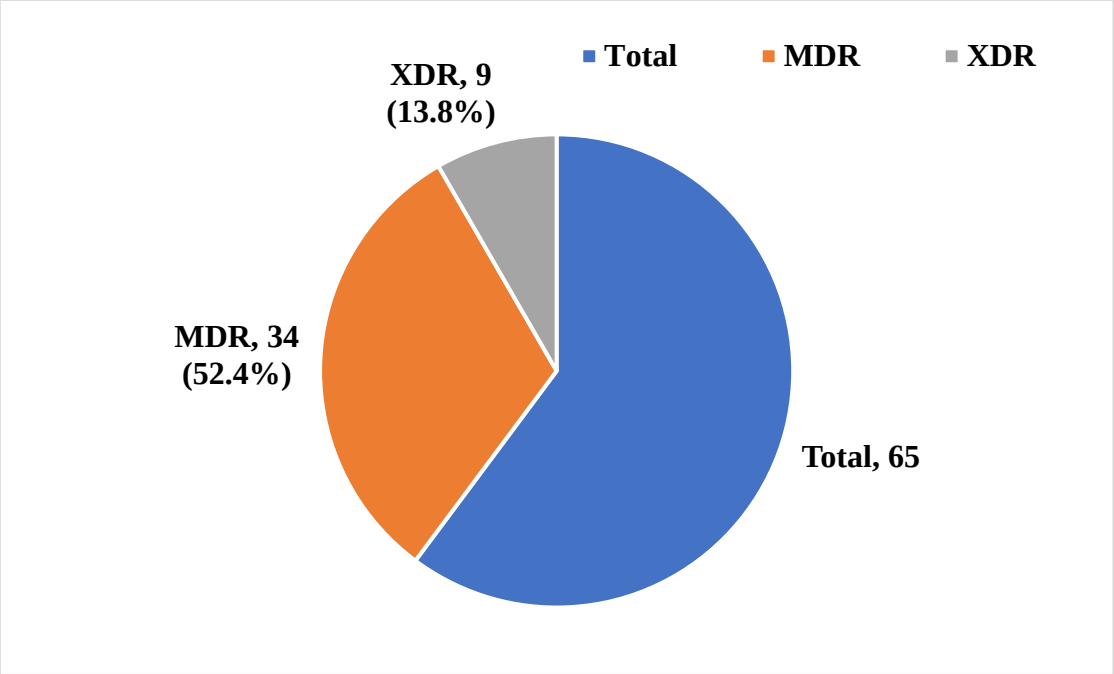
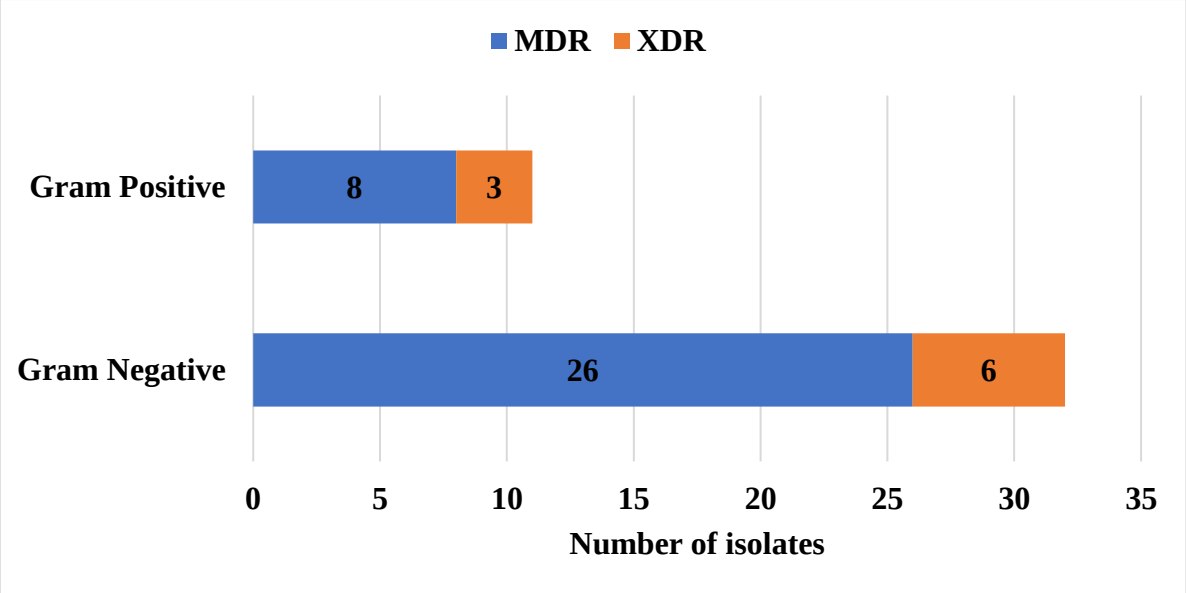


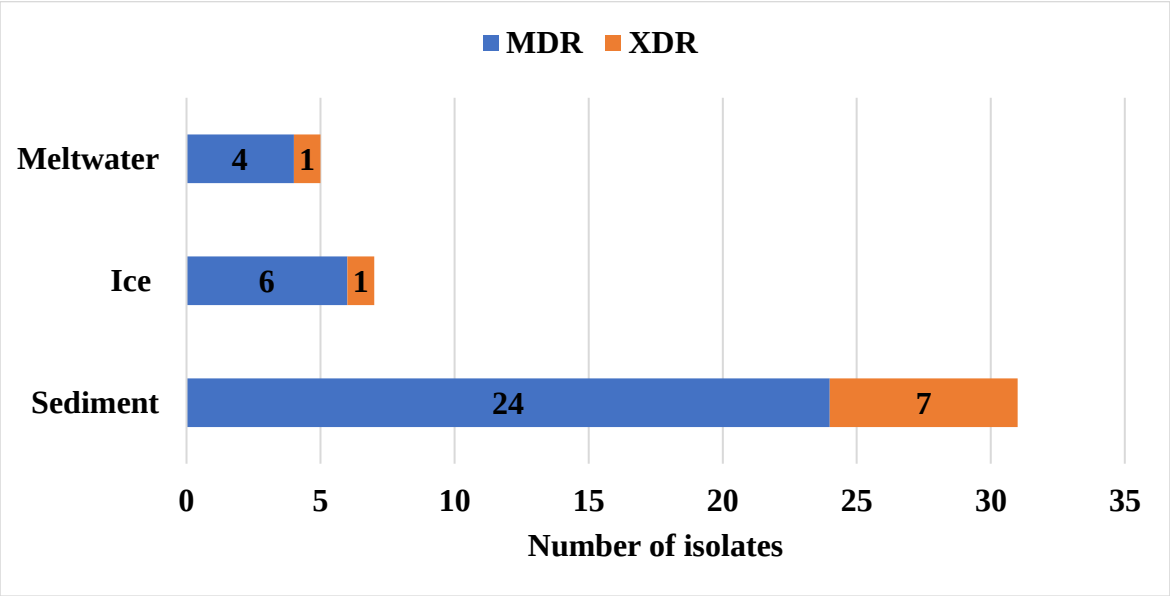
Figure A4. Phylogenetic tree of isolates (1492R) retrieved from Passu glacier by Maximum Likelihood method constructed in MEGA X software



FigureA5. Number and percentage of Multi-drug resistant (MDR) and Extensive-drug resistant (XDR) bacteria among total isolates



FigureA6. Number of Gram-Positive and Gram-Negative Multi-drug resistant (MDR) and Extensive-drug resistant (XDR) bacteria among total isolates



FigureA7. Number of Multi-drug resistant (MDR) and Extensive-drug resistant (XDR) bacteria among glacier Sediment, Ice, and Meltwater isolates

Appendix 1. List of Multi-drug resistant (MDR) and Extensive-drug resistant (XDR) bacteria among Gram-negative and Gram-positive isolates retrieved from glacier

Gram-negative		Gram-positive	
MDR	**XDR**	**MDR**	**XDR**
HP2 Alcaligenes faecalis	*HP20 Flavobacterium antarcticum*	*HP14 Bacillus xiamenesis*	*HP19 Staphylococcus equorum*
HP3 Rahnella inusitata	*HP21 Brevundimonas diminuta*	*HP18 Bacillus pumilus*	*HP22 Leucobacter aridicollis*
HP4 Alcaligenes faecalis	*HP50 Serratia marcescens*	*HP19 Staphylococcus equorum*	*LP2 Arthrobacter psychrochitiniphilus*
HP12 Flavobacterium suaedae	*HP55 Alcaligenes sp.*	*HP22 Leucobacter aridicollis*	
HP16 Alcaligenes faecalis	*HPI1 Serratia sp.*	*HP23 Leucobacter sp.*	
HP20 Flavobacterium antarcticum	*LPW6 Pseudomonas brenneri*	*HP51 Leucobacter komagatae*	
HP21 Brevundimonas diminuta		*HP53 Carnobacterium maltaromaticum*	
HP50 Serratia marcescens		*LP2 Arthrobacter psychrochitiniphilus*	
HP52 Rahnella inusitata			
HP54 Brevundimonas bullata			
HP55 Alcaligenes sp.			
LP5 Acinetobacter calcoaceticus			
LP7 Pseudomonas psychrophila			
LP9 Rahnella aquatilis			
LP10 Alcaligenes aquatilis			
LP11 Pseudomonas fragi			
HPI1 Serratia sp.			

HPI3 Serratia proteamaculans
HPI4 Serratia sp.
HPI7 Alcaligenes sp.
HPI8 Alcaligenes faecalis
LPI1 Serratia plymuthica
LPW1 Serratia sp.
LPW6 Pseudomonas brenneri
LPW7 Arthrobacter sp.
LPW8 Pseudomonas paralactis

Appendix 2. Translational variations and CARD identities of glacier bacteria harbored ARGs and *IntI1* compared to the reference ARGs circulating among clinical bacteria

ARGs	Amino acids alterations	Reference ARGs Strains	CARD Identity
blaCTX-M-15	No variation	*Escherichia coli* *Enterobacter hormaechei*	99.74%
blaCMY-4	No variation	*Citrobacter portucalensis* *Escherichia coli* *Proteus mirabilis* *Salmonella enterica* *Vibrio parahaemolyticus*	99.39%
blaOXA-1	7 amino acids variations Leu160→ Val, 161Gln→ Trp, 162Asn→ Glu, 163Gly→ Asn, 165Phe→ Cys, 166Glu→ Arg, 170Ile→ His	*Providencia alcalifaciens* *Avibacterium paragallinarum* *Morganella morganii* *Pseudomonas aeruginosa* *Escherichia coli*	97.36%
blaTEM-1	No variation	*Citrobacter werkmanii* *Serratia marcescens* *Enterobacter cloacae* *Raoultella ornithinolytica* *Salmonella enterica*	99.8%
blaNDM-1	Single amino acid variations 61Asp→ Tyr	*Alcaligenes aquatilis* *Acinetobacter junii* *Citrobacter freundii* *Shewanella putrefaciens* *Raoultella planticola* *Escherichia coli*	99.0%
blaSHV	2 amino acids variations 80Val→ Leu, 84Arg→ Pro	*Klebsiella pneumoniae* *Pseudomonas aeruginosa*	98.9%
mecA	No variation	*Staphylococcus aureus* *Staphylococcus epidermidis* *Staphylococcus haemolyticus* *Staphylococcus pseudintermedius* *Staphylococcus saprophyticus*	99.6%
aac6-Ib3	No variation	*Escherichia coli* *Serratia marcescens* *Enterobacter hormaechei* *Salmonella enterica* *Raoultella planticola* *Morganella morganii* *Pseudomonas aeruginosa* *Citrobacter freundii*	97.86%

sul2	2 amino acids variations 4Ala→ Thr, 62Pro→ Thr	*Escherichia coli* *Citrobacter werkmanii* *Salmonella enterica* *Providencia alcalifaciens* *Enterobacter cloacae*	98.9%
tetA	3 amino acids variations 4Pro→ Arg, 52Leu→ Val, 54Phe→ Ile	*Aeromonas caviae* *Edwardsiella tarda* *Citrobacter werkmanii* *Escherichia coli* *Pseudomonas aeruginosa* *Salmonella enterica*	99.0%
tetB	No variation	*Avibacterium paragallinarum* *Uncultured bacterium* *Actinobacillus pleuropneumoniae* *Pasteurella multocida* *Escherichia coli*	98.8%
qnrB	3 amino acids variations (6Ile→ Asn, 103Thr→ Ser, 128Arg→ Pro	*Pantoea agglomerans* *Shigella flexneri* *Aeromonas veronii* *Salmonella enterica* *Escherichia coli* *Enterobacter cloacae*	99.3%
gyrA	2 amino acids variations 5Phe→ Tyr, 46Asp→ Glu	*Acinetobacter baumannii*	98.4%
Class 1 integron integrase	2 amino acids variations 60Val→ Asp, 61Phe→ Leu	*Serratia marcescens* *Edwardsiella tarda* *Aeromonas caviae* *Raoultella planticola* *Providencia alcalifaciens* *Citrobacter freundii*	98.9%

Appendix 3. Bacteria retrieved from Passu glacier, *16S ribosomal RNA* partial sequences

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Appendix 4. Antibiotic-resistant genes, detected among Passu glacier's bacteria, nucleotides, and amino acid sequences

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RESEARCH

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

Sabir Nawaz[1] · Muhammad Rafiq[2] · Ian L. Pepper[3] · Walter Q. Betancourt[3] · Aamer Ali Shah[1] · Fariha Hasan[1]

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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*, *intl2*, and *intl3*. 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 *intI1*. 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 · ß-lactamase

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

✉ Fariha Hasan
farihahasan@yahoo.com

Sabir Nawaz
sabirnawaz@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

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