Characterization of antibiotic-resistant bacteria in two non-integrated fish farming sites

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2023

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A thesis submitted in partial fulfillment of the requirements for the Degree of

Master of Philosophy

In

Microbiology

by

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2023

بسم الله الرحمن الرحيم

Dedication

This humble effort is dedicated to my parents, siblings, and teachers who have always loved and encouraged me and without whom none of this would have been possible.

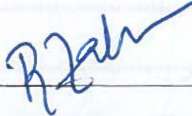
Declaration

I hereby declare that this thesis is the outcome of my original work and has not been submitted for any other degree in this university or elsewhere previously.

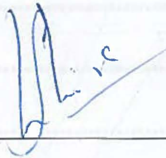
Muhammad Hamza

CERTIFICATE

This thesis, submitted by Muhammad Hamza to the Department of Microbiology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan, is accepted in its present form as satisfying the requirements for the degree of Master of Philosophy in Microbiology.

Supervisor: 

Prof. Dr. Rabaab Zahra

External Examiner: 

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Dated: 02-11-2023

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List of Abbreviations

AMR	Antimicrobial resistance
HGT	Horizontal gene transfer
PCR	Polymerase chain reaction
ARG	Antibiotic-resistance gene
ARB	Antibiotic-resistant bacteria
ESBLs	Extended-spectrum beta-lactamases
QNR	Quinolone resistance
PMQR	Plasmid-mediated quinolone resistance
BEA	Bile esculin agar
NDM	New Delhi Metallo β-lactamases
Oxa-48	Oxacillinase-48
MDR	Multi-drug resistant
VIM	Veron Integron metallo β-lactamases
IMP	Imipenem Resistant Pseudomonas
MIC	Minimum inhibitory concentration
CFU	Colony Forming Unit
UTI	Urinary tract infections
KPC	*Klebsiella pneumoniae* Carbapenemases

Acknowledgment

All praise is to Allah Almighty, whose blessings made it possible for me to earn this M. Phil. in microbiology. Praise be to Prophet Muhammad (PBUH), a guide for all students of wisdom and all of humanity.

My sincere thanks go out to my honorable supervisor **Prof. Dr. Rabaab Zahra** for her unwavering support, direction, helpful advice, persistent encouragement, and active supervision that made it possible for me to complete this assignment successfully.

A very special thank you to **Dr. Ayaz Ali Khan** Post Doc. EMBARK, who helped and supported me during my working tenure.

I am speechless in my appreciation for my devoted Parents, Family, and especially my Siblings, who have always been there for me. I will always be in debt to them for what they have done for me and will never be able to repay them.

Many thanks to my senior lab colleagues **Shahid Mehmood**, **Shahid Ali, Abdul Rauf Tareen**, **Rida toufiq**, **Nauman Kabir**, and **Muhammad Ismail** for their steadfast assistance and support throughout my research.

I want to thank my friends **Umair**, **Asfandyar**, **Ihtihsam**, **Haziq,** and **Awaise** who have been by my side during the entire journey.

I am also grateful to my other friends and lab fellows **Alizay**, **Eman**, **Fizza**, and **Shahid bhai** for their assistance throughout my research project.

Although not all are mentioned, none is forgotten.

Muhammad Hamza

Abstract

Abstract

Antibiotic resistance genes (ARGs) are emerging pollutants that pose a significant threat to public health. The utilization of antibiotics in aquaculture fosters both the rise of antibiotic-resistant bacteria and the dissemination of antibiotic resistance genes (ARGs) in the fish farming environment. The present investigation centered on the prevalence of antimicrobial resistance genes (ARGs) within Gram-negative bacteria and their corresponding susceptibility to antimicrobial agents. A total of thirty-five samples were collected from fish seed hatcheries located in Islamabad and Faisalabad, Pakistan. These samples consisted of eighteen water samples and seventeen sediment samples. The samples were subjected to plating on various selective media and media supplemented with antibiotics, after which they were incubated at 37ºC for 24 hours. A total of 992 bacterial isolates were obtained from a set of thirty-five water and sediment samples. Among them, a subset of 15.5% (n=143) were identified as Gram-negative bacteria, while 12.6% (n=116) were Gram-positive bacteria. Additionally, 8.1% (n=75) of the isolates belong to *Enterococci*, whereas a significant majority of 63.8% (n=588) were antibiotic-resistant Gram-negative bacteria. The highest CFU count was reported in sediment samples which were 1x107 CFU/mL for Gram-negative bacteria. The conventional PCR was used to screen for genes encoding extended-spectrum beta-lactamase (ESBLs), carbapenemases, tetracycline resistance, polymyxin resistance, and plasmid-mediated quinolone resistance (PMQR).

Of the 35 samples, fourteen types of ARGs were identified within 79 isolates. The isolates correspond to *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Acinetobacter, baumannii*, *E. coli*, *Serratia marcescens*, *Citrobacter freundii*, *Proteus mirabilis*, *Edwardsiella tarda*, and *Klebsiella oxytoca.* Among the PMQR genes, aac(6')-Ib-cr was found as the most common gene associated with ciprofloxacin resistance, accounting for 11.11%, followed by *qnr*B in 7.9% of isolates. The presence of *qnr*S was observed in 2.3% of isolates, Conversely, found no prevalence of *qnr*A, *qnr*C, and *qnr*D. Moreover, a coexistence of more than one PMQR genes were detected in 1.6% of the isolates. The prevalent gene in ESBL-producing isolates was $bla_{CTX\text{-}MU}$, found in 6.1% of the isolates, followed by bla_{SHV} in 3.8% of cases. Furthermore, $bla_{CTX\text{-}M9}$ was found positive in 2.2% of isolates, but bla_{TEM} was reported in just 0.7% of isolates. Regarding polymyxin resistance genes, *mcr-1* was found in only 0.9% of the isolates, with no prevalence of other polymyxin resistance genes. Among the carbapenem resistance genes, $bla_{OXA\text{-}}$

$_{48}$ was found in 7.2% of isolates, followed by 4.5% carrying *bla*$_{\text{VIM}}$ and 2.7% carrying *bla*$_{\text{NDM}}$. From oxytetracycline-resistant isolates, *tet*A was found, the most prevalent, appearing in 10.3% of isolates, followed by *tet*B in 6% and *tet*E in 5.1% of isolates. Furthermore, a co-occurrence of more than one tetracycline resistance gene was detected in 3.4% of the isolates. The multiple antibiotic resistance (MAR) index for isolates positive for antibiotic resistance determinants were found to vary from 0.2 to 1. These findings highlight the prevalence of multidrug-resistant bacteria and antimicrobial resistance genes in fishponds, posing a substantial threat to both human and animal health, as well as the environment. As a result, comprehensive surveillance measures are required to halt the spread of antibiotic resistance.

Chapter 1

Introduction

Introduction

Antimicrobial resistance poses a significant public health challenge, compromising the effectiveness of antibiotic treatments and making them ineffective against potentially life-threatening bacterial infections (Wang *et al.,* 2015). It is commonly accepted that several infectious diseases have long been treated with antibiotics in both humans and animal husbandry. In addition to infectious disease treatment, different medical therapies such as organ transplantation, open heart surgery, and cancer therapy have been made possible through antibiotics (Katz & Baltz, 2016). Proper antibiotic use has proven beneficial, but there are still negative consequences linked with antibiotic overuse. The usage of antimicrobials and the emergence of resistant bacteria have always had a close temporal and spatial relationship (Marshall & Levy, 2011). Antimicrobial-resistant bacteria were discovered among patients and in the hospital environment, where the majority of the use of antimicrobials occurred, during the start of the antimicrobial era (Chevrette *et al.,* 2019). The utilization of antimicrobials in veterinary medicine and aquaculture led to the emergence of antimicrobial-resistant zoonotic pathogens within these settings. Consequently, it leads to the transmission of bacteria harboring antimicrobial resistance genes from industrially raised animals to humans as well as in the environment. Serotyping and plasmid genetic analysis initially showed the presence of this passage. Still, it wasn't confirmed unless the introduction of new techniques like molecular analysis and DNA sequencing verified the use of antimicrobials and its passage (Cabello *et al.,* 2016).

The utilization of antimicrobials in different settings is linked to the growing human population that requires increased food production to meet the rising demand for sustenance (Nations, 2016). According to Gitz and Meybeck (2016), based on an estimate provided by the Food and Agriculture Organization (FAO), it is projected that there would be a significant need to increase global food production by 70% if the world population reaches 9 billion by the year 2050 (Gitz & Meybeck, 2016). Aquaculture, as a vital and profitable industrial activity, holds considerable significance in addressing the issues of the current millennium and serves as an affordable source of protein (Cidad *et al.,* 2018; Colgrave *et al.,* 2021). The global demand for seafood has substantially increased aquaculture production, encompassing cold and warm waters, with over 85 million tonnes produced in 2019 (Deekshit *et al.,* 2023). Aquaculture forecasts for 2030 predict a 27% increase in fish and seafood consumption and a doubling in production (Pepi & Focardi,

2021). In order to address the growing demand and sustain the growth within the aquaculture sector, the utilization of antibiotics in aquaculture is a widely acknowledged activity to treat disease prophylactically and therapeutically (Ibrahim *et al.,* 2020). Antimicrobials in aquaculture are frequently provided to whole populations, including those who are sick, healthy, and carrying the disease, through a procedure known as metaphylaxis. (Romero *et al.,* 2012). Antibiotics are frequently given to animals through their food (Rico *et al.,* 2013; Wang *et al.,* 2015), and between 30 and 90 percent are excreted as either feaces or urine (Sarmah *et al.,* 2006). As a result, fish waste and discarded food containing antibiotics, their byproducts, and degradation materials are released into the nearby environment (Sørum, 2005). This is especially the case in high-density fish farming, where more than 70% of the antibiotics administered through feed disperse into the surroundings (Le & Munekage, 2004; Rico *et al.,* 2013; Sebesvari *et al.,* 2012; Suzuki & Hoa, 2012). Moreover, farmers involved in aquaculture production often have an insufficient understanding of antibiotic utilization (Ali *et al.,* 2016; Cabello, 2006a) . Consequently, the excessive or improper utilization of antibiotics in aquaculture frequently occurs (Heuer *et al.,* 2009a; Sapkota *et al.,* 2008).

The consumption of antimicrobial compounds in aquaculture has significantly increased globally in recent years. In 2017, the global consumption of antibiotics reached 93 million tonnes (MT), with the aquaculture sector accounting for almost 10 MT. The estimated growth rate is expected to reach 33% by 2030. In 2017, the most considerable proportion of consumption was observed in China, which accounted for 58% of the total. Subsequently, India accounted for 11%, Indonesia for 9%, and Vietnam for 5% (Schar *et al.,* 2020). Furthermore, according to Schar et al. (2020), it is projected that the global output of aquacultural products will account for 5.7% of the total consumption of 236 MT. by the year 2030 (Schar *et al.,* 2020). The approval of specific antibiotics for use in aquaculture in several major aquaculture-producing countries has led to extensive use. From 2008 to 2018, a study conducted by Lulijwa et al. (2020) revealed that oxytetracycline, florfenicol, and sulphadiazine were utilized by more than 70% of the nations under investigation. Additionally, amoxicillin, erythromycin, and enrofloxacin were found to be employed by 55% of the same nations throughout the same period (Lulijwa *et al.,* 2020). To treat fish diseases, the FDA agencies of some countries that produce aquaculture have authorized the use of antibiotics such as tetracycline, trimethoprim, oxytetracycline, florfenicol, sulphadiazine, amoxicillin, erythromycin, enrofloxacin (Baoprasertkul *et al.,* 2012; Lozano *et al.,* 2018).

According to a study conducted by Lulijwa et al. (2020), a total of 67 antibiotics were employed by the top 15 aquaculture-producing countries over the period from 2008 to 2018 (Lulijwa *et al.,* 2020). The implementation of more stringent laws and the growing worldwide consciousness have decreased antibiotic use in various economically significant production systems, particularly those aimed at the foreign market (Ali *et al.,* 2016; Henriksson *et al.,* 2015). To assess the impact of regulations and aquaculture practices on antibiotic resistance, it is imperative to carry out nationwide monitoring of antibiotic resistance genes (ARGs), a measure that has already been put into operation in certain nations (Dejsirilert *et al.,* 2011; Karp *et al.,* 2017). But, the studies unveiled the utilization of formerly banned antibiotics, namely chloramphenicol, rifamycin, penicillin, and many others within China, Vietnam, and Thailand (Lulijwa *et al.,* 2020). The utilization of antibiotics in aquaculture has been found to raise concerns regarding food safety owing to the presence of antibiotic residues. This, in turn, can adversely affect aquaculture products' marketability, as indicated by many studies(Chen *et al.,* 2015, 2018; Heuer *et al.,* 2009b; Sapkota *et al.,* 2008). Moreover, the potential existence of antibiotic residues in aquaculture products has the potential to impact the safety of wild fish. This is due to the possibility of ingestion of antibiotic by wild fish, which can result in adverse consequences for the safety of captured fishing products (Boxall *et al.,* 2004; Li *et al.,* 2012).

In fish farms and the surrounding aquatic habitats, there is a high prevalence of resistant bacteria to aquaculture antimicrobials. Furthermore, antimicrobial residues discovered in the sediments of fish farms (Cabello, 2006a; Maki *et al.,* 2006). The surplus antimicrobial residues may accumulate in fish farms since ponds are rarely drained before harvest. This type of accumulation could create selective pressure that favors the selection and growth of antimicrobial-resistant microorganisms. Moreover, genes that confer antibiotic resistance may continue to exist in the environment for multiple years following the cessation of antibiotic usage (Tamminen *et al.,* 2011). Typically, the widespread use of antibiotics for prophylactic purposes in aquaculture intensifies the probability of resistance arising from increased selective pressure, making the medication progressively ineffective (Aarestrup, n.d.; Schwarz *et al.,* 2005). As a result, antibiotic-resistant strains of *Escherichia coli* (S. Q. A. Shah *et al.,* 2014; Tuševljak *et al.,* 2013), *Acinetobacter* spp., *Aeromonas* spp. (Agersø & Petersen, 2007a), Streptococci and Enterococci (Osman *et al.,* 2017), Vibrio spp. (Kitiyodom *et al.,* 2010; Tuševljak *et al.,* 2013; Zulkifli *et al.,* 2009), Salmonella spp.,

Edwardsiella spp., and *Streptococcus* spp. (Tuševljak *et al.,* 2013) have been observed in aquaculture.

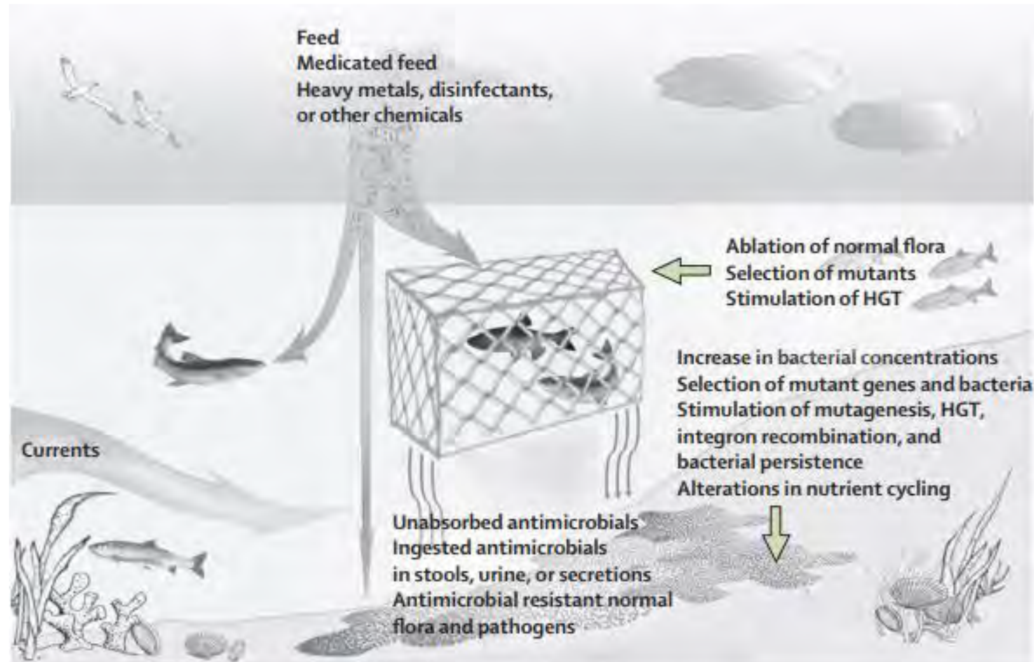


Figure 1.1: Antimicrobials in feed and their residues in the environment

For many years, animal manure used as fertilizer in fishponds has a prebiotic effect on the pond ecosystem, rather than probiotic effect on fish (Minich *et al.,* 2018). On integrated farms, livestock such as chickens and pigs are raised intensively, and antimicrobial drugs are employed as growth stimulants as well as preventative and therapeutic treatment (McKernan *et al.,* 2021). Although manure application helps recycle nutrients from one biological system to another (Ugwumba *et al.,* 2010). At the same time, animal manure is also a source of antibiotic residues and antibiotic-resistant bacteria (Agersø & Petersen, 2007b; Petersen *et al.,* 2002). Direct consumption of antimicrobial-resistant bacteria found in fish and related products has the potential to transmit resistant resistance genes to people from aquaculture environments (Akinbowale *et al.,* 2006).

The ingestion of fish products containing antibiotic residues has the potential to cause adverse drug reactions (ADR). It can also contribute to the emergence of antibiotic resistance in clinically relevant bacterial pathogens (X. Liu *et al.,* 2017). Furthermore, some studies have presented empirical data indicating that the occurrence and dissemination of antimicrobial resistance (AMR)

within aquaculture environments could potentially contribute to the evolution of AMR in human diseases. (Rhodes *et al.,* 2000). Furushita et al. (2003) emphasized that in Japan, there was a noteworthy resemblance between the genes associated with tetracycline resistance in bacteria identified in farmed fish and those isolated from clinical samples. This similarity suggests a potential common origin for these genes. Laboratory experiments were conducted on marine strains of *Photobacterium*, *Vibrio*, *Aeromonas*, and *Pseudomonas* to investigate the transferability of tetracycline resistance to *E. coli* via conjugation. The findings of these experiments suggest the potential for the transfer of resistance determinants from marine bacteria to gut-associated bacteria in humans (Furushita *et al.,* 2003).

Likewise, research has revealed that a comparable set of antibiotic resistance genes (ARGs) may be identified in bacterial populations found in fish and those isolated from human clinical samples. Indeed, approximately 50% of the antimicrobial resistance genes (ARGs) detected in fish infections exhibit similarities to those found in human diseases. This observation suggests that bacteria from diverse habitats, such as aquatic environments and hospital settings, can exchange identical resistance genes (Furushita *et al.,* 2003; Rhodes *et al.,* 2000). Before being detected and spread among human and animal pathogens, numerous antibiotic resistance genes (ARGs) were initially discovered in bacteria found in aquatic environments. These include various emerging plasmid-mediated quinolone-resistance (PMQR) genes in aquatic *Vibrio*, *Shewanella*, and *Aeromonas*. (Cabello *et al.,* 2013b).

Concerning potential hazards to human health, the existence of antibiotic remnants can give rise to allergic reactions, such as those associated with penicillin, as well as other grave ailments, including cancer, anaphylactic shock, nephropathy , bone marrow toxicity, mutagenic effects, and reproductive disorders (Arsène *et al.,* 2022). The utilization of antibiotics in aquaculture also poses a potential repercussion on the well-being of individuals employed within the sector. Occupational health risks have been documented among individuals employed in agricultural settings, including farm laborer's, feed mill workers, and cage farm workers. These risks are mostly attributed to their exposure to airborne dust particles that contain antibiotics (Arsène *et al.,* 2022; Cabello, 2006b). In these particular cases, the introduction of antibiotics into the body through ingestion and contact with the skin has the potential to disturb the balance of normal microflora, facilitate the proliferation of bacteria that are resistant to antibiotics, and potentially lead to complications such

as allergic reactions and toxicity (Cerniglia & Kotarski, 2005; Salyers *et al.,* 2004; White & McDermott, 2014). Furthermore, it should be noted that fish can potentially serve as reservoirs for zoonotic pathogens. These pathogens can infect fish and people through direct contact within the aquaculture environment or gastrointestinal transmission (Gauthier, 2015). Some of the common fish pathogens that can infect fish handlers include *Aeromonas hydrophilia*, *Mycobacterium marinum*, and *Photobacterium damselae*. Meanwhile, foodborne illnesses from fish consumption mainly involve *Listeria monocytogenes*, *Aeromonas*, and *Clostridium* spp (Herrera *et al.,* 2006). This study offers valuable insights into the occurrence of resistance genes within fishponds, which are vital sources of sustenance and livelihood for numerous communities in Pakistan. Understanding the prevalence and distribution of the resistance genes becomes imperative in assessing potential hazards to human health and the environment due to consuming contaminated fish or contacting polluted water-carrying resistant bacteria. Additionally, the findings contribute to formulating public health strategies and interventions required to halt the spread of antibiotic resistance (Cabello et al., 2013c; Imtiaz et al., 2021).

Aims and Objectives

Aim of study

The aim of the study was to assess the prevalence of antibiotic-resistant bacteria and antibiotic-resistance genes in water and sediment samples of fishponds.

Objectives

1. To determine the microbial load (CFU/mL) in water and sediment samples of fishponds.
2. To determine the prevalence of antibiotic-resistant bacteria in water and sediment samples of fishponds.
3. Molecular screening of antibiotic-resistance genes in the resistant bacteria isolated from water and sediment samples of fishponds.
4. Antimicrobial susceptibility testing of isolates positive for resistance genes.

Chapter 2

Literature Review

Literature Review

Antibiotics are agents employed to eradicate or impede the replication of bacteria. Their predominant application lies in human and livestock medicine, where they are utilized to address various ailments (Etebu & Arikekpar, 2016). The term "antibiotic" originates from the ancient Greek word "antibiosis," which can be translated as "against life." Antibiotics were chemical compounds synthesized by a specific bacterial strain, exhibiting toxicity towards a wide range of other bacterial species (Choo *et al.,* 2023). Antibiotics may be bacteriostatic agents that exhibit the capacity to inhibit the proliferation of bacteria or bactericidal which can kill the bacteria (Lobritz *et al.,* 2015).

2.1 Classification of antibiotics

There are numerous approaches for classifying antibiotics, but the most common groupings are based on chemical composition, mode of action, and pharmacokinetic features. They can also be divided into groups based on their mode of administration (topical, parenteral, and oral). Contrarily, if antibiotics share structural similarities, their efficacy will be similar, as well as their potential for adverse and allergic side effects (Etebu & Arikekpar, 2016). The major antibiotics are mentioned below, along with details on their application and mode of action.

2.1.1 Polymyxin

Polymyxins, including polymyxins B and E (colistin), represent a class of antibiotics that frequently apply to control Gram-negative bacterial infections. They are classified as non-ribosomal peptides, heterogeneous compounds that are enzymatically produced by non-ribosomal peptide synthetases. The mechanism underlying the pharmacological effects of polymyxins entails their specific interaction with the lipopolysaccharide moiety present in the bacterial cell membrane. This interaction disrupts the membrane's structural integrity, ultimately resulting in the compromised containment of intracellular contents and subsequent leakage and causing the death of the bacterial cell (Gales *et al.,* 2001).

2.1.1.1 Colistin

Colistin exhibits efficacy against a diverse spectrum of gram-negative bacteria, encompassing notable pathogens such as *Pseudomonas aeruginosa* and *Acinetobacter* spp. It is a member of the polymyxin class, which exhibits its primary mode of action by targeting the cell wall (Gogry *et al.,* 2021; Ledger *et al.,* 2022) and induces alterations in the cytoplasmic membrane's permeability, ultimately leading to the death of bacterial cells. The bacterial outer membrane is traversed via a self-promoted mechanism, which involves the displacement of divalent cations and subsequently increased membrane permeability. This facilitates enhanced absorption of the antibiotics, resulting in their bactericidal properties (Gales *et al.,* 2001). Recent investigations have additionally proposed another mechanism of action for colistin and polymyxin B, which involves suppressing crucial respiratory enzymes within the bacterial inner membrane. The potential bactericidal activity of these antibiotics may be attributed to their secondary mode of action. But, the exact mechanism is still not fully understood (Deris *et al.,* 2013; J. Liu *et al.,* 2021).

2.1.2 Beta lactams

Beta-lactam antibiotics, renowned for their bactericidal properties, exert their therapeutic effects by impeding the final phase of bacterial cell wall synthesis. These compounds are frequently employed in clinical settings and exhibit a comparable molecular characteristic to penicillin, cephalosporins, carbapenems, monobactams, and beta-lactamase inhibitors (Drawz & Bonomo, 2010; Tenover, 2006). The discovery of penicillin in 1928 revolutionized the battle against microbial infectious diseases. The medication exhibits negligible toxicity, and time-dependent bactericidal effect, and excellent systemic distribution. However, the usage and efficacy of beta-lactams is restricted in some therapeutic contexts due to the rising prevalence of antibiotic resistance (Payne *et al.,* 2007). Beta-lactam inhibitors and cephalosporins exhibit various applications in treatment. In contrast, carbapenems are specifically employed to treat nosocomial infections and multidrug-resistant (MDR) bacterial strains. Synthetically modified derivatives of penicillin, namely aminopenicillins, and ureidopenicillins, exhibit remarkable efficacy in treating numerous infections. However, their effectiveness is compromised in cases where resistance arises as a result of beta-lactamase production (Ben-David *et al.,* 2012).

2.1.3 Cephalosporin

Cephalosporins are broad-spectrum antibiotics used against both Gram-positive and Gram-negative organisms. They are classified into five generations, with 7-aminocephalosporanic acid being the basic compound (Srinivas *et al.,* 2023). First-generation cephalosporins are susceptible to Gram-positive cocci and certain Gram-negative bacteria. Second-generation cephalosporins target *Bacteroid* spp., *Moraxella catarrhalis*, and *Haemophilus influenza*. Third generation cephalosporins have reduced activity against most Gram-positive organisms but show a higher spectrum against *Haemophilus influenza*, *Enterobacteriaceae*, and *Neisseria* spp. Fourth generation cephalosporins share a similar spectrum, but also susceptible to Gram-negative antibiotic-resistant bacteria. Penicillin-resistant pneumococci and Methicillin-resistant Staphylococci are also susceptible to fifth-generation cephalosporins (El-Shaboury *et al.,* 2007).

2.1.3.1 Cefotaxime

Cefotaxime is a third-generation cephalosporin antibiotic that has been facing resistance due to extended spectrum -lactamases (ESBLs). Despite being more effective against Gram-negative β-lactamases, cefotaxime is often used against *Klebsiella pneumoniae*, *Enterobacter* species, and *E coli*. (Tekiner & Özpınar, 2016). The challenge of multi-drug resistance is made more difficult by the genes encoding ESBLs being located on identical plasmids that induce resistance to sulfonamides, aminoglycosides, and quinolones. Cefotaxime is primarily used against Gram-negative aerobic bacteria like *Proteus vulgaris*, *Enterobacter*, *Citrobacter*, *Haemophilus*, *Neisseria*, and *Moraxella*. Most third-generation cephalosporins have high β-lactamase resistance, making them effective therapeutic agents against bacterial meningitis caused by susceptible pathogens (Bourjilat *et al.,* 2011; F. Zhang, 2020).

2.1.4 Carbapenems

Carbapenems are broad-spectrum antibiotics with unique properties, providing protection against Extended Spectrum β-lactamases. They are widely considered effective in treating bacterial diseases, but resistance to these antibiotics is a global health issue (Nicolau, 2008). Due to the development of cephalosporin resistance in *Enterobacteriaceae*, several types, such as imipenem, meropenem, doripenem, ertapenem, panipenem, and biapenem, are now widely used globally (Codjoe & Donkor, 2017).

2.1.4.1 Meropenem

Meropenem is a carbapenem antibiotic with a wide therapeutic index, used in treating skin infections, bacterial meningitis, urinary tract infections, and nosocomial pneumonia, severe community acquired pneumonia, and cystic fibrosis. It has broad-spectrum against Gram-negative and Gram-positive bacteria, including AmpC-producing *Enterobacteriaceae* and extended-spectrum beta-lactamase. It shows effectiveness against several anaerobes, such as *Peptostreptococcus* spp., *Prevotella* spp., *Clostridium difficile*, and *C. perfringens*. Meropenem has time-dependent bactericidal activity. It can resist hydrolysis by beta-lactamases such as AmpC beta-lactamases and ESBLs. However, the activity of meropenem can be altered by serine carbapenemases, metallo-beta-lactamases, and oxacillinases. Carbapenem resistance can also be caused by the active efflux of carbapenems, and overexpression of tripartic efflux pumps may result in carbapenem resistance leading to multidrug resistance (Walsh, 2010).

2.1.5 Tetracycline

Tetracycline antibiotics have an extensive range of effectiveness, encompassing a diverse array of Gram-positive and Gram-negative bacteria, and obligate intracellular bacteria. It exhibits a distinct affinity for bacterial ribosomes and interacts with 16S ribosomal RNA (rRNA) target within the 30S ribosomal subunit. This interaction effectively halts the translation process by impeding aminoacyl-transfer RNA (Grossman, 2016a). Tetracyclines are commonly employed in the therapeutic treatment of urinary tract, respiratory tract, and intestinal infections. Additionally, they are utilized in treating chlamydia, particularly in individuals who exhibit allergies to β-lactams and macrolides. However, the utilization of tetracyclines for these purposes has experienced a decline in popularity over time, primarily due to the extensive emergence of resistance among the microorganisms responsible for these infections (Sloan & Scheinfeld, 2008).

2.1.6 Quinolones and Fluoroquinolones

Quinolones are a subclass of antibiotics that work by blocking the DNA replication enzyme topoisomerase, most frequently topoisomerase II (DNA gyrase). DNA gyrase stretches supercoiled DNA molecules in closed-circular super helical twists, causing temporary damage while reattaching phosphodiester linkages. The DNA strand can now be duplicated by RNA or DNA polymerases. Based on the types of bacteria they are effective against and their chemical

composition, quinolones are often divided into four generations. The second-generation quinolones known as fluoroquinolones, which include ciprofloxacin, norfloxacin, and levofloxacin, are effective against both Gram-positive and Gram-negative pathogens (Yan & Bryant, 2023). Fluoroquinolones were developed by inserting a fluorine atom at carbon number six in order to increase their activity. Fluoroquinolones have been used successfully in clinical applications since the 1980s. Their mode of action is to prevent the DNA synthesis by interfering with the activity of DNA gyrase and topoisomerase, preventing replication and transcription. (Andraud *et al.,* 2011).

2.1.6.1 Ciprofloxacin

Ciprofloxacin, a fluoroquinolone antibiotic, is effective against Gram-negative bacteria like *Salmonella* spp., *E. coli*, *Neisseria*, and *Shigella* spp. It targets DNA gyrase, disrupting DNA supercoiling and SOS response. It is most effective against *P. aeruginosa*, with about 30% of resistance reported. Factors contributing to resistance include mutations in DNA gyrase and topoisomerase IV, and expression of efflux pumps. Ciprofloxacin is also used against Gram-positive bacteria. A spike in ciprofloxacin resistance has been observed in hospitals acquired urinary tract infections associated with *E.coli*. Fluoroquinolones are prioritized due to their ability to treat critical infections like septicemia, but rising resistance a serious concern (Blaettler *et al.,* 2009; Redgrave *et al.,* 2014).

2.2 Antibiotic resistance

The emergence of antibiotic resistance can be attributed to various factors, including horizontal gene transfer, selective pressure, and mutations. The phenomenon of acquired resistance arises when bacteria that are initially susceptible to certain substances acquire external genetic material or undergo chromosomal mutations within their own genome through horizontal gene transfer mechanisms, such as DNA uptake, transduction, and/or conjugation. Bacterial organisms have evolved various strategies that mitigate the effect of antimicrobial agents (Li, 2005). These mechanisms include the production of enzymes that can neutralize or degrade antimicrobial compounds, the changes in cellular permeability to limit the entry of these agents, the utilization of alternative metabolic pathways to bypass their detrimental effects, and the direct elimination of antimicrobials from the bacterial system (Nathan & Cars, 2014). The phenomenon of antibiotic resistance has been observed since ancient times, as genes conferring resistance have been found to naturally occur in environments. The investigation of ecosystems devoid of human influence

has unveiled the existence of resistance as an inherent phenomenon. Ecological niches, including soil, water, and other habitats, offer an unmatched gene pool significantly more diversified than humans and domestic animal microbiota (Roca *et al.,* 2015). The most obvious feature of the environmental microbiome is its extensive diversity that provides countless genes that can be acquired and used by pathogens. Resistance has been developed in nearly every class of antibiotic. When pathogens acquire new ARGs, the level of resistance, pathogenicity island, virulence, transmission potential and co-selection opportunities are affected that can enhance the burden of resistance. ARGs when acquired by the pathogens, new resistant genotypes spread in the environment, and they are more difficult to treat, and the situation is alarming (Lim & Webb, 2005).

2.3 Mechanism of antibiotic resistance

Antibiotic resistance develops when bacteria develop the ability to defend themselves against antibiotic actions, rendering the medications useless. Antibiotic resistance can occur in a variety of ways.

1. Mutations: Genetic changes in bacterial populations, such as mutations, can result in antibiotic resistance. Changes in the genes that code for an antibiotic's target, for example, can alter the target's structure, making it resistant to the treatment (Ogawara, 2019).
2. Horizontal gene transfer: Bacteria have the ability to aquire antibiotic resistance genes from different bacteria through mechanisms like conjugation, transduction, or transformation. Horizontal gene transfer is a technique of transmitting antibiotic resistance genes among bacterial populations (Kakoullis *et al.,* 2021).
3. Efflux pumps: Bacteria have the ability to aggressively pump antibiotics out of cells, preventing inhibiting antibiotic concentrations from building up (Uddin *et al.,* 2021).
4. Biofilm formation: Some bacteria have the ability to form biofilms, which are complicated bacterial colonies encased in a protective matrix. Biofilms can be difficult to penetrate because they inhibit antibiotics from functioning as effectively against bacteria (Pang *et al.,* 2019). Controlling the spread of antibiotic-resistant microorganisms is difficult because antibiotic resistance pathways can interact and amplify one another. To minimize the formation and spread of antibiotic resistance, it is necessary to use antibiotics responsibly and implement methods such as limiting their use in agriculture and stimulating the development of novel antibiotics (Cascioferro *et al.,* 2020).

2.3.1 Colistin resistance

The acquisition of colistin resistance in *Enterobacteriaceae* is attributed to the structural alterations of bacterial lipopolysaccharide through the incorporation of 4-amino-4-deoxy-l-arabinose (L-Ara4N) or phosphoethanolamine (pEtN). This phenomenon, known as intrinsic resistance, involves the modification of the lipopolysaccharide structure. Acquired resistance arises because of chromosomal mutations occurring in genes that regulate the two-component regulatory system, namely *PhoPQ* and *PmrAB*. Additionally, resistance can be conferred through the presence of *mrgB* and plasmid-associated genes, specifically mcr-1 to mcr-10, which encode a cluster of pEtN transferases (C. Wang *et al.,* 2020).

2.3.1.1 Prevalence of colistin resistance in fishponds

Colistin, an antibiotic of utmost significance, is predominantly employed as a therapeutic intervention for the treatment of Gram-negative bacteria infection that exhibit resistance to multiple drugs (Kalová *et al.,* 2021; Shen *et al.,* 2020). Numerous publications have documented the prevalence of *mcr-1*-positive bacteria that have been isolated from various aquaculture settings. A study conducted in China has documented a significant occurrence of the *mcr-1* resistance gene, ranging from 28.0% to 87.3%, in both earthen and film line ponds inhabited by P. vannamei and its associated environmental samples. (Campos *et al.,* 2016; Y.-Y. Liu *et al.,* 2016). Similarly, a comprehensive investigation carried out by (Shen *et al.,* 2019) involved the analysis of 143 colistin-resistant isolates, unveiled a prevalence rate of 39.2% for the mcr-1 resistance gene in aquatic supply.

2.3.2 Beta lactams resistance

β-lactams are the antibiotics that are most commonly utilized. The identification of the three main mechanisms of antibiotic resistance has been identified. Modifying the drug's interaction with Penicillin-binding proteins, thereby reducing its affinity. Existence of efflux pumps, which can effectively eliminate antibiotics, and the hydrolytic activity of β-lactamases. Hydrolysis occurs, leading to the opening of the ring and subsequent inactivation of the beta-lactam ring (De Angelis *et al.,* 2020). There exists a wide range of enzymes referred to as beta-lactamases that possess the ability to deactivate various beta-lactam antibiotics. The classification of 19 lactamases is determined by their molecular structure and/or functional characteristics (Sawa *et al.,* 2020). The

categorization of β-lactamases is based on three primary functional groups: the SHV family, the TEM family, and the CTX family. These groups were named after their respective characteristics, such as the Sulfhydryl variable (SHV) family, which is known for its variability in sulfhydryl groups. The TEM family, on the other hand, was named after the first patient in which it was identified. Lastly, the CTX family predominantly hydrolyzes Cefotaxime, making it substrate specific. Gram-negative bacteria produce all four primary types of β-lactamases. A limited proportion of β-lactamases identified in Gram-positive bacteria belong to group B (Arredondo *et al.,* 2020).

2.3.3 Extended spectrum β-lactamases resistance

Extended spectrum β-lactamases (ESBLs) are enzymes that are capable of hydrolyzing and inactivating a broad range of β-lactam antibiotics. Clavulanic acid exerts inhibitory effects on the functioning of these enzymes. The emergence of β-lactam resistance, facilitated by β-lactamases, is attributed to alterations in the enzyme-substrate interaction spectrum. To date, a total of over 200 Extended-Spectrum Beta-Lactamases (ESBLs) have been identified. Studies on extended-spectrum beta-lactamase (ESBL)-related phenomena have been documented in nearly 30 nations, providing substantial evidence for the widespread occurrence of ESBL-producing bacteria on a global level (Livermore, 2008). During the latter part of the 1990s, it was discovered that Extended-Spectrum Beta-Lactamases (ESBLs) were prevalent among a majority of bacteria acquired within the community, particularly those responsible for gastroenteritis and urinary tract infections (Sawa *et al.,* 2020). The most prevalent extended-spectrum beta-lactamases (ESBLs) that can be effectively inhibited by clavulanic acid include narrow-spectrum SHV and TEM-type beta-lactamases, as well as PER, CTX-M, IBC, and VEB type beta-lactamases. TEM-1, a beta-lactamase enzyme, are the predominant variant discovered within the *Enterobacteriaceae* family. Its initial recognition dates back to 1965 when it was isolated from an *Escherichia coli* strain (Tamma *et al.,* 2021). The initial identification of the non-SHV, non-TEM CTX-M family occurred in *Enterobacteriaceae* in the year 1992. During the period from 1989 to 2001, a group of approximately 20 CTX-M enzymes, collectively known as the CTXM family, were identified in various isolates of enterobacteria. According to Tamma et al. (2021), *Salmonella typhimurium*, *Klebsiella pneumoniae*, and *Escherichia coli* strains have been identified as the predominant origins of CTX-M-1 (Pana & Zaoutis, 2018).

2.3.3.1 Prevalence of ESBLs in fish ponds

Beta-lactam antibiotic resistance has been documented to primarily arise from the enzymatic activity of beta-lactamas. *Vibrio* spp. are prominent pathogens in the field of aquaculture, known for their possession of gene conferring resistance to β-lactam antibiotics (Y. Wang *et al.,* 2006). The upregulated transcription of the chromosomal ampC beta-lactamase gene also leads to a robust resistance against β-lactam antibiotics (Slama *et al.,* 2010). The presence of beta-lactam resistance genes blaSHV-12 and blaTEM-52 has been detected in the fecal matter of Gilthead seabream (Sousa *et al.,* 2011). The manifestation of extended-spectrum beta-lactamase (ESBL) signifies the inherent ability of fish pathogens to withstand the effects of third and fourth-generation cephalosporin antibiotics, thereby posing a significant challenge in terms of control and management (Verner-Jeffreys *et al.,* 2009). The extended-spectrum beta-lactamase genes, namely bla_{TEM}, bla_{SHV}, and bla_{OXA}, were observed in *Aeromonas* isolates obtained from cultured rainbow trouts (Vega-Sánchez *et al.,* 2014).

2.3.4 Tetracycline resistance

Multiple mechanisms have been identified through which bacteria acquire resistance to tetracycline. One mechanism involves genetic mutations that modify the permeability of outer membrane porins or lipopolysaccharides, disrupting the regulation of innate efflux system. The other mechanism includes energy-dependent efflux proteins, i.e., the *tet*A, *tet*B, and *tet*E genes have been identified as the predominant efflux pumps involved in tetracycline resistance. The efflux pump expelled tetracycline molecules from within the bacterial cell and served to impede the accumulation of tetracycline. This hinders tetracycline's ability to bind to the ribosome, thereby exerting inhibitory effects. In conjunction with efflux pumps, ribosomal protection proteins serve as another mechanism for conferring resistance to tetracycline. The *tet*M gene, which encodes the ribosomal protection protein, is widely recognized as the predominant determinant responsible for conferring resistance to tetracycline (Grossman, 2016b; Ndi & Barton, 2011).

2.3.4.1 Prevalence of tetracycline resistance gene in fishponds

The tetracycline genes prevalence in the aquatic fish farming environment has been demonstrated in numerous studies (Jacobs & Chenia, 2007). Basically, tetracycline-producing microflora in the environmental niche has led to the evolution of excessive tetracycline resistance genes (Schmidt

et al., 2001). Many studies reported the prevalence of *tet*A-G genes in fish pathogens (Miranda *et al.,* 2003). Moreover, *tet*A, *tet*B, *tet*D, and *tet*G were frequently discovered in aquatic farms of Korea (Jang *et al.,* 2018). The frequent administration of oxytetracycline has been observed during aquaculture disease outbreaks (Jacobs & Chenia, 2007). In their study, (Nonaka *et al.,* 2007) discovered a novel oxytetracycline resistance gene, *tet*34, within a *Vibrio* strain obtained from cultured yellowtail. In a study conducted by (Agersø & Guardabassi, 2005), a new tetracycline resistance determinant called *tet*39 was discovered in an *Acinetobacter* strain obtained from freshwater trout farms. This determinant was found to be located on a transferable plasmid. Moreover, *Vibrio* strains harboring *tet*M and *Aeromonas* strains carrying *tet*E also reported from different fish farms (Agersø & Guardabassi, 2005; Nonaka *et al.,* 2007).

2.3.5 Carbapenem resistance

Carbapenems are the last resort for many infections, but their increased use has led to the emergence of carbapenem resistance in clinical strains. The expression of carbapenemase is the most frequent mechanism of carbapenem resistance, and a wide variety of beta-lactams. All carbapenem antibiotics are ineffective against carbapenemase-producing bacteria, particularly those that produce *Klebsiella pneumoniae* carbapenemase (KPC) and New Delhi metallo β-lactamase-1 (NDM-1). This poses major risks to human health (Yang *et al.,* 2016). The *Klebsiella pneumoniae* carbapenemase family expressed by the bla_{KPC} gene is the most common carbapenemase. Other carbapenemases linked to *Enterobacteriaceae* outbreak include IMP, NDM, VIM, and OXA-48 enzymes (Navon-Venezia *et al.,* 2006). Carbapenem is unable to access PBPs due to reduced outer membrane permeability, such as OprD, a porin in *Pseudomonas* ssp., which allows the entry of carbapenem into the periplasmic space where PBPs are found. The active efflux pump uses proton motive force for the development of resistance against antibiotics. When they are overexpressed in the case of meropenem, it leads to carbapenem resistance coupled with multi-drug resistance. This happens because aminoglycosides, cephalosporins, penicillin, and quinolones are typical substrates of overexpressed efflux pumps. Since carbapenem resistance is now a major global issue, investigations on identifying isolates that produce carbapenemase in clinical settings are being published more frequently (Anderson & Boerlin, 2020; Meletis, 2016; Walsh, 2010) .

2.3.5.1 Prevalence of carbapenemases in fishponds

The study conducted by (Xu *et al.,* 2020) sheds light on the prevalence of carbapenem-resistant *Enterobacteriaceae* (CRE) in river fish from of Vietnam, revealing the presence of NDM-1, NDM-4, NDM-5, OXA48, and KPC carbapenemases at a rate of 6.8%. Additionally, the detection of NDM-5-producing *Enterobacteriaceae* in freshwater fish in Vietnam adds to these findings. Notably, a separate study in India demonstrated the dissemination of NDM-5 carbapenemase within freshwater aquaculture environments by the high-risk clones of ST167 and ST361 (Dwivedi *et al.,* 2023). In Nigeria, research unveiled the existence of VIM resistance genes within bacterial isolates from shellfish found in the Iko and Douglas river estuaries. Another study investigated fishpond water inlets and fresh fish, identifying the prevalence of OXA-48, NDM, and KPC carbapenemases (Hamza *et al.,* 2020).

2.3.6 Quinolones and Plasmid mediated quinolone resistance

Quinolones are antibacterial drugs that have steadily evolved over the last 20 years. Quinolones have potent antibacterial activity and are easy to use due to their broad antifungal spectrum. Quinolones are widely used to treat a variety of infectious diseases. Quinolones are more commonly used than cephalosporins and penicillin (Manenzhe *et al.,* 2015; B. Zhang *et al.,* 2014). Quinolone resistance occurs when quinolones cannot access their targets due to mutations in topoisomerase IV and gyrase, resulting in a reduced concentration of medicines in the cytoplasm. When quinolones are actively transported out of the cell or quinolone absorption is impeded, the drug concentration is reduced, leading to quinolone resistance (Hooper & Jacoby, 2015). Quinolones target two essential bacterial enzymes: DNA topoisomerase IV and DNA gyrase. Gyrase is made up of two GyrB and two GyrA subunits, whereas topoisomerase IV is made up of two ParE and two ParC subunits. Amino acid changes in topoisomerase IV or gyrase mediate quinolone resistance. Typically, the mutations are discovered in a specific domain of ParC and GyrA. Around 1998, three distinct PMQR (plasmid-mediated quinolone resistance) mechanisms were discovered. Topoisomerase IV and gyrase are safeguarded by pentapeptide proteins encoded by plasmid-mediated genes *qnr*D, *qnr*C, *qnr*B, *qnr*A, and *qnr*S. These genes are typically integrated into sul1-type integrons and are commonly connected to transposons found on plasmids. Another plasmid-mediated resistance mechanism involves aminoglycoside acetyltransferase

aac(6′)-Ib acetylates. The third mode of action is mediated by overexpressed efflux pumps mediated by the OqxAB and OepAB genes (Jacoby *et al.,* 2015a).

2.3.6.1 Prevalence of PMQR genes in fishponds

Aeromonas serves as a pivotal reservoir of mobile genetic elements within aquaculture, wherein the acquisition of quinolone resistance stands as a prominent mechanism. The development of resistance can be attributed to mutations occurring in DNA gyrase and DNA topoisomerase. In a study conducted by (Chenia, 2016), a notable prevalence of plasmid-borne qnr alleles was observed in *Aeromonas* isolates derived from freshwater fish in South Africa. This finding serves as a compelling indication of a substantial degree of resistance exhibited by these organisms towards fluoroquinolone antibiotics. In a study conducted by (Lunn *et al.,* 2010), it was observed that plasmid-mediated transferable quinolone resistance (PMQR) determinants, namely *qnr*A, *qnr*B, and *qnr*S, exhibited a predominant presence within the *Enterobacteriaceae* bacterial family. Moreover, a significant proportion of qnr plasmids exhibit a close association with integrons, thereby enabling them to bestow resistance against a diverse range of antimicrobial agents. The increased drug resistance observed among fish pathogens can be attributed to the increased mutations in the gyrA gene, which have arisen due to the widespread utilization of quinolones in aquaculture facilities (Li, 2005). Moreover, the coexistence of multiple plasmid-mediated qnr genes within marine bacteria also reported in aquatic reservoir (Jacoby & Hooper, 2013).

Chapter 3

Materials and Methods

Materials and Methods

The present study was carried out to check the prevalence of antibiotic-resistance genes in the aquaculture environment and conducted in the Department of Microbiology, Quaid-i-Azam University, Islamabad.

3.1 Study Sites and sampling information

Eighteen pounds from two fish seed hatcheries located in Islamabad and Faisalabad, Pakistan were selected to sample in November and December. The fishes reared in the selected ponds were Rohu Gulfam and Tilapia.

1. In Faisalabad, 25 samples were collected, consisting of both water and sediment. Twelve samples were found to have monoculture cultivation of Tilapia, while the other samples contained a combination of Gulfam and Rahu fish species.
2. In Islamabad, 10 samples were collected from the fish seed hatchery ponds, all of which demonstrated the presence of both Rohu and Gulfam fish species being cultivated.

Information regarding the source, date, and time of sample collection is shown below in Table 3.1.

Figure 3.1: Map showing the location of fish seed hatcheries

Table 3.1: Detail list of sampling sites, coordinates sample types, time and date of their collection

S.No	Sample ID	Date of collection	Address	Coordinates	Time of collection
RH1	Water	11/04/2022	Rawal Town, Islamabad	33.6828° N, 73.1150° E	10:30 am
RH2	Water	11/04/2022	Rawal Town, Islamabad	33.6828° N, 73.1150° E	10:45 am
RH3	Water	11/04/2022	Rawal Town, Islamabad	33.6828° N, 73.1150° E	11:00 am
RH4	Water	11/04/2022	Rawal Town, Islamabad	33.6828° N, 73.1150° E	11:15 am
RH5	Water	11/04/2022	Rawal Town, Islamabad	33.6828° N, 73.1150° E	11:30 am
RHS 1	Sediments	11/04/2022	Rawal Town, Islamabad	33.6828° N, 73.1150° E	10:30 am
RHS 2	Sediments	11/04/2022	Rawal Town, Islamabad	33.6828° N, 73.1150° E	10:45 am
RHS 3	Sediments	11/04/2022	Rawal Town, Islamabad	33.6828° N, 73.1150° E	11:00 am
RHS 4	Sediments	11/04/2022	Rawal Town, Islamabad	33.6828° N, 73.1150° E	11:15 am
RHS 5	Sediments	11/04/2022	Rawal Town, Islamabad	33.6828° N, 73.1150° E	11:30 am
H1	Water	21/11/2022	Satiyana Road, Faisalabad	31.3832° N, 73.1269° E	1:10 pm
H2	Water	21/11/2022	Satiyana Road, Faisalabad	31.3832° N, 73.1269° E	1:30 pm
H3	Water	21/11/2022	Satiyana Road, Faisalabad	31.3832° N, 73.1269° E	1:45 pm
H4	Water	21/11/2022	Satiyana Road, Faisalabad	31.3832° N, 73.1269° E	1:55 pm
H5	Water	21/11/2022	Satiyana Road, Faisalabad	31.3832° N, 73.1269° E	2:10 pm
H6	Water	21/11/2022	Satiyana Road, Faisalabad	31.3832° N, 73.1269° E	2:20 pm
HS1	Sediments	21/11/2022	Satiyana Road, Faisalabad	31.3832° N, 73.1269° E	1:10 pm
HS2	Sediments	21/11/2022	Satiyana Road, Faisalabad	31.3832° N, 73.1269° E	1:30 pm
HS3	Sediments	21/11/2022	Satiyana Road, Faisalabad	31.3832° N, 73.1269° E	1:45 pm
HS4	Sediments	21/11/2022	Satiyana Road, Faisalabad	31.3832° N, 73.1269° E	1:55 pm
HS5	Sediments	21/11/2022	Satiyana Road, Faisalabad	31.3832° N, 73.1269° E	2:10 pm
HS6	Sediments	21/11/2022	Satiyana Road, Faisalabad	31.3832° N, 73.1269° E	2:20 pm
H7	Water	26/12/2022	Satiyana Road, Faisalabad	31.3832° N, 73.1269° E	11:45 am

H8	Water	26/12/2022	Satiyana Road, Faisalabad	31.3832° N, 73.1269° E	12:03 pm
H9	Water	26/12/2022	Satiyana Road, Faisalabad	31.3832° N, 73.1269° E	12:20 pm
H10	Water	26/12/2022	Satiyana Road, Faisalabad	31.3832° N, 73.1269° E	12:38 pm
H11	Water	26/12/2022	Satiyana Road, Faisalabad	31.3832° N, 73.1269° E	12:50 pm
H12	Water	26/12/2022	Satiyana Road, Faisalabad	31.3832° N, 73.1269° E	1:02 pm
H13	Water	26/12/2022	Satiyana Road, Faisalabad	31.3832° N, 73.1269° E	1:15 pm
HS7	Sediments	26/12/2022	Satiyana Road, Faisalabad	31.3832° N, 73.1269° E	11:45 am
HS8	Sediments	26/12/2022	Satiyana Road, Faisalabad	31.3832° N, 73.1269° E	12:03 pm
HS9	Sediments	26/12/2022	Satiyana Road, Faisalabad	31.3832° N, 73.1269° E	12:20 pm
HS10	Sediments	26/12/2022	Satiyana Road, Faisalabad	31.3832° N, 73.1269° E	12:38 pm
HS11	Sediments	26/12/2022	Satiyana Road, Faisalabad	31.3832° N, 73.1269° E	12:50 pm
HS12	Sediments	26/12/2022	Satiyana Road, Faisalabad	31.3832° N, 73.1269° E	1:02 pm

3.2 Sample collection

Water samples were obtained from three distinct locations within each pond at a subsurface depth of approximately 1 meter. These individual samples were then combined to create a pooled water sample. Additionally, 2 liters of water samples were collected in autoclavable bottles specifically for DNA extraction purposes.

Regarding sediment collection, a falcon tube was inserted roughly 5 cm into the bed of the pond. The sediment and the falcon tube were carefully extracted by applying gentle suction while using a gloved hand to cover the top of the falcon tube. To prevent sediment disturbance, the water above the sediment was cautiously decanted. Three grab samples were collected and subsequently combined into a single sediment sample.

3.3 Processing of the samples

One gram of sediments was thoroughly mixed with 9 ml of sterile normal saline solution to process the sediment sample. It was assumed that the bacteria present in the sample were evenly distributed between the liquid and solid components. Subsequently, stepwise dilutions of both water and sediment samples were performed. From each relevant dilution, 100 µl was plated onto eight distinct media, using a sterile glass spreader for even distribution. The plates were then incubated at a temperature of 37 degrees Celsius for a duration of 24 hours.

Figure 3.2: Representing the site of sample collection

3.4 Preparation of antibiotic solution

3.4.1 Stock solution

The antimicrobial agent stock solution was prepared according to the EUCAST recommendation, with a minimum concentration of 1000 µg/ml. The stock solutions of Cefotaxime, Ciprofloxacin, Meropenem, Colistin, and Oxytetracycline were prepared using the following formula.

$$\text{Weight (mg)} = \frac{\text{Volume(ml).Concentration}\left(\frac{\mu g}{ml}\right)}{\text{Assay potency}\left(\frac{\mu g}{mg}\right)}$$

3.5 Growth media and antibiotic-selective media

A total of eight different types of media were prepared to facilitate the growth of various organisms.

- MacConkey agar for the growth of Gram-negative organisms
- Mannitol salt agar for the growth of Gram-positive organisms
- Bile esculin agar for the growth of *Enterococci*
- MacConkey agar supplemented with 1mg/ml Cefotaxime for the screening of ESBL producers.
- MacConkey agar supplemented with 0.25mg/L Meropenem for the screening of Carbapenemase-producers.
- MacConkey agar supplemented with 1mg/L Colistin for the screening of polymyxin-resistant bacteria.
- MacConkey agar supplemented with 0.125mg/L Ciprofloxacin for the screening of fluoroquinolones-resistant bacteria.
- MacConkey agar supplemented with 4mg/L Oxytetracycline for the screening of tetracycline-resistant bacteria.

3.5.1 Media Preparation

Media was prepared as instructed by manufacturers:

- To prepare MacConkey agar, the media was dissolved in 1 liter of distilled water, with a quantity of 51.5 grams.

- For Mannitol salt agar, a total of 111g media was added in 1 liter of distilled water.
- In the formulation of Bile esculin agar, the 63.5g media was added to 1 liter of distilled water.

Following the mixing, the media underwent autoclaving, where it was exposed to a temperature of 121 degrees Celsius for 15 minutes while maintaining a pressure of 15 psi. After sterilization, the media was allowed to cool and poured into petri-plates in a laminar air flow hood. The media containing antibiotics were prepared by pouring sterile antibiotic solutions into temperature-adjusted media at 48-50°C and poured into petri plates in a laminar flow hood.

3.6 Colony forming units (CFU/ml)

To determine the number of colony-forming units (CFUs), the colonies obtained were manually counted. The concentration of bacteria within the original culture was calculated by assuming that each colony originated from a single bacterium (Brugger *et al.,* 2012). After overnight incubation, the plates were examined to count the colony-forming units. The sample was plated in microliters (µL), and the following formula was used for bacterial count .

$$\text{CFU/ml} = \frac{\text{No.of colonies on plate}}{\text{Volume of sample} \cdot \text{Dilution}}$$

3.7 Membrane filtration assembly

A membrane filter is a barrier either permeable or semi-permeable, that allows several substances in the sample to pass through it while restricting the others selectively. The principle of separation is based on the size and charge of the contaminant. A driving force such as concentration gradient, pressure difference, and potential field are required to move the substances across the membrane.

For DNA extraction, 2 liters of drinking water sample was allowed to pass through a membrane filter paper of 0.2µm pore size adjusted over a filtration assembly. The filter paper was folded using sterile forceps and put in a properly labelled falcon and kept at -21°C.

3.8 Morphological identification and colony preservation

The colonies obtained from each media plate were examined to determine their color, size, and shape. Presumptive identification of the organisms and any suspected species were recorded. Each colony was suspended in 500ul nutrient broth and transferred to a labelled Eppendorf tube. The

tubes were then placed in a shaking incubator at 37°C for 24 hours. After the incubation, 500µl of 40% glycerol was added to each Eppendorf tube, which was then vortexed and stored at a temperature of -81°C.

3.9 Biochemical Testing

Isolates carrying ESBLS, carbapenemases, PMQR, mcr, and tet genes were identified through biochemical testing.

3.9.1 Catalase Test

This test is used to distinguish between Catalase-positive and Catalase-negative Gram-negative bacteria. Catalase enzyme breaks down hydrogen peroxide into water and oxygen.

Reagent: Hydrogen peroxide

Procedure: 1-2 colonies of bacteria were added on a glass slide with the help of a sterilized loop followed by a few drops of 3% hydrogen peroxide. Bubbles formation indicate positive result.

3.9.2 Oxidase Test

The oxidase test uses cytochrome c oxidase to reduce oxygen to water by transferring electrons and tetramethyl-p-phenylene-diamine-dihydrochloride is used as an electron acceptor. If the donor is oxidized, a purple color appears due to indophenol.

Reagent: Tetramethyl-p-phenylene-diamine-dihydrochloride

Procedure: A filter paper was moistened in 1 % oxidase reagent and left for drying. Then, picked 1-2 bacterial colonies from an agar plate using a sterilized wire loop and streak them on the same filter paper. Observe any color changes. The changes of color to deep blue or purple represent positive oxidase test.

3.9.3 Citrate Test

The citrate test is used to differentiate *Enterobacteriaceae*. Bacteria that use citrate produce citrase enzyme, which breaks down citrate to create bicarbonate ions. This causes the pH to increase to above 7, and bromothymol blue turns dark blue in response.

Media: Simmon's Citrate Agar

Procedure: We inoculated the Simmons citrate agar tube slant using a sterilized wire loop and noted any color changes after 24 hours of incubation. A change in color from green to Prussian blue indicated positive test results, while no change was associated with negative test results.

3.9.4 Urease Test

The urease test is used to detect the presence of the urease enzyme. This enzyme hydrolyzes urea into ammonia and carbon dioxide, resulting in an alkaline pH shift and causing the phenol red indicator to change to pink.

Media: Urea agar

Procedure: We streaked the bacteria on the slant using a sterilized wire loop, incubated it for 24 hours, and noted any color changes. A change in color from yellow to pink indicated positive test results, while no change was associated with negative test results.

3.9.5 Triple-Sugar-Iron Test

The triple-sugar-iron test is used to determine whether an organism can utilize glucose, lactose, and sucrose. This causes the pH to become acidic, resulting in a yellow color formation.

Media: Triple Sugar Iron media

Procedure: A pure colony is isolated from a 24-hour incubated culture using a sterile needle. The needle is then stabbed into the center of a slant, and the sample is streaked on the slant of the tube using a wire loop. Subsequently, the tube is incubated for 24 hours at 37°C within an incubator.

Red Slant/ Red base: No fermentation of carbohydrates.

Red slant/Yellow base: Glucose fermentation only.

Yellow slant/Yellow base: Fermentation of glucose, sucrose, and lactose.

Carbon dioxide production: Cracks or bubbles in the base and slant represents CO_2 gas production.

Blackening of media: Production of hydrogen sulfide.

3.9.6 Methyl Red test

MR test identifies bacteria's ability to use the mixed-acid fermentation pathway in glucose metabolism. This pathway generates persistent acids. The process involves significant acid production during glucose fermentation, leading to a noticeable pH reduction.

Media: MR-VP media

Reagents: Methyl red indicator

Procedure: To conduct the test, 2-3 colonies are picked up with a sterilized wire and mixed in the broth. The mixture is incubated for 24 hours before adding reagents. 4-5 drops of Methyl red indicator are added. Positive isolates have exhibited a bright red color, whereas negative isolates have appeared yellow.

3.9.7 Voges Proskauer test

The Voges-Proskauer test identifies the 2,3-butanediol fermentation pathway, which converts glucose to acetoin. A positive VP test shows a crimson color, while a negative result shows no color change.

Reagents: Voges-Proskauer Reagent A (Barritt's Reagent) and Potassium hydroxide (Voges-Proskauer Reagent B)

Procedure: After preparing the broth, a sterilized wire was used to collect 2-3 colonies, which were then mixed in the broth. The mixture was incubated for 24 hours before adding reagents. Subsequently, 0.6mL of 5% α-naphthol was added, followed by the addition of 0.2mL of 40% KOH. The tube was gently agitated to ensure proper exposure of the medium to atmospheric

oxygen. The tube was allowed to remain undisturbed for a duration of 10-15 minutes. A positive result is indicated by a pink-red color at the surface within 15 minutes.

3.9.8 Sulfide-Indole-Motility Test

The Sulfide-Indole-Motility Test is employed to determine specific traits of bacterial species based on their capacity for sulfur reduction, indole synthesis, and motility. The test is used for the differentiation of gram-negative enteric bacilli.

Media: SIM media

Reagent: Kovac's reagent

Procedure: Using a sterilized wire loop, took a pure colony from a 24-hour incubated culture and then stabbed into the center of the medium. The medium was incubated at 37 degrees Celsius for 48 hours. After the incubation period, the sample was examined for motility and H2S production. Subsequently, the indole reagent was added, and observations were made for any indications of indole production.

H_2S production: Positive results shows blackening at the inoculation line or appear throughout the medium. No blackening of media in negative results.

Motility: Fuzzy growth from the stab line indicates positive results. While restricted growth along the stab line indicates negative results.

Indole: Red ring formation after the addition of the indole reagent. Negative results show no color change.

3.10 Antimicrobial susceptibility testing

Isolates carrying PMQR, ESBLS, carbapenemases, mcr, and tet genes were analyzed for antibiotic susceptibility testing against nine antibiotics (Ceftazidime, Meropenem, Aztreonam, Nalidixic acid, Fosfomycin, Cefepime, Piperacillin, amikacin, and tetracycline) using Kirby Bauer disc diffusion following CLSI (2023) guidelines. If not possible by CLSI (2023) guidelines, we followed EUCAST (2023) guidelines.

Procedure

Take 3 to 5 isolated colonies from agar plates less than 18 to 24 hours old and suspended them in normal saline. Adjusted the turbidity with 0.5 McFarland standard and used a glass spreader to distribute the suspension over the MHA agar plate. Then dispensed antibiotics onto the plates using forceps, placing the discs apart from each other. The plates were incubated for 18-24 hours at 37°C. After incubation, measured the zone of inhibition in millimeters, and compared the readings with the zone diameter interpretative chart following CLSI or EUCAST guidelines.

Table 3.2: Generic name, class, and dose of antibiotics used

Sr.No	Antibiotics	Class of antibiotics	Dose per disc (ug)	Symbol
1	Meropenem	CARBAPENEMS	10	MEM
2	Aztreonam	Monobactams	30	ATM
3	Fosfomycin	Fosfomycin	200	FOT
4	Ceftazidime	Cephalosporins	30	CAZ
5	Cefepime	Cephalosporins	30	FEP
6	Amikacin	Aminoglycoside	30	AK
7	Tetracycline	Tetracyclines	30	TE
8	Piperacillin	Penicillins	30	PRL
9	Nalidixic acid	Quinolones	30	NA

3.11 DNA extraction

DNA extraction was performed using the boiling colony method. The preserved colonies were refreshed on MacConkey media plates and incubated overnight at 37 degrees Celsius. A loopful of the culture grown overnight was taken and suspended in 200µL of double distilled water using a sterile loop in an Eppendorf tube. The Eppendorf tubes were appropriately labelled. A water bath was set to a temperature of 100ºC, and the Eppendorf tubes were placed in the water bath for a duration of 15 minutes. After 15 minutes, the Eppendorf tubes were allowed to cool and then centrifuged for 2 minutes at 14,000 rpm to obtain a cell pellet. The pellet was discarded, and the supernatant was collected as DNA.

3.12 Molecular characterization

3.12.1 Primers for PCR screening of antibiotic resistance genes (ARGs)

The primers for screening antibiotic resistance genes were chosen by referring to published sequence data.

Table 3.3: List of primers used for PCR detection of antibiotic-resistant genes.

Primers	Nucleotide Sequence	Product Size	Reference
mcr-1-F	5'-AGTCCGTTTGTTCTTGTGGC-3'	320bp	(Rebelo *et al.*, 2018)
mcr-1-R	5'-AGATCCTTGGTCTCGGCTTG-3'		
mcr-2-F	5'-CAAGTGTGTTGGTCGCAGTT-3'	715bp	(Rebelo *et al.*, 2018)
mcr-2-R	5'-TCTAGCCCGACAAGCATACC-3'		
mcr-3-F	5'-AAATAAAAATTGTTCCGCTTATG-3'	929bp	(Rebelo *et al.*, 2018)
mcr-3-R	5'-AATGGAGATCCCCGTTTTT-3'		
mcr-4-F	5'-TCACTTTCATCACTGCGTTG-3'	1116bp	(Rebelo *et al.*, 2018)
mcr-4-R	5'-TTGGTCCATGACTACCAATG-3'		
mcr-5-F	5'-ATGCGGTTGTCTGCATTTATC-3'	1644bp	(Rebelo *et al.*, 2018)
mcr-5-R	5'-TCATTGTGGTTGTCCTTTTCTG-3'		
Tet A-F	5'-GCGCGATCTGGTTCACTCG-3'	164bp	(Aminov *et al.*, 2002)
Tet A-R	5'-AGTCGACAGYRGCGCCGGC-3'		
Tet B-F	5'-TACGTGAATTTATTGCTTCGG-3'	206bp	(Aminov *et al.*, 2002)
Tet B-R	5'-ATACAGCATCCAAAGCGCAC-3'		
Tet D-F	5'-GGAATATCTCCCGGAAGCGG-3'	187bp	(Aminov *et al.*, 2002)
Tet D-R	5'-CACATTGGACAGTGCCAGCAG-3'		
Tet E-F	5'-GTTATTACGGGAGTTTGTTGG-3'	199bp	(Aminov *et al.*, 2002)
Tet E-R	5'-AATACAACACCCACACTACGC-3'		
qnrA-F	5'-AGAGGATTTCTCACGCCAGG-3'	580bp	(Cattoir *et al.*, 2007)
qnrA-R	5'-TGCCAGGCACAGATCTTGAC-3'		
qnrB-F	5'-GGMATHGAAATTCGCCACTG-3'	264bp	(Cattoir *et al.*, 2007)
qnrB-R	5'-TTTGCYGYYCGCCAGTCAGG-3' S'-		
qnrS-F	5'-GCAAGTTCATTGAACAGGGT-3'	428bp	(Cattoir *et al.*, 2007)
qnrS-R	5'-TCTAAACCGTCGAGTTCGGCG-3'		
qnrC-F	5'-GCAGAATTCAGGGGAGTGAT-3'	180bp	(Ciesielczuk *et al.*, 2013)
qnrC-R	5'-AACTGCTCCAAAAGCTGCTC-3'		
qnrD-F	5'-CGAGATCAATTTACGGGGAATA-3'	581bp	(Ciesielczuk *et al.*, 2013)
qnrD-R	5'-AACAAGCTGAAGCGCCCTG-3'		

aac (6')-Ib-cr-F	5'-TTGCGATGCTCTATGAGTGGCTA-3'	482bp	(Eftekhar & Seyedpour, n.d.)
aac (6')-Ib-cr-R	5'-CTCGAATGCCTGGCGTGTTT-3'		
NDM-F	5'-GGTTTGGCGATCTGGTITTC-3'	621bp	(Poirel *et al.,* 2011)
NDM-R	5'-CGGAATGGCTCATCACGATC-3'		
OXA-48-F	5'-GCGTGGTTAAGGATGAACAC- 3	438bp	(Poirel *et al.,* 2011)
OXA-48-R	5'-CATCAAGTTCAACCCAACCG -3'.		
KPC-F	5'-CGTCTAGTTCTGCTGTCITG-3	789bp	(Poirel *et al.,* 2011)
KPC-R	5'-CTTGTCATCCTTGTTAGGCG-3'		
VIM-F	5'- GTTTGGTCGCATATCGCAAC -3'	389bp	(Pitondo-Silva *et al.,* 2016)
VIM-R	5'- AATGCGCAGCACCAGGATAG -3'		
IMP-F	5'-GAATAGAATGGTTAACTCTC-3	188bp	(Askari *et al.,* 2019)
IMP-R	5'-CCAAACCACTAGGTTATC-3 '		
CTX-MU-F	5'-CGCTTTGCGATGTGCAG-3'	551bp	(Suleyman *et al.,* 2016)
CTX-MU-R	5'-ACCGCGATATCGTTGGT 3'		
CTX-M9-F	5'-GCAGTACAGCGACAATACCG-3'	356bp	(Amosun *et al.,* 2021)
CTX-M9-R	5'-TATCATTGGTGGTGCCGTAG-3'		
SHV-F	5'-TCAGCGAAAAACACCTTG-3'	471bp	(Chandra & Goswami, n.d.)
SHV-R	5'-TCCCGCAGATAAATCACC-3'		
TEM-F	5'-CTTCCTGTTTTTGCTCACC-3'	632bp	(Dehshiri *et al.,* 2018)
TEM-R	5'-AGCAATAAACCAGCCAGC-3'		

3.12.2 Primer dilution

The concentrated primer solution was diluted by adding 10 µl of the stock solution to 90 µl of PCR water. The resulting diluted solution was then utilized for the PCR reaction.

3.13 PCR for extended spectrum beta-lactamases (ESBLs) encoding genes

3.13.1 Multiplex PCR recipe for the detection of bla_{SHV} and bla_{TEM}

The Multi gene OptiGene thermocycler from Labnet International was used to perform a multiplex PCR for detecting the bla_{SHV} and bla_{TEM} genes. The PCR reaction mixture had a total volume of 12µl. It consisted of 6µl of Thermo Scientific DreamTaq Green PCR Master Mix 2X, 0.3µl of each forward primer, 0.3µl of each reverse primer, 2.8µl of PCR water, and 2µl of template DNA.

3.13.2 Condition for PCR reaction

The PCR reaction comprised of the following steps. Step 1 was initial denaturation which was carried out for 5min at 94ºC. Step 2 was DNA denaturation carried out at 94ºC for 30sec followed by 40 cycles. Annealing was carried out at 52ºC for 40sec and extension at 72ºC for 50 sec. Step 3 was the final extension and it was performed at 72ºC for 10min.

3.13.3 Multiplex PCR recipe for the detection of $bla_{CTX\text{-}MU}$ and $bla_{CTX\text{-}M9}$

Multiplex PCR assay was performed for the detecting two genes: $bla_{CTX\text{-}M9}$ (for group 9 of the target gene) and $bla_{CTX\text{-}MU}$ (for all groups of CTX-M) using The Multi gene Opti Gene thermocycler from Labnet International. The PCR reaction mixture had a total volume of 12µl, consisting of 6µl of Thermoscientific Dream Taq Green PCR Master Mix 2X, 0.3µl of each forward primer, 0.3µl of each reverse primer, 2.8µl of PCR water, and 2µl of template DNA.

3.13.4 Condition for PCR reaction

For 15 minutes the initial denaturation was carried at 95°C. DNA denaturation was carried out at 94ºC for 30sec followed by 30 cycles. Annealing was carried out at 56ºC for 30sec and extension at 72ºC for 50 sec. The final extension was performed at 72ºC for 10min.

3.14 PCR for the detection of carbapenems genes

3.14.1 Multiplex PCR recipe for the detection of $bla_{oxa\text{-}48}$, bla_{NDM} and bla_{KPC} gene

Multiplex PCR was done for the detection of $bla_{oxa\text{-}48}$, bla_{NDM}, and bla_{KPC} using Multi gene Opti Gene (Labnet International) thermocycler. The PCR reaction mixture was 12µl, which contained 6µl master mix, 0.3µl forward primereach, 0.3 µl reverse primer each, 2.2µl of PCR water and 2µl of templet DNA.

3.14.2 Condition for PCR reaction

The PCR reaction comprised of the following steps. Step 1 was initial denaturation which was carried out for 10min at 94ºC. Step 2 was DNA denaturation carried out at 94ºC for 30sec followed by 36 cycles. Annealing was carried out at 52ºC for 40sec and extension at 72ºC for 50 sec. Step 3 was the final extension and it was performed at 72ºC for 5min.

3.14.3 Uniplex PCR for the detection of *bla*$_{VIM}$ gene

Using the Multi gene Opti Gene thermocycler from Labnet International, a uniplex PCR assay was conducted to detect the *bla*$_{VIM}$ gene.The PCR reaction mixture was prepared with a volume of 12µl. It comprised 6µl of master mix, 0.3µl of the forward primer, 0.3µl of the reverse primer, 3.4µl of PCR water, and 2µl of the template DNA.

3.14.4 Condition for PCR reaction

The PCR reaction comprised of the following steps. Step 1 was initial denaturation which was carried out for 10min at 95ºC. Step 2 was DNA denaturation carried out at 94ºC for 60sec followed by 33cycles. Annealing was carried out at 55ºC for 40sec and extension at 72ºC for 50 sec. Step 3 was the final extension and it was performed at 72ºC for 10min.

3.14.5 Uniplex PCR recipe for the detection of *bla*$_{IMP}$ gene

Uniplex PCR was done for the detection of the *bla*$_{IMP}$ gene using the Multi gene Opti Gene (Labnet International) thermocycler. The PCR reaction mixture was 12µl, which contained 6µl of master mix, 0.3µl of forward primer, 0.3µl of reverse primer, 3.4µl of PCR water, and 2µl of DNA templet.

3.14.6 Condition for PCR reaction

The PCR reaction was carried out at the following conditions. Step 1 was initial denaturation whichwas carried out for 10min at 95ºC. Step 2 was DNA denaturation carried out at 94ºC for 60sec followed by 33 cycles. Annealing was carried out at 55ºC for 40sec and extension at 72ºC for 50 sec. Step 3 was the final extension and it was performed at 72ºC for 10min

3.15 PCR for tetracycline resistance genes

3.15.1 Multiplex PCR recipe for detection of *tet*A, *tet*B and *tet*E genes

The Multi gene Opti Gene thermocycler from Labnet International was employed to perform a multiplex PCR assay for detecting three genes: *tet*A, *tet*B and *tet*E. The PCR reaction mixture had a total volume of 12µl, consisting of 6µl of the master mix, 0.3µl of each forward primer, 0.3µl of each reverse primer, 2.2µl of PCR water, and 2µl of template DNA.

3.15.2 Condition for PCR reaction

The PCR reaction comprised of the following steps. Step 1 was initial denaturation which was carried out for 5min at 94ºC. Step 2 was DNA denaturation carried out at 94ºC for 5sec followed by 25 cycles. Annealing was carried out at 61ºC for 30sec and extension at 61ºC for 30 sec. Step 3 was the final extension and it was performed at 61ºC for 7min.

3.15.3 Uniplex PCR recipe for the detection of *tet*D gene

For the detection of the *tet*D gene, a uniplex PCR assay was conducted using the Multi gene Opti Gene thermocycler from Labnet International. The PCR reaction mixture was carefully prepared, resulting in a total volume of 12µl. The mixture consisted of 6µl of Thermo Scientific Dream Taq Green PCR Master Mix 2X , 0.3µl of the forward primer, 0.3µl of the reverse primer, 3.4µl of PCR water, and 2µl of the template DNA.

3.15.4 Condition for PCR reaction

The The PCR reaction comprised of the following steps. Step 1 was initial denaturation which was carried out for 5min at 94ºC. Step 2 was DNA denaturation carried out at 94ºC for 5sec followed by 25 cycles. Annealing was carried out at 68ºC for 10 sec and extension at 68ºC for 10 sec. Step 3 was the final extension and it was performed at 68ºC for 7min.

3.16 PCR for acquired plasmid-mediated quinolone resistance genes

3.16.1 Multiplex PCR for detection of *qnr*A, *qnr*B and *qnr*S gene

A multiplex PCR assay was carried out using the Multi gene Opti Gene thermocycler from Labnet International to detect the presence of the *qnr*A, *qnr*B and *qnr*S genes. The PCR reaction mixture, with a total volume of 12µl, consisted of 6µl of the master mix, 0.3µl of each forward primer, 0.3µl of each reverse primer, 2.8µl of PCR water, and 2µl of DNA template.

3.16.2 Condition for PCR reaction

The PCR reaction was carried out at the following conditions. Step 1 was initial denaturation which was carried out for 10min at 95ºC. Step 2 was DNA denaturation carried out at 95ºC for 1min

followed by 35 cycles. Annealing was carried out at 54ºC for 1min and extension at 72ºC for 1min. Step 3 was the final extension, and it was performed at 72ºC for 10min.

3.16.3 Multiplex PCR for detection of *qnr*C and *qnr*D genes

The Multi gene Opti Gene thermocycler from Labnet International was utilized to conduct a multiplex PCR assay for the simultaneous detection of the qnrC and qnrD genes. The PCR reaction mixture had a volume of 12µl, consisting of 6µl of the master mix, 0.3µl of each forward primer, 0.3µl of each reverse primer, 2.8µl of PCR water, and 2µl of DNA template.

3.16.4 Condition for PCR reaction

The PCR reaction was carried out at the following conditions. Step 1 was initial denaturation which was carried out for 15min at 95ºC. Step 2 was DNA denaturation carried out at 95ºC for 30sec followed by 35 cycles. Annealing was carried out at 63ºC for 90sec and extension at 72ºC for 90sec. Step 3 was the final extension, and it was performed at 72ºC for 10min.

3.16.5 Uniplex PCR recipe for the detection of *aac (6')-Ib-cr* gene

For the detection of the *aac (6')-Ib-cr* gene, a uniplex PCR assay was conducted using the Multi gene Opti Gene thermocycler from Labnet International. The PCR reaction mixture was carefully prepared, resulting in a total volume of 12µl. The mixture consisted of 6µl of the master mix, 0.3µl of the forward primer, 0.3µl of the reverse primer, 3.4µl of PCR water, and 2µl of the template DNA.

3.16.6 Condition for PCR reaction

The PCR reaction was carried out at the following conditions. Step 1 was initial denaturation which was carried out for 5min at 94ºC. Step 2 was DNA denaturation carried out at 94ºC for 1min followed by 30 cycles. Annealing was carried out at 54ºC for 1min and extension at 72ºC for 1min. Step 3 was the final extension, and it was performed at 72ºC for 10min.

3.17 PCR for the detection of colistin-resistance genes

3.17.1 Multiplex PCR for detection of *mcr-1, mcr-2,mcr-3,mcr-4, and mcr-4* genes

A multiplex PCR assay was carried out using the Multi gene Opti Gene thermocycler from Labnet International to detect the presence of the *mcr-1, mcr-2, mcr-3, mcr-4, and mcr-5* genes. The PCR reaction mixture, with a total volume of 12µl, consisted of 6µl the master mix, 0.3µl of each forward primer, 0.3µl of each reverse primer, 2µl of PCR water, and 1µl of DNA template.

3.17.2 Condition for PCR reaction

The PCR reaction was carried out at the following conditions. Step 1 was initial denaturation which was carried out for 15min at 94ºC. Step 2 was DNA denaturation carried out at 94ºC for 30sec followed by 33 cycles. Annealing was carried out at 58ºC for 1min and extension at 72ºC for 1min. Step 3 was the final extension, and it was performed at 72ºC for 10min.

3.18 Gel electrophoresis for identification of PCR products

The amplified products underwent analysis through gel electrophoresis, utilizing a 1.5% agarose gel that was enriched with Ethidium bromide.

3.18.1 Preparation of Gel

A 1x TBE buffer was prepared by combining distilled water and a 10x TBE buffer in a 9:1 ratio. To create a 1.5% agarose gel, 3g of agarose was mixed with 200ml of the 1x TBE buffer followed by heating until fully dissolved. A gel tray was prepared with the appropriate number of combs. After adding 10µl of ethidium bromide to the agarose solution, the mixture was poured into the gel tray and left to solidify for about an hour.

3.18.2 Gel electrophoresis for ARG's

The PCR product, with a volume of 12µl, was combined with a 1.5% agarose gel. Additionally, a thermos scientific Gene reader 100bp DNA ladder was included as a size marker. The gel electrophoresis apparatus was set to a voltage of 110V, and the samples were run for a duration of

1 hour in a horizontal configuration. To visualize the gel, a gel documentation system (Syngege) was employed to check for the presence of the desired amplified PCR products.

Chapter 4

Results

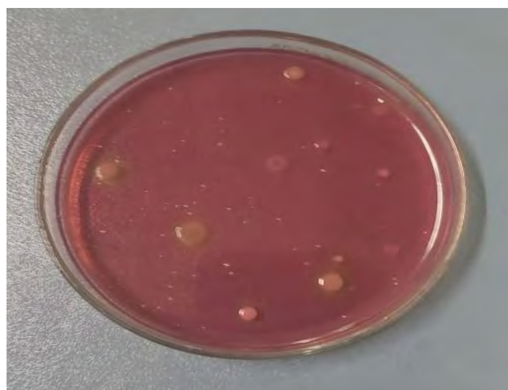
Results

In this study sampling was done from fish seed hatcheries of Islamabad and Faisalabad. A total of 35 samples were collected during the study. Out of which 992 isolates were obtained and examined at Zahra lab at Department of Microbiology Quaid -i-Azam University Islamabad.

4.1 Analysis of water samples from fishponds

4.1.1 Growth of bacteria on different media

The water samples underwent culture on distinct selective media, followed by overnight incubation at 37°C. This incubation led to the growth of bacterial colonies. Isolated pure colonies were obtained and counted for all the samples. The growth pattern observed on different media plates is shown in figure 4.1.



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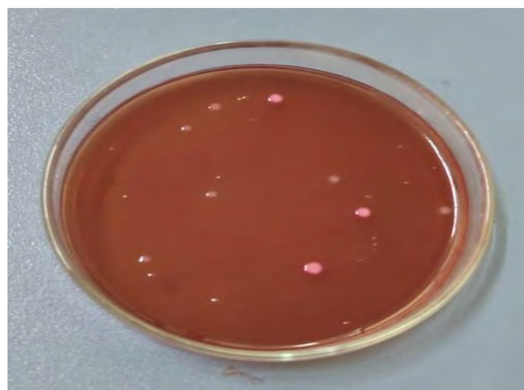

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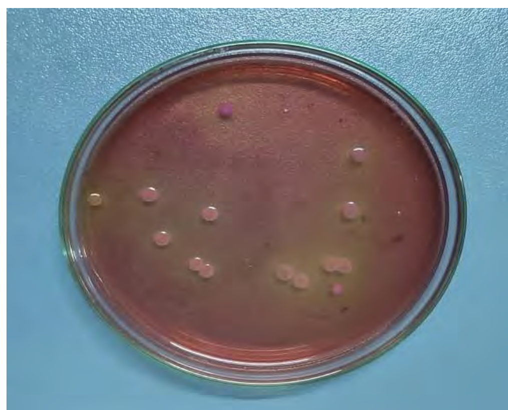

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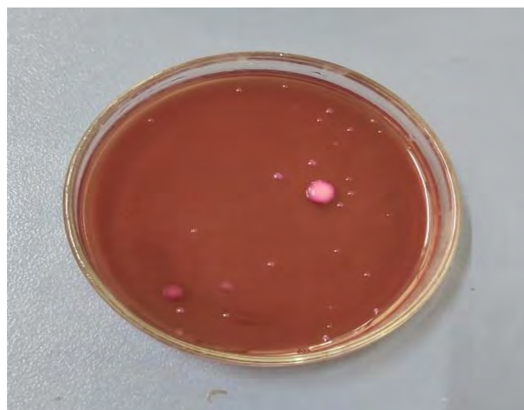


D

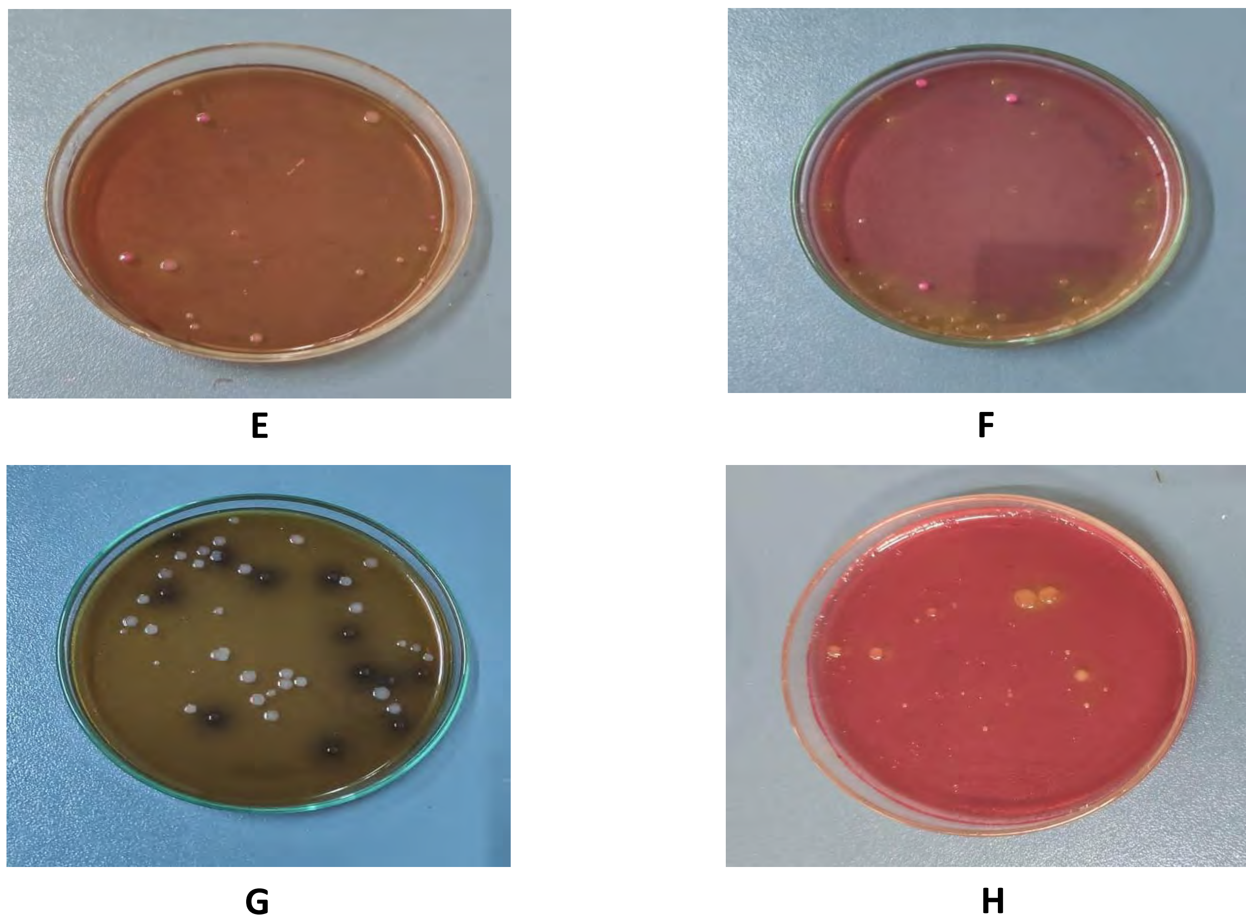


Figure 4.1: Growth of bacteria on different media (A): MacConkey agar; (B): MacConkey agar supplemented with Meropenem; (C): MacConkey agar supplemented with Ciprofloxacin; (D): MacConkey agar supplemented with Colistin; (E): MacConkey agar supplemented with Cefotaxime; (F): MacConkey agar supplemented with oxytetracycline; (G): Bile esculin agar; (H): Mannitol salt agar

4.1.2 Isolation of bacteria from water samples of fishponds

A total of 362 bacteria were isolated from 18 water samples. Out of 362, 11.9% (n = 43) of the bacteria were Gram-positive, 8.3% (n = 30) were Enterococci, 16.6% (n = 60) were Gram-negative, and 63.2% (n = 229) were antibiotic-resistant Gram-negative bacteria. A total of 229 resistant isolates were found, and of those, 42 (18.3%) were resistant to Colistin, 45 (19.7%) were resistant to Ciprofloxacin, 52 (22.7%) were resistant to Cefotaxime, 50 (21.8%) were resistant to Oxytetracycline, and 40 (17.5%) were resistant to Meropenem (Figure 4.2).

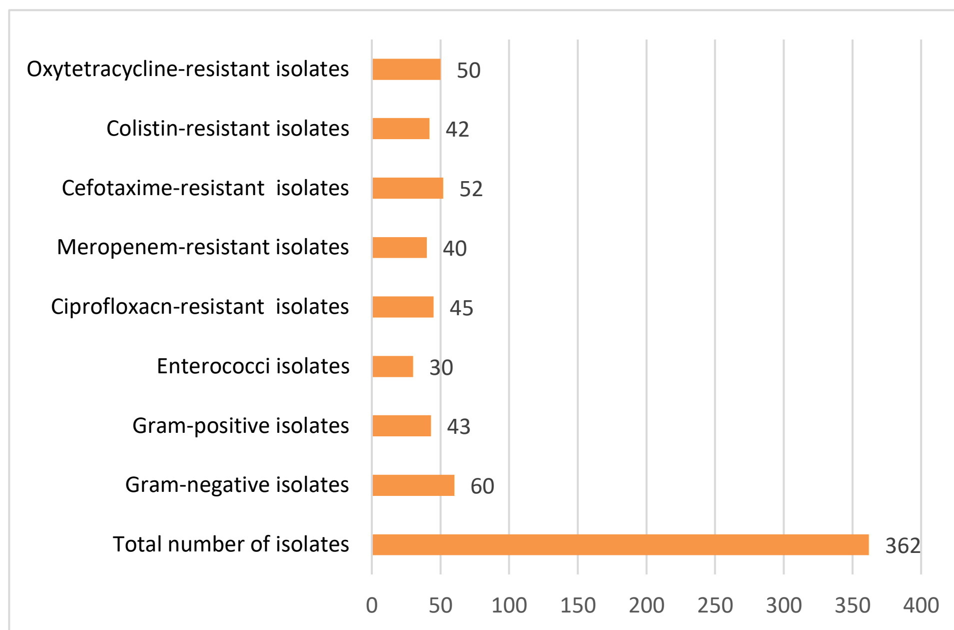


Figure 4.2: Number of bacteria isolated from fishponds water samples on Mannitol salt agar, Bile Esculin agar, McConkey agar, and McConkey agar supplemented with different antibiotics.

4.1.3 Bacterial load in the water sample

After overnight incubation of plates at 37°C, the bacterial load on all plates were calculated. The highest bacterial load for Gram-negative bacteria in water samples was recorded in the FF12 (FF=Fish seed hatchery, Faisalabad water sample) sample i.e., $8.8x10^4$ CFU/mL, and the lowest bacterial load was recorded in the FR2 (FR=Fish seed hatchery, Rawalpindi water sample) sample i.e., $9.8x10^3$ CFU/mL (Figure 4.3.A).

For Gram-positive bacteria, the highest bacterial load in water samples was recorded in the FF9 sample i.e., $6.1x10^2$ CFU/mL. The lowest bacterial load was recorded in the FF5 sample i.e., $1.4x10^2$ CFU/mL (Figure 4.3.B).

$2.3x10^2$ CFU/mL was recorded in the FR5 sample which was the highest bacterial load for the Enterococcus in water samples, and the lowest bacterial load was recorded in the FF8 sample i.e., $7.0x10^1$ CFU/mL (Figure 4.3.C).

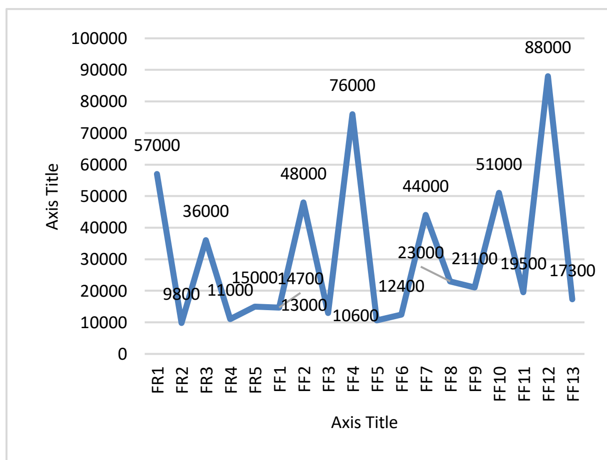
For the Meropenem-resistant bacteria, the highest bacterial load was $3.1x10^2$ CFU/mL in the FF12 sample of the water, while the lowest bacterial load was recorded in the FF6 sample i.e., $6.0x10^1$ CFU/mL (Figure 4.3.D).

The highest bacterial load for the Ciprofloxacin-resistant bacteria in water samples was recorded in the FF10 sample i.e., $2.4x10^2$ CFU/mL and the lowest bacterial load was recorded in the FF4 sample i.e., $3.0x10^1$ CFU/mL (Figure 4.3.E).

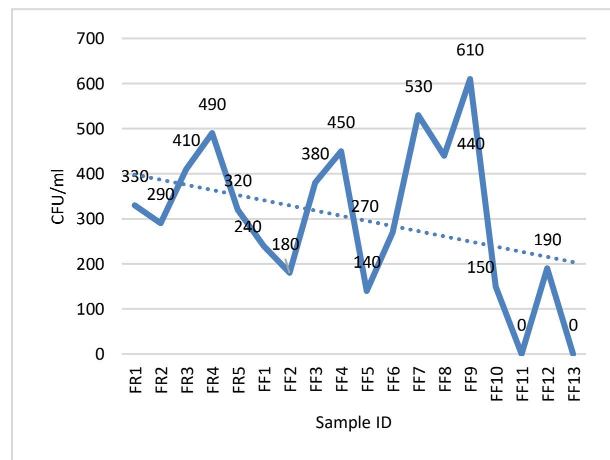
The highest bacterial load for the Cefotaxime-resistant bacteria in water samples was recorded in the FF10 sample i.e., $3.1x10^2$ CFU/mL, and the lowest bacterial load was recorded in the FF1 sample i.e., $7.0x10^1$ CFU/mL (Figure 4.3.F).

The highest bacterial load was $5.1x10^2$ CFU/mL in the FF9 sample of water for the Colistin-resistant bacteria. The lowest bacterial load was recorded in the FF3 sample i.e., $4.0x10^1$ CFU/mL (Figure 4.3.G).

For the oxytetracycline-resistant organisms, the highest bacterial load of $3.9x10^2$ CFU/ml was recorded in the water sample (FF12) and $1.4x10^2$ CFU/mL was recorded as the lowest bacterial load in the FF10 sample as shown in Figure no (4.3.H).



A



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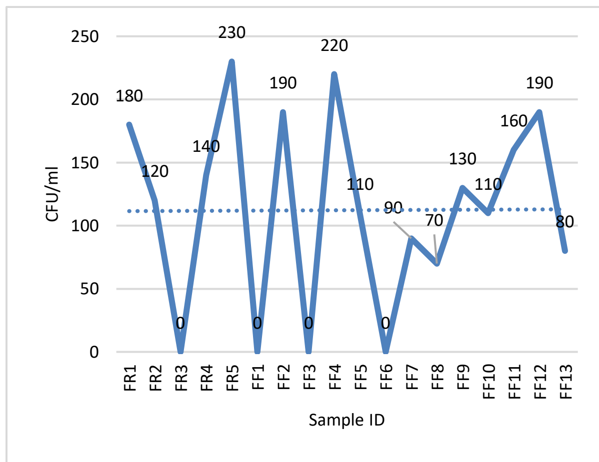

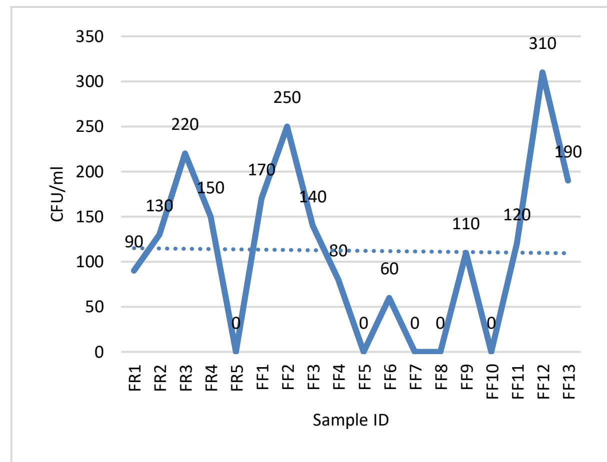

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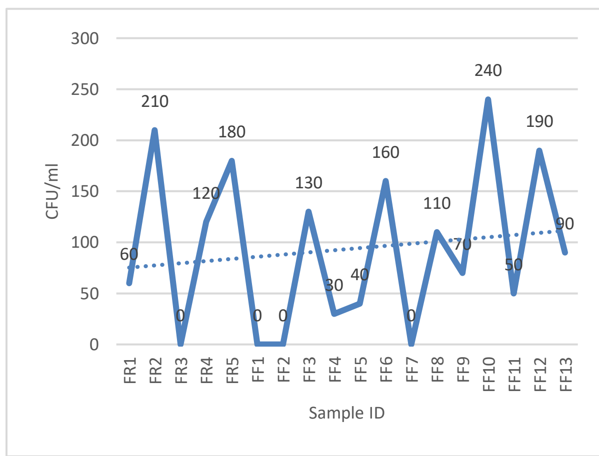

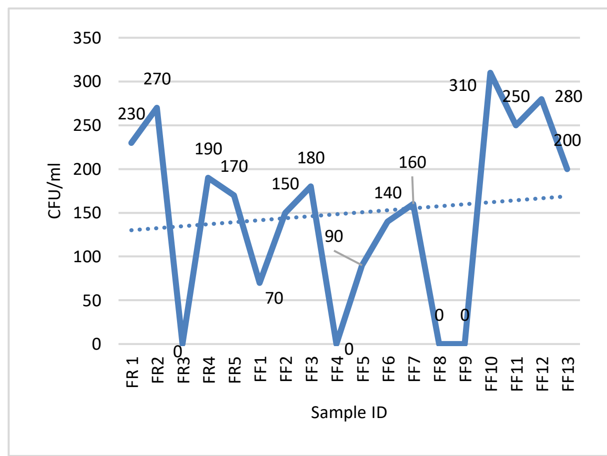

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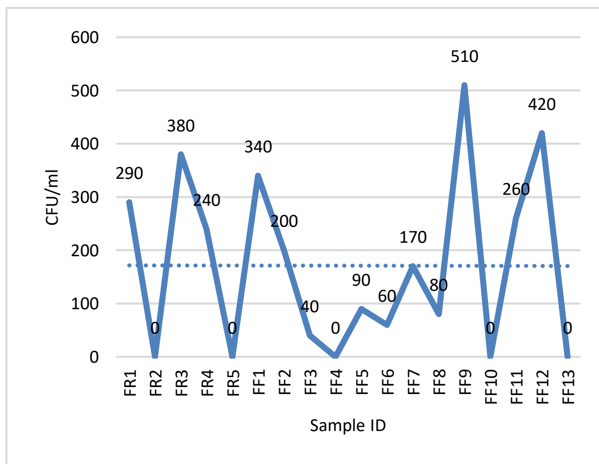
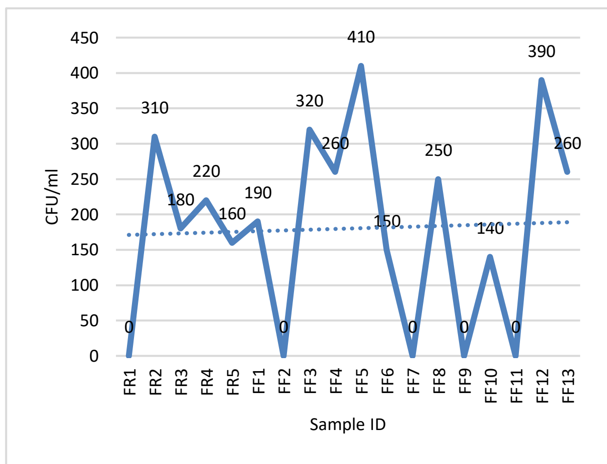




G

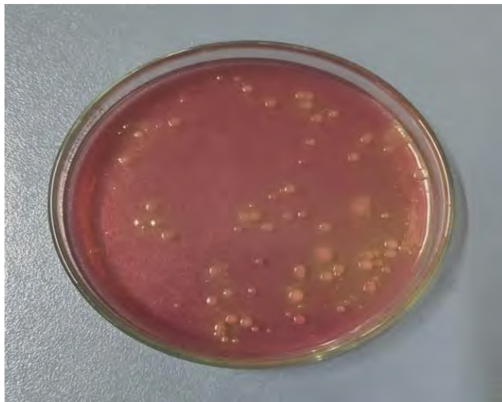
H

Figure 4.3: CFU/ml of A. Gram-negative bacteria, B. Gram-positive bacteria, C. *Enterococcus*, D. Meropenem-resistant, E. Ciprofloxacin-resistant, F. Cefotaxime-resistant, G. Colistin-resistant isolates, and H. Oxytetracycline-resistant isolates in sediment samples

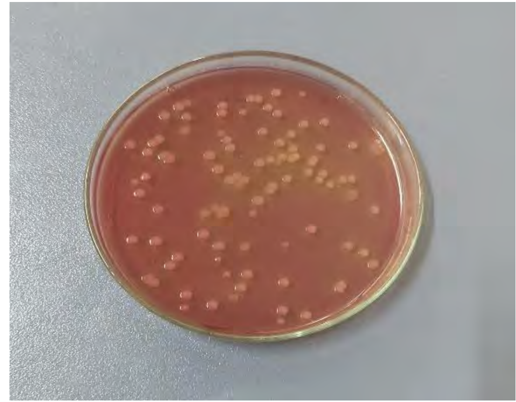
4.2 Analysis of sediment samples from fishponds

4.2.1 Growth of bacteria on different media

The water samples were processed, and dilutions were made. The dilutions were spread on different media plates. The plates were then incubated at 37ºC overnight, resulting in the growth of bacterial colonies (as shown in figure 4.4).



A



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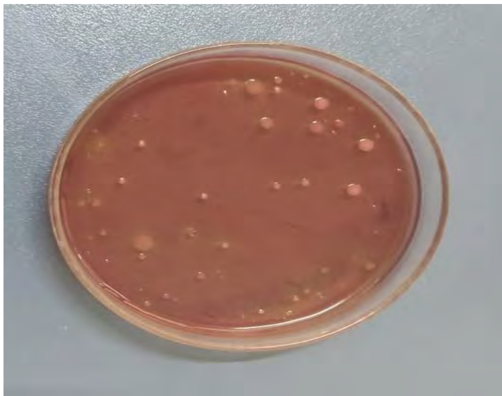

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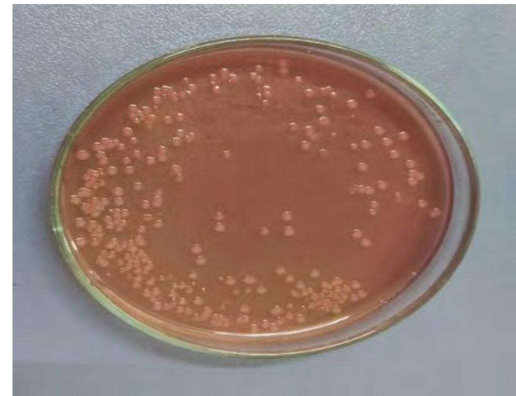

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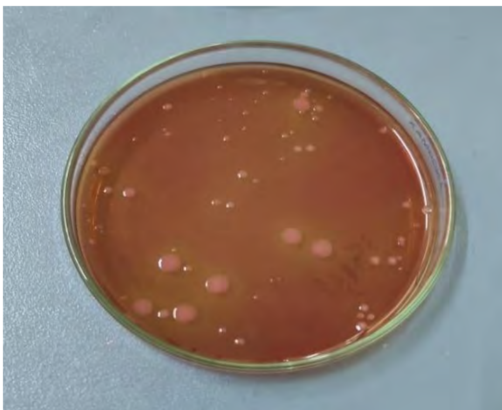

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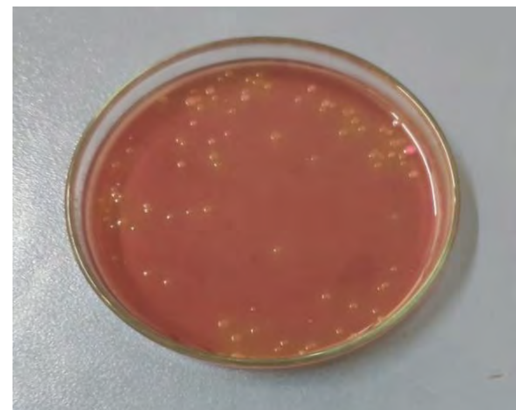


F

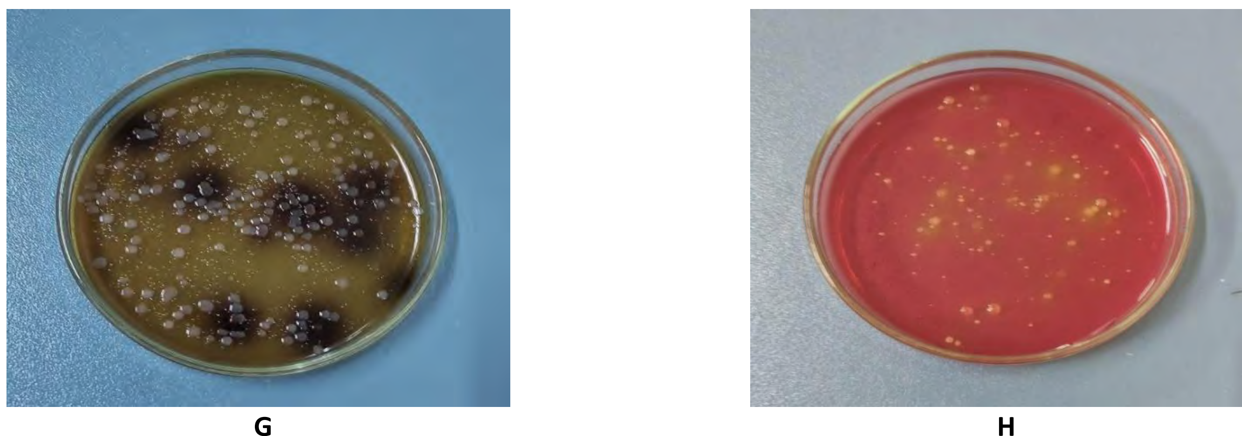


Figure 4.4: Growth of bacteria on different media (A): MacConkey agar; (B): MacConkey agar supplemented with Meropenem; (C): MacConkey agar supplemented with Ciprofloxacin; (D): MacConkey agar supplemented with Colistin; (E): MacConkey agar supplemented with Cefotaxime; (F):

4.2.2 Isolation of bacteria from sediment samples of fishponds

In sediment samples, 555 bacteria were identified, and among these, 11.5% (n=64) were Gram-positive bacteria, 8.8% (n=49) were *Enterococci*, 15% (n=83) were Gram-negative and 64.7% (n=359) were antibiotic-resistant Gram-negative bacteria. Colistin resistance was found in 17.2% (n=62/359), Ciprofloxacin resistance in 22.6% (n=81), Cefotaxime resistance in 22% (n=79), Oxytetracycline resistance in 18.7% (n=67) and Meropenem resistance in 19.5% (n=70) (Figure 4.5).

Figure 4.5: Number of bacteria isolated from fishpond sediment samples on Mannitol salt agar, Bile Esculin agar, McConkey agar, and McConkey agar supplemented with different antibiotics.

4.2.3 Bacterial load in the water sample

After overnight incubation of plates at 37°C, the bacterial load on all plates were calculated. The highest bacterial load for Gram-negative organisms in sediment samples was recorded in the FFS6 (FFS= Fish seed hatchery, Faisalabad sediment sample) sample i.e., $1x10^6$ CFU/ml, and the lowest bacterial load for Gram-negative organisms was recorded in the FFS1 sample i.e., $1.2x10^5$ CFU/ml (Figure 4.6.A).

$2.5x10^4$ CFU/mL was recorded in the FRS3 (FRS= Fish seed hatchery, Rawalpindi sediment sample) sample which was the highest bacterial load for Gram-positive organisms in sediment samples of fishponds (figure 4.6.B).

For Enterococcus, the highest bacterial load was $4.8x10^3$ CFU/mL in the FFS2 sample, while in FRS2, the lowest bacterial load for Enterococcus organisms was recorded i.e., $1.7x10^3$ CFU/mL (figure 4.6.C).

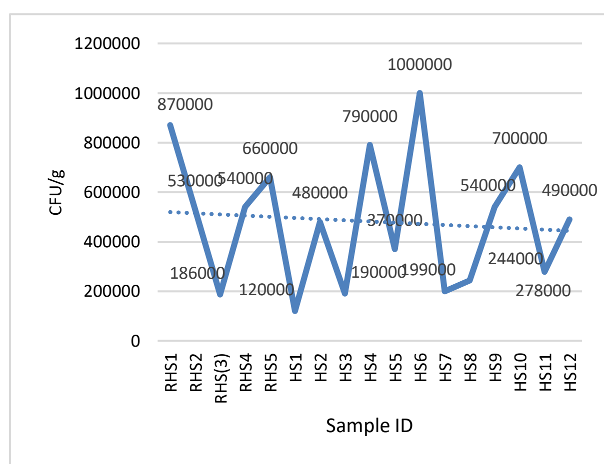
In the FFS12 sample of sediment, the highest bacterial load for the Meropenem-resistant organisms was recorded i.e., 6.9×10^3 CFU/mL, and the lowest bacterial load was recorded in the FFS6 sample i.e., 11.9×10^3 CFU/mL (Figure 4.6.D).

8.2×10^3 CFU/mL was recorded in the FFS6 sample of the sediment which was the highest bacterial load for the Ciprofloxacin-resistant organisms. The lowest bacterial load for ciprofloxacin-resistant organisms was recorded in the FRS3 sample i.e., 101×10^3 CFU/mL (Figure 4.6.E).

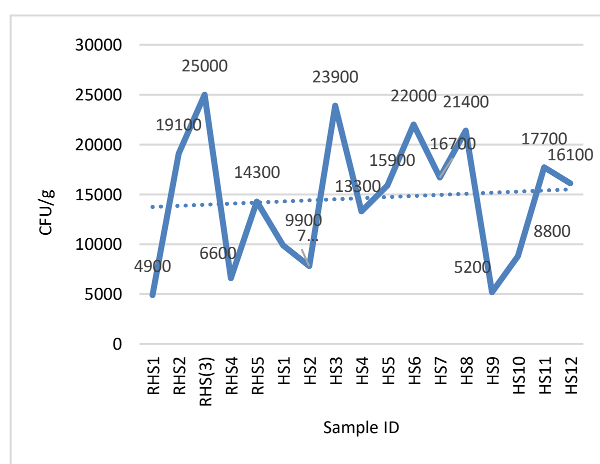
The highest bacterial load for the Cefotaxime-resistant organisms in sediment samples was recorded in the FFS12 sample i.e., 6.2×10^3 CFU/mL, and the lowest bacterial load was recorded in the FFS5 sample i.e., 1.49×10^3 CFU/mL (Figure 4.6.F).

In the FRS4 sample, the highest bacterial load for the colistin-resistant organisms in sediment i.e., 4.9×10^3 CFU/mL. In the FF2 sample, the lowest bacterial load was recorded i.e., 1.05×10^3 CFU/mL (figure 4.6.G).

The highest bacterial load of 8.5×10^3 CFU/ml was recorded for the oxytetracycline-resistant organisms in sediment sample FFS5 and 1.3x103 CFU/mL was recorded i.e., 1.05×10^3 as the lowest bacterial load in the FRS4 sample (figure 4.6.H).



A



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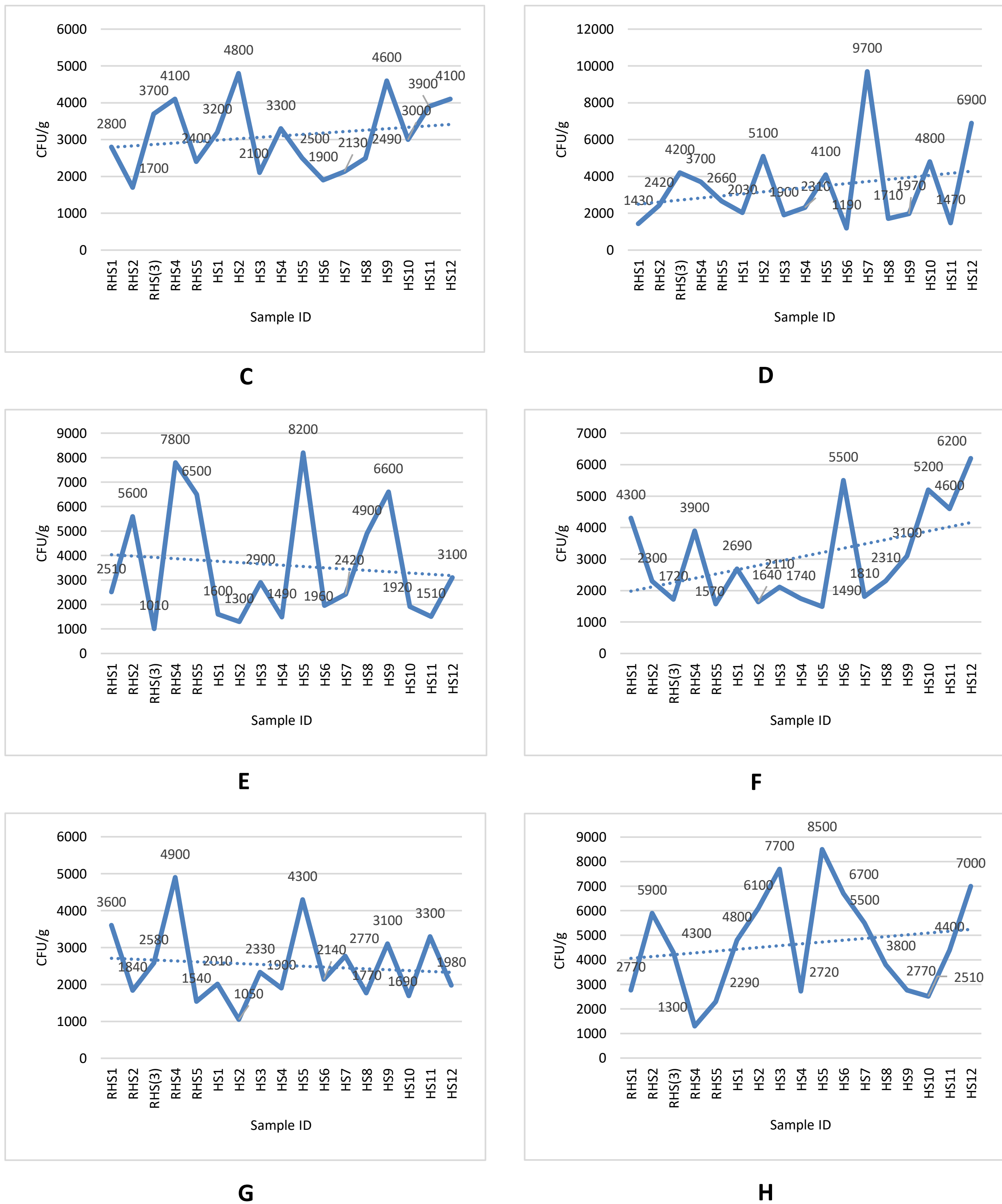


Figure 4.6: CFU/ml of A. Gram-negative bacteria, B. Gram-positive bacteria, C. *Enterococcus*, D. Meropenem-resistant, E. Ciprofloxacin-resistant, F. Cefotaxime-resistant, G. Colistin-resistant isolates, and H. Oxytetracycline-resistant isolates in sediment samples

4.3 Molecular identification of antibiotic resistance genes in different fishponds samples

4.3.1 Molecular identification of PMQR- genes in samples of fishponds:

Two multiplex and one single-plex PCR assays were performed to detect (*qnr*A, *qnr*B, and *qnr*S), (*qnr*C and *qnr*D), and (*aac*(*6*')-Ib-*cr*) in ciprofloxacin-resistant isolates. PCR products of 264bp, 428bp, and 482bp were observed for *qnr*B, *qnr*S, and aac(6')-Ib-cr respectively, which can be seen in Figures 4.7 and 4.8 below.

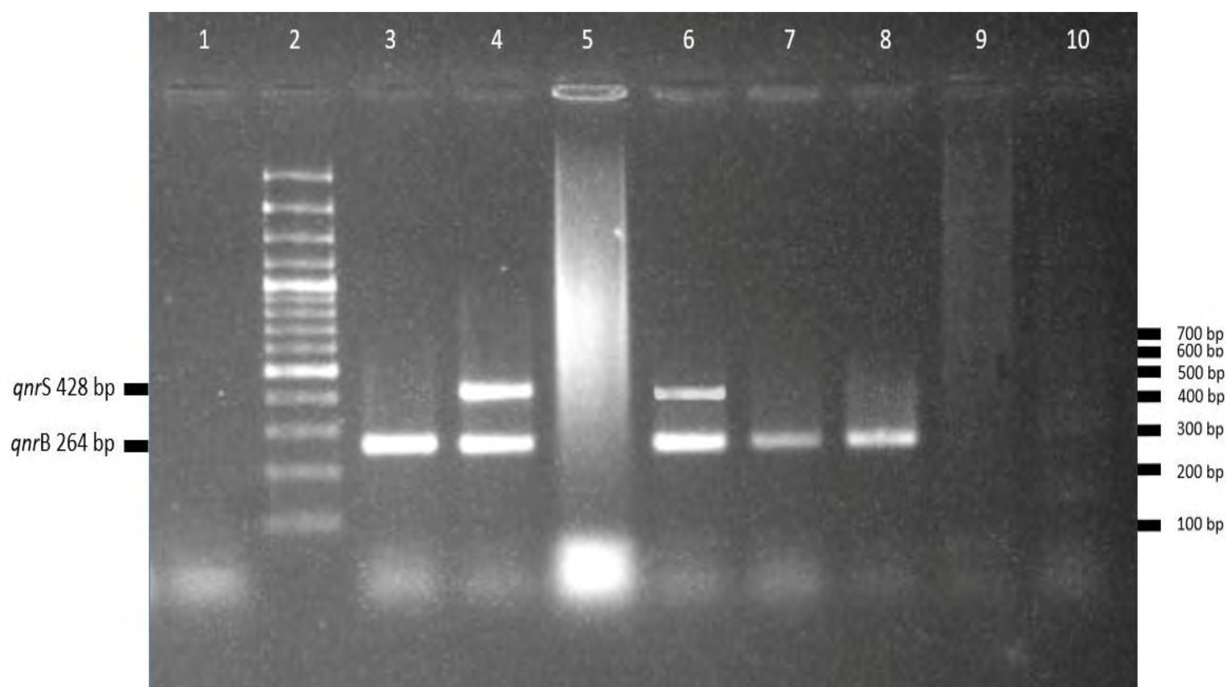


Figure 4.7: Representative gel pictures of PCR products for PMQR genes, lane 4: control for *qnr*B and *qnr*S gene, lane 6: represents *qnr*S at 428bp and *qnr*B at 264bp, lane 2: 100-bp DNA ladder marker, lane 3,7, and 8: represents *qnr*B at 264bp.

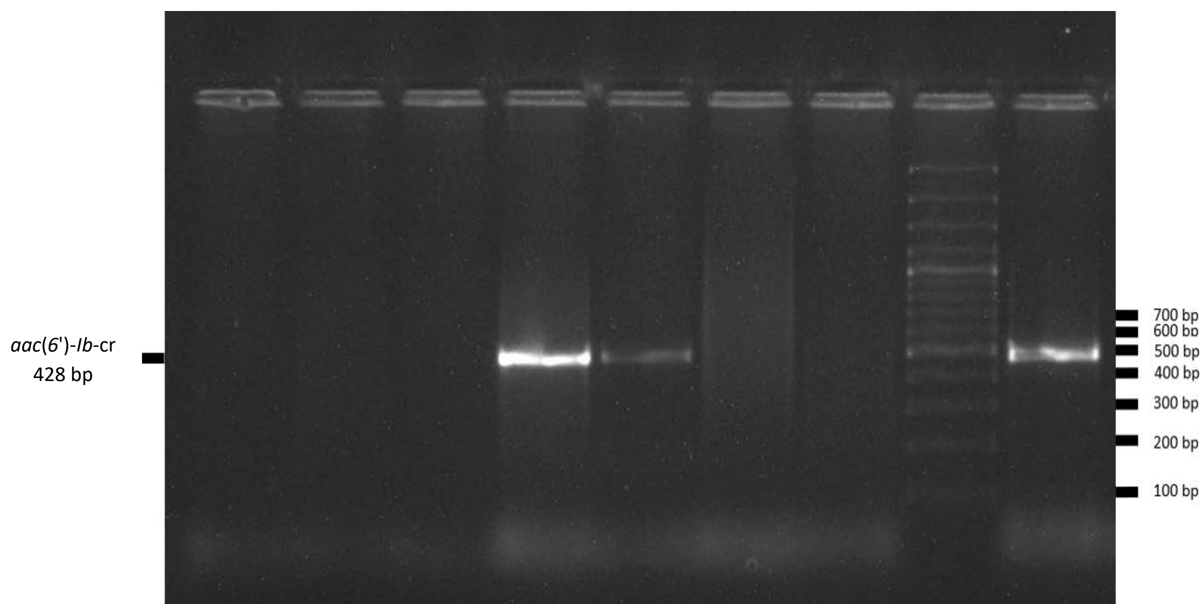


Figure 4.8: Representative gel pictures of PCR products for *aac(6′)-Ib*-cr gene, lane 9: control for aac(6')-Ib-cr, lane 8: 100-bp DNA ladder marker, lane 4, and 5: represents *aac*(*6*')-*Ib*-cr at 482bp

4.3.2 Prevalence of PMQR genes

In water samples from Islamabad, out of 16 isolates, 6.25% (n=1) isolates carried genes for *qnr*B, 12.5% (n=2) isolates carried aac*(6′)-Ib*-cr. While no prevalence of *qnr*S, *qnr*C, and *qnr*D was found in water samples from Islamabad (Figure 4.9).

In water samples of fishponds from Faisalabad, out of 29 isolates, 10.3% (n=3) isolates carried *qnr*B, while no isolates were found to have *qnr*C, *qnr*S, and *qnr*D. 10.3% (n=3) isolates had aac*(6′)-Ib*-cr (Figure 4.9).

In the sediment samples, out of 30 isolates from Islamabad, 6.7% (n=2), 3.3% (n=1) and 10% (n=3) isolates carried *qnr*B, *qnr*S, and *aac*(*6*')-Ib-*cr*. No prevalence of *qnr*C and *qnr*D was found(Figure 4.10).

From Faisalabad, out of 51 isolates, no isolate was found positive for *qnr*C, and *qnr*D in sediment samples. 7.8% (n=4) isolates carried *qnr*B, 4% (n=2) isolates had *qnr*S, whereas 11.8% (n=6) isolates had aac*(6′)-Ib*-cr (Figure 4.10).

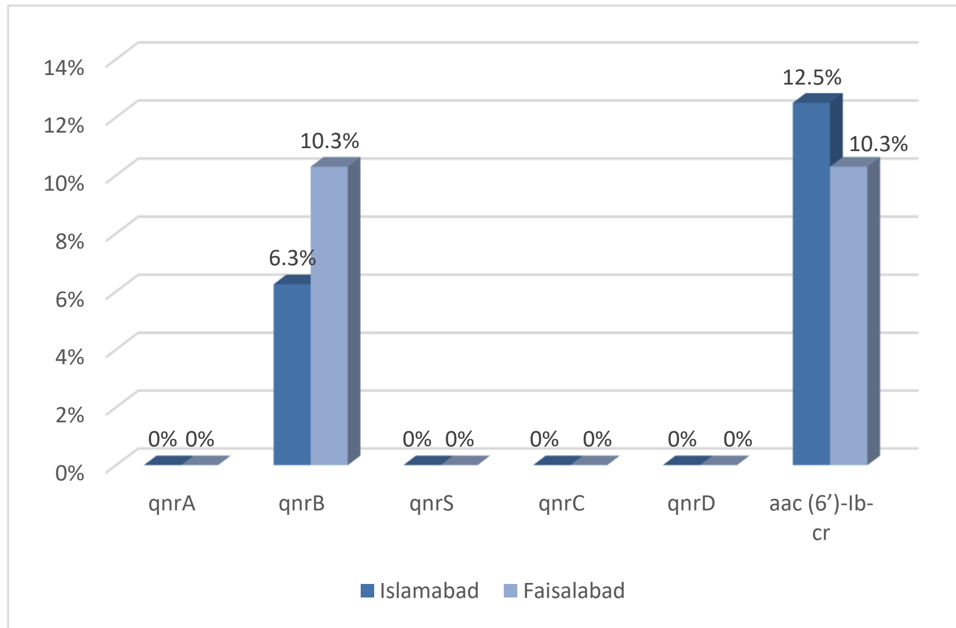


Figure 4.9: Percentages occurrence of PMQR genes in Ciprofloxacin-resistant isolates from water samples of Faisalabad and Islamabad

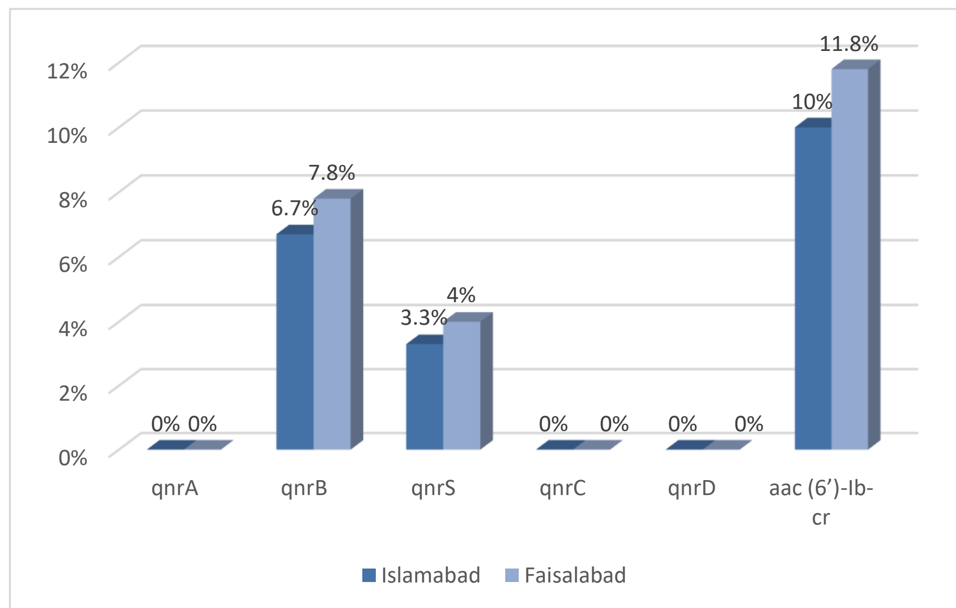


Figure 4.10: Percentages of occurrence of PMQR genes in Ciprofloxacin-resistant isolates from sediment samples of Faisalabad and Islamabad

4.3.3 Molecular identification of ESBL-encoding genes in samples of fishponds:

Two multiplex PCR assays were performed for the detection of bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}MU}$ and $bla_{CTX\text{-}M9}$. PCR products of size 632bp, 471bp, 551bp, and 356bp were detected for bla_{TEM}, blaSHV, and blaCTX-MU, and blaCTX-M9, respectively which can be seen in Figures 4.11 and 4.12 below.

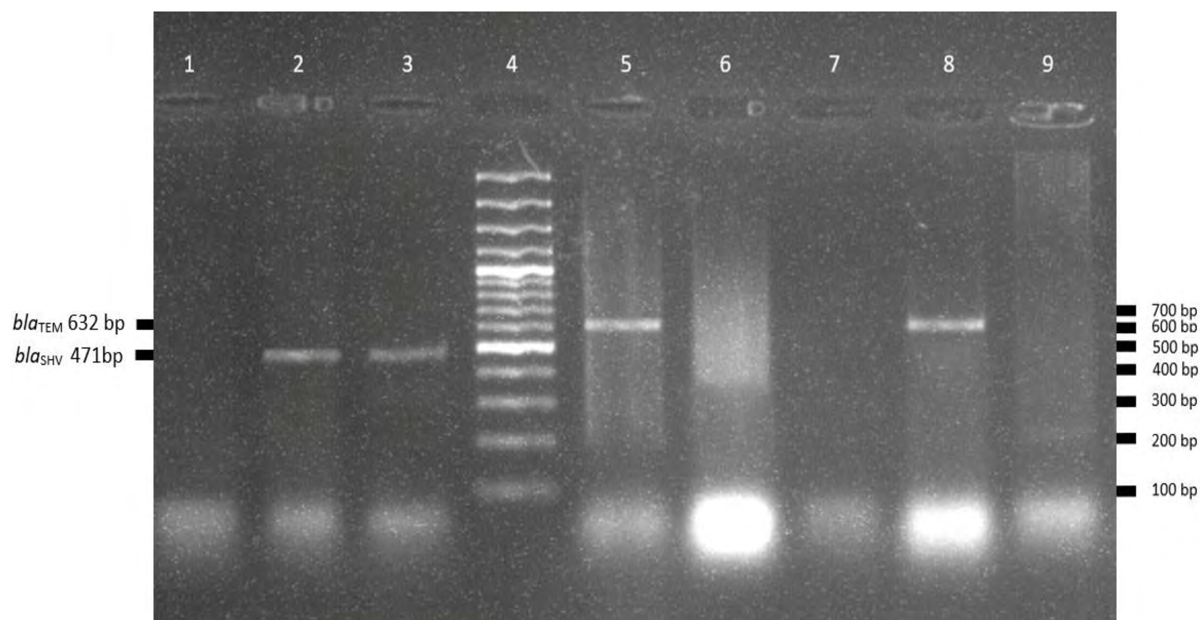


Figure 4.11: Representative gel pictures of PCR products for ESBL genes in fishponds samples. Lanes 3 and 5 were positive controls for bla_{SHV} and bla_{TEM} genes respectively. Lane (4), 100-bp DNA ladder marker, lane 2 bla_{SHV} 471 bp. lane 8, bla_{TEM} at 428bp.

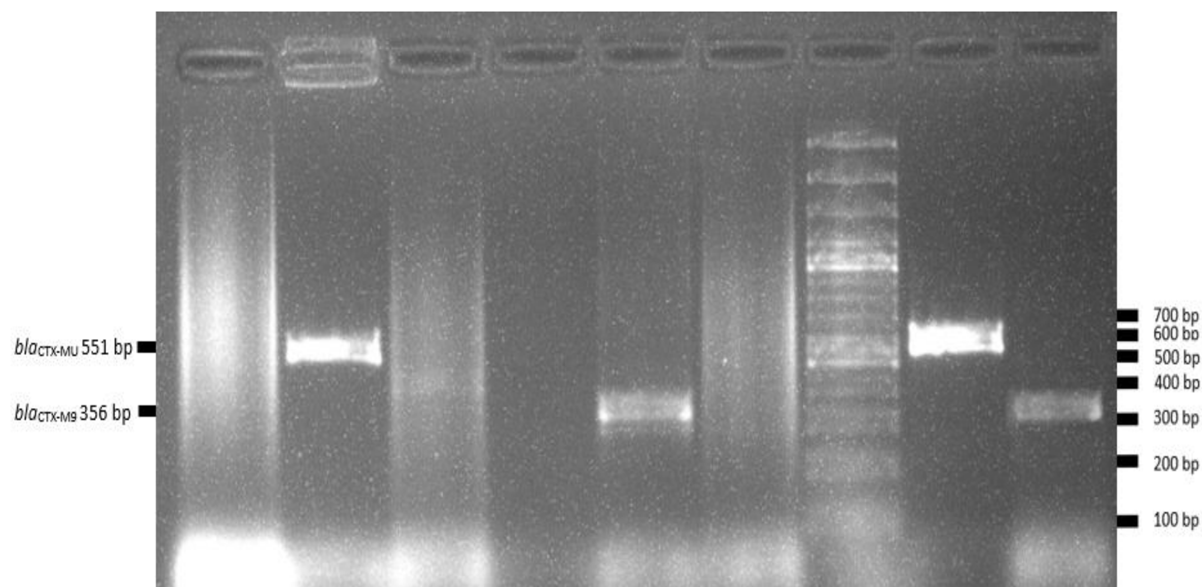


Figure 4.12: Representative gel pictures of PCR products for PMQR genes, lanes 8 and 9: control for *bla*$_{CTX-MU}$ and *bla*$_{CTX-M9}$ gene, lane 2: represents *bla*$_{CTX-MU}$ at 551 bp, lane 7: 100-bp DNA ladder marker, lane 5: represents *bla*$_{CTX-M9}$ at 356bp

4.3.4 Prevalence of ESBLs genes

In water samples from Islamabad, out of 20 isolates, 3.7% (n=1) isolates were found positive for *bla*$_{SHV}$. While no isolate was found positive for *bla*$_{CTX-M9}$, *bla*$_{TEM,}$ and *bla*$_{CTX-MU}$ (Figure 4.13).

From Faisalabad, out of 32 isolates from water samples of fishponds, 3.12% (n=1) isolates carried genes for *bla*$_{CTX-MU}$., 3.12% (n=1) isolates carried *bla*$_{CTX-M9}$. 6.2% (n=2) isolates carried genes for *bla*$_{SHV}$, whereas *bla*$_{TEM}$ was not found in any isolates (Figure 4.13).

In sediment samples of fishponds from Faisalabad, out of 27 isolates, 11.11% (n=3) isolates carried genes for *bla*$_{CTX-MU}$, and 3.7% (n=1) isolates carried *bla*$_{CTX-M9}$. 3.7% (n=1) isolates were found positive for *bla*$_{TEM,}$ while all the isolates were found negative for *bla*$_{SHV}$ (Figure 4.14).

From Faisalabad, out of 52 isolates from sediment samples, 7.7% (n=4) of isolates carried the *bla*$_{CTX-MU}$ gene while no isolate had *bla*$_{TEM}$. 2% (n=1) isolates had *bla*$_{CTX-M9}$, whereas 3.8% (n=2) isolates had the *bla*$_{SHV}$ gene (Figure 4.14).

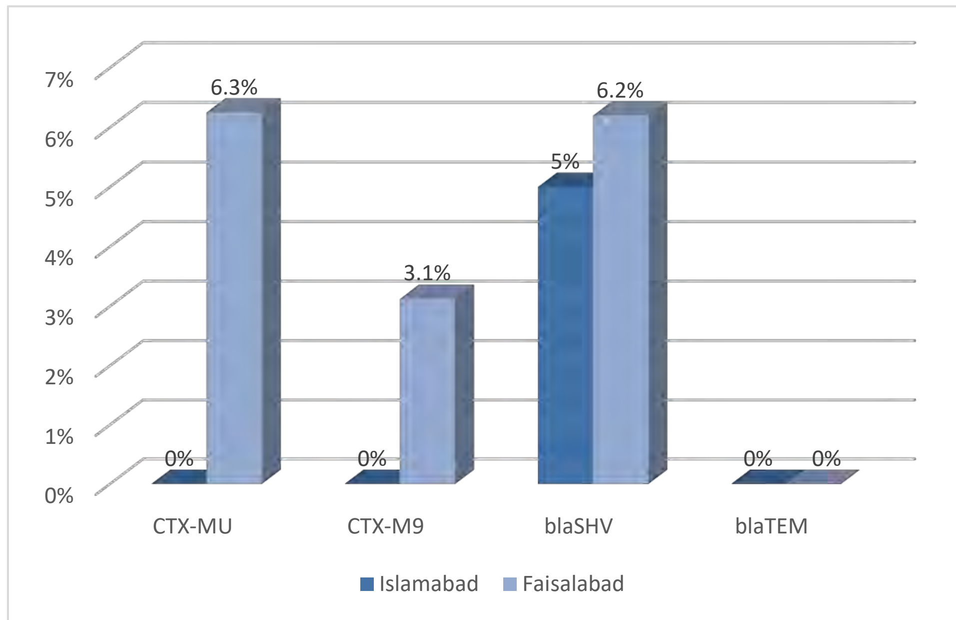


Figure 4.13: Percentages of occurrence of ESBLS genes in Cefotaxime-resistant isolates from water samples of Faisalabad and Islamabad

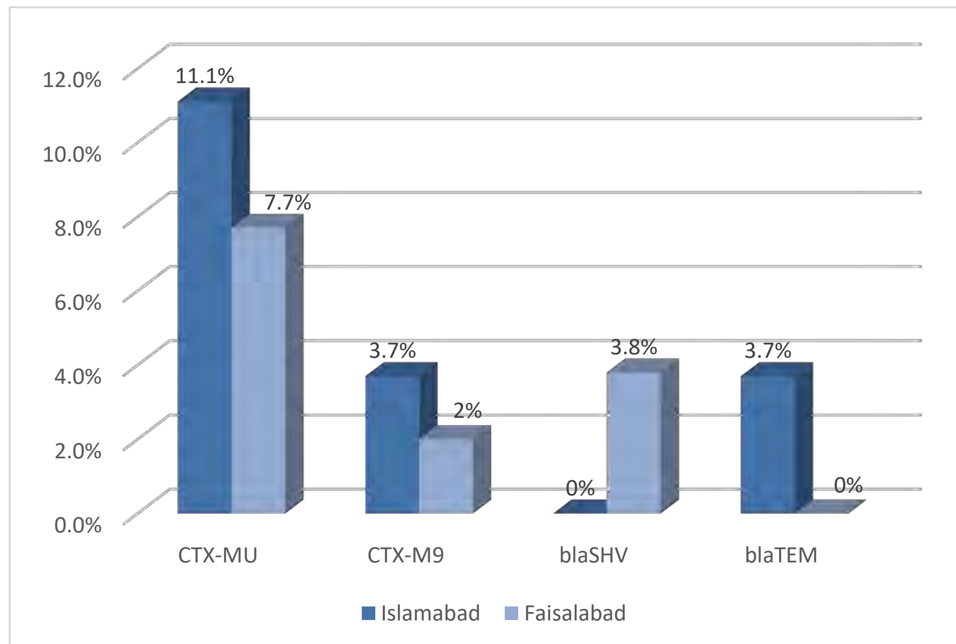


Figure 4.14: Percentages of occurrence of ESBLS genes in Cefotaxime-resistant isolates from sediment samples of Faisalabad and Islamabad

4.3.5 Molecular identification of Carbapenemases encoding genes in samples of fishponds:

PCR was conducted for the identification of carbapenemase-encoding genes in Meropenem-resistant isolates. A Multiplex-PCR was carried out for bla_{NDM}, bla_{KPC}, and bla_{OXA-48}. Two single-plex-PCR reactions were carried out for bla_{IMP} and bla_{VIM}. PCR products of size 438bp, 621bp, 798bp, 181bp, and 389bp were observed for bla_{OXA-48}, bla_{NDM}, bla_{KPC}, $bla_{IMP,}$ and bla_{VIM} respectively, which can be seen in Figures 4.15 and 4.16.

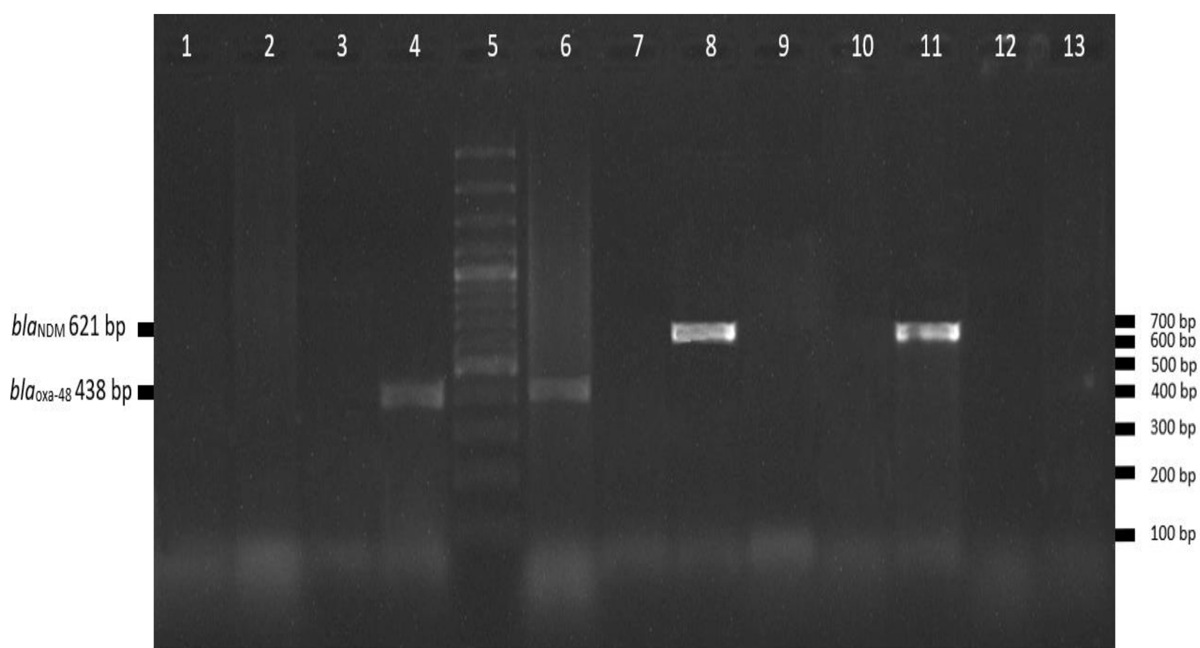


Figure 4.15: Representative gel pictures of PCR products for carbapenemase-encoding genes. lane 5: 100-bp DNA ladder marker, Lanes 6 and 8 represent controls for bla_{NDM} and $bla_{oxa-48.}$ lane 64: bla_{OXA-48} at 438 bp, lane 11: represents bla_{NDM} at 621bp.

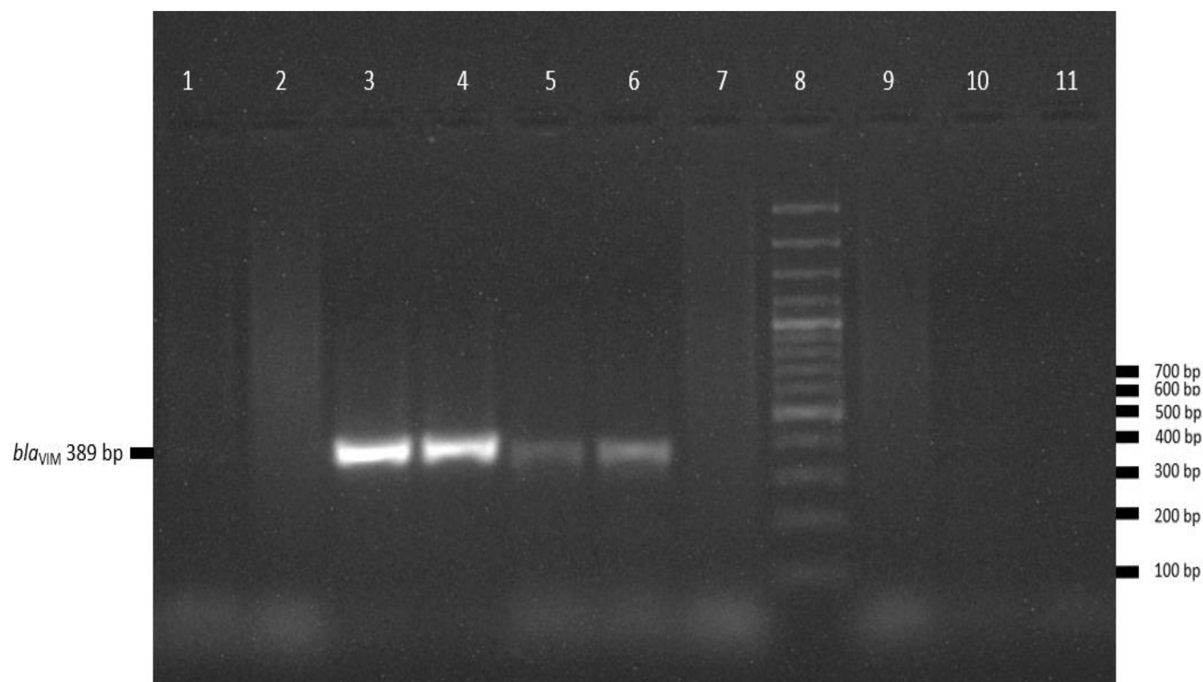


Figure 4.16: Representative gel pictures of PCR products for carbapenemase-encoding genes. lanes 3,4 and 5: *bla*$_{VIM}$ gene at 382-bp. Lane 8: 100-bp DNA ladder marker. Lane 6 represents a control for *bla*$_{VIM}$

4.3.6 Prevalence of Carbapenemases genes

In water samples from Islamabad, out of 15 Meropenem-resistant isolates, 6.7% (n=1/15) isolate had *bla*$_{VIM}$. No isolates were found positive for *bla*$_{oxa}$-48, *bla*$_{KPC}$, *bla*$_{NDM}$, and *bla*$_{IMP}$ (Figure 4.17).

From Faisalabad, out of 25 isolates from water samples of fishpond, 8% (2/25) isolates were found positive for *bla*$_{oxa-48}$, 4% (1/25) were found positive for *bla*$_{NDM}$ gene, and no isolate was found positive for *bla*$_{KPC}$, and *bla*$_{IMP.}$ (Figure 4.23. B). 4% (1/25) isolates were found positive for *bla*$_{VIM.}$ (Figure 4.17).

In sediment samples from Islamabad, 4% (1/25) were found positive for *bla*$_{NDM}$ gene and 4% (1/25) had *bla*$_{VIM}$. While no isolates were found positive for *bla*$_{IMP,}$ and *bla*$_{OXA-48}$, and *bla*$_{KPC}$ genes (Figure 4.18). In Meropenem-resistant isolates, out of 45 isolates from water samples of fishpond, 13.3% (6/45) isolates were found positive for *bla*$_{oxa-48}$, 2.2% (1/45) were found positive for *bla*$_{NDM}$ gene and 4.4% (2/45) isolates were found positive for *bla*$_{VIM.}$ No isolate was found positive for *bla*$_{KPC}$, and *bla*$_{IMP}$ genes (Figure 4.18).

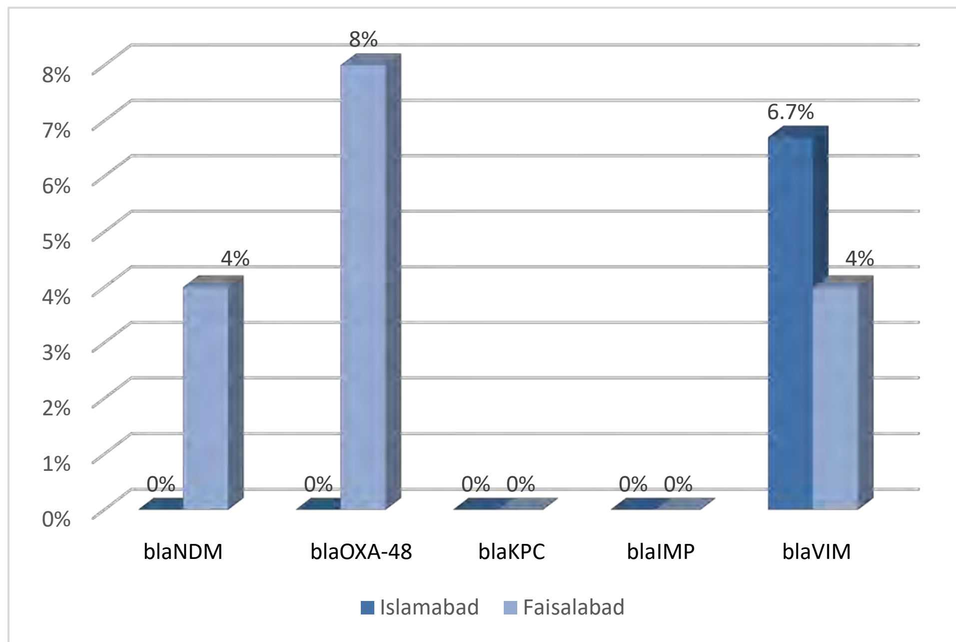


Figure 4.17: Percentages of occurrence of carbapenemase encoding genes in Meropenem-resistant isolates from sediment samples of Faisalabad and Islamabad

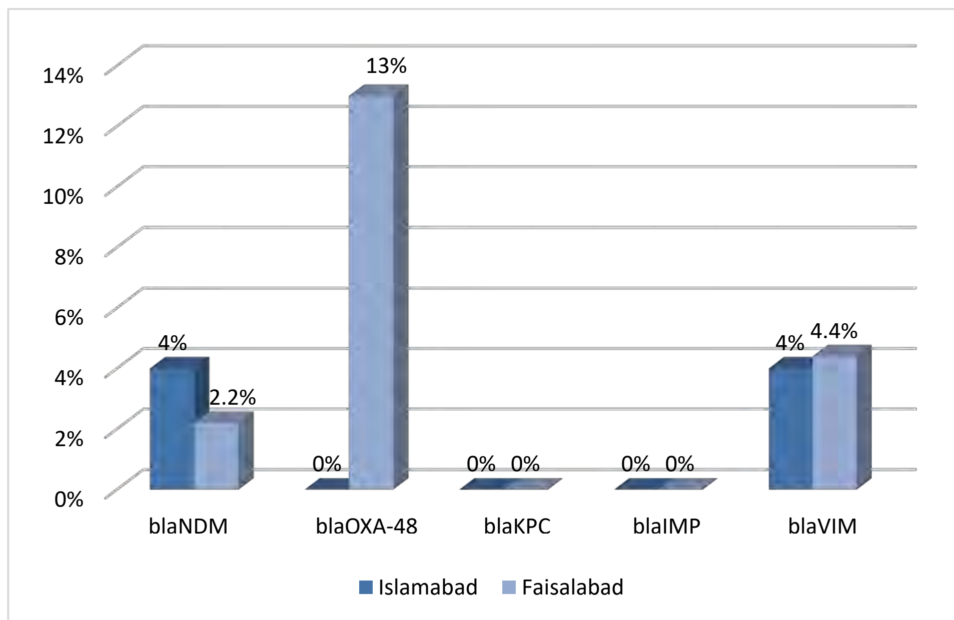


Figure 4.18: Percentages of occurrence of carbapenemase encoding genes in Meropenem-resistant isolates from sediment samples of Faisalabad and Islamabad

4.6.7 Molecular identification of Colistin-resistance genes in samples of fishponds:

PCR was conducted for the identification of genes responsible for colistin-resistance (*mcr*). A Multiplex-PCR was carried out for *mcr-1, mcr-2, mcr-3, mcr-4, and mcr-5*. PCR products of size 320bp, 715bp, 929bp, 1116bp, and 1144bp were observed for *mcr-1, mcr-2, mcr-3, mcr-4, and mcr-5* respectively (Figure 4.19).

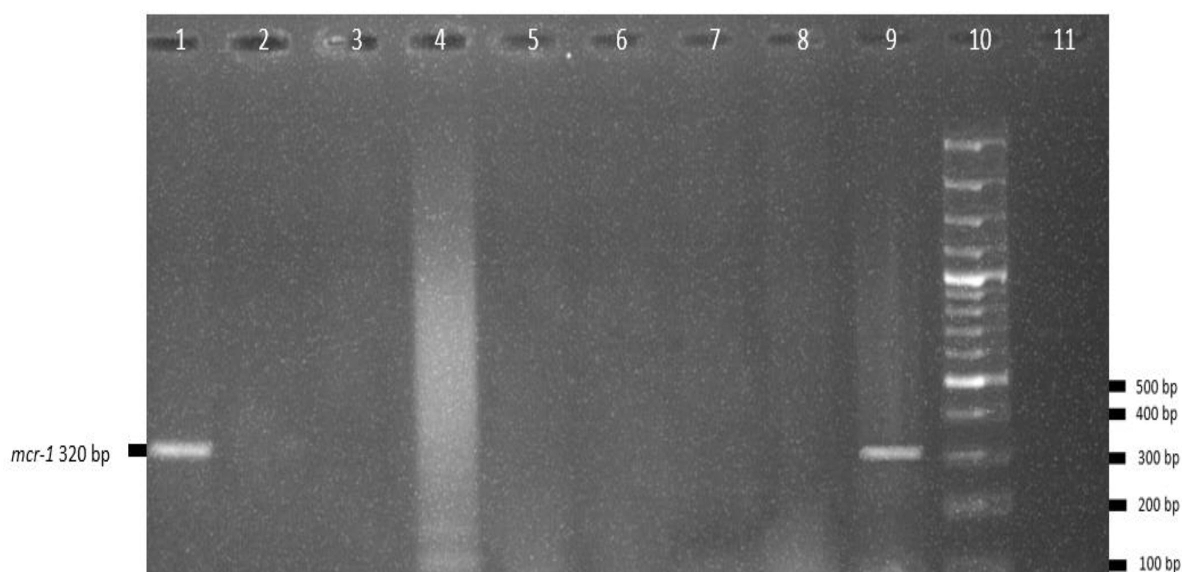


Figure 4.19: Representative gel pictures of PCR products for the Colistin-resistance gene. Lane 9 represents a control for *mcr-1*, lane 1: *mcr-1* gene at 320-bp. Lane 10: 100-bp DNA ladder marker.

4.3.8 Prevalence of colistin-resistance genes

From water samples of Faisalabad, and Islamabad, no isolate found positive for colistin resistance genes.

In sediment samples from Islamabad, out of 22 colistin-resistant isolates, 4.5% (1/22) isolates were found positive for *mcr-1*, while no isolates were found positive for other mcr genes (Figure 4.20).

From Faisalabad, out of 66 colistin-resistant isolates from water and sediment samples, no Isolate was found positive for *mcr-1 ,mcr-2, mcr-3, mcr-4, and mcr-5.*

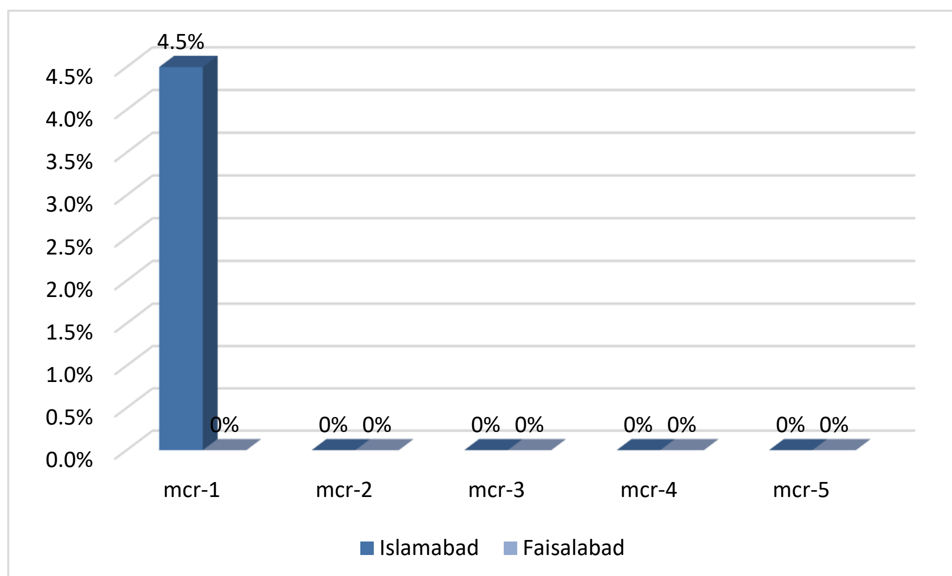


Figure 4.20: Percentages of occurrence of Colistin-resistance genes from sediment samples of Faisalabad and Islamabad

4.3.9 Molecular identification of tetracycline resistance genes samples of fishponds:

PCR was conducted for the identification of tetracycline-resistance genes in oxytetracycline-resistant isolates. A Multiplex-PCR was carried out for *tet*A, *tet*B and *tet*E. One single-plex-PCR reaction was carried out for *tet*D. PCR products of 164bp, 206bp, 187bp, and 199bp were observed for *tet*A, *tet*B, *tet*D, and *tet*E respectively, as seen in Figures 4.21 and 4.22.

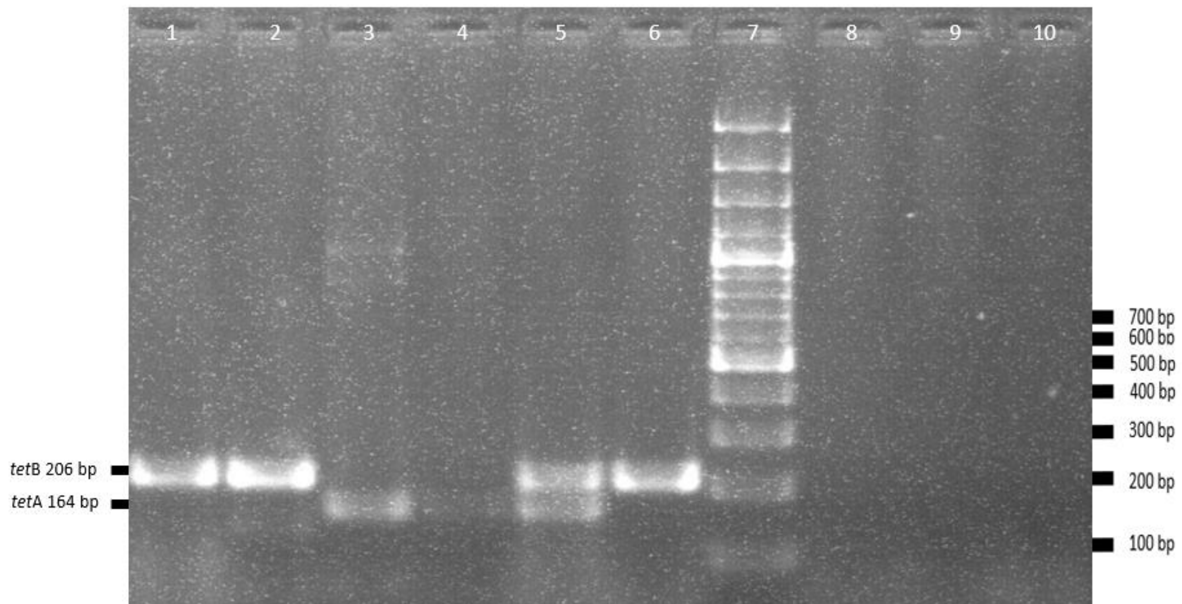


Figure 4.22: Representative gel pictures of PCR products for tetracycline-resistance genes. Lanes 6 and 5 represent a control for *tet*B and *te*tA. Lane 3: *tet*A gene at 164-bp. Lane 1, and 2: *tet*B gene at 206-bp. Lane. 7: 100-bp DNA ladder marker

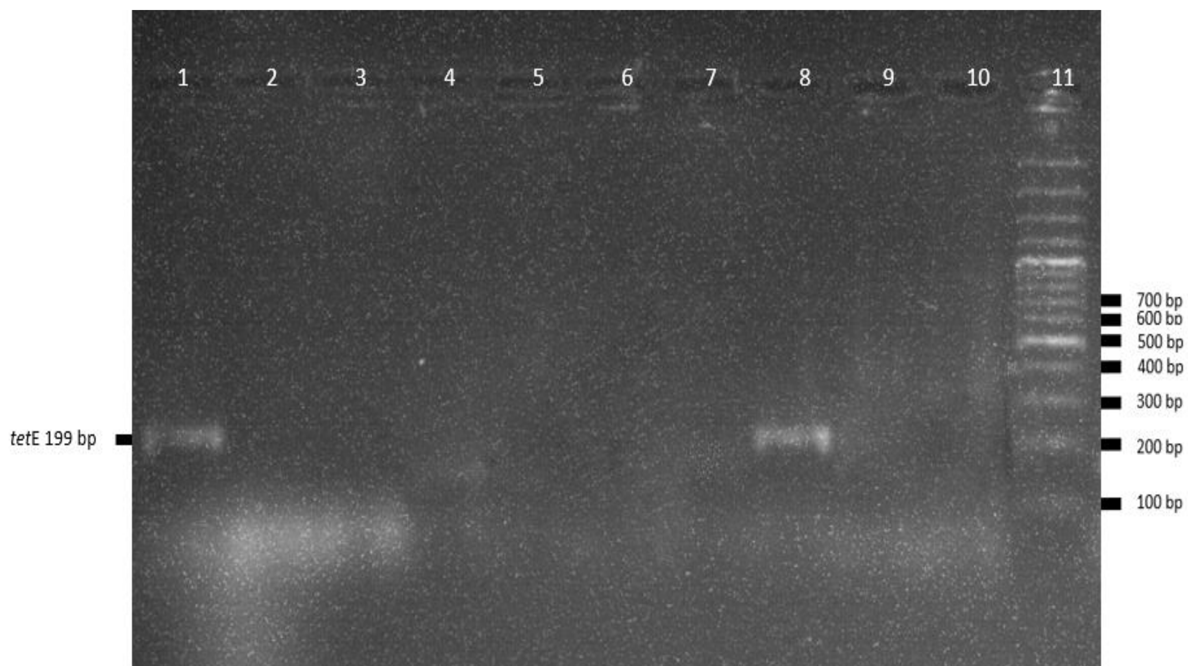


Figure 4.21: Representative gel pictures of PCR products for tetracycline-resistance genes. Lane 8 represents a control for *tet*E. Lane 1: *tet*E gene at 199-bp. Lane 11: 100-bp DNA ladder marker Lane

4.3.10 Prevalence of tetracycline resistance genes

In water samples from Islamabad, out of 17 tetracycline-resistant isolates, 11.8% (2/17) isolates were found positive for *tet*A, 5.9% (1/17) for *tet*E gene, and no isolate was found positive for *tet*B and *tet*D (Figure 4.23).

From Faisalabad, out of 33 tetracycline-resistant isolates from water samples of fishpond, 6.1% (2/33) isolates were found positive for *tet*A, 3% (1/33) were found positive for *tet*E gene, and 6.1% (2/33) had *tet*B gene. While no isolate was found positive for the *tet*D gene (Figure 4.23).

In sediment samples from Islamabad, 12.5% (3/24) isolates were found positive for *tet*A, 8.3% (2/24) for *tet*E, and 4.1% (1/24) had *tet*B gene. While no isolate was found positive for *tet*D gene (Figure 4.24).

In tetracycline-resistant isolates, out of 43 isolates from sediment samples of fishpond, 11.6% (5/43) isolates were found positive for *tet*A, 4.7% (2/43) were found positive for *tet*E gene and 9.3% (4/43) isolates were found positive for *tet*B. No isolate was found positive for *tet*D gene (Figure 4.24).

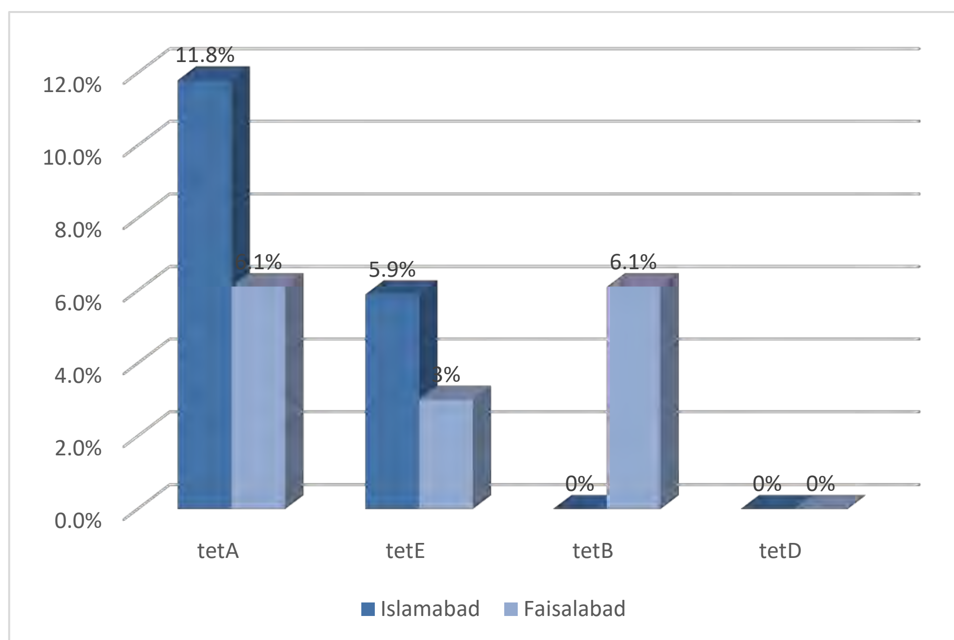


Figure 4.24: Percentages of occurrence of tetracycline-resistance genes in isolates from water samples of Faisalabad and Islamabad

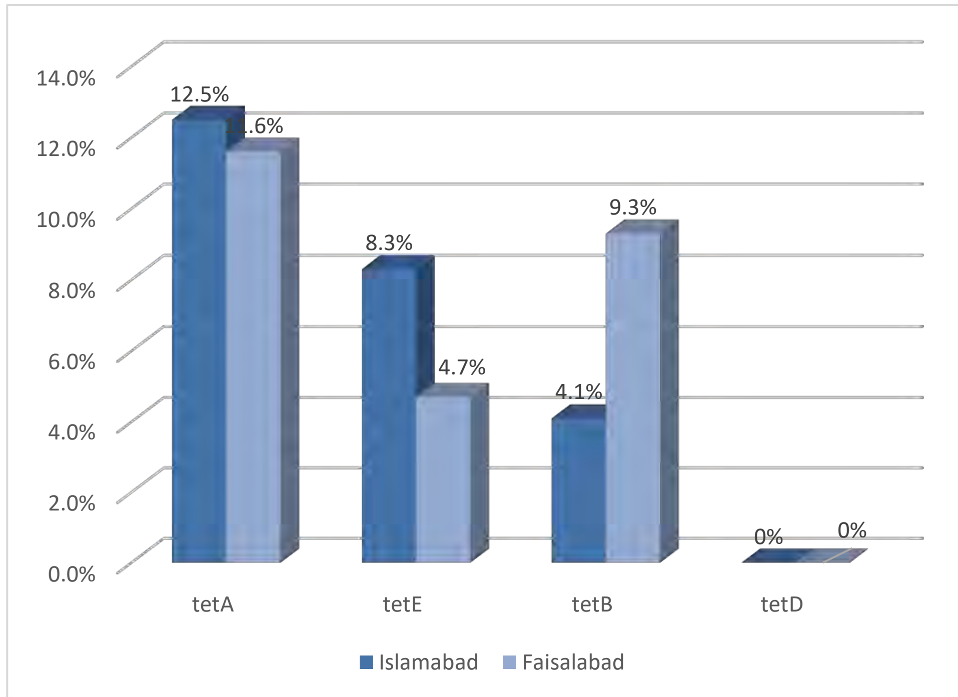
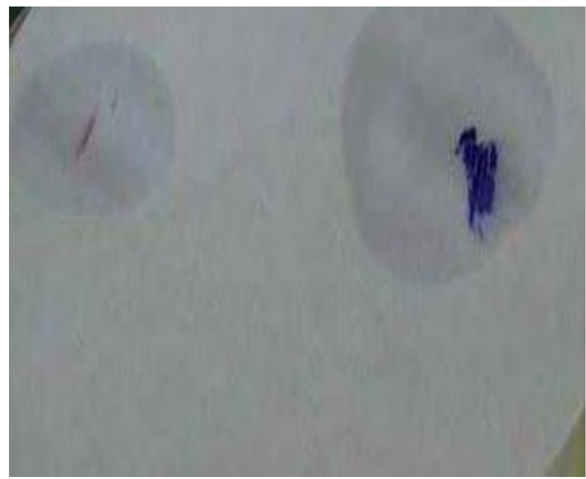


Figure 4.25: Percentages of occurrence of tetracycline-resistance genes in isolates from sediment samples of Faisalabad and Islamabad

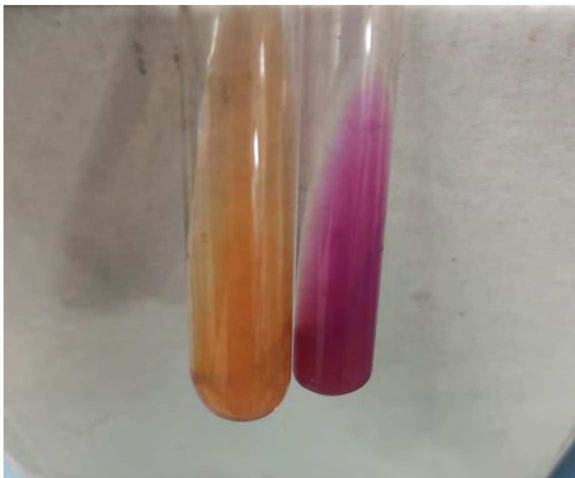
4.4 Biochemical test

Various biochemical tests were conducted to identify the isolates carrying resistance genes. A collection of ten distinct bacterial species was identified among the isolates carrying resistance genes. These species encompass *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Acinetobacter, baumannii*, *E. coli*, *Serratia marcescens*, *Citrobacter freundii*, *Proteus mirabilis*, *Edwardsiella tarda*, and *Klebsiella oxytoca*. The results enumerating these identified isolates are showcased in Table 4.1.

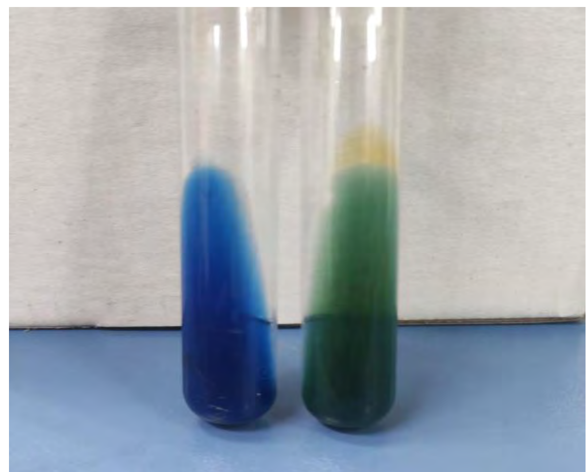
A



B

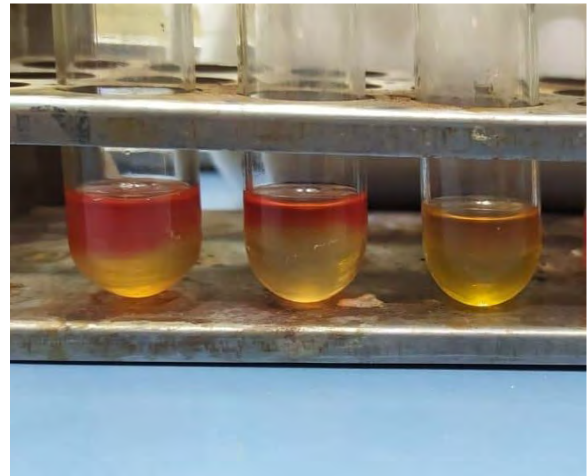


C

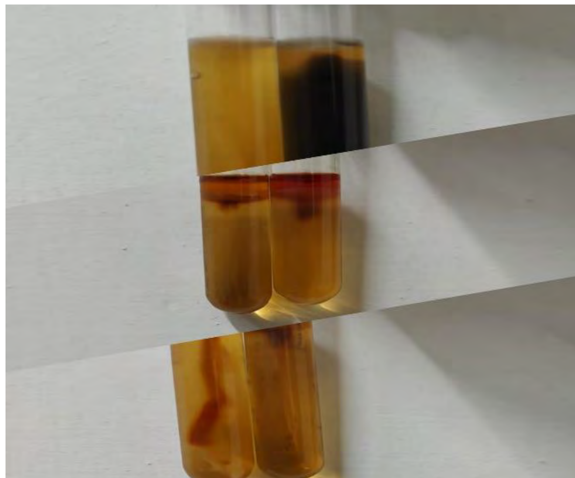


D

E



F



G

H

Figure 4.26: Illustrative depiction of biochemical tests. In (A), the formation of bubbles signifies a positive catalase test. In (B), the appearance of purple-colored lines indicates a positive oxidase test result. In (C), the transition of media color to pink indicates a positive urease test. In (D), the presence of a deep Prussian blue color signifies a positive citrate test result. In (E), the emergence of a pink-red color indicates a positive Voges-Proskauer test result. In (F), the manifestation of a red color indicates a positive methyl red test result. In (G), the presence of a cherry red colored ring signifies a positive Indole test result, the region with increased growth around the growth line indicates a positive motility test, and the blackening of the media indicates a positive sulfide gas test result. In (H), a red slant with a red butt signifies no glucose fermentation and a yellow slant with a yellow butt signifies fermentation of all sugars.

Table 4.1: Biochemical identification of genotypically resistant isolates

Sample ID	Colony morphology	Oxidase	Citrate	Sulfide	Indole	Motility	TSI	Urease	MR	VP	Catalase	Lactose Fermenter	Organism
HS3O(3)	Small, round, and wheatish	-	+	+	-	+	K/A	+	+	-	+	-	*Proteus mirabilis*
HS2O(6)	Round, regular margin, and pink	-	+	-	-	+	K/A	-	-	+	+	+	*Enterobacter cloacae*
RH1O(3)	Pale and round	-	-	+	+	+	K/A	-	+	-	+	-	*Edwardsiella* spp.
RH3Cip(1)	Small, round, and wheatish	-	+	-	+	-	A/A	-	-	+	+	+	*Klebsiella oxytoca*
HS3M(4)	Small, round and wheatish	+	+	-	-	+	K/K	-	-	-	+	-	*Pseudomonas aeruginosa*
HS4O(2)	Dark pink in center, light pink boundary, mucoid, round	-	+	-	-	-	A/A	-	-	+	+	+	*Klebsiella pneuomoniae*
RH1O(4)	Large, round, and smooth	-	+	-	-	+	K/A	D +	+	+	+	-	*Serratia marcescens*
RHS1O(4)	light pink, small, and round	-	+	-	-	-	K/NC	D +	+	-	+	+	*Citrobacter ferundii*
RHS1O(3)	Pink, dry, small, regular margin	-	-	-	-	-	A/A	+	+	+	+	+	*E.coli*
RHS1Cef(3)	Dry, and small	-	+	-	-	-	K/A	D +	-	-	+	-	*Acinetobacter baumanii*

4.5 Relative abundance of resistance genes carrying isolates

4.5.1 Relative abundance of PMQR genes carrying isolates

Amongst the isolates that exhibited positive results for ciprofloxacin resistance genes, the distribution across distinct bacterial species unfolded as follows: *E. coli* encompassed 20% (5 out of 25), *Citrobacter Ferundii* made up 8% (2 out of 25), *Proteus mirabilis* constituted 16% (4 out of 25), *Enterobacter cloacae* accounted for 8% (2 out of 25), , *Klebsiella oxytoca* accounted for 4% (1 out of 25), and *Klebsiella pneumoniae* dominated at 44% (11 out of 25). These ratios are visually depicted in Figure 4.26.

4.5.2 Relative abundance of ESBLs carrying isolates

Among the isolates that tested positive for carbapenem resistance genes, the distribution across different bacterial species was as follows: *Proteus mirabilis* for 17.6% (3 out of 17), *E. coli* for 17.6% (3 out of 17), *Citrobacter freundii* for 29.4% (5 out of 17), *Acinetobacter baumannii* for 23.5% (4 out of 17), and *Serratia marcescens* for 11.8% (2 out of 17). These proportions are visually presented in Figure 4.26.

4.5.3 Relative abundance of carbapenemases carrying isolates

Of the genotypically positive isolates carrying carbapenem resistance genes, their distribution across different bacterial species was as follows: *Pseudomonas aeruginosa* for 33.3% (5 out of 15), *Klebsiella pneumoniae* for 20% (3 out of 15), and *Enterobacter cloacae* accounted for 6.7% (1 out of 15). Notably, *Citrobacter freundii, E. coli,* and *Serratia marcescens* shared same relative abundance of 13.3% (2 out of 15) as depicted in Figure 4.26.

4.5.4 Relative abundance of colistin resistance genes carrying isolates

Among the entire set of colistin-resistant isolates, a solitary isolate, constituting 0.9% (1 out of 104), exhibited genotypic resistance to colistin, and this isolate was identified as *Proteus mirabilis* (Figure 4.26).

4.5.5 Relative abundance of tetracycline resistance genes carrying isolates

In the group of isolates displaying positive outcomes for tetracycline resistance genes, the distribution among distinct bacterial species emerged as follows: *E. coli* encompassed 47.6% (10 out of 21), *Klebsiella pneumoniae* constituted 19% (4 out of 21), *Citrobacter freundii* comprised 14.3% (3 out of 21), and *Proteus mirabilis* accounted for 9.5% (2 out of 21). While *Enterobacter cloacae, Klebsiella pneumoniae oxytoca* and *Edwardsiella ssp.* represented equivalent abundance of 4.8% (1 out of 21). These proportions are visually illustrated in Figure 4.27.

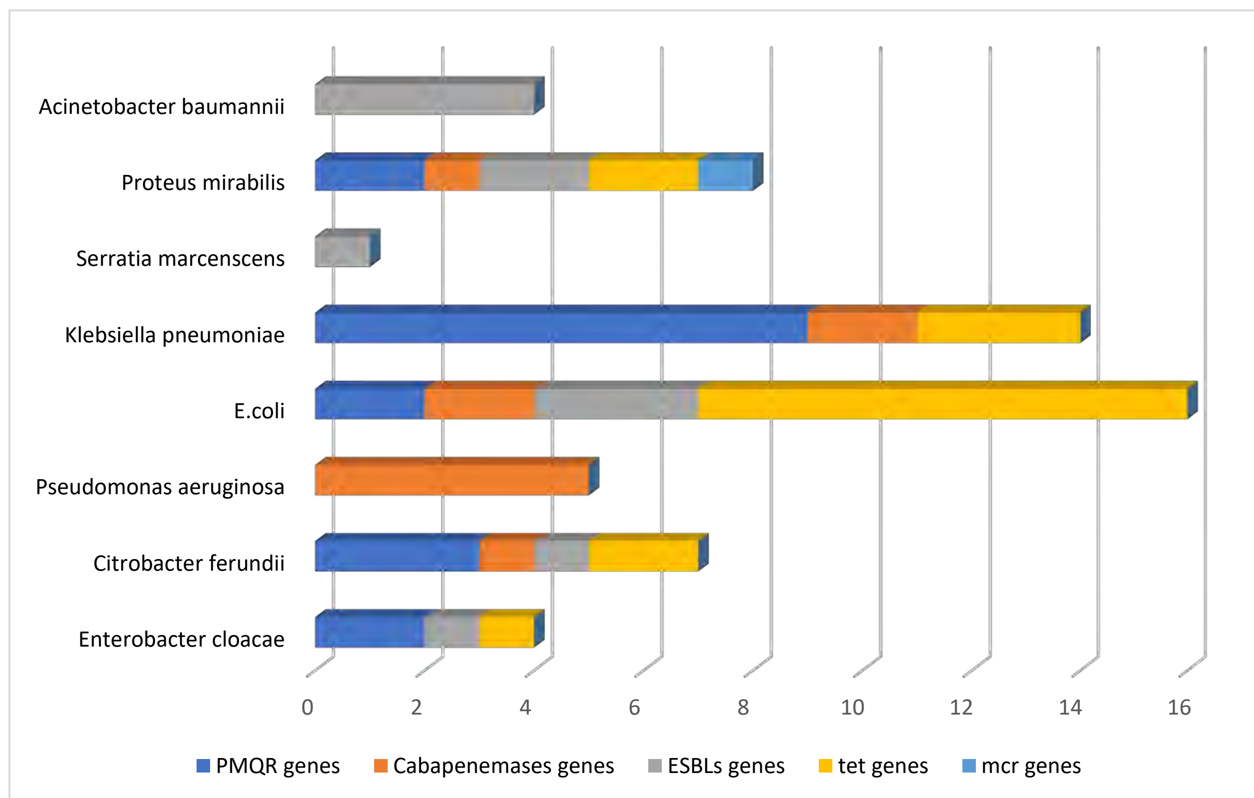


Figure 4.27: Representing the relative abundance of positive isolates for resistance genes

4.6 Antibiotic susceptibility testing (AST)

A total of eighty-two isolates were found genotypically positive for various resistance genes. Among the total 79 isolates, 18.3% (15/82) were found to possess carbapenem resistance genes, while tet genes were present in 39.2% (24/82) of the isolates. Additionally, 20.7% (17/82) of the isolates carried ESBL genes, 1.2% (1/82) were found positive for mcr gene, and the presence of PMQR genes was detected in 30.5% (25/82) of the isolates. These isolates, which carried resistance genes, were subjected to an analysis of their susceptibility to nine distinct antibiotics. Following the incubation process, the resulting zones of inhibition (as depicted in Figure 4.8) were interpreted according to the guidelines established by CLSI and EUCAST, as illustrated in Figure 4.28.

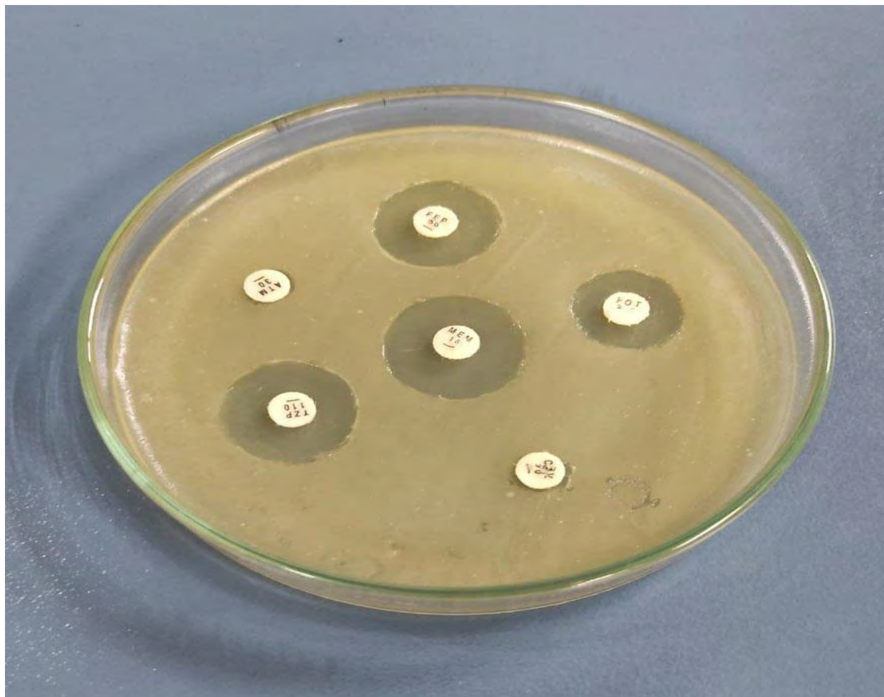


Figure 4.27: Petri plate showing antimicrobial susceptibility pattern

4.6.1 Antibiogram profile of PMQR genes-carrying isolates

The percentages of PMQR carrying isolates showing resistance were as follows: 20% (5/25) against meropenem, 48% (12/25) against aztreonam, 12% (3/25) against Fosfomycin, 36% (9/25) against cefepime, 56% (14/25) against ceftazidime, 52% (13/25) against amikacin, 60% (15/25) against piperacillin, 40% (10/25) against tetracycline, and 48% (12/25) against nalidixic acid. Moreover, the multiple antibiotic resistance index ranged between 0.1 to 0.7 for these isolates.

In addition, 28% (7/25) of PMQR carrying isolates exhibited intermediate resistance to meropenem, 16% (4/25) to aztreonam, 4% (1/25) to Fosfomycin, 24% (6/25) to cefepime, 36% (9/25) to ceftazidime, 36% (9/25) to amikacin, 28% (7/25) to tetracycline, and 32% (8/25) to nalidixic acid (Figure 4.28). Furthermore, 52% (13/25) of ciprofloxacin-resistant isolates were sensitive to meropenem, 36% (9/25) to aztreonam, 84% (21/25) to Fosfomycin, 40% (10/25) to cefepime, 8% (2/25) to ceftazidime, 12% (3/25) to amikacin, 32% (8/25) to piperacillin, 32% (8/25) to tetracycline, and 20% (5/25) to nalidixic acid (Figure 4.29).

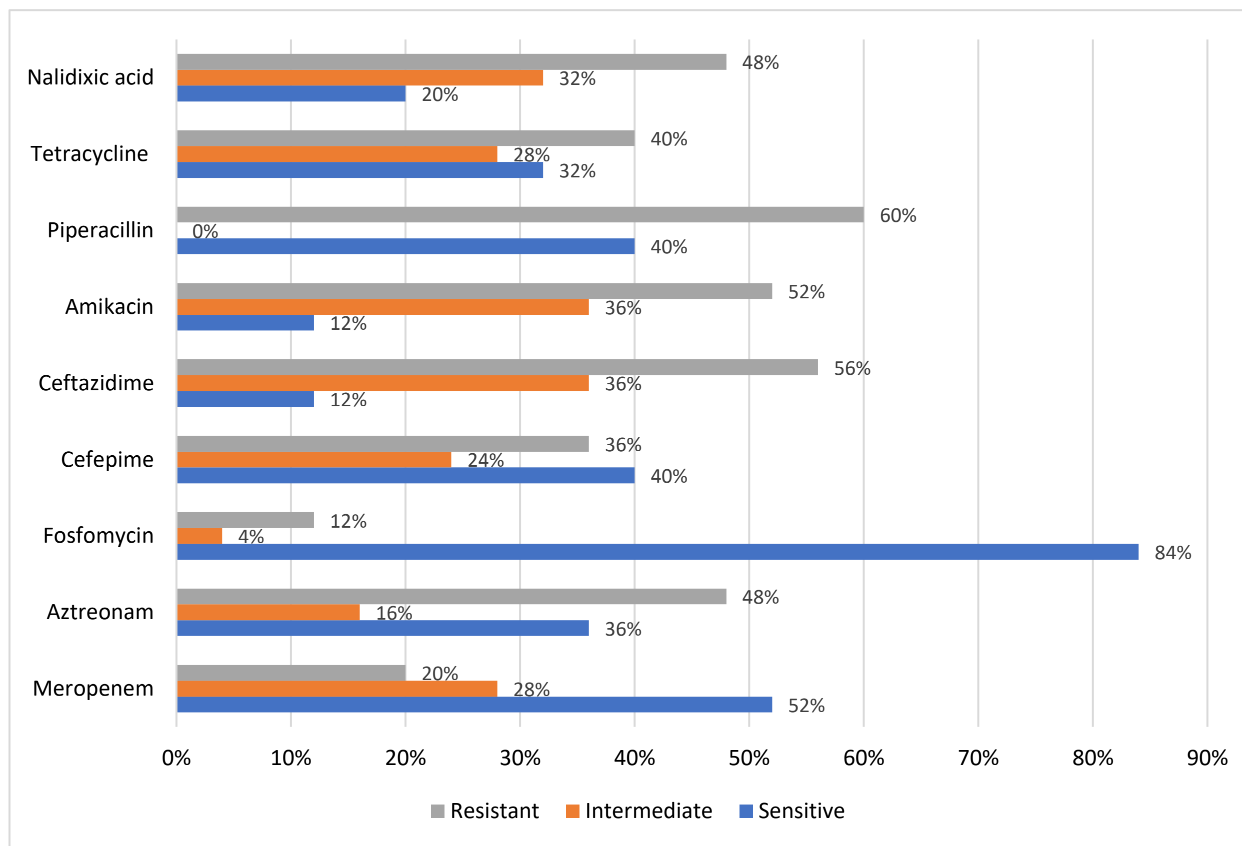


Figure 4.29: Antibiogram of PMQR genes carrying isolates

4.6.2 Antibiogram profile of ESBLs genes-carrying isolates

The resistance patterns among ESBLs positive isolates were as follows: 17.6% (3/17) exhibited resistance to meropenem, 92.3% (12/13) to aztreonam, 30.8% (4/13) to Fosfomycin, 70.6% (12/17) to cefepime, 82.4% (14/17) to ceftazidime, 47.1% (8/17) to amikacin, 100% (17/17) to piperacillin, 35.3% (6/17) to tetracycline, and 30.8% (4/13) to nalidixic acid. Additionally, the MAR index of these isolates varied from 0.2 to 0.8.

For ESBLs carrying isolates, intermediate resistance frequencies were recorded: 29.4% (7/17) for meropenem, 7.7% (1/13) for aztreonam, 23.5% (/13) for Fosfomycin, 29.4% (5/17) for cefepime, 17.6% (3/17) for ceftazidime, 23.5% (4/17) for amikacin, 23.5% (4/17) for tetracycline, and 53.5% (7/13) for nalidixic acid.

Furthermore, 53% (9/17) of ESBLs positive isolates demonstrated sensitivity to meropenem, while 46.1% (6/13) were sensitive to Fosfomycin, 29.4% (5/17) to amikacin, 41.2% (7/17) to tetracycline, and 15.3% (2/13) to nalidixic acid (Figure 4.30)

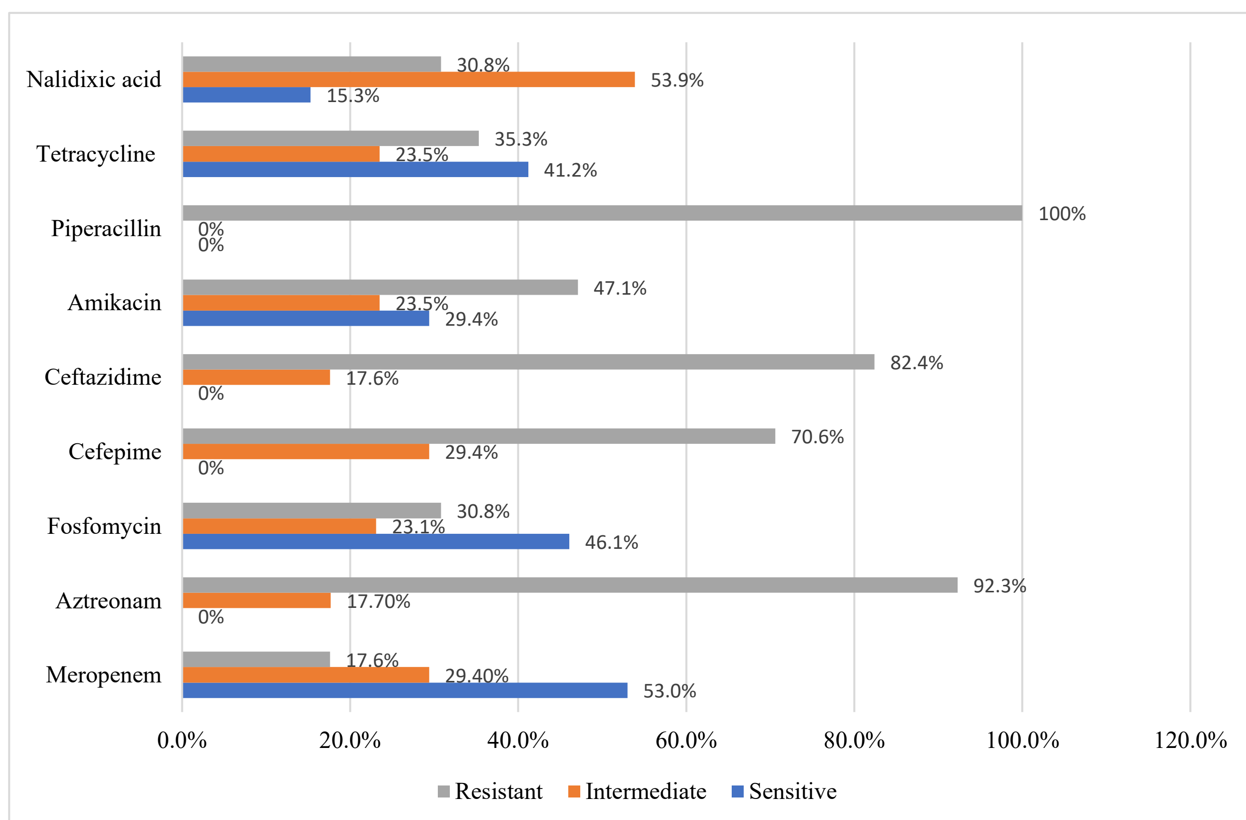


Figure 4.30: Antibiogram of ESBLS carrying isolates

4.6.3 Antibiogram profile of carbapenemases carrying isolates

Among carbapenem genes carrying isolates, the resistance percentages were as follows: 100% (15/15) for meropenem, 6.7% (1/15) for aztreonam, 20% (2/10) for Fosfomycin, 66.7% (10/15) for cefepime, 80% (12/17) for ceftazidime, 40% (6/15) for amikacin, 100% (10/10) for piperacillin, 40% (4/10) for tetracycline, and 30% (3/10) for nalidixic acid. Moreover, the MAR index for these isolates ranged from 0.2 to 1.

For carbapenem genes carrying isolates, intermediate resistance rates were observed: 13.3% (2/15) for aztreonam, 10% (1/10) for Fosfomycin, 33.3% (5/15) for cefepime, 20% (3/15) for ceftazidime, 46.7% (7/15) for amikacin, 50% (5/15) for tetracycline, and 30% (3/10) for nalidixic acid.

Furthermore, among carbapenem genes carrying isolates, susceptibility was noted: 80% (12/15) for aztreonam, 70% (7/10) for Fosfomycin, 13.3% (2/15) for amikacin, 10% (1/10) for tetracycline, and 40% (4/10) for nalidixic acid (Figure 4.31).

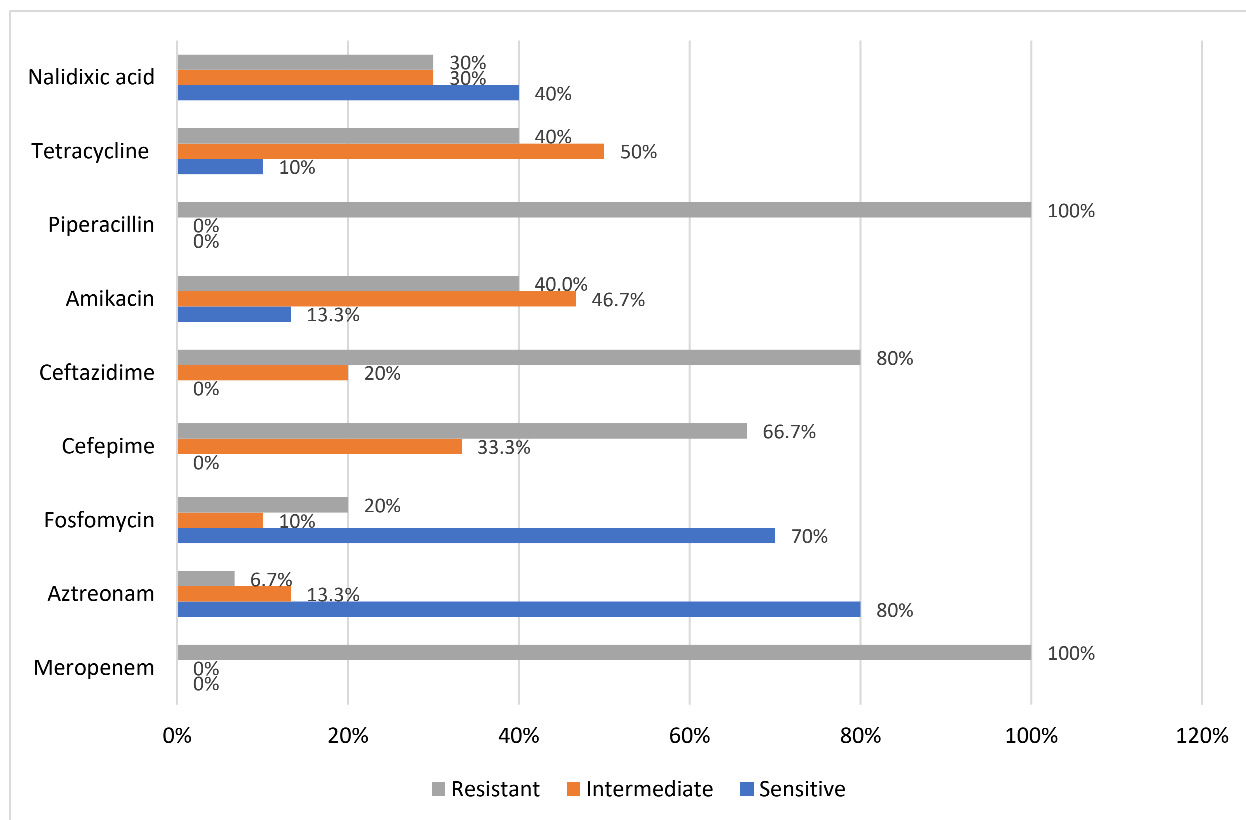


Figure 4.31: Antibiogram of carbapenemases carrying isolates

4.6.4 Antibiogram profile of *tet* genes carrying isolates

Among tetracycline resistance genes positive isolates, resistance rates were observed as follows: 28.6% (6/21) for meropenem, 33.3% (7/21) for aztreonam, 14.3% (3/21) for Fosfomycin, 42.9% (9/21) for cefepime, 47.6% (10/21) for ceftazidime, 52.4% (11/21) for amikacin, 57.1% (12/21) for piperacillin, 100% (21/21) for tetracycline, and 9.5% (2/21) for nalidixic acid. Additional the MAR index of these isolates varied from0.3 to 0.7.

Similarly, for the same isolates, intermediate resistance was evident: 33.3% (7/21) for meropenem, 19% (4/21) for aztreonam, 23.8% (5/21) for Fosfomycin, 33.3% (7/21) for cefepime, 23.8% (5/21) for ceftazidime, 19% (4/21) for amikacin, and 52.4% (11/21) for nalidixic acid.

Furthermore, within the same isolates, susceptibility rates were observed: 38.1% (8/21) for meropenem, 47.6% (10/21) for aztreonam, 62% (13/21) for Fosfomycin, 23.8% (5/21) for cefepime, 28.6% (6/21) for ceftazidime, 28.6% (6/21) for amikacin, 42.9% (9/21) for piperacillin, and 38.1% (8/21) for nalidixic acid (Figure 4.32).

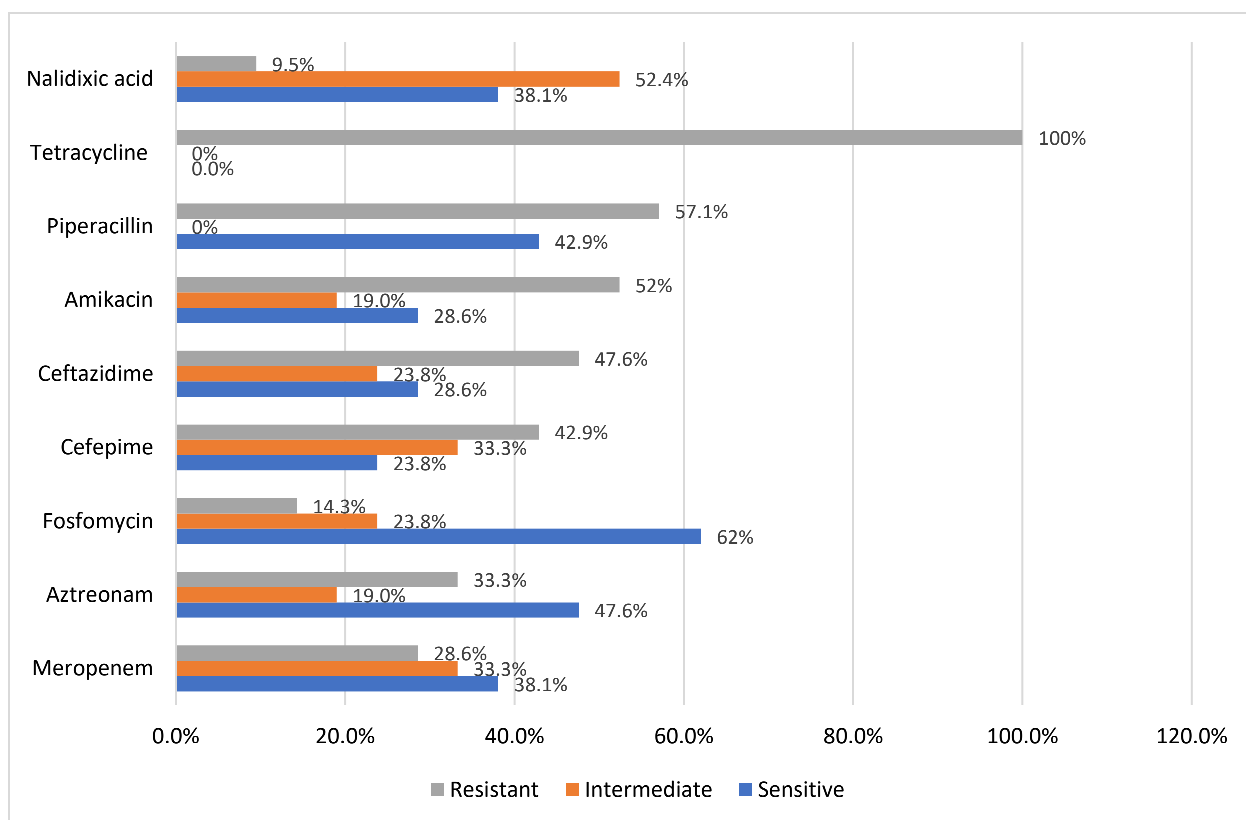


Figure 4.32: Antibiogram of *tet* genes carrying isolates

4.6.5 Antibiogram profile of colistin resistance genes carrying isolates

The *mcr-1* gene was only detected in Proteus mirabilis among the colistin-resistant isolates. This strain exhibited resistance to meropenem, ceftazidime, and piperacillin while demonstrating intermediate resistance to amikacin, fosfomycin, and cefepime. Conversely, it displayed sensitivity to tetracycline, nalidixic acid, and aztreonam.

Chapter 5

Discussion

Discussion

The emergence of antibiotic-resistant bacteria (ARB) and antibiotic-resistance genes (ARGs) in the aquaculture environment is a major concern for environmental health. In the present study, the prevalence of ARGs in two fish farm regions of Pakistan was found to be higher than expected and the previous study, even though there was no known history of antimicrobial usage in the studied sites. There is a possibility that some cases may be due to natural resistance. Other cases may be due to the misuse of antimicrobials in animal husbandry along with the subsequent application of animal and poultry waste, containing antibiotic residues in fish ponds (Kivaria, 2006; Nonga *et al.,* 2010). These ARGs in aquaculture act as reservoirs and disseminate to human-associated bacteria, ultimately contributing to the development of MDR bacteria (Furushita *et al.,* 2003; Singh *et al.,* 2022).

In our study, we observed the prevalence of Gram-negative bacteria especially *Enterobacteriaceae* in the fishponds, which carried a variety of clinically significant antibiotic-resistance genes encoding carbapenem, cephalosporin, tetracycline, polymyxin, and plasmid-mediated quinolone resistance. Only 13.9% (n=82) of bacterial isolates tested positive for resistance genes, with most of them showing phenotypic resistance to multiple drugs. Other isolates did not found positive for any of the tested genes, suggesting the possibility of other genes which were not screened in the present study (Hamza *et al.,* 2020a). The resistant Gram-negative bacteria isolated predominantly include *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Acinetobacter, baumannii*, *E. coli*, *Serratia marcescens*, *Citrobacter freundii*, *Proteus mirabilis*, *Edwardsiella tarda*, and *Klebsiella oxytoca*. This predominance was similar to earlier studies conducted on fish farms (Adeyemi *et al.,* 2022; Niyi-David *et al.,* n.d.). Additionally, the presence of *Staphylococcus* spp. and *Enterococci* was also found within this study which poses a threat to both fish and consumers (Torimiro *et al.,* 2014).

Beta-lactams are broad-spectrum antibacterial agents, that have extensive usage in both human and veterinary medical practices (S. Q. Shah *et al.,* 2012). Many different β-lactamases are reported in Gram-negative bacteria in aquaculture, but *bla*$_{TEM}$, *bla*$_{SHV}$, and *bla*$_{CTX-M}$ type-lactamases are the most prevalent ones (Bradford, 2001). Based on research outcomes, 13% (n=17) of the total 131 isolates were identified as positive for ESBLs genes. Among these, the prevalence

of *bla*$_{CTX-MU}$ was the highest, observed in 6.1% (8/131) of the isolates, followed by *bla*$_{SHV}$ detected in 3.8% (5/131) of the isolates. Additionally, 2.2% (3/131) of the isolates were found to carry *bla*$_{CTX-M9}$, while only 0.7% of the isolates exhibited the presence of *bla*$_{TEM}$. The findings receive validation from a study (Hamza *et al.,* 2020b) that identified ESBLs genes in bacteria originating from integrated fish farming in the Giza governorate. In our investigation, the identification of the *bla*$_{TEM}$ gene was attributed to *Acinetobacter baumannii*. This correlation is consistent with a prior study conducted in Pakistan (S. Q. Shah *et al.,* 2012), which also reported the presence of *bla*$_{TEM}$ in *Acinetobacter baumannii*, *Pseudomonas* spp., and *Enterobacter cloacae* within the fish farming environment. Similarly, the same genes were identified in the water of fish farms in Egypt (Ishida *et al.,* 2010a). These outcomes collectively underscore the resistance to extensively used antibiotics, such as β-lactams, raising significant concerns due to their broad treatment applicability and low toxicity (Bush & Bradford, 2016).

Carbapenems, among the beta-lactams, exhibit exceptional effectiveness against Gram-positive and Gram-negative bacteria, making them a potent class of antibiotics (Meletis, 2016a). In the present study, *Pseudomonas aeruginosa,* and members of the *Enterobacteriaceae* family were identified as the most prevalent Gram-negative bacteria carrying carbapenem-resistance genes which is a matter of concern because the World Health Organization has identified carbapenem-resistant bacteria, including *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacteriaceae*, as high-priority agents that present a considerable threat to human health (Meletis, 2016b). In this study, 14.5% (16/110) isolates genotypically positive for carbapenem resistance genes from which 7.2% (8/110) isolates carried *bla*$_{OXA-48}$, 4.5% (5/110) were found positive for *bla*$_{VIM}$, and 2.7% (3/110) carried *bla*$_{NDM}$. But, no isolate found positive for *bla*$_{KPC}$ and *bla*$_{IMP}$ from fishponds. Xu *et al.,* 2020 also reported the prevalence of *bla*$_{OXA-48}$, *bla*$_{KPC}$, *bla*$_{VIM}$, and *bla*$_{IMP}$ in a fish farming environment of China (Xu *et al.,* 2020). In comparison to this study, no prevalence of *bla*$_{KPC}$ and *bla*$_{IMP}$ in our study. In the previous study of the Pakistan which was conducted in Lahore District, about 500 km from New Delhi, the *bla*$_{NDM-1}$ was not found to be associated with the presence of ertapenem resistance (S. Q. Shah *et al.,* 2012). But, a study carried out in India reported the prevalence of *bla*$_{NDM1}$ on plasmids in different members of *Enterobacteriaceae* in clinical isolates as well as from drinking water in the Indian subcontinent, New Delhi (Walsh *et al.,* 2011). The most likely reason for the observed carbapenem resistance

genes is the anthropogenic contamination of the aquatic environment with a reservoir of the β-lactam resistance genes (S. Q. Shah *et al.,* 2012).

Tetracycline is the most frequently used therapeutic agent in veterinary medicine as well as in aquaculture worldwide (Ishida *et al.,* 2010b). Within this investigation, a total of 21.4% (29/117) of the examined isolates demonstrated the presence of at least one tetracycline resistance determinant. In a prior investigation carried out in 2012 in Pakistan, no prevalence of tetracycline resistance genes was identified within the fish farm environment (S. Q. Shah *et al.,* 2012). Among the determinants, *tet*A was the prevalent one, occurring in 10.3% (12/117) of the isolates, followed by *tet*B in 6% (7/117), and *tet*E in 5.1% (6/117) of isolates. Ishida *et al.,* 2010c reported the presence of *tet*A, *tet*B, *tet*E, and *tet*D in their investigation of fish farms in Egypt (Ishida *et al.,* 2010c). Unlike their study, none of the isolates in our investigation carried the *tet*D gene. Moreover, 3.4% (4/117) isolates showed co-existence of more than one tetracycline resistance gene which was also previously reported by (Ishida *et al.,* 2010c). Moreover, tetracycline resistance genes were also found in Gram-negative bacteria from fish farms in Tanzania, and China (Dang *et al.,* 2006; S. Q. A. Shah *et al.,* 2012), as well as from aquaculture sources in Australia (Akinbowale *et al.,* 2007). This prevalence of tetracycline resistance genes in Gram-negative bacteria from fishponds holds great clinical significance because study conducted (Furushita *et al.,* 2003) highlighted that in Japan, genes responsible for tetracycline resistance in bacteria found in farmed fish and clinical isolates exhibited significant similarity, indicating that they may have originated from the same source.

Broad-spectrum antibiotics such as quinolones and fluoroquinolones (FQs) are often used to treat both Gram-positive and Gram-negative infectious diseases in both human and veterinary medicine (Manenzhe *et al.,* 2015; B. Zhang *et al.,* 2014). Our study reported 27 of 126 (21.4%) isolates tested positive for PMQR genes. The ciprofloxacin-resistant isolates showed various degrees of detection for different PMQR genes. Out of them, aac(6')-Ib-cr was identified as the most prevalent PMQR determinant, which results in resistance to kanamycin, tobramycin, netilmicin, amikacin, and ciprofloxacin (Varela *et al.,* 2015). It was followed by *qnr*B with 10 isolates (7.9%), and *qnr*S with 3 isolates (2.3%), while there was no isolate found positive for *qnr*A, *qnr*C, and *qnr*D. According to (Ishida *et al.,* 2010b), they found a high prevalence of *qnr*A, *qnr*B, *qnr*S, and

aac(6')-Ib-cr genes in the fish farm in the Egypt. In contrast to their work, none of the isolates in our investigation were found positive for the *qnr*A gene.

Colistin is a last-resort antibiotic primarily utilized as a treatment for multidrug-resistant Gram-negative bacterial infections (Kalová *et al.,* 2021; Shen *et al.,* 2020). Many articles reported the isolation of *mcr-1-positive* bacteria from aquaculture environments. The present study found a prevalence of (0.9%) for the *mcr-1* resistance gene with no detection of other mcr genes which is a matter of concern due to its potential impact on food safety and public health (Antunes *et al.,* 2018). It suggests the use of colistin in the animal production setting especially in poultry farming as the major driver for the emergence of colistin-resistant bacteria (Campos *et al.,* 2016; Y.-Y. Liu *et al.,* 2016). A study in China also reported the high prevalence of the *mcr-1* resistance gene (28.0%–87.3%) in earthen and film line ponds of *P. vannamei* and its environmental samples. Unlike their study, none of the isolates in our investigation carried the mcr-4 gene (Que *et al.,* 2022). Likewise, in a study conducted by (Shen *et al.,* 2019) revealing a mcr-1 resistance gene prevalence of 39.2% (56/143).

In this study, the multiple antibiotic resistance (MAR) index value observed >0.2 signifies anthropogenic contamination of antibiotics, because the MAR index value less than 0.2 indicates no use of antibiotics (Sughra *et al.,* 2021). Similarly, a study conducted in Pakistan also discovered MAR index values greater than 0.2 in the aquaculture environment (S. Q. A. Shah *et al.,* 2014). It is hypothesized unregulated utilization of antimicrobials in animal husbandry, coupled with the introduction of animal and poultry waste containing antibiotic residues into fishponds, is linked to the development of antibiotic resistance in aquaculture settings. Subinhibitory quantities of antimicrobials favor the selection of antimicrobial resistance genes (ARGs), and these genes can persist in aquatic environmental bacteria even without intense selection pressure (He *et al.,* 2022; Ogunlaja *et al.,* 2022). These genes can be released into the environment through diverse routes. This release could lead to human exposure, particularly among individuals who come into direct contact with contaminated water or sediments, including workers and individuals participating in recreational activities around fishponds, and pose a human health problem (Cabello *et al.,* 2013a).

Chapter 6

Conclusion and future prospect

Conclusion

This investigation found prevalence of many *Enterobacteriaceae* from fish farms, which were subsequently discovered to possess diverse forms of antibiotic resistance genes. It confirms and broadens the conclusions derived from other studies about the prevalence of antimicrobial resistance genes in fish farming settings. Significantly, many of these resistance genes have also been detected in clinical bacterial isolates derived from human sources, suggesting a potential transmission route from fish farms to human populations. These findings of our study highlight the significance of fish farms as possible reservoirs for zoonotic bacteria and their associated antibiotic-resistance genes, posing a substantial public health risk. Aquaculture serves as an effective means of organic waste recycling, yet misusing antimicrobials in livestock and poultry farming threatens its integrity. To mitigate pathogen transmission, pretreatment of waste before introduction to fishponds is advisable. Furthermore, robust regulations and enforcement are imperative to govern antimicrobial use, while adhering to recommended veterinary medicine dosages in livestock can mitigate adverse effects on aquaculture.

Future prospect

The present study "Characterization of antibiotic-resistant bacteria in two non-integrated fish farming sites" can be carried out in further different directions such as:

- The sampling areas should be expanded along with the sample size.
- Identifying the virulent components in the isolates of fishponds samples collected from various locations.
- Identification of sources responsible for the antibiotic resistant bacteria such as feed and inlet water.

Chapter 7

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Chapter 8

Appendices

Appendices

Table 8.1: Bacterial load in different water samples of fishponds

Sample ID	Bacterial count (CFU/ml)							
	Gram-Negative	***Enterococci***	**Gram-positive**	**Meropenem resistant**	**Ciprofloxacin resistant**	**Cefotaxime resistant**	**Colistin resistant**	**Oxytetracycline resistant**
RH1	5.7x10^4	1.8x10^2	3.3x10^2	9.0x10^1	6.0x10^1	2.3x10^2	2.9x10^2	0
RH2	9.8x10^3	1.2x10^2	2.9x10^2	1.3x10^2	2.1x10^2	2.7x10^2	0	3.1x10^2
RH3	3.6x10^4	0	4.1x10^2	2.2x10^2	0	0	3.8x10^2	1.8x10^2
RH4	1.11x10^4	1.4x10^2	4.9x10^2	1.5x10^2	1.2x10^2	1.9x10^2	2.4x10^2	2.2x10^2
RH5	1.5x10^4	2.3x10^2	3.2x10^2	0	1.8x10^2	1.7x10^2	0	1.6x10^2
H1	1.47x10^4	0	2.4x10^2	1.7x10^2	0	7.0x10^1	3.4x10^2	1.9x10^2
H2	4.8x10^4	1.9x10^2	1.8x10^2	2.5x10^2	0	1.5x10^2	2.0x10^2	0
H3	1.3x10^4	0	3.8x10^2	1.4x10^2	1.3x10^2	1.8x10^2	4.0x10^1	3.2x10^2
H4	7.6x10^4	2.2x10^2	4.5x10^2	8.0x10^1	3.0x10^1	0	0	2.6x10^2
H5	1.06x10^4	1.1x10^2	1.40x10^2	0	4.0x10^1	9.0x10^1	9.0x10^2	4.1x10^2
H6	1.24x10^4	0	2.70x10^2	6.0x10^1	1.6x10^2	1.4x10^2	6.0x10^2	1.5x10^2
H7	4.4x10^4	9.0x10^1	5.3x10^2	0	0	1.6x10^2	1.7x10^2	0
H8	2.33x10^4	7.0x10^1	4.4x10^2	0	1.1x10^2	0	8.0x10^1	2.5x10^2
H9	2.11x10^4	1.3x10^2	6.1x10^2	1.1x10^2	7.0x10^1	0	5.1x10^2	0
H10	5.1x10^4	1.1x10^2	1.5x10^2	0	2.4x10^2	3.1x10^2	0	1.4x10^2
H11	1.95x10^4	1.6x10^2	0	1.2x10^2	5.0x10^1	2.5x10^2	2.6x10^2	0
H12	8.8x10^4	1.9x10^2	2.8x10^2	2.4x10^2	1.9x10^2	2.8x10^2	4.2x10^2	3.9x10^2
H13	1.73x10^4	8.0x10^1	0	1.9x10^2	9.0x10^1	2.0x10^2	0	2.6x10^2

Table 8.2: Bacterial load in different sediment samples of fishponds

Sample ID	Bacterial count (CFU/ml)							
	Gram-Negative	*Enterococci*	Gram-positive	Meropenem resistant	Ciprofloxacin resistant	Cefotaxime resistant	Colistin resistant	Oxytetracycline resistant

RHS1	8.7x10^5	2.8x10^3	4.9x10^3	1.43x10^3	2.51x10^3	4.3x10^3	3.6x10^3	2.77x10^3
RHS2	5.3x10^5	1.7x10^3	1.91x10^4	2.42x10^3	5.6x10^3	2.3x10^3	1.84x10^3	5.9x10^3
RHS(3)	1.86x10^5	3.7x10^3	2.5x10^4	4.2x10^3	1.01x10^3	1.72x10^3	2.58x10^3	4.3x10^3
RHS4	5.4x10^5	4.1x10^3	6.6x10^3	3.7x10^3	7.8x10^3	3.9x10^3	4.9x10^3	1.3x10^3
RHS5	6.6x10^5	2.4x10^3	1.43x10^4	2.66x10^3	6.5x10^3	1.57x10^3	1.54x10^3	2.29x10^3
HS1	1.20x10^5	3.2x10^3	9.9x10^3	2.03x10^3	1.6x10^3	2.69x10^3	2.01x10^3	4.8x10^3
HS2	4.8x10^5	4.8x10^2	7.8x10^4	5.1x10^3	1.3x10^3	1.64x10^3	1.05x10^3	6.1x10^3
HS3	1.90x10^5	2.1x10^3	2.39x10^4	1.9x10^3	2.9x10^3	2.11x10^3	2.33x10^3	7.7x10^3
HS4	7.9x10^5	3.3x10^3	1.33x10^4	2.31x10^3	1.49x10^3	1.74x10^3	1.9x10^3	2.72x10^3
HS5	3.7x10^5	2.5x10^3	1.59x10^4	4.1x10^3	8.2x10^3	1.49x10^3	4.3x10^3	8.5x10^3
HS6	1.0x10^6	1.9x10^3	2.2x10^4	1.19x10^3	1.96x10^3	5.5x10^3	2.14x10^3	6.7x10^3
HS7	1.99x10^5	2.13x10^3	1.67x10^4	9.7.x10^3	2.42x10^3	1.81x10^3	2.77x10^3	5.5x10^3
HS8	2.44x10^5	2.49x10^3	2.14x10^4	1.71x10^3	4.9x10^3	2.31x10^3	1.77x10^3	3.8x10^3
HS9	5.4x10^5	4.6x10^3	5.2x10^3	1.97x10^3	6.6x10^3	3.1x10^3	3.1x10^3	2.77x10^3
HS10	7.0x10^5	3.0x10^3	8.8x10^3	4.8x10^3	1.92x10^3	5.2x10^3	1.69x10^3	2.51x10^3
HS11	2.78x10^5	3.9x10^3	1.77x10^4	1.47x10^3	1.51x10^3	4.6x10^3	3.3x10^3	4.4x10^3
HS12	4.9x10^5	4.1x10^3	1.61x10^4	6.9x10^3	3.1x10^3	6.2x10^3	1.98x10^3	7.0x10^3

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