# Antibiotic Susceptibility and Antibiotic Resistance Genes in *Enterococcus* Species Isolated from Feces of Bats

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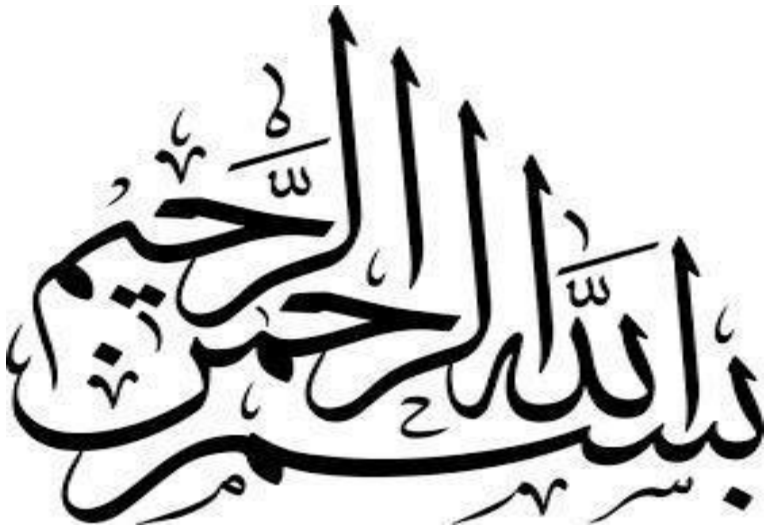


***In the Name of Allah***

***AlmightyThe Most***

***Beneficent***

***The Most Merciful***

# DECLARATION

I hereby declare that this dissertation is a presentation of my own work and that it has not been submitted anywhere for any award, if received, I will acknowledge in a written statement to authorities, otherwise, I will be liable for the cancellation of my dissertation.

**SUNDAS SHAFQAT**

# CERTIFICATE OF APPROVAL

This thesis **"Antibiotic Susceptibility and Antibiotic Resistance Genes in *Enterococcus* Species Isolated from Feces of Bats"** by **Sundas Shafqat** ishereby approved by Department of Zoology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad in partial fulfillment of the requirement for the degree of Master of Philosophy in Zoology.

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# DEDICATION

This dissertation is dedicated to

***Mohammad Danish***

who has been a source of strength, support, patience, and motivation for me throughout this entire experience.

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# Abbreviations

| | |
|---|---|
| *E. faecium* | *Enterococcus faecium* |
| *E. faecalis* | *Enterococcus faecalis* |
| AST | Antimicrobial susceptibility test |
| VRE | Vancomycin Resistance Enterococcus |
| UTI | Urinary tract infection |
| PCR | Polymerase Chain Reaction |
| NARC | National Agriculture Research Institute |
| AHS | Animal Health Section |
| ASI | Animal Science Institute |
| BPW | Buffered Peptone Water |
| BHI | Brain Heart Infusion broth |
| SBA | Slanetz and Bartley Agar |
| SIM | Sulfur Indole Motility |
| MHA | Muller Hinton Agar |
| QC | Quality Control |
| ARE | Ampicillin-Resistant Enterococci |

| | |
|---|---|
| AC | Acute cholangitis |

# Abstract

Enterococci are Gram positive, catalase negative, facultative anaerobic cocci (single, paired or chained), and are found in intestinal tract of many animals including bats. The present work was aimed at studying the prevalence of *Enterococcus* species specifically *E. faecalis* and *E. faecium* in feces of fruit bats. A total of 95 fecal samples were collected from fruit bats (*Pteropus medius*) from G11/3 sector of Islamabad, Pakistan. From those 95 samples, 43 *Enterococcus* isolates were recovered. Further testing showed 11 of the isolates were *E. faecalis* and 32 were *E. faecium.* After isolation and confirmation, AST was done for 43 Enterococcal isolates. Among 11 *E. faecalis* isolates, the drugs for which the sensitivity values were high were nitrofurantoin (100%), pencillin (100%), chloramphenicol (100%) ampicillin (91%), teicoplanin (91%) and fosfomycin (81%) while the drugs to which the resistance values were greater than 50% were quinupristin (55%) and rifampicin (55%). Among the 32 *E. faecium* isolates, the drugs which were highly effective towards *E. faecium* were ampicillin (91%), fosfomycin (88%) and vancomycin (84%) while the antibiotics against which high resistance was observed were rifampicin (75%), norfloxacin (34%), Linezolid (34%), quinupristin (28%) and doxycycline (25%). Thus, both *Enterococcus* species showed highest resistance towards rifampicin and quinupristin. After AST, PCRs were done for antibiotic resistance genes for tetracyclines and quinolones. Among the six tetracycline resistance genes of Enterococci, *tetM* was found in one isolate of *E. faecalis* and eight isolates of *E. faecium* respectively while other tetracycline genes were not detected by PCR in any of the positive isolates. The quinolones resistance genes *oqxA qnrA, qnrB,* and *qnrS* were not observed in any of the isolates.

**CHAPTER 1**

# INTRODUCTION

## 1.1 Introduction to *Enterococcus*

### 1.1.1 History

Thiercelin discovered *enterococcus* in 1899; he named them "Enterocoque" and characterized these bacteria as saprophytic, Gram-positive diplococci that are intestine-born. He also investigated the pathogenicity of this bacteria in humans. He also noted that septicemia can result from the bacterium moving from the intestines to the bloodstream. Similarly, a 37-year-old patient was found to have a hemolytic microorganism linked to endocarditis by a different team of researchers. Initially identified as *Micrococcus zymogenes*, this isolate was later identified as *Enterococcus faecalis* (Sherman, 1937).

In order to emphasize the isolate's gastrointestinal origin, MacCallum and Hastings gave it the name *Streptococcus faecalis* when they discovered it from a patient with endocarditis in 1906. The organism could ferment sugars like mannitol and lactose as well as clot milk, but not raffinose. *E. faecalis* was classified as a group D streptococcus because it had Lancefield group D cell wall antigens (Coia & Cubie, 1995). When *S. faecium* was first identified in 1919, it was noted that it had a distinct sugar fermentation pattern from *S. faecalis*. *S. durans* was discovered twenty years later as this organism had less advanced fermentation abilities. Streptococci were divided into four groups: enterococcus, lactic, phylogenetic, and viridians.

The organisms categorized as *enterococci* were capable of growing at both 10 and 45 ˚C, grow in an environment with high sodium chloride concentration (6.5 % NaCl), tolerate elevated pH in media and survive at high level of temperatures i.e at 60 °C for 30 minutes. According to the initial classification, *S. faecalis* was regarded as identical to *S. faecium;* however, it wasn't until the middle of the 1960s that it received official recognition as a separate species. *S. faecium var. casseliflavus*, a species that produces yellow pigment and is motile, was discovered in 1957 following the introduction of *S. avium* (Lebreton et al., 2014).

The *Enterococcus* genus was distinguished from Streptococci in 1984 due to the improvements in genomic techniques, particularly DNA-DNA and DNA-ribosomal RNA (rRNA) hybridization studies.These studies suggested that the genus *Enterococcus* should be used to reclassify *S. faecalis* and *S. faecium*. Evidence showing their remote relationship to other streptococci and non-enterococcal streptococci (*S. bovis* and *S. equinus)* belonging to serological group D led to this reclassification (Schleifer et al., 1984). The sequencing of the 16S rRNA gene further confirmed the distinction between enterococci and streptococci, as well as lactococci and other Gram-positive bacteria (Ludwig et al., 1985).

### 1.1.2 *Enterococcus* species

In the *Enterococcus* genus, about 50 different species have been identified. These are categorized into five categories, which include *E. faecalis, E. faecium, E. avium, E. gallinarurum, E. cecorum,* and a group of unclassified species (Euzéby, 1997). In 1997, there were only 17 species known, however the development of quick and inventive molecular techniques for bacterial identification and differentiation has led to the expansion of the *Enterococcus* genus They isolated these new species from a wide range of sources. From the red-billed wood hoopoe's uropygial gland, *E. phoeniculicola* was identified as a distinct species (Law-Brown & Meyers, 2003).

Environmental samples of *E. rotai* and *E. ureilyticus* were collected (Sedláček et al., 2013). Due to its isolation from seawater, *E. aquimarinus* was discovered to be a new species. Human stools were used to isolate *E.caccae* (Carvalho et al., 2006). In Thailand, fermented tea leaves were used to isolate *E. camelliae* (Sukontasing et al., 2007). In their natural environments, these bacteria are widely distributed among many host species, including people, animals, insects, and plants (Cox et al., 2007).

Accordingly, it is postulated that enterococci are related to mammals, reptiles, birds, and insects, as well as their last common ancestor (Gilmore et al., 2013). The aquatic plants, soil, terrestrial vegetation, beach sand, sediments and other water bodies such as streams and rivers are additional enteric habitats in which they can also survive.

Unlike the relatively stable temperature conditions found in the gastrointestinal (GI) tract of warm-blooded animals, they can also be considered inhabitants of heterothermic environments, where temperatures fluctuate. They constitute about 1% of the gut microbiota and largely populate the GI tract (Tedim et al., 2015). They primarily exist in the stomach, oral cavity, small and large intestines, and gastrointestinal tract. They serve as commensal organisms and may aid in gut metabolic pathways and digestion (Byappanahalli et al., 2012).

They belong to the gastrointestinal niche's commensal population. Humans frequently host *E. faecalis* and *E. faecium*, while *E. avium* and *E. durans* are uncommon. There are typically 107 colony-forming units (CFU) of enterococci per microgram in the colon. The genital tract can also host enterococci (Huycke, 2014). It's important to note that enterococci can differ in composition between different animal species, possibly because of dietary differences. They act as markers to detect fecal contamination in food and water because they are excreted in both human and animal feces (Graf et al., 2015).

## 1.2 Diseases caused by *Enterococci*

Enterococci can cause opportunistic infections in both animals as well as humans especially when the host's immune system is weakened or when the integrity of the intestinal epithelium is compromised (Berg, 1996) *E. faecalis* and *E. faecium* are the main agents causing infections in humans and *E. faecalis* contributes to 80% of these incidents (Huyckee et al., 1998). Additional *Enterococcus* species that can infect humans include *E. durans,E. gallinarum,E. avium,E. casseliflavus,E. mundtii* and *E. raffinosus* (Olawale et al., 2011).Meningitis, endocarditis, urinary tract infections (UTI), pelvic and abdominal infections, skin and subcutaneous infections, joint and bone-related infections, bacteremia, and meningitis are among the most frequently observed infections caused by enterococci (Sood et al., 2008).

In wild animals the resident enterococci are seen as a cause for antimicrobial resistance (AMR), specifically vancomycin resistance (VMR). Researchers in Spain found *E. faecalis, E. faecium, E. casseliflavus* and *E. gallinarum* in the feces of wild small mammals, with *E. faecalis* and *E. faecium* being the prevalent enterococcal species. *Rattus rattus*, one of the mammals examined, was found to be a possible source of acquired VMR (Lozano et al., 2015). The feces of Iberian

wolves and Iberian lynx were used to isolate *E. faecium, E. casseliflavus,* and *E. gallinarum* from a small occurance of VMR (Gonçalves et al., 2011).

*E. faecalis* is regarded as the well-known cause of infectious diseases such as urinary tract infections, sepsis, and abdominal infections throughout the world, and due to its drug resistance, its treatment is becoming increasingly challenging day by day (Xioayu Ma et al., 2021). Urinary tract infection (UTI) is the most prevalent infectious condition that results in morbidity in humans. The antibiotics used to treat UTI brought on by *E. faecalis* include ampicillin, trimethoprim, sulfamethoxazole and ciprofloxacin (Mireles et al., 2015). Acute cholangitis (AC), a fatal infectious condition, is brought on by gallstones, bile duct stenosis and bile duct stones. Other bacteria, including *Enterococcus*, have also been isolated from AC patients. Fever, abdominal pain and jaundice are some of its most typical symptoms (Karasawa et al., 2020).

There is an increasing correlation between enterococcal spondylitis (ES), formerly known as enterococcal vertebral osteoarthritis (EVOA) in poultry. It has been increasingly associated with *E. cecorum.* Most disease outbreaks were discovered in flocks of broiler chickens raised in intensive production systems. The osteomyelitis caused by *E. cecorum* infected birds manifested as necrosis in the femoral head (FHN) and compression of the spinal cord in the thoracic vertebrae, which inferred with their ability to move. In addition to culling and carcass condemnation, outbreaks of this disease can result in significant financial losses in a short amount of time, as well as significant morbidity and death rate (Dolka et al., 2016).

*E. faecalis and E. faecium* are the most frequent cause of opportunistic infections, which can range in severity from mild to severe infections. Nowadays, numerous strains of bacteria that are resistant to antibiotics are the main cause of hospital-acquired infections in humans (O'Dea et al., 2019). Numerous illnesses in poultry, primarily in turkeys, chicken, ostriches, ducks, and pigeons, are brought on by enterococcus. The *Enterococcus* species that are linked to diseases in domesticated birds include *E. faecalis, E. cecorum, E. hirae, E. faecium*, and *E. durans.* The illnesses brought on by these *Enterococcus* species include omphalitis, endocarditis, meningitis, fibrinous arthritis, pulmonary hypertension syndrome in broiler chickens, enterococcal spondylitis, femoral head (Dolka et al., 2016)

Animal GI tracts are most likely enterococci's main reservoir (Gilmore et al., 2013). Animals can contract enterococci just like humans do (Aarestrup et al., 2014). Cattle have also been reported to experience age-dependent enterococcal colonization. *E. faecalis, E. faecium,* and *E. avium* make up the majority of the enterococcal flora in pre-ruminant calves. *E. cecorum* slowly replaces this flora. Enterococci, primarily *E. faecalis* and, to a lesser extent, *E. raffinosus*, are found on the tonsils of pre-ruminating calves (Devriese et al., 1992). Additionally, in wild geese enterococci have also been found (Han et al., 2011) and conventional methods have found enterococci in cattle raised in Ethiopia (Bekele et al., 2009)

Enterococci have been found on bodies of insects in medical facilities, and there are concerns about the possibility of human contamination from this source (Fotedar et al., 1992; Pai et al., 2004). It is unknown how insects contribute to the spread of dangerous or resistant enterococci. House flies and cockroaches have been shown by research to potentially act as carriers and reservoirs for antibiotic-resistant and potentially dangerous enterococci in enclosed pig farming environments (Ahmed et al., 2011). House flies caught in fast food establishments frequently carry bacteria that are resistant to antibiotics (Macovei et al., 2008). Newly discovered *Enterococcus* species include *E. termitis*, which was found in a termite's digestive system, and some isolates of *E. rotai*, which were found in mosquitoes (Sedláček et al., 2013)

## 1.3 Bats as reservoirs of enterococci

The second-largest mammalian order after rodents is the Chiroptera, which includes bats. With more than 1,400 species, they can be found almost everywhere in the world (Irving et al., 2021). Bats have a unique ability to fly, are widely distributed, have a long lifespan, and have a variety of feeding methods (Carrillo-Araujo et al., 2015; Irving et al., 2021). Bats are vital participants in the global ecosystem and humans gain advantages from them in various manners (Irving et al., 2021). Bats act as a natural host and reservoir for a variety of microorganisms and viruses, some of which are highly pathogenic to humans, while also displaying strong immunity to a large number of pathogens (Allocati et al., 2016; Hayman, 2016). There is a ton of evidence that they have served as hosts for recently emerging zoonotic diseases.

Humans often come into contact with bats. One of the primary reasons for this is the changes made by humans to the bats' natural habitat, which forces them to find new places to live and

come in contact with humans and other animals (Daszak et al., 2000). Considering this, having a good understanding about microbiota, particularly harmful microorganisms, should be crucial for public health. Despite many beneficial roles that bats play in the ecosystem, they are also a source of multi-drug resistant microorganisms and can spread resistant bacteria to humans as well as contribute to their spread in the environment (Federici et al., 2022).

The common roosting location known as a camp is where flying foxes (*Pteropus spp*.) make vast colonies of hundreds to millions of individuals (Nakamoto & Kinjo, 2012). Due to the timing of fruit production, numerous fruit-eating animals, like fruit bats, demonstrate seasonal reliance on the distribution and availability of food (Nakamoto & Kinjo, 2012; Páez et al., 2018; Devnath et al., 2022)

In spite of not being fully understood, bats are known hosts of zoonotic diseases, as evidenced by events in the field of epidemiology in 2020 that pointed to bats as a source of novel viruses like SARS-CoV-2 (Allocati et al., 2016b; Cyranoski, 2020). Similar to this, it is still unclear how significant the bats gut microbiota is in terms of their susceptibility to drugs and the possible risk to both animal and human health. The Enterobacteriaceae family, particularly *E. coli,* is the source of the drug-resistant variants that have received the most attention till now. Other microorganisms, such as Campylobacter, have occasionally been reported to exhibit resistance. The susceptibility of different microorganism groups, such as the *Enterococcus* genus, which is the second most significant group of indicator bacteria in susceptibility tests, has not been thoroughly studied ("The European Union Summary Report on Antimicrobial Resistance in Zoonotic and Indicator Bacteria from Humans, Animals and Food in 2017/2018," 2020). The current study and other scholarly studies show the significant presence of *Enterococcus,* especially *E. faecalis*, in bats' gastrointestinal environments (Claudio et al., 2018). However, this presence varies and may be impacted by seasonal variations in the metabolic functions of the bat gut. Notably, *E. faecalis* and *E. faecium* are the two main agents that cause human nosocomial infections (Niu et al., 2016; Torres et al., 2018). According to findings from related studies, it appears that bat-derived *E. faecalis* strains may also be important in promoting resistance in the environment.

## 1.4 Occurrence of *Enterococcus* species in various animal species

Researchers have also looked into the enterococci prevalence in wild birds. The most common *Enterococcus* specie documented in the analysis of fecal samples from common buzzard was *E. faecium* (48.4 %), followed by *E. faecalis* (16.1 %), *E. hirae,* and *E. durans* (each 12.9 %) (Radhouani et al., 2012). *E. faecalis, E. faecium, E. hirae,* and *E. casseliflavus* were the most common enterococci found in different insect species as well as in the digestive tracts of cockroaches and flies, including some fish species and their aquatic ecosystem (Di Cesare et al., 2012; Michel et al., 2007). In addition to serving as a reservoir for enterococci in the GI tract, they also spread resistance in the surrounding environment (Zurek & Ghosh, 2014).

In the past, *E. faecalis* made up between 80 to 90% of clinical samples of enterococci while 5 to 10% of that samples was made up by *E. faecium* (Treitman et al., 2005). The occurance of invasive vancomycin-resistant *E. faecium* (VRE) infections currently closely resembles the proportions of *E. faecium* infections in the overall enterococcal infections in the US, which have exceeded 30% (Hidron et al., 2008). Since 2000, an increase in the occurrence of VRE in the hospitals across Europe is reported (Schouten et al., 2000; Werner et al., 2008). Although prevalence rates in most European nations are lower than in the US, invasive infections caused by vancomycin-resistant *E. faecium* is increased over the past six years in a number of countries, namely Turkey, Germany, Slovenia, Israel, Greece and Ireland (EARSS annual report 2007). Since both VRE and methicillin-resistant Staphylococcus aureus (MRSA) are being targeted by Dutch hospitals' "search and destroy" strategies and careful antibiotic use, the Netherlands has historically low VRE prevalence among bloodstream isolates (1%).

Importantly, the rapid increase of VRE during the 1990s paralleled the emergence of ampicillin-resistant *E. faecium* (ARE) in the United States during the early 1980s (Coudron et al., 1984; Top, Willems, & Bonten, 2008). Similar increases in ARE have been seen in various European nations, although with a delay of ten years (Top et al., 2007). Although the prevalence rates of VRE are low, invasive ARE has significantly increased in the Netherlands, according to data from recent nationwide research (Pincus et al., 2022).

Additionally, research to identify the ARE reservoir in the intestine found the rates of carrying it was 35% in hematological along with geriatric wards. The proportion of ARE in all enterococcal bacteremia increased from 4% in 1994 to 20% in 2005 (de Regt et al., 2008). Enterococcal

epidemiology has been studied using a variety of genotyping techniques, including multilocus sequence typing, amplified fragment length polymorphism analysis, and pulsed field gel electrophoresis. It's interesting to note that a distinct disparity between isolates from those who were hospitalized and those who weren't was found (Leavis et al., 2006).

## 1.5 Antibiotic resistance in bacteria

Innate resistance, also known as natural resistance, is the most basic type of antimicrobial resistance in bacteria. This is a characteristic that a species, a particular strain, or an entire bacterial group always has. Due to its ability to naturally resist a certain class of antibiotic group, a given microorganism doesn't respond to that antibiotic. This resistance can be related to the lack of an antibiotic receptor, low affinity, impermeable cell walls, or enzyme production. (van Hoek et al., 2011)

Bacterial susceptibility can either be primary or secondary. Resistance developed without coming into contact with a drug and results from a spontaneous mutation is primary resistance. This type of resistance is encoded within the chromosome and is unique to different bacterial species. Although it is very uncommon for a bacteria to acquire mutation, in the presence of an antibiotic, the mutants gain a competitive advantage over general population, which increases their chances of survival and domination over the susceptible populations. These mutations can be passed to other microorganisms or spread to different ecological niches within the same organism. Over their evolutionary history, bacteria have developed diverse mechanisms to combat the impact of antibacterial agents, including antibiotics, in order to defend themselves. Bacteria that have acquired resistance genes develop various mechanisms that make them partially or completely resistant to an antibiotic. (Acar & Röstel, 2001)

Numerous theories explaining bacterial resistance to antibiotics have been given based on other scientific studies starting in the mid of 20th century. Bacteria are currently thought to develop resistance to antibiotics through various mechanisms, including actively expelling the antibiotic from the bacterial cell, making enzymatic changes to the antibiotic's structure, altering the components targeted by the antibiotic within the cell, increasing the expression of an enzyme that counters the antibiotic's effects, changing the permeability of the bacterial cell membranes, generating a different metabolic pathway, elevating the concentration of a substance that

counteracts the antibiotic, decreasing the action of an enzyme that trigger the activation of the antibiotic's precursor, making modifications to regulatory systems that are not directly linked to the antibiotic's primary function, or reducing the demand for the end product of the suppressed metabolic pathway (Giedraitienė et al., 2011)

Numerous research studies show that bacteria use two major genetic strategies to facilitate natural defense against antibiotics: horizontal gene transfer and gene mutation, which are both closely related to the mechanism of action of an antibiotic. (Munita & Arias, 2016).

Secondary resistance mechanisms, which emerge when a microorganism is exposed to an antibiotic, it is more complicated than primary resistance. This type of resistance is extrachromosomal. The small circular DNA molecules known as plasmids in the cytoplasm are home to the genes in charge of this phenomenon. Multiple antimicrobial resistance genes may be found on one plasmid. Resistance-encoding genes can be transferred by plasmids from one bacterial cell to the other. Transduction and conjugation are the main methods used to transfer plasmids. Plasmids are transferred directly between two or more bacterial cells during conjugation using the protein strands those cells have produced.

Bacteria from various species and genera, often from distant phylogenetic branches, can participate in conjugation. This method of transferring resistance between saprophytic and pathogenic bacteria is unfavorable. Plasmids are transferred during the process known as transduction, which is facilitated by bacteriophages. The DNA enters the bacterium when the bacteriophage attaches to transmembrane receptorl. In order to make copies of DNA and proteins of the virus, the bacteriophage takes advantage of the host cell's metabolic processes. The bacterial cell eventually experiences lysis, a process referred to as the lytic cycle, as new bacteriophages are formed inside the bacterial cell.

Phage DNA holds the capability to be integrated into the bacterial chromosome, leading to the formation of a prophage, a process referred to as lysogeny (van Hoek et al., 2011). Within the genome,there are two genetic elements capable of repositioning; two distinct categories can be identified: insertion sequences (IS) and transposons (Tn). The DNA fragments carrying a coding gene for an enzyme i.e transposase, flanked on both ends by sequences that are inverted repeats are called Insertion sequences. This transposase enzyme facilitates the movement of insertion

elements to various loci within the DNA. Additionally, transposons may harbor resistance genes and are termed 'jumping genes'. A classification can be made between composite transposons that comprise of two IS flanking genes responsible for conferring antibiotic resistance or other traits unrelated to transposon mobility (such as Tn10). There are non-composite transposons as well, such as Tn3, which contain genes with additional features enveloped by inverted sequences. The transposition process for non-composite transposons involves replication and requires the involvement of the gene products.

Conjugative transposons exhibit a distinction from typical transposons as they have capacity to move both within the DNA of a single cell and between different cells. The plasmid or bacterial chromosome can be found fused with these transposons. Triggered by specific cues, these transposons adopt circular configurations that lack replication capabilities. The process of transfer bears resemblance to that of conjugative plasmids (van Hoek et al., 2011). Integrons play an important role in development of bacterial multi-drug resistance. Both plasmids and bacterial chromosomes may contain these components. Integrons are unique, specialized genetic material carriers that have the innate capacity to combine resistance genes into cassettes. Notably, recipient cells receive all of these cassettes together in this composite form. (Giedraitienė et al., 2011).

### 1.1.1 1.5.1 Antimicrobial resistance in *Enterococcus* species

In clinical settings, the approach to managing severe enterococcal infections in critically ill individuals, those presenting sepsis indications, and patients afflicted with conditions like endocarditis, meningitis, and osteoarthritis involves employing a combination therapy strategy. This strategy integrates a cell wall-activating agent, such as ampicillin or penicillin, in conjunction with a synergistic aminoglycoside, such as gentamicin at elevated dosages. This therapeutic approach is employed. Vancomycin is also thought to be the last resort in cases where resistance to a particular antimicrobial agent from these antibiotic classes develops. Alternative treatments, such as linezolid, daptomycin, quinupristin/dalfopristin, and tigecycline, are available for infections caused by vancomycin-resistant *Enterococcus* strains. (Chibebe et al., 2013).

In veterinary medicine, the therapeutic approach to addressing infections caused by *Enterococcus* species predominantly revolves around the utilization of single-agent therapy employing antibiotics. The choice of antibiotics is made on the outcomes of antimicrobial susceptibility testing, the localized infection site, and the affected animal species (Stępień-Pyśniak et al., 2021). This practice is closely linked to the limited options available for antibiotic use in animals, particularly those raised for human consumption (Stępień-Pyśniak et al., 2021; Ma et al., 2021). Despite the treatment's apparent efficacy, it is notable that there haven't been any randomized, controlled studies that evaluate its therapeutic efficacy.

Over the past ten years, there has been an increased occurrence of documented instances detailing the involvement of *Enterococcus* spp. in the pathogenesis of avian ailments. These organisms inherently possess resistance to several antibiotics commonly employed in therapeutic strategies, including sulphonamides, all generations of cephalosporins, and β-lactam antibiotics. However, they exhibit relatively lower levels of resistance to quinolones, lincosamides, and aminoglycosides (Leclercq, 1997). The prevalence of *Enterococcus* strains with multi-drug resistance has significantly increased as a result of excessive antimicrobial agent use.

Enterococci naturally resist β-lactam antibiotics due to their weak interaction with penicillin-binding proteins, such as PBP4 and PBP5 in *E. faecalis* and *E. faecium,* respectively (Sifaoui et al., 2001). The extent of this resistance depends on the particular β-lactams type. For instance, penicillin is the most effective against enterococci, cephalosporins have the lowest activity while carbapenems have a moderately lower effectiveness when treating enterococci infections. There's another mechanism of resistance linked to penicillin-binding proteins, occasionally seen in bacteria that develop significantly greater resistance to β-lactams as compared to wild strains. PBP5 overproduction in specific penicillin-resistant strains of *E. hirae* is an example of how excessive production of surface proteins like PBP5 can contribute to bacterial resistance to β-lactams. The *psr* gene controls the expression of the *pbp5* gene in *E. hirae* (Ligozzi et al., 1993). PBP5 production rises as a result of disruption or mutation of the *psr* gene, saturating all of the protein's available molecules. Certain enterococci use a completely unique and less frequent mechanism to resist β-lactam antibiotics. This process involves the β-lactamases production, an enzyme that functions to breakdown an antibiotic's β-lactam ring. This degradation renders the antibiotic ineffective since it is unable to block the enzymatic activities of surface PBPs. The

gene that controls the expression of β-lactamases is frequently found together with a gene in charge of gentamicin resistance on a plasmid. In general, β-lactamases are only occasionally produced. The Minimum Inhibitory Concentration (MIC) values for penicillin and ampicillin may therefore match those of bacteria susceptible to these antibiotics when the bacterial population is low (Murray, 2000).

The most prevalent vancomycin resistance is VanA phenotype which can be either acquired or induced. This resistance is characterized by its ability to counteract elevated levels of vancomycin (with MIC, range of 64 to 100 μg/mL) and teicoplanin (with MIC ranging from 16 to 512 μg/mL). This particular resistance pattern is most frequently encountered in species such as *E. faecium, E. faecalis, E. hirae, E. durans, E. avium, E. casseliflavus, E. raffinosus,*and *E. mundtii*. It is also observed, though less frequently, in *E. gallinarum* (Boyd et al., 2008).

The VanB phenotype maintains susceptibility to teicoplanin under laboratory conditions (MIC 0.5-1 μg/mL) while exhibiting inducible resistance to vancomycin across a range of concentrations (MIC 4-1024 μg/mL). It uses the dipeptide D-Ala-D-Lac instead of D-Ala-D-Ala, which is similar to the VanA phenotype. Several species, including *E. faecium, E. faecalis, E. durans,* and *E. gallinarum,* exhibit this phenotype (Leclercq et al., 1992). By carefully examining the DNA sequences of the genes encoding the VanB ligase, researchers have discovered three subtypes of this phenotype*: VanB1, VanB2* and *VanB3* (Courvalin, 2006). However, there is currently no evidence linking a particular sub-type and the extent of vancomycin and teicoplanin resistance.

*VanC* resistance, also known as Type C resistance, is a type of innate resistance seen in motile enterococci species, including *E. flavescens* (*vanC3*), *E. casseliflavus* (*vanC2*) and *E. gallinarum* (*vanC1*) (Navarro & Courvalin, 1994; Leclercq et al., 1992). Both inducible as well as constitutive characteristics exists in this resistance. In addition to D-Ala-D-Ala fragments, 1:3 ratios of D-Ala-D-Ser peptides also exists. This phenotype is distinguished by low-level resistance to vancomycin while retaining teicoplanin susceptibility.

*VanD* resistance, also known as Type D resistance, develops as an outcome of peptidoglycan precursors synthesis that end in D-alanyl-D-lactate. The described strains' genetic make-up has so far shown diversity (Depardieu et al., 2003). *VanD* enterococci exhibit intrinsic resistance to

both lower concentrations of teicoplanin and relatively high vancomycin. It is important to note that VanD and other Vancomycin resistant phenotypes such as VanE, VanG and VanL are extremely rare, and there are no records of clinical strains displaying these resistance patterns.

Tetracyclines disrupt bacterial cell energy processes and prevent protein synthesis via blocking the activity of 30S ribosomal subunit. Specific efflux pumps can actively expel antibiotics from the interior of the cell as a result of the activation of the *tetK* and *tetL* genes and changes in ribosome structure. *tetM, tetO* and *tetS* gene-derived proteins, which work to sheild the ribosome, have an impact on resistance.The ribosome must first bind resistance proteins before the ribosome's conformation can change, which then limits the attachment of tetracyclines. The main gene responsible for tetracycline resistance, *tetM* is typically found on the chromosome and is frequently spread by transposons like Tn916 or other transposons. However, conjugative plasmids can also transmit it (Bentorcha et al., 1991).

Quinolones exert their antibacterial effect by interacting with essential enzymes, specifically type II topoisomerases, namely DNA gyrase and topoisomerase IV, that are integral to bacterial DNA replication. DNA gyrase is comprised of two subunits, denoted as gyrA and gyrB. In gram-positive bacteria, particularly, topoisomerase IV serves as the principal target of quinolones; it is composed of two subunits known as parC and parE, exhibiting structural similarity to gyrA and gyrB. Particular point mutations in their chromosomes are primarily responsible for the enterococci bacteria's resistance to fluoroquinolones. Therefore, horizontal gene transfer mechanisms cannot spread the genetic elements causing fluoroquinolone resistance to other bacterial strains. The occurance of these mutations in bacterial genome depends on how frequently it has been exposed to antibiotics, especially those in the quinolone class. Predominant mutations frequently involve alterations in the genes responsible for encoding the topoisomerase II and IV enzymes. There is an intermediate level of quinolone resistance in *E. faecalis* when only parC is mutated without corresponding gyrA mutations. Notably, this variant has a higher MIC of quinolones as compared to *E. faecalis* strains with neither *parC* nor *gyrA* gene mutations. When compared to *E. faecalis* strains with mutations in the *parC* and *gyrA* genes, the MIC is still lower (Jacoby, 2005).

In a study which tested 911 *Enterococcus* isolates from the poultry sources exhibited trimethoprim/sulfamethoxazole to be most resistant, followed by tylosin , doxycycline and the combination of lincomycin with spectinomycin (Stępień-Pyśniak et al., 2018). According to a different study conducted in Poland 227 enterococci isolated from clinical samples, animals, water bodies (fresh and sea water) and fresh food showed highest drug susceptibility towards penicillin, ampicillin, teicoplanin, vancomycin and linezolid (Gawryszewska et al., 2017). These isolates frequently showed high level of resistance to rifampin and tetracycline. Another study regarding antimicrobial resistance in *enterococcus* isolates obtained from infected dogs with UTI in Italy along with healthy dogs with infection in gastrointestinal tract gave evidence regarding high levels of resistance to various antibiotic groups such as aminoglycosides, fluoroquinolones, oxacillin, clindamycin, tetracycline, and quinupristin-dalfopristin. The highest resistant enterococci included streptomycin (94.1%) which is an aminoglycoside (Stępień-Pyśniaket al., 2021). Human *Enterococcus* isolates in Italy exhibited high level of resistance to high doses of gentamicin and streptomycin, as well as ampicillin and imipenem (Boccella et al., 2021)

## 1.6 Bats as source of transmission of antibiotic resistance

In bacterial flora of wildlife and in environmental bacterial communities, selective pressure is exerted when antibiotics are released in the environment that encourages the horizontal-gene transfer (Ashbolt et al., 2013). Bats are capable of acting as reservoirs for antibiotic-resistant genes and plasmids because of their extensive flying and roaming abilities (Garcês et al., 2019) and they can widely spread these bacteria and their genes to populations of humans and other domesticated animals. However, little information is available on bat bacterial flora and their AMR profiles (Nowakiewicz et al., 2020). AMR pollution, which can happen when wildlife is exposed to human waste such as disposed food, water treatment facilities, and aquaculture facilities with antimicrobial residues, may be to blame for the AMR acquisition of microorganisms by bats (Kraemer et al., 2019). According to current evidence, the main cause of the rise in AMR is selection pressure. Nevertheless, the additional factors like biocides/ heavy metals may also contribute to the emergence of AMR. It currently appears that AMR emerges under selection and is largely caused by antibiotics. AMR pollution should be considered to exist when medically important AMR genes and antibiotic-resistant bacteria are found in wildlife that are not treated by antibiotics.

As wild mammals, bats typically don't construct specialized shelters. Instead, they use man-made habitats and natural caves as places to rest or hibernate (Leivers et al., 2019). They are forced to use both urban and rural environments, like buildings and their roofs, as nesting and foraging sites for their breeding due to deforestation and food insecurity (Voigt et al., 2016). When people and domesticated animals are present in these areas, the likelihood of direct and indirect interaction, as well as the exchange of microflora, increases. In areas where bats are commonly found, anthropogenic activities like forest cutting, hunting and cave exploration can increase the risk of zoonotic infections linked to bats.

In Poland, *E. faecalis* was recently isolated from bat guano samples.The isolates showed high resistance towards tetracycline and kanamycin (Nowakiewicz et al., 2021). Two *Enterococcus* isolates were found in rectal swabs from bats in another Spanish study, and both of these isolates showed resistance towards quinupristin-dalfopristin, ciprofloxacin, and erythromycin (O'Mahony et al., 2005).

The Habitats Directive protects bats in Poland and other EU countries, making it illegal to intentionally disturb, capture, or kill bats or damage or destroy their nesting or resting places. As a result, even to enter a bat colony and gathering data without coming into contact with the animals requires permission from the relevant legal authorities. Thus, these are the first findings in Europe addressing resistance in *E. faecalis* isolated from bats. The results of our study can only be contrasted with those of other researchers who have looked at different kinds of wildlife mammals.

The resistance patterns found in the isolates from bats appear to be somewhat similar to those of *E. faecalis* isolated from domesticated and wild animals. Tetracycline and macrolide resistance is common, and the presence of *tetM* and *ermB* genes is confirmed by molecular methods, which are frequently the cause of this resistance in humans and other animals strains that have received targeted therapy (Torres et al., 2018).

The isolates from bats also exhibited high levels of resistance to all tested aminoglycosides, which varied from over 40% to 30% based on the antimicrobials such as macrolides and tetracyclines. We have only found ant (6)-Ia gene, which is typically in charge of resistance to

excessive concentrations of streptomycin, in half of the HLSR strains (Ramirez & Tolmasky, 2010).

The ability of bacteria to adjust to their environment and long time survival is increased by factors like sex pheromones as well as aggregation factor which is supported via horizontal gene transfer. This can serve for resistance and virulence genes (Chaje cka-Wierzchowska et al., 2017).

**Aims and objectives**

Since the bats can be an important source of antibiotic resistance to livestock and humans, the current project had the following aims and objectives:

1. To investigate antibiotic resistance in *Enterococcus* species isolated from feces of bats.

2. To study selected antibiotic resistance genes in these isolates.

**CHAPTER 2**

# MATERIALS AND METHODS

## 2.1 Sample size and sample collection

This study was conducted at the Animal Health Section (AHS) Animal Sciences Institute, National Agriculture Research center (NARC), Islamabad and at the Department of Zoology Quaid-i-Azam University, Islamabad. Fecal samples from bats (Indian flying fox (*Pteropus medius*) were collected from only one roosting site i.e., G11/3 sector of Islamabad, Pakistan. The samples were collected by the coordinating units and received at AHS from 8-june-2021 to 29-november-2021. A total of 95 samples were received and analyzed for the presence of E*nterococcus.*

For sample collection, plastic sheets were spread under the trees in the evening. Next day in the early morning fresh bat droppings from the plastic sheets were collected in pre-sterilized falcon tubes. Then the falcon tubes were placed in the plastic box having ice boxes to maintain cold chain. No bats were caught for the current study and fecal samples were obtained only if deposited naturally from the bat. After sampling in the field, the samples were transported at -4℃ to the laboratory within 24 hours of collection. The samples were processed immediately after assigning unique identification numbers in the laboratory.

## 2.2 Sample analysis

Samples were first suspended in Buffered Peptone Water (Cat. No. CM0509, OXOID) and 1ml of suspension was inoculated into 10 ml Brain Heart Infusion Broth (Cat. No. EBH1210318023, OXOID) containing 6.5% NaCl and incubated for 24 hours at 37°C as *Enterococcus* can survive in 6.5% NaCl (Yilema et al., 2017). After incubation, a loopful of samples was streaked on a differential medium that is Slanetz and Bartley Agar (Cat. No. CM0377, OXOID) and incubated for 24 hours at 37°C. After 24 hours two types of colonies were detected on the medium with different samples, one with red colonies with the golden reflection that are characteristics of *E. faecalis* and another one with white or pink colonies indicates *E. faecium*. Isolated colonies from the differential medium were subcultured on Slanetz and Bartley Agar (Cat. No. CM0377, OXOID) and incubated for 24 hours at 37°C and stock solutions of the culture were made by

using 70% Glycerol from Slanetz and Bartley Agar. Then Grams' staining, motility and different biochemical tests were performed such as catalase, Simmon citrate test were performed following standard protocols (FAO Regional Antimicrobial Resistance Monitoring and Surveillance Guideline, Vol 1).

***Table 2.1****:* Colony characteristics of *E. faecalis* and *E. faecium* on Slanetz and Bartley agar

| **Colony characteristics** | **Results for *E. faecalis*** | **Results for *E. faecium*** |
|---|---|---|
| Colony shape | Circular | Circular |
| Colony color | Bright red with golden reflection | white/ pink colonies |
| Elevation | Convex | Convex |
| Edges | Entire | Entire |
| Surface | Smooth | Smooth |

**Confirmatory tests**

Suspected isolates were confirmed by performing different confirmatory tests:

- Gram's staining
- Catalase test
- Motility test
- Simmon citrate test

### 2.2.1 Grams' Staining

A drop of distilled water was put on a clean slide and one suspected colony was picked and spread on the slide in the way that the smear was not too thick nor too thin. Slide was heat-fixed by passing over the flame thrice and was stained by Grams' staining as follows: few drops of crystal violet were poured on the slide and were left for 1min and the slide was washed with distilled water. Gram's iodine was added, slide was washed after 1 min, decolorizer was added and after 30sec slide was washed at the end safranin was added and was washed after 2 min and the slides were observed under microscope at 100X lens under oil immersion.

### 2.2.2 Catalase test

In a clean slide a suspected *Enterococcus* colony was separated with the help of disposable inoculating loop and a drop of 3% $H_2O_2$ was put on the colony. Bubble production indicates positive catalase test.

### 2.2.3 Motility

Motility of *E. faecalis* and *E. faecium* was examined with the help of SIM medium. A suspected *Enterococcus* colony was stabbed into the 27ml tube containing SIM medium and incubated for 24 hours. After incubation, growth of non-motile *Enterococcus* species was limited to stabbed region only while growth of motile species was observed in whole medium sometimes, Sulphur production was also observed along with separated growth.

### 2.2.4 Citrate Test

Take test tubes containing Simmons' citrate agar slants. Label the test tubes with media name, sample ID and your group name. Maintaining aseptic conditions, carefully retrieve 3 to 4 isolated colonies of bacteria with a sterile inoculating loop and streak it over the entire surface of the citrate agar slant. Repeat this procedure for the remaining test tubes. Incubate the inoculated tube at 37 °C for 24 hours. Examine the Simmons' citrate slants' color after the incubation. Positive citrate results are indicated by a bright blue coloration, while negative citrate results are indicated by a lack of color change.

### 2.2.5 Molecular characterization of *Enterococcus*

**Isolation of genomic DNA**

Genomic DNA was extracted from all *Enterococcus* confirmed cultures following a standard procedure as described in following sections. The DNA extracts served as templates for amplification through PCR with primers that are specific for the targeted genes.

**DNA extraction methods**

For this study, the DNA was extracted by a modified boiling method as well as genomic kit method. The procedure is described as follows:

**Boiling lysis method**

The boiling method for the extraction of DNA from bacterial culture is a typical extraction method used in labs. This is the modified technique for Gram positive bacterial DNA extraction that proposes lysis of bacterial cells by boiling with lysis buffer.

In this method, colonies from isolated bacterial culture were used. A few colonies were immersed and mixed in 200µl lysis buffer taken in the Eppendorf tube. It is then boiled for 10 minutes over a hot plate (Dashti et al., 2009). Immediately after boiling, the Eppendorf tubes are placed in an ice bath for over 5-10 minutes and centrifuged at 12,500 rpm for 5 minutes at room temperature. The DNA containing supernatant is transferred to a new Eppendorf tube and processed for PCR or stored at –20°C for future use.

**Genomic DNA Purification Protocol**

**Before starting**

For the DNA extraction of Gram-positive bacteria lysis buffer should be prepared before the procedure. 10 ml of lysis buffer is prepared by adding 2 ml of 10 mM EDTA, 4ml of 50 mM Tris-HCl, 4 ml of 3% Triton X-100 at pH 8.0.

**Procedure**

Take 180 µL of Gram-positive bacteria lysis buffer in an Eppendorf tube of 1.5 to 2.0 ml and add 4 to 5 colonies from the pure culture of bacteria. Vortex so the mixture is homogenized. Set

the dry water bath at 37°C and incubate for 30 minutes. After 30 minutes, add 200 µL of Lysis Solution and 20 µL of Proteinase K in the tube. Keep votexing or pipetting to obtain a uniform suspension. Set the dry water bath at 56°C and incubate the sample for 30 minutes while vortexing occasionally until the bacterial cells are completely lysed. Now add 20 µL of RNase A Solution, mix it by vortexing and incubate it for 10 minutes at room temperature. 400 µL of 50% ethanol will be added and mixed again. After that the prepared lysate will be transferred to GeneJET Genomic DNA Purification Column inserted in a collection tube provided in the genomic kit used for DNA extraction in the laboratory. Centrifuge the column of 1 minute at 6000 g. The collection tube containing the flow-through solution will be discarded. Move the purification column to a new 2 ml collection tube (provided in the kit). Remember to seal the bag containing the GeneJET Genomic DNA Purification Columns tightly after each use. Now add 500 µL of Wash Buffer 1 with added ethanol and centrifuge it at 8000 g for 1 minute. Discard the fluid and add the purification column back to the collecting tube. Add 500 µL of Wash Butter II (with added ethanol) to the genomic purification column and centrifuge it at maximum speed for 3 minutes (If after the centrifugation you can see some residual fluid in the purification column you can give an additional spin for 1 min after discarding the fluid in the collection tube but this step is optional). Now add the purification column to an autoclaved 1.5 ml Eppendorf tube and to elute genomic DNA, add 200 µL of elution buffer to the GeneJET Genomic DNA Purification Column membrane. Centrifuge for 1 minute at 8000 g, After 2 minutes of room temperature incubation. Repeat the elution step with an additional 200 µL of elution buffer for maximum DNA yield. Please be aware that adding less Elution Buffer will result in less DNA being eluted in the end. This is especially true if more concentrated DNA is needed or if it needs to be isolated from a small amount of starting material. Purification column is discarded. Use the purified DNA right away in applications downstream, or store at -20 °C.

### 2.2.6 PCR for identification

PCR was carried out for identification of *E. faecalis* and *E. faecium* targeted on conserved gene sequences.

**Master mix recipe**

**For *E. faecalis***

A singleplex confirmatory PCR was conducted for *ddl* gene in *E faecalis.*

**Table 2.2***:* Ingredients and Volume used for the *E. faecalis*

| **Ingredients** | **Volume for one reaction** |
|---|---|
| NFW | 12.7µl |
| 10X buffer | 2.5µl |
| 25mm $MgCl_2$ | 2.0µl |
| 10uM Primer forward | 1.0µl |
| 10uM Primer reverse | 1.0µl |
| 10mM dNTPs | 0.5µl |
| Taq polymerase | 0.3µl |
| Template | 5µl |

The total volume for each reaction was 25ul.The primer sequence is mentioned in **Table 2.2.**

**For *E. faecium***

A singleplex confirmatory PCR was conducted for *sodA* gene in *E faecium.*

**Table 2.3***:* Ingredients and Volume used for the *E. faecium*

| **Ingredients** | **Volume for one reaction** |
|---|---|
| NFW | 13.5µl |
| 10X buffer | 2.5µl |
| 25mm $MgCl_2$ | 2.5µl |
| 10uM Primer forward | 1.25µl |
| 10uM Primer reverse | 1.25µl |
| 10mM dNTPs | 0.5µl |
| Taq polymerase | 0.5µl |
| Template | 3.0µl |

The total volume for each reaction was 25ul.The primer sequence is mentioned in **Table 2.3.**

**Thermal profile for PCR**

| Genes | Initial denaturation | Denaturation | Annealing | Extension | Final extension | Cycles | References |
|---|---|---|---|---|---|---|---|
| *ddl* gene *E. faecalis* | 94° C for 8 minutes | 94° C for 45 seconds | 44° C for 45 seconds | 72° C for 45 seconds | 72° C for 11 minutes | 35 cycles | Dutka-Malen et al., 1995 |
| *sodA* gene *E. Faecium* | 95° C for 4 minutes | 95° C for 30 seconds | 55° C for 1 minute | 72° C for 1 minute | 72° C for 7 minutes | 30 cycles | Jackson et al., 2004 |

### 2.2.7 Antimicrobial susceptibility test:

Antimicrobial susceptibility test was done against 17 antibiotics. For AST, the Disk Diffusion method was used, in which a 90 mm Petri plate containing 25 ml of MHA (Cat. No.012821501, Liofelchem) was swabbed with the colony suspension equivalent to 0.5 McFarland Standard. Antibiotic disks (OXOID) were dispensed into the swabbed Petri plate and incubated for 16-18 hours at 37° C in the incubator. After the incubation zone of inhibition formed by each antibiotic was analyzed. All the procedures and interpretation of results were done following standard protocols (CLSI,2020). AST was measured as per zone of inhibition in three profiles i.e., Resistance, Intermediate and Sensitive according to the values given by CLSI, 2020 as shown in **Table 2.4.**

**Table 2.4***:* Reference values of AST results

| Antibiotics | Sensitivity (mm) | Intermediate (mm) | Resistance (mm) |
|---|---|---|---|
| **Ampicillin** | 17 | 16 | 15 |

| | | | |
|---|---|---|---|
| **Teicoplanin** | 14 | 11-13 | 10 |
| **Norfloxacin** | 17 | 13-16 | 12 |
| **Vancomycin** | 17 | 15-16 | 14 |
| **Chloramphenicol** | 18 | 13-17 | 12 |
| **Ciprofloxacin** | 18 | 16-18 | 12 |
| **Rifampicin** | 20 | 17-19 | 16 |
| **Fosfomycin** | 16 | 13-15 | 12 |
| **Erythromycin** | 23 | 14-22 | 13 |
| **Linezolid** | 23 | 21-22 | 20 |
| **Levofloxacin** | 17 | 14-16 | 13 |
| **Doxycycline** | 16 | 13-15 | 12 |
| **Quinupristin** | 19 | 16-18 | 15 |

| | | | |
|---|---|---|---|
| **Penicillin** | 16 | 15 | 14 |
| **Minocycline** | 19 | 15-18 | 14 |
| **Tetracycline** | 19 | 15-18 | 14 |
| **Nitrofurantoin** | 17 | 15-16 | 14 |

After AST, molecular detection of antibiotic resistance genes was done by using the PCRs. Those antibiotics were selected against which the isolates recovered from Islamabad showed resistance. PCRs were done for detection of the resistance genes against the two classes of antibiotics which includes tetracyclines, and quinolones against which the resistance was noted mostly.

### 2.2.8 Molecular characterization:

**Plasmid DNA Extraction:**

According to previous studies, the antibiotic resistance genes to be examined were plasmid-borne; hence plasmid DNA extraction was used. Eleven *Enterococcus* isolates were chosen for this study. *Enterococcus* isolates were revived from the stock solution for plasmid DNA extraction. After carefully mixing the stock solutions with a vortex, 20µl of the stock solution was streaked on Slanetz and Bartley agar and incubated for 24 hours at 37° C. After incubation, inoculate 5 ml LB broth with 1 to 4 colonies. Incubate for 12-16 hours at 37◦C while shaking at 200-250rpm to make a bacterial culture suspension. After that, the suspension was centrifuged, supernatant was discarded, and the pellet was taken for plasmid DNA extraction. Plasmid DNA was extracted using ThermoScientific, GeneJET Plasmid Miniprep Kit (CAT. NO. K0502, ThermoFisher Scientific) as follows: pellet was resuspended in 25µl of Resuspension solution in a microcentrifuge tube. Pellet was properly mixed by pipetting. Then 250 µl of Lysis solution was added and inverted 4-6 times to gently mix it in. After inverting 4-6 times, 350 µl of neutralizing solution was added and mixed. The supernatant was placed into a new sterile

GeneJET Spin column and centrifuged for 1 minute at 13000 rpm at room temperature following centrifugation. The flow-through was removed, and the column was reinserted within the spin column. The DNA was then washed twice with 500 µl of wash solution, centrifuged for 1 minute, and the flow discarded after each wash. The wash solution residues were then removed by centrifuging the GeneJET Spin column for 1 minute.The column was moved into a new centrifuge tube after centrifugation, and 50 µl of elution buffer was added to the center of the column so that DNA could be eluted from the column into the centrifuge tube. The tube was incubated for 2 minutes at room temperature after adding the elution buffer, and then centrifuged for 2 minutes, the column was discarded, and the recovered plasmid DNA was kept at -20°C till the next process.

**Polymerase Chain Reaction:**

After the extraction of plasmid DNA from 11 of the resistant isolates, PCRs were performed for detection of antibiotic resistance genes. PCR recipe for *oqxA, tetM, tetC, tetG, and* genes are shown in table 2.5 while two multiplex PCR were done for *qnrA, qnrB, qnrS genes* table as shown in table 2.6 *tetO, tetS* as shown in table 2.7 and thermal cycles of all genes in table 2.6

**Table 2.5***:* PCR recipe for *oqxA, tetM, tetC, tetG, and tetL* genes

| **Reagents** | ***oqxA*** | ***tetM*** | ***tetC*** | ***tetG*** | ***tetL*** |
|---|---|---|---|---|---|
| DNA | 2.5 µl | 2.5 µl | 2.5 µl | 2.5 µl | 5 µl |
| 10X buffer (KCl) | 2.5 µl | 2.5 µl | 2.5 µl | 2.5 µl | 2.5 µl |
| 25mm $MgCl_2$ | 1.5 µl | 1.5 µl | 1.5 µl | 2.5 µl | 1.5 µl |
| 10 mM dNTPs | 0.5 µl | 0.5 µl | 0.5 µl | 0.5 µl | 0.5 µl |
| 10 µM of Forward primer | 2.5 µl | 2.5 µl | 2.5 µl | 1.0 µl | 2.5 µl |
| 10 µM Reverse primer | 2.5 µl | 2.5 µl | 2.5 µl | 1.0 µl | 2.5 µl |
| Taq DNA polymerase (Cat No. 10342053, Thermo Fisher Scientific) | 0.5µl | 0.5 µl | 0.5 µl | 0.5 µl | 0.5 µl |
| Water | 12.5 ul | 12.5 µl | 12.5 µl | 14.5 µl | 10 µl |
| **Total** | **25 ul** | **25 ul** | **25 ul** | **25 ul** | **25 ul** |

**Table 2.6***:* PCR recipe for *qnrA, qnrB, qnrS* Genes

| **Reagents** | ***qnrA qnrB, qnrS* Genes** |
|---|---|
| DNA | 5 µl |

| | |
|---|---|
| 10X buffer (KCl) | 2.5 µl |
| 25mm $MgCl_2$ | 1.5 µl |
| 10 mM dNTPs | 0.5 µl |
| 10 uM qnrA-F primer | 2.5 µl |
| 10 uM qnrA-R primer | 2.5 µl |
| 10 uM qnrB-F primer | 2.5 µl |
| 10 uM qnrB-R primer | 2.5 µl |
| 10 uM qnrS-F primer | 2.5 µl |
| 10 uM qnrS-R primer | 2.5 µl |
| Taq DNA polymerase | 0.5µl |
| **Total** | **25µl** |

**Table 2.7***:* PCR recipe for *tetO, tetS genes*

| **Reagents** | ***tetO, tetS*** |
|---|---|
| DNA | 5 µl |
| 10X buffer (KCl) | 2.5 µl |
| 25mm $MgCl_2$ | 1.5 µl |

| | |
|---|---|
| 10 mM dNTPs | 0.5 µl |
| 10 uM tetO-F primer | 2.5 µl |
| 10 uM tetO-R primer | 2.5 µl |
| 10 uM tetS-F primer | 2.5 µl |
| 10 uM tetS-R primer | 2.5 µl |
| Taq DNA polymerase | 0.5µl |
| Water | 5 µl |
| **Total** | **25 µl** |

The PCR conditions for all reactions are mentioned in ***Table 2.6.***

**Table 2.8**: Thermal Profiles of PCR

| Genes | Initial denaturation | Denaturation | Annealing | Extension | Final Extension | Cycles | References |
|---|---|---|---|---|---|---|---|
| *tetO, tetS* | 95℃ for 5min | 95℃ for 59 seconds | 55℃ for 59 sec | 72℃ for 59 seconds | 72℃ for 10min | 35 cycles | Aarestrup et al (2000). |
| *tetL* | 95℃ for 5min | 95℃ for 55 seconds | 54℃ for 60 sec | 72℃ for 55 seconds | 72℃ for 10min | 35 cycles | Aarestrup et al (2000). |
| *tetG* | 95℃ for 10 min | 95℃ for 45 sec | 55℃ for 60 sec | 72℃ for 30 seconds | 72℃ for 8min | 35 cycles | Stanton and Humphrey (2003). |
| *tetC* | 95℃ for 3min | 95℃ for 59 seconds | 68℃ for 30 sec | 72℃ for 30 sec. | 72℃ for 10min | 40 cycles | Miranda et al (2003). |
| *tetM* | 94℃ for 5min | 94℃ for 59 seconds | 45℃ for 60 sec | 72℃ for 59 sec | 72℃ for 10min | 35 cycles | Aarestrup et al (2000). |
| *oqxA* | 94℃ for 5min | 94℃ for 60 seconds | 57℃ for 55 sec | 72℃ for 60 minutes | 72℃ for 10min | 30 cycles | Qui et al (2019) |
| *qnrA, qnrB, qnrS* | 94℃ for 4 min | 94℃ for 45 sec | 53℃ for 45 sec | 72℃ for 60 sec | 72℃for 7min | 32 cycles | Robicsek et al (2006) |

In the case of all the PCRs, gel electrophoresis was done on 1.5% gel and the DNA bands were visualized in a DNA gel-doc system after staining with ethidium bromide.

**Table 2.9***:* List of antibiotics, symbols, and their concentration

| Class | Antibiotics | Symbol | Concentration (µg) |
|---|---|---|---|
| **Penicillin** | Ampicillin | AMP | 5 |
| | Penicillin | P | 10 |
| **Lipoglycopeptides** | Teicoplanin | TEC | 30 |
| **Glycopeptides** | vancomycin | VA | 30 |
| **Phenicols** | Chloramphenicol | C | 30 |
| | Norfloxacin | NOR | 30 |
| **Fluoroquinolones** | Levofloxacin | LEV | 5 |
| | Ciprofloxacin | CIP | 5 |
| **Ansamycins** | Rifampicin | RD | 5 |
| **Fosfomycin** | Fosfomycin | F | 200 |
| **Macrolides** | Erythromycin | E | 15 |
| **Oxazolidinones** | Linezolid | LZD | 30 |
| **Streptogramins** | Quinupristin | QD | 15 |

| Class | Antibiotics | Symbol | Concentration (µg) |
|---|---|---|---|
| **Tetracyclines** | Doxycycline | DO | 30 |
| | Minocycline | MH | 30 |
| | Tetracycline | TE | 30 |
| **Nitrofurantoins** | Nitrofurantoin | F | 300 |

**Table 2.10***:* List of genes and primers

| Genes | Primer sequences | Product size(bp) | References |
|---|---|---|---|
| ***ddl -F***<br>***ddl -R*** | ATCAAGTACAGTTAGTCT<br>ACGATTCAAAGCTAACTG | 941 | Dutka-Malen et al., 1995 |
| ***sodA-F***<br>***sodA-R*** | GAAAAAACAATAGAAGAATTAT<br>TGCTTTTTTGAATTCTTCTTTA | 215 | Jackson et al., 2004 |
| ***tetC*-F**<br>***tetC*-R** | CTTGAGAGCCTTCAACCCAG<br>TGGTCGTCATCTACCTGCC | 417 | Miranda *et al* 2002 |
| ***tetG*-F**<br>***tetG*-R** | TTGTTTGAGAGCATTGCCTGC<br>TTCAAGCCGGCTTGGAGAG | 171 | Stanton *et al* 2003 |
| ***tetL*-F**<br>***tetL*-R** | ATTACACTTCCGATTTCGG<br>CATTTGGTCTTATTGGATCG | 475 | Aarestrup *et al* 2000 |

| | | | |
|---|---|---|---|
| ***tetM*-F**<br>***tetM*-R** | GTTAAATAGTGTTCTTGGAG<br>CTAAGATATGGCTCTAACAA | 657 | Aarestrup *et al* 2000 |
| ***tetO*-F**<br>***tetO*-R** | CAATATCACCAGAGCAGGCT<br>GATGGCATACAGGCACAGAC | 634 | Aarestrup *et al* 2000 |
| ***tetS*-F**<br>***tetS*-R** | TGGAACGCCAGAGAGGTATT<br>ACATAGACAAGCCGTTGACC | 661 | Aarestrup *et al* 2000 |
| ***oqxA*-F**<br>***oqxA*-R** | TACTCGGCGTTAACTGATTA<br>GATCAGTCAGTGGGATAGTTT | 671 | Robicsek *et al* 2006;<br>Qiu *et al* 2019 |
| ***qnrA*-F**<br>***qnrA*-R** | GATCGGCAAAGGTTAGGTCA<br>ATTTCTCACGCCAGGATTTG | 516 | Robicsek *et al* 2006;<br>Qiu *et al* 2019 |
| ***qnrB*-F**<br>***qnrB*-R** | GATCGTGAAAGCCAGAAAAGG<br>ACGATGCCTGGTAGTTGTCC | 469 | Robicsek *et al* 2006;<br>Qiu *et al* 2019 |
| ***qnrS*-F**<br>***qnrS*-R** | TAAATTGGCACCCTGTAGGC<br>ACGACATTGTCAACTGCAA | 417 | Robicsek *et al* 2006;<br>Qiu *et al* 2019 |

**CHAPTER 3**

# RESULTS

The results were recorded at each step and are given with details in subsequent sections.

## 3.1 Sample enrichment

After overnight incubation, BHI broth turned into a turbid and slightly dark brown color as shown in fig 3.1.

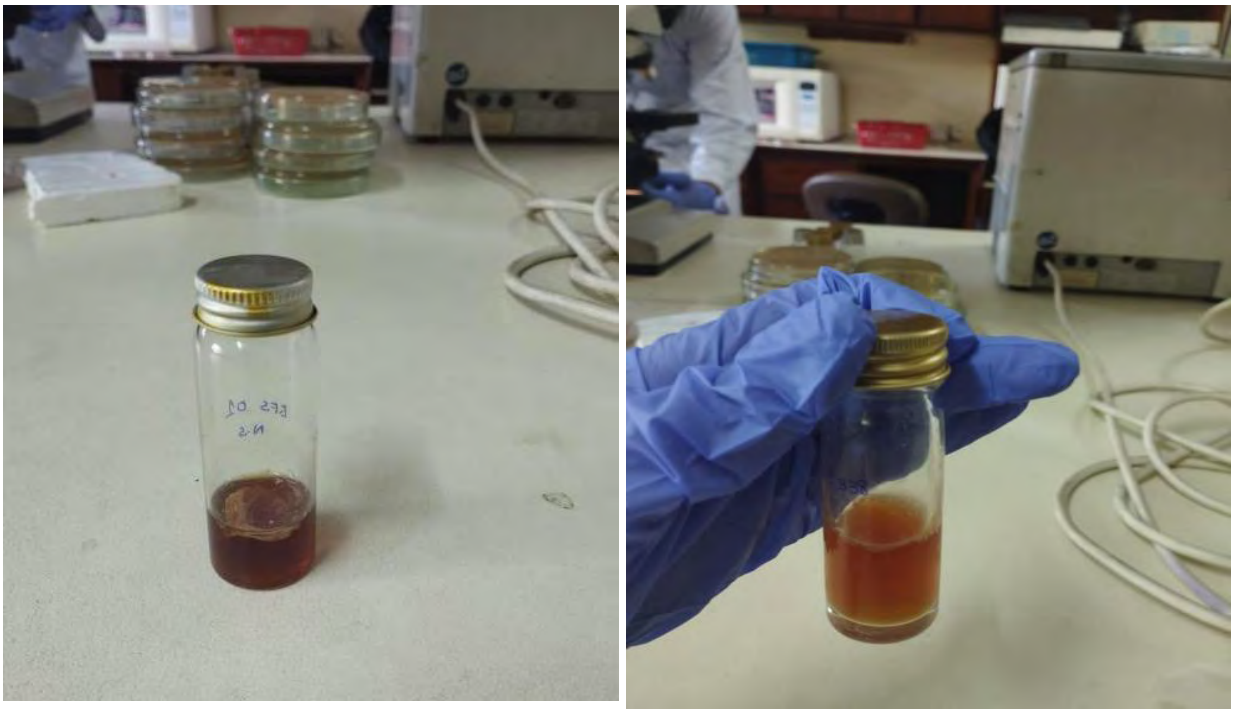
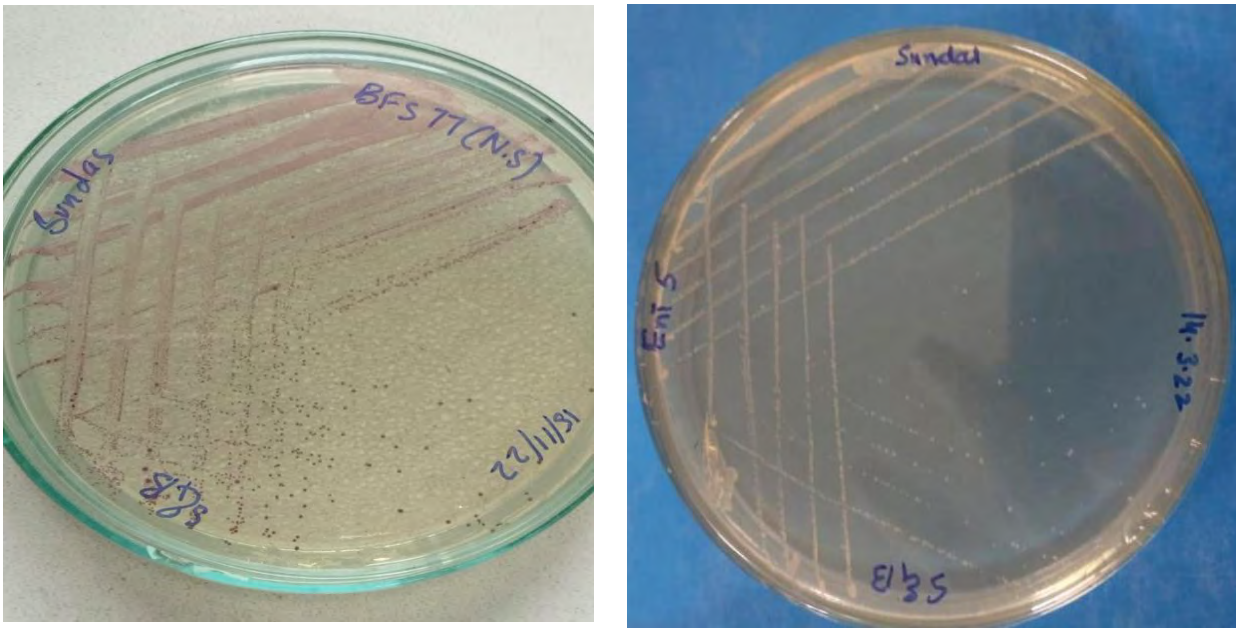


**Figure 3.1:** Brain Heart Infusion broth before and after inoculation of bacterial culture

## 3.2 Isolation of Enterococci

Red and white colonies with clear zones were specified as *E. faecalis* and *E. faecium.* The culture thus obtained was pure culture of *E. faecalis* and *E. faecium* which was processed for confirmatory tests as shown in fig 3.2.




***E. faecalis*** ***E. faecium***

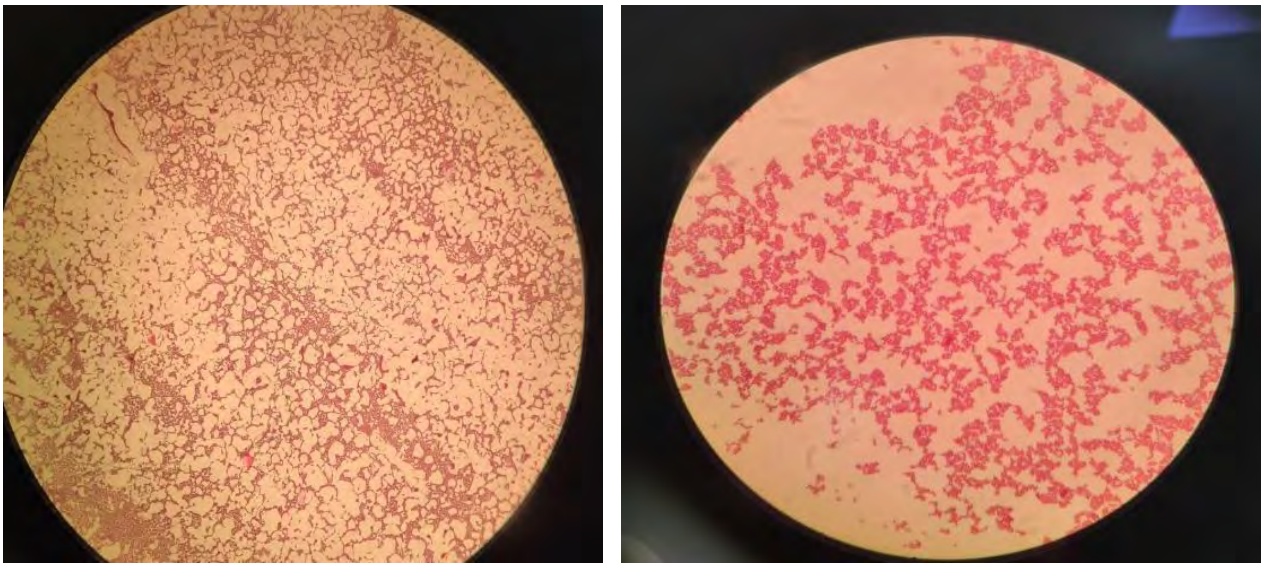
**Figure 3.2:** Growth of *Enterococcus* species on Slanetz and Bartley Agar colonies with Golden colonies with red center that are characteristics of *E. faecalis* and another one with white or pink colonies indicates *E. faecium* respectively.

## 3.3 Biochemical identification

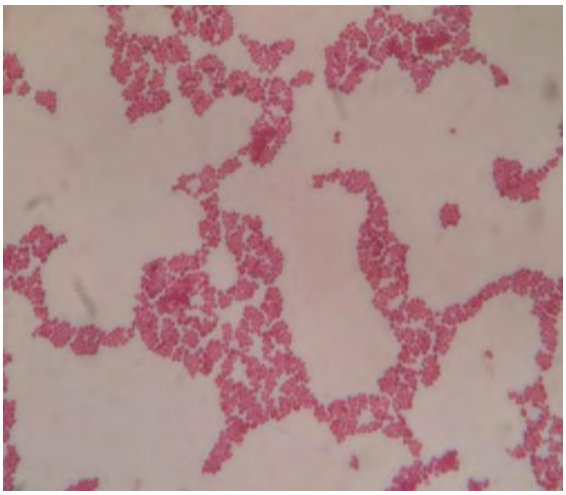
All the isolates that produced growth characteristic for *Enterococcus* were further confirmed by three biochemical tests: catalase test, citrate test and SIM test. Grams' staining was also performed for the confirmation. Following results are given:

### 3.3.1 Grams' staining

After Grams' staining purple-colored Gram-positive *Enterococci* were clearly observed when slides were examined under microscope as shown in **Figure 3.3**



(A) (B)



(C)

**Figure 3.3:** Gram-positive *Enterococcus* A: at 4X; B and C : at 100X

### 3.3.2 Catalase test result

The formation of bubbles soon after the inoculum was mixed with hydrogen peroxide was the indication of catalase positive results. All the suspected isolates were negative for catalase. This confirmed the existence of *Enterococcus* species. (see **Figure 3.4**).

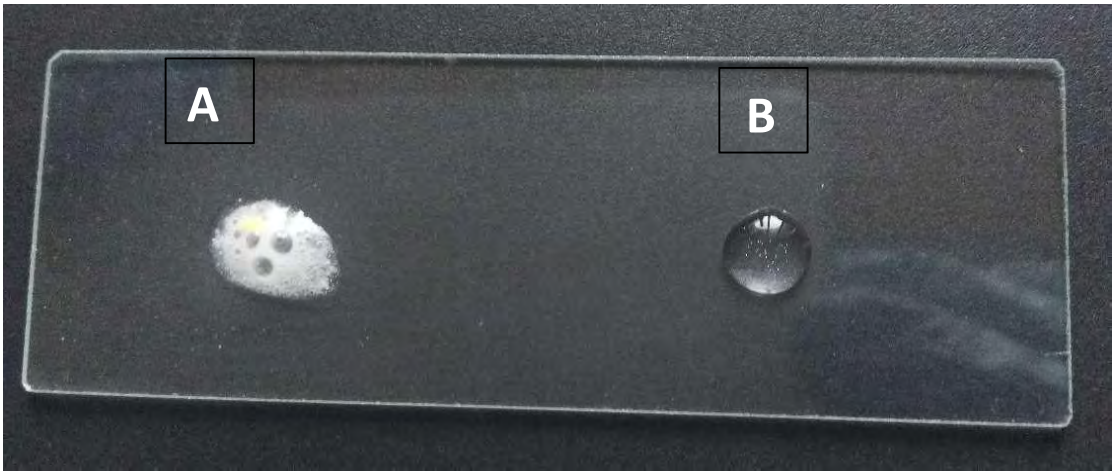



**Figure 3.4:** (A)Catalase positive Staphylococcus along with (B) Catalase negative Enterococcus

### 3.3.3 SIM result

Motility was analyzed by using SIM medium in which *Enterococcus* showed no motility as shown in the Fig 3.5

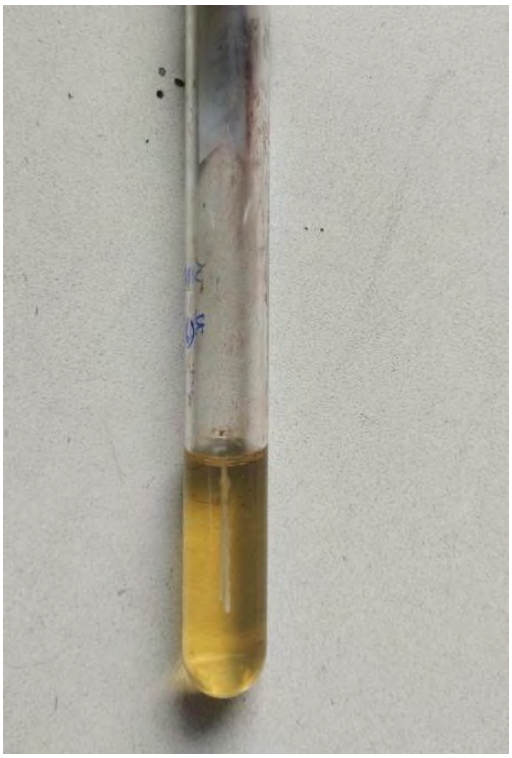


**Figure 3.5:** Non-Motile *Enterococcus*

### 3.3.4 Citrate test result

After 24 hours incubation of inoculated Simmon Citrate slants no color change was observed. All the suspected isolates were negative for the citrate test. This confirmed the existence of *Enterococcus* species. *Salmonella* is used as a positive control in this test hence color change can be seen in the Fig 3.6

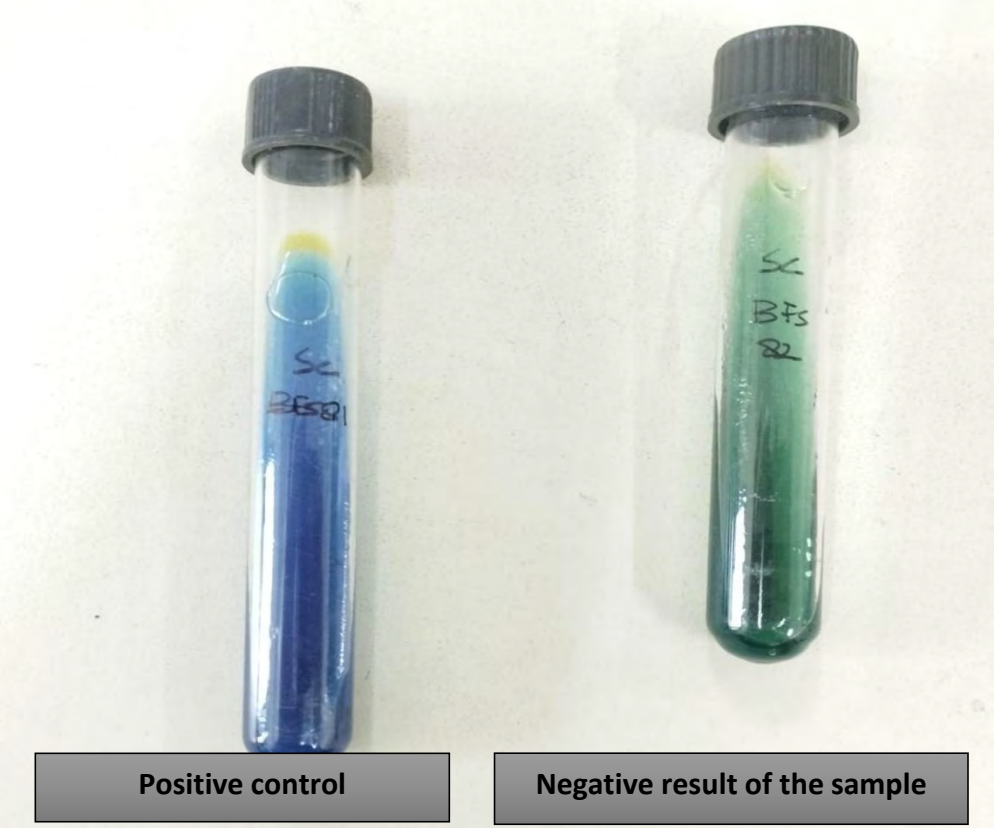



**Figure 3.6:** Citrate test for Enterococcus

### 3.5 Molecular characterization of *Enterococcus* species

Confirmatory PCRs were done for *E. faecalis* and *E. faecium* respectively. Out of 43 E*nterococcus* isolates 32 were *E. faecium* as shown in Fig 3.7 and 11 isolates were confirmed as *E. faecalis* as shown in Fig 3.8

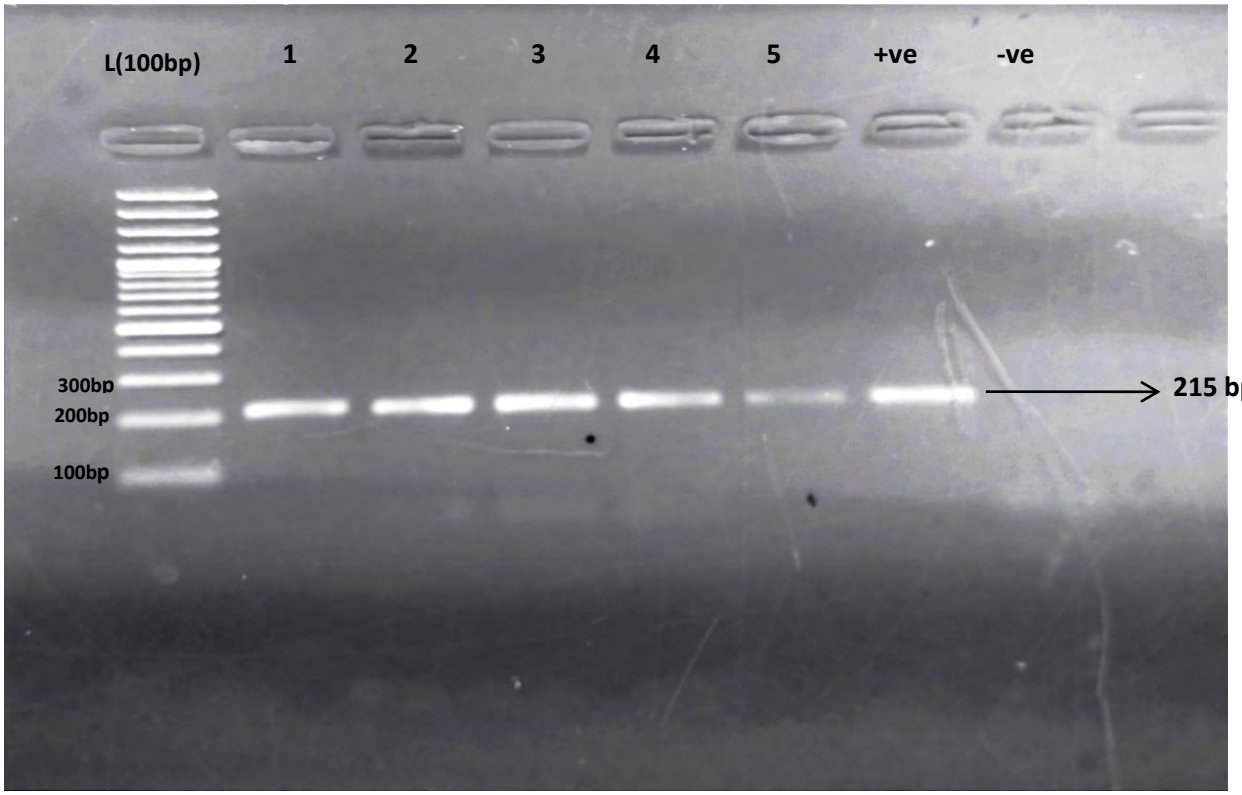



**Figure 3.7:** Agarose gel electrophoresis of PCR products for the identification of *E. faecium*. 1-5 show *E. faecium* positive samples (215 bp), +ve and -ve indicate positive and negative controls respectively L signifies DNA size marker (100bp DNA ladder, ThermoFisher Scientific).

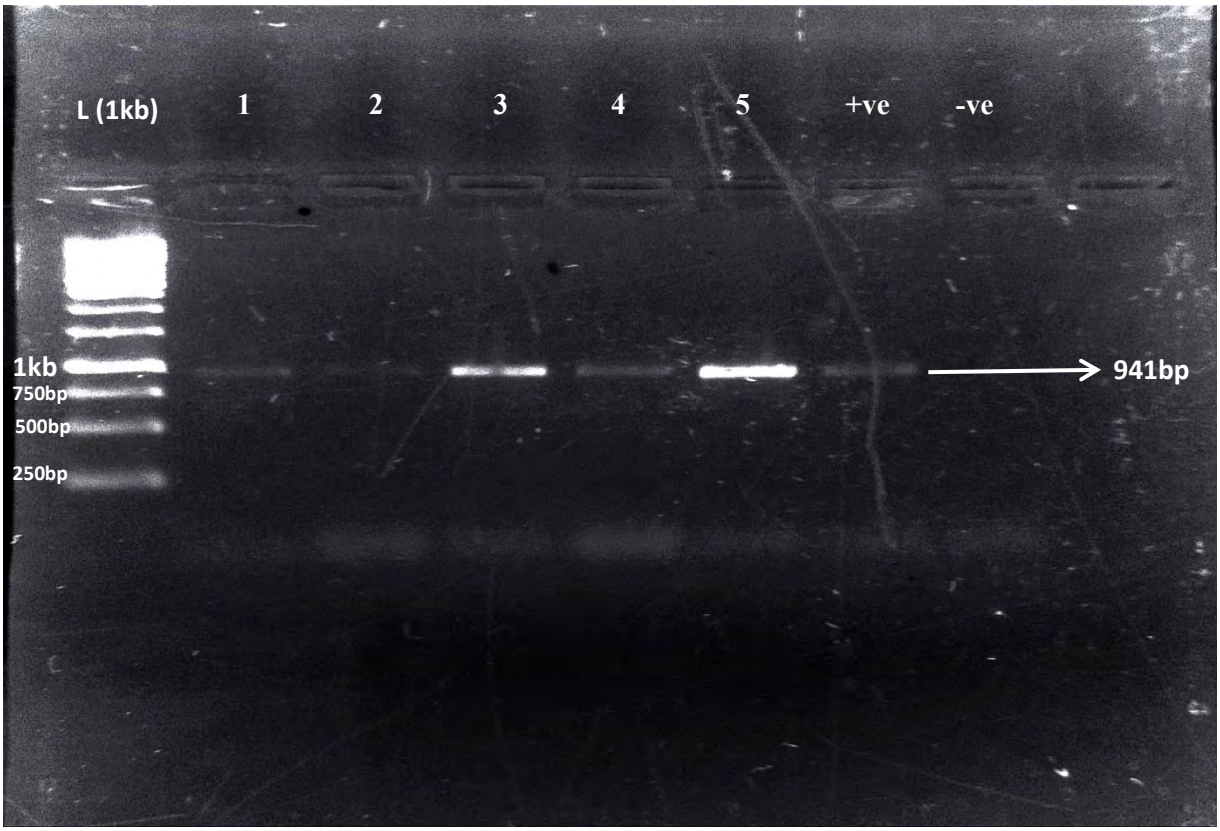



**Figure 3.8:** Agarose gel electrophoresis of PCR products for the identification of *E. faecalis,* 1-5 show *E. faecalis* positive samples(941bp) *+ve and -ve* indicate positive and negative controls respectively. L signifies DNA size marker (1kb DNA ladder, ThermoFisher Scientific).

Results of the processed samples for *Enterococcus* are shown in **Table 3.1**

**Table 3.1**:. Results of isolation of *Enterococcus* from fecal samples

| **City** | **No. of fecal samples received** | **Samples positive for *Enterococcus*** | **Positive for *E. faecalis*** | **Positive for *E. faecium*** |
|---|---|---|---|---|
| Islamabad | 95 | 43 | 11 | 32 |

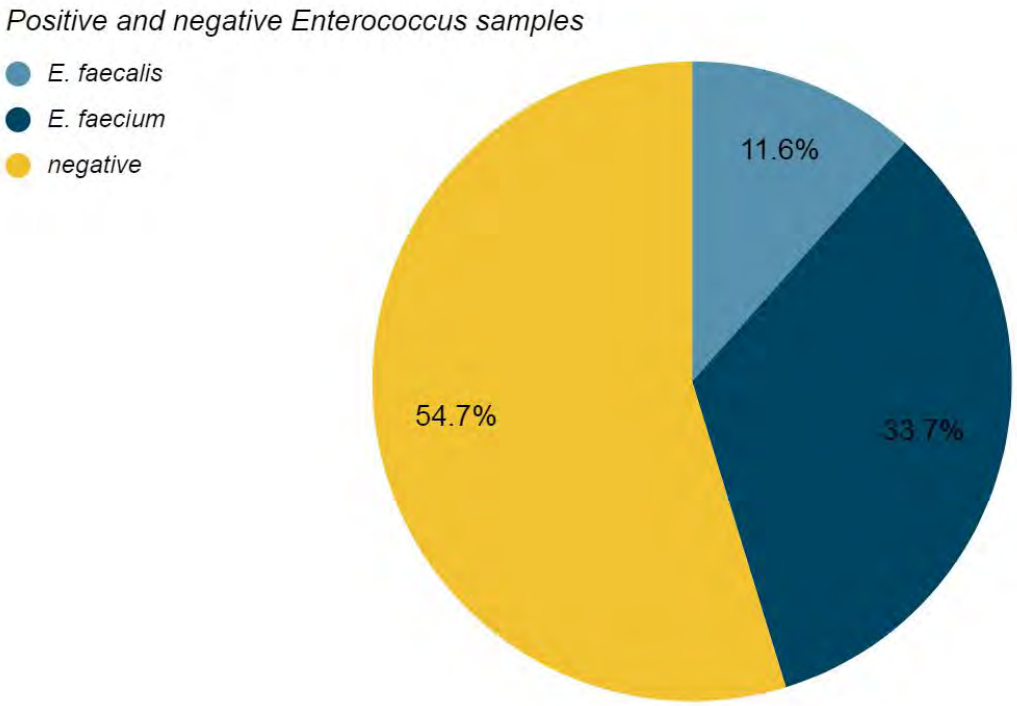



**Figure 3.9:** Pie graph representing %age of positive or negative fecal samples.

### 3.6 Antimicrobial susceptibility test

Antimicrobial susceptibility test was done against 17 antibiotics that showed zone of inhibition in the pattern as shown in the Fig. 3.10

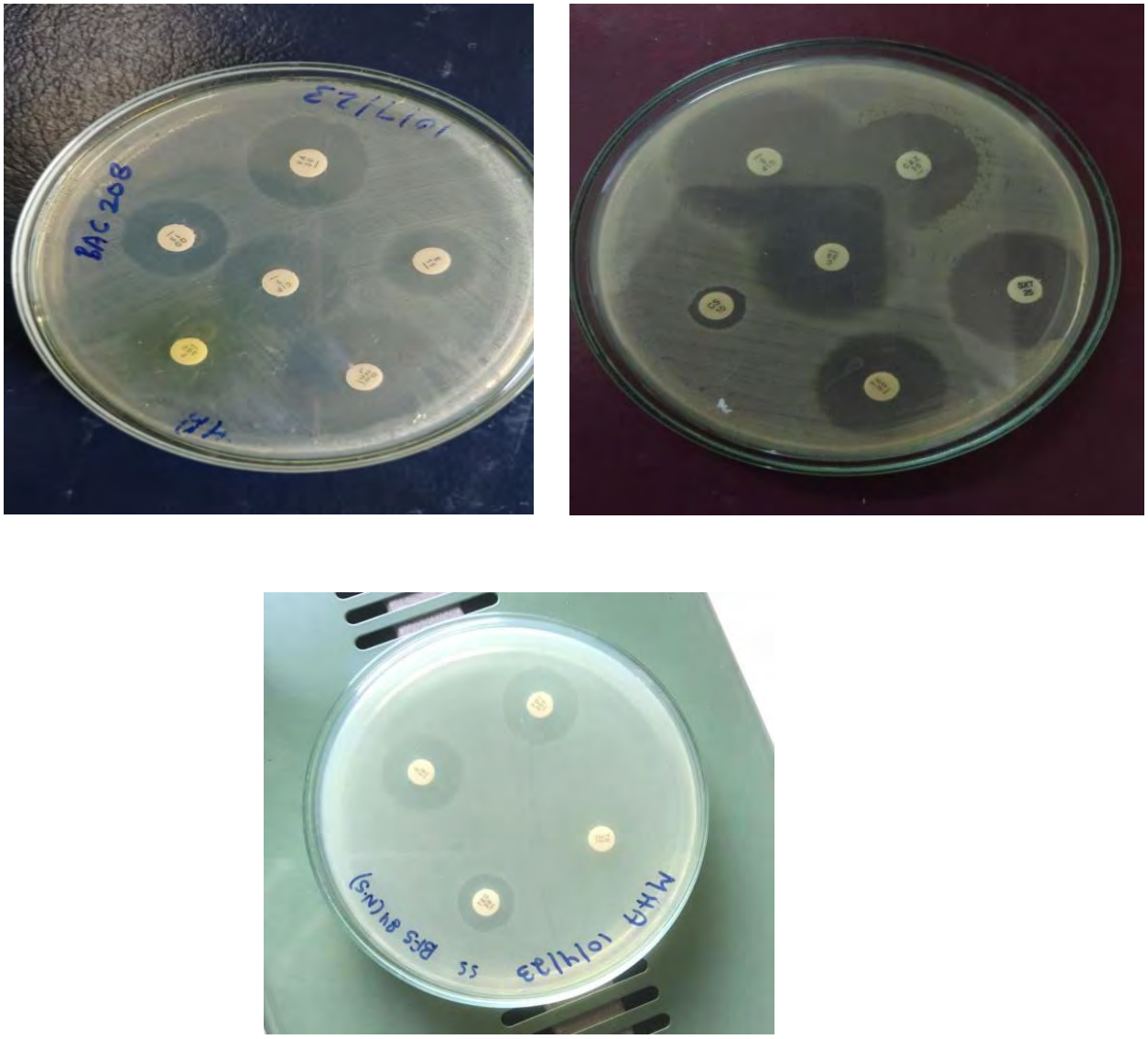


**Figure 3.10:**: Zone of inhibitions formed as result of different antibiotics.

### 3.7 Antibiotic susceptibility testing

Results of AST of *E. faecalis* and *E. faecium* are shown in tables and table respectively.

#### 3.7.1 Results of AST of *E. faecalis:*

All 11 *E. faecalis* isolates were resistant towards different antibiotics. The drug for which the sensitivity rate was the highest was nitrofurantoin (100%) while the drug to which the resistance profile was the highest was quinupristin (55%). Resistance,

intermediate and sensitivity values of other antibiotics are shown below in **table 3.2**

**Table 3.2**: Results of AST of 11 *E. faecalis* isolates

| **Antibiotics** | **Resistance Isolates** | **Intermediate Isolates** | **Sensitive Isolates** |
|---|---|---|---|
| Ampicillin | 1(9%) | 0(0%) | 10(91%) |
| Teicoplanin | 0(0%) | 1(9%) | 10(91%) |
| Norfloxacin | 0(0%) | 4(36%) | 7(64%) |
| Vancomycin | 1(9%) | 4(36%) | 6(55%) |
| Chloramphenicol | 0(0%) | 0(0%) | 11(100%) |
| Ciprofloxacin | 0(0%) | 4(36%) | 7(64%) |
| Rifampicin | 6(55%) | 5(45%) | 0(0%) |
| Fosfomycin | 0(0%) | 2(18%) | 9(81%) |
| Erythromycin | 0(0%) | 3(27%) | 8(73%) |
| Linezolid | 1(9%) | 2(18%) | 8(73%) |
| Levofloxacin | 1(9%) | 1(9%) | 9(82%) |
| Doxycycline | 2(19%) | 0(0%) | 9(81%) |
| Quinupristin | 6(55%) | 3(27%) | 3(27%) |

| | | | |
|---|---|---|---|
| Penicillin | 0(0%) | 0(0%) | 11(100%) |
| Minocycline | 0(0%) | 1(9%) | 10(91%) |
| Tetracycline | 1(9%) | 1(9%) | 9(82%) |
| Nitrofurantoin | 0(0%) | 0(0%) | 11(100%) |

### 3.7.2 Results of AST of *E. faecium*

All 32 *E. faecium* isolates were multi-drug resistance. *E. faecium* was highly susceptible to fosfomycin (88%) while *E. faecium* has shown high resistance towards rifampicin (75%). Resistance, intermediate and sensitivity values of other antibiotics is shown below in table 3.4.2

**Table 3.3**: Results of AST of 32 *E. faecium* isolates

| **Antibiotics** | ***Resistance* Isolates** | **Intermediate Isolates** | **Sensitive Isolates** |
|---|---|---|---|
| Ampicillin | 0(0%) | 3(9%) | 29(91%) |
| Teicoplanin | 2(6%) | 15(47%) | 15(47%) |
| Norfloxacin | 11(34%) | 15(47%) | 6(19%) |
| Vancomycin | 1(3%) | 4(13%) | 27(84%) |
| Chloramphenicol | 0(0%) | 7(22%) | 25(78%) |
| Ciprofloxacin | 1(3%) | 18(56%) | 13(41%) |
| Rifampicin | 24(75%) | 4(13%) | 4(13%) |

| | | | |
|---|---|---|---|
| Fosfomycin | 1(3%) | 3(9%) | 28(88%) |
| Erythromycin | 9(28%) | 16(50%) | 7(22%) |
| Linezolid | 11(34%) | 4(13%) | 17(47%) |
| Levofloxacin | 9(28%) | 15(47%) | 8(25%) |
| Doxycycline | 8(25%) | 2(6%) | 22(69%) |
| Quinupristin | 9(28%) | 16(50%) | 7(22%) |
| Penicillin | 2(6%) | 5(16%) | 25(78%) |
| Minocycline | 0(0%) | 13(41%) | 19(59%) |
| Tetracycline | 2(6%) | 7(22%) | 23(72%) |
| Nitrofurantoin | 10(31%) | 10(31%) | 12(38%) |

### 3.8 Results of PCR of *tetM* gene

Out of 11 isolates of *E. faecalis* only one showed resistance to *tetM* gene with 657bp while 8 isolates of *E. faecium* showed resistance to *tetM* gene. No resistance was shown towards other tetracycline genes used in this study.

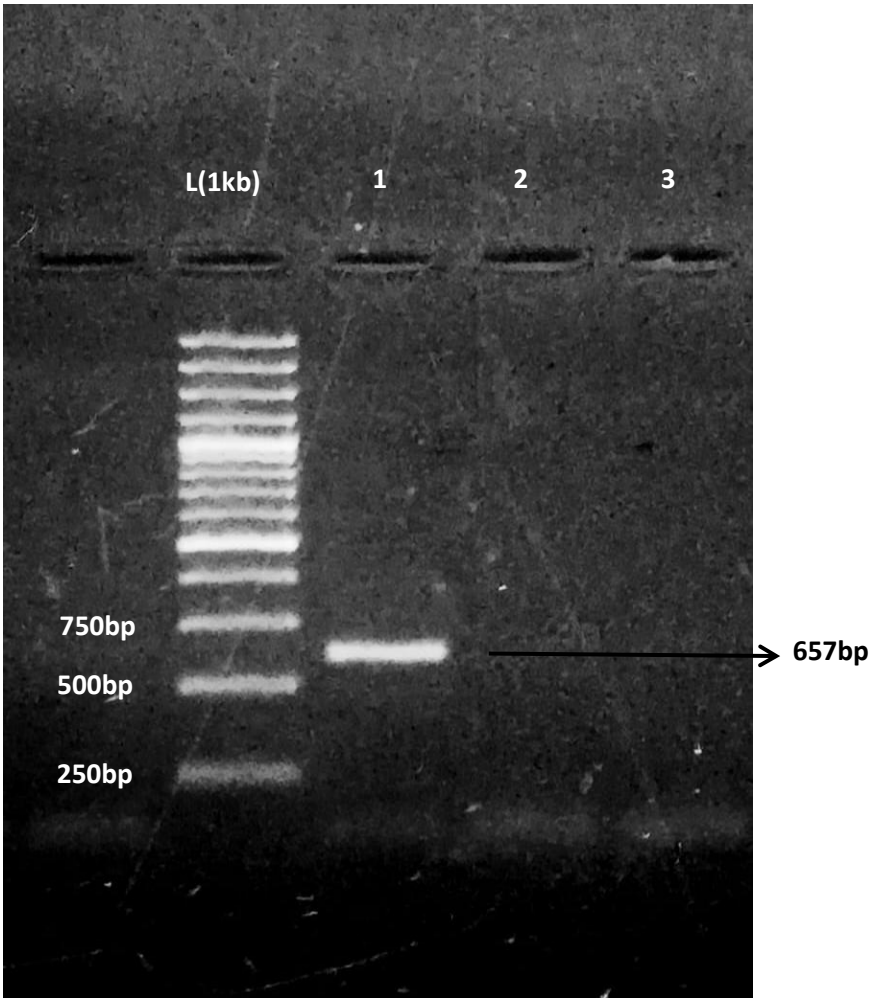



**Figure 3.11:** Agarose gel electrophoresis of PCR products for the detection of *tetM* gene (657bp),1-3 are samples, L signifies DNA size marker (100bp DNA ladder, ThermoFisher Scientific).

### 3.8.1 Results for oqxA, qnrA, qnrB, qnrS genes

Quinolones resistance genes were not detected from any isolate after PCR as shown in **Figure 3.12.**

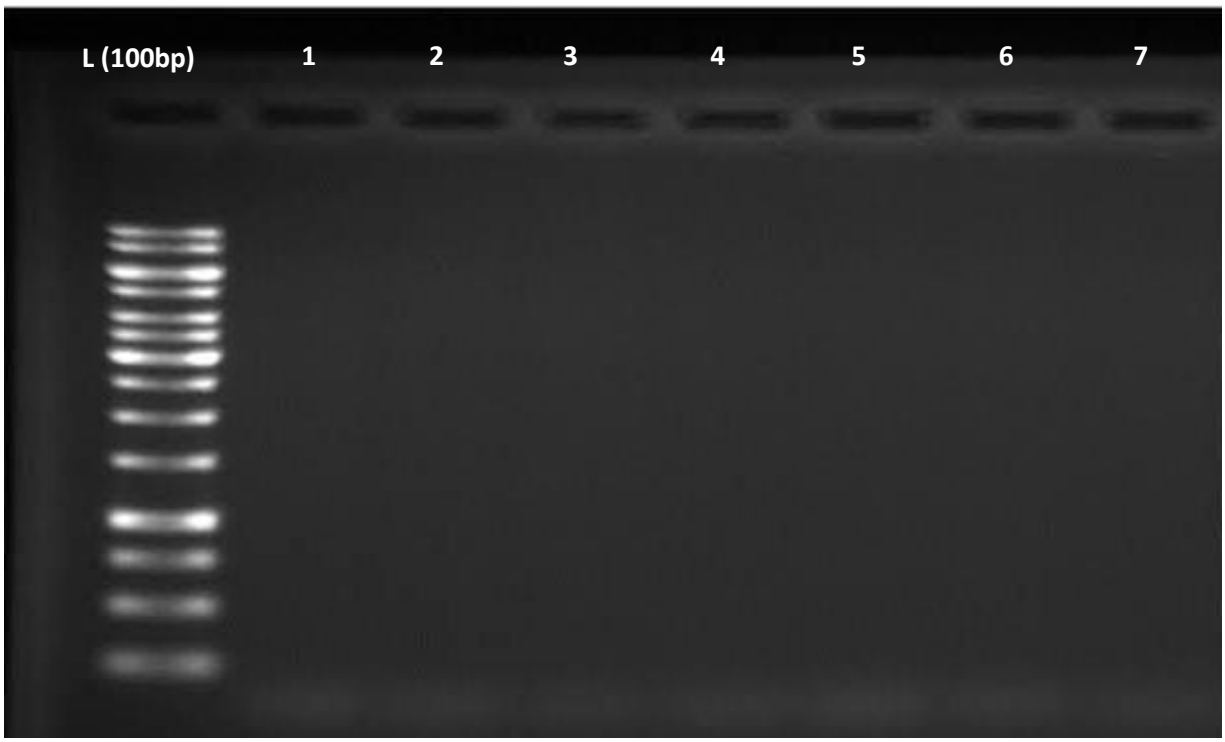



**Figure 3.12:** Agarose gel electrophoresis of PCR product for detection of oqxA (671bp), qnrA (516bp), qnrB (469bp), qnrS (417bp) genes, L signifies DNA size marker (100bp DNA ladder, ThermoFisher Scientific).

**Table 3.4:** Number and percentage of resistance genes detected in *Enterococcus* species

| S. No. | *Gene* | Positive *Enterococcus* Isolates Out of 43 | Positive *E. faecium* Isolates Out of 32 | Positive *E. faecalis* Isolates Out of 11 |
|---|---|---|---|---|
| **1** | *tetC* | 0 | 0 | 0 |
| **2** | *tetG* | 0 | 0 | 0 |
| **3** | *tetL* | 0 | 0 | 0 |
| **4** | *tetM* | 9(21%) | 8(25%) | 1(9%) |
| **5** | *tetO* | 0 | 0 | 0 |

| | | | | |
|---|---|---|---|---|
| **6** | *tetS* | 0 | 0 | 0 |
| **7** | *oqxA* | 0 | 0 | 0 |
| **8** | *qnrA* | 0 | 0 | 0 |
| **9** | *qnrB* | 0 | 0 | 0 |
| **10** | *qnrS* | 0 | 0 | 0 |

**Table 3.5**: Antimicrobial Resistance Pattern and Occurrence of Resistance Genes in *E. faecium*

| S. No. | Sample ID | Resistance pattern | No. Of antibiotics | Genes detected |
|---|---|---|---|---|
| 1 | BFS-81 | TEC, NOR, RD, P, F | 5 | Nil |
| 2 | BFS-338 | TEC, NOR, CIP, RD, LZD, LEV, QD, P, F | 9 | Nil |
| 3 | BFS-76 | NOR, Q, TE, DO | 4 | *tetM* |
| 4 | BFS-77 | NOR, RD, LZD, F | 4 | Nil |
| 5 | BFS-79 | NOR, RD, E, LEV, TE, F, DO | 7 | *tetM* |
| 6 | BFS-171 | NOR, VA, LZD, QD | 4 | Nil |
| 7 | BFS-03 | NOR, LZD, Q, DO | 4 | *tetM* |
| 8 | BFS-328 | NOR, RD, E, LZD | 4 | Nil |

| S. No. | Sample ID | Resistance pattern | No. Of antibiotics | Genes detected |
|---|---|---|---|---|
| 9 | BFS-329 | NOR, RD, DO | 3 | *tetM* |
| 10 | BFS-181 | NOR, RD, E, LEV, QD | 5 | Nil |
| 11 | BFS-217 | NOR, RD, E, LEV, DO | 5 | *tetM* |
| 12 | BFS-81(N.S) | RD, LZD, QD | 3 | Nil |
| 13 | BFS-85(N.S) | RD | 1 | Nil |
| 14 | BFS-89(N.S) | RD, E, LZD, LEV, QD | 5 | Nil |
| 15 | BFS-04 | RD, F | 2 | Nil |
| 16 | BFS-02 | RD | 1 | Nil |
| 17 | BFS-05 | RD, QD | 2 | Nil |
| 18 | BFS-333 | RD | 1 | Nil |
| 19 | BFS-84 | RD | 1 | Nil |
| 20 | BFS-321 | RD, LZD | 2 | Nil |
| 21 | BFS-78 | RD, FOT, E, F | 4 | Nil |

| S. No. | Sample ID | Resistance pattern | No. Of antibiotics | Genes detected |
|---|---|---|---|---|
| 22 | BFS-337 | RD, LEV | 2 | Nil |
| 23 | BFS-170 | RD, E, F | 3 | Nil |
| 24 | BFS-173 | RD, LEV, F | 3 | Nil |
| 25 | BFS-190 | RD, DO | 2 | *tetM* |
| 26 | BFS-202 | RD, E, LZD, LEV, QD, F | 6 | Nil |
| 27 | BFS-208 | RD, DO | 2 | *tetM* |
| 28 | BFS-204 | E, LZD, DO | 3 | *tetM* |

**Table 3.6**: Antimicrobial Resistance Pattern and Occurrence of Resistance Genes in *E. faecalis*

| S. No. | Sample ID | Resistance pattern | No of antibiotics | Genes resistance |
|---|---|---|---|---|
| 1 | BFS-08 | AMP, RD, LEV, QD | 4 | Nil |
| 2 | BFS-05 | VA, RD, LZD, QD | 4 | Nil |
| 3 | BFS-84 | RD, DO, QD, TE | 4 | Nil |
| 4 | BFS-209 | RD | 1 | Nil |
| 5 | BFS-187 | RD, QD | 2 | Nil |
| 6 | BFS-09 | RD, DO, QD | 3 | *tetM* |

| S. No. | Sample ID | Resistance pattern | No of antibiotics | Genes resistance |
|---|---|---|---|---|
| 7 | BFS-186 | QD | 1 | Nil |

**CHAPTER 4**

# DISCUSSION

*Enterococcus* are Gram's positive, catalase-deficient, facultative anaerobic cocci found in the guts of humans and other animals, including bats (Fisher & Phillips, 2009). Enterococci, which were previously regarded as commensal organisms, have drawn a lot of attention because they are opportunistic agents that can cause a variety of pathological conditions, especially in hospital environment (Nowakiewicz et al., 2021) There are numerous *Enterococcus* species, but the two most common disease-causing species are *E. faecium* and *E. faecalis* (Sood *et al* 2008).

In the present study, 95 fecal samples from Islamabad, Pakistan were analyzed to investigate the occurance of *E. faecium* and *E. faecalis* in fruit bats. Forty-three *Enterococcus* isolates were recovered, out of which 11 were *E. faecalis* and 32 were *E. faecium.* The data is similar as compared to previous studies given that 26 (84%) of 31 specimens recovered, bats do serve to be natural hosts. 20 out of the 26 people tested positive for enterococci from the strongly reducing group. (Mundt, 1963). A study was conducted in Thailand to know the prevalence of *Enterococcus* species, AMR and virulence genes in Enterococci isolated from rectal and carcass swabs of pig, pork and humans. The results revealed that the most prevalent species was *E. faecium* with 74.3% prevalence while *E. faecalis* was at second number with 25.7% prevalence (Thu et al., 2019).

A major obstacle in effectively treating enterococcal infections lies in their resistance to commonly used antibiotics (Hammerum, 2012). Notably, the concerning feature is that resistant enterococci can easily exchange antimicrobial resistance (AMR) genes with both i.e other species of Gram-positive bacteria and fellow enterococci (Beukers et al., 2017). The objective of this study was also aimed to analyze antibiotic sensitivity of *Enterococcus* isolates in bats. AST was done against 17 antibiotics, which revealed that multiple drug resistance (MRD) exists in some *Enterococcus* isolates. MDR is defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories. *Enterococcus* has shown the most resistance against 5 out 17 antibiotics which were rifampicin (75%) followed by quinupristin (55%), erythromycin (28%), norfloxacin (34%) and levofloxacin (28%). The resistance patterns of *Enterococcus* strains derived from different animal groups in Poland showed comparable resistance to rifampicin

(Nowakiewicz et al., 2021b). This result provided additional evidence that the level of drug resistance is more influenced by environmental factors than by the evolution of resistance mechanisms due to selective pressure from direct exposure to the drug. A recent study from Poland documented the identification of *E. faecalis* from bat guano samples, in accoradance to his other study (Nowakiewicz et al., 2021b), in agreement with the conclusions proposed by Kristich et al. (2014). The strains isolated from these samples showed a rate of 69.4% tetracycline resistance, which was notably high (Nowakiewicz et al., 2021a). Similar to this, a different study carried out in Spain discovered *Enterococcus* strains isolated from rectal swabs obtained fron bats. Both of these isolates showed resistance to quinupristin-dalfopristin, but only one of them was found to be resistant to erythromycin (García et al., 2022). As in our research *Enterococcus* has not shown very low resistance against vancomycin. Similar to this, none of the enterococcal strains in a different study involving wild mammals showed resistance to glycopeptides. This finding raises the possibility that there may be vancomycin-resistant enterococci among the population of fecal enterococci found in wild animals. However, compared to vancomycin-susceptible strains, these resistant strains might be present in relatively low concentrations. It might be difficult to find these resistant strains when using culture methods without supplementation (Poeta et al., 2007).

In current research antibiotics resistance against rifampicin and quinupristin-dalfopristin is very high, as QD resistance is higher than all other antibiotic resistance recorded in the present study just as similar to a study conducted in China. A combination of two synthetic streptogramins named QD was created to treat infections in people brought on by VRE and multidrug-resistant *E. faecium* (Wang et al., 2016). Furthermore, a different investigation involving wild mammals revealed erythromycin and tetracycline resistance (Poeta et al., 2007). The most common isolates in our study were found to be *E. faecium.* Notably, *E. faecium* isolates were more likely to be multidrug-resistant (MDR) than *E. faecalis* isolates. In contrast, to the findings from a study carried out in a children's hospital in Iran and other countries (Sattari-Maraji et al., 2019). According to another research, the common resistance of the bacteria to medications intended to treat enterococci may be the cause of the increased prevalence of *E. faecium* species (Gawryszewska et al., 2016). Ampicillin resistance was not found in our research. Although *Enterococcus* species frequently develop ampicillin resistance, there was a very low level of ampicillin resistance found in our study. This contrasts with the higher ampicillin resistance

levels frequently observed in human isolates. Notably, *E. faecalis* usually shows susceptibility to this β-lactam and isolates from human hospital settings also showed susceptibility. 1.6% of these hospital-derived isolates exhibited AMP resistance, which is a very low percentage (García et al., 2022).

Similar to findings from other categories of farmed and wild animals, fluoroquinolone-resistant strains were significantly less common, as was the case in a study conducted in Poland (Nowakiewicz et al., 2021b) only two strains in that study demonstrated significant resistance to the antibiotic ciprofloxacin at a concentration of 32 μg/ml, and efflux pumps encoding genes were not found. Strangely, in the same study, comparable results were found for *E. faecalis* strains obtained from commercial poultry (Nowakiewicz et al., 2021b) While simultaneously recording a 30% resistance rate in *E. faecium* isolates against ciprofloxacin, this poultry-related research also noted *E. faecalis* isolates with low percentage of resistance. The primary mechanism of quinolone resistance, which typically does not spread through horizontal gene transfer (which would make it easier to spread), but instead arises as a result of mutations might be connected to this divergence (García-Solache & Rice, 2019).

The presence of tetracycline and quinolone resistance genes in *Enterococcus* species was examined in the current study. The results showed that only the *tetM* gene was found among the six tetracycline resistance genes studied. More specifically, 9 out of 43 isolated strains carried the *tetM* gene. The other five tetracycline resistance genes were not found by PCR. Our findings support earlier research showing that zoonotic strains and infections acquired in hospitals frequently show resistance to tetracycline. This resistance is frequently encoded by the *tetM* gene, which is frequently found in strains originating from humans and other animals receiving targeted therapy (Torres et al., 2018; Farman et al., 2019). According to this study, tetracycline resistance in enterococci is frequently caused by the *tetM* gene (del Campo et al., 2003; Kühn, 2003).

In the present study quinolones resistance genes *qnrA, qnrB, qnrS and oqxA* were not detected from any of the isolates but quinolones resistance was observed in AST. It might be due to the reason that quinolones resistance in *Enterococcus* isolates of present study might not be caused by these genes, but possibly other resistance genes were responsible for resistance, as in a study quinolones resistance was linked with *gyrA* and *parC* genes in *E. faecalis* isolated from clinical

samples of UTI patients (Kanematsu et al., 1998). In another research in Sweden, mutation in *gyrA and parC* genes was associated with quinolones resistance genes in *E. faecium* isolated from clinical samples (el Amin et al., 1999).

The release of antibiotics can exert selective pressure in the environment, encouraging the spread of resistant genes among bacterial communities in the environment and the bacterial flora of wildlife (Allen et al., 2010). Bats act as reservoirs for plasmids and genes linked to antibiotic resistance (McDougall et al., 2019). Bats have the potential to spread these bacteria and their genes widely among humans and domesticated animal populations due to their extensive flying and roving abilities. The bacterial flora of bats and their antimicrobial resistance (AMR) profiles, however, are poorly understood (Nowakiewicz et al., 2020). AMR pollution, which can happen when wildlife is exposed to human activities such as food wastage, water treatment facilities, and aquaculture facilities with antimicrobial residues, may be the cause of bats acquiring AMR microorganisms (Kraemer et al., 2019). Bats typically don't build specialized shelters. Instead, they use man-made habitats and natural caves as places to rest or hibernate (Leivers et al., 2019). The reduction of forested areas and challenges related to food availability compel bats to inhabit urban and rural environments, including buildings and their interiors, for roosting and breeding purposes (Voigt et al., 2015). The possibility of both direct and indirect contact is increased by being close to people and domesticated animals, which promotes the exchange of microflora. Exploration of bat-inhabited areas, hunting of wild animals, and deforestation all increase the risk of zoonotic infections linked to bats.

# CONCLUSION

In conclusion, a total of 43 *Enterococcus* isolates were obtained from the feces of bats collected from a selected area of Islamabad. Among these, 11 were identified as *E. faecali*s, while 32 were categorized as *E. faecium*. Antimicrobial susceptibility testing (AST) results showed that a significant percentage of both *E. faecalis* and *E. faecium* isolates had tetracycline, macrolide, and quinolone resistance. However, only tetracyclines resistance genes *tetM* (21%) were detected, but no quinolones resistance genes were found in any of the *Enterococcus* isolates. So, it is important that while prescribing treatment for infections caused by the *Enterococci* in humans, it is essential to avoid those antibiotics against which enterococcal species have shown high rates of resistance as isolates resistant to these antibiotics might have passed from bats to humans. Furthermore, since a very limited number of resistance genes have been detected in the *Enterococci* in this study, it is important to conduct more studies focusing on resistance genes other than those investigated in this study.

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