Antibiotic Sensitivity and Antibiotic Resistance Genes in *Enterococcus* Species Isolated from Chicken Feces

KAINAT ABBAS

02022011005

Department of Zoology

Faculty of Biological Science

QUAID-I-AZAM UNIVERSITY ISLAMABAD

2022

Antibiotic Sensitivity and Antibiotic Resistance Genes in *Enterococcus* Species Isolated from Chicken Feces

By

Kainat Abbas

02022011005

Session 2020-2022

This dissertation is submitted in the partial fulfillment of the requirements for the degree of

MPhil

In

Zoology

(Specialization in Animal Microbiology)

Department of Zoology, Quaid-i-Azam University Islamabad

2022

In the Name of Allah Almighty

The 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.

KAINAT ABBAS

CERTIFICATE OF APPROVAL

This thesis **"Antibiotic Sensitivity and Antibiotic Resistance Genes in *Enterococcus* Species Isolated from Chicken Feces"** by **KAINAT ABBAS** is hereby 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.

Supervisor: _____

(Dr.Saeed-ul-Hassan Khan)

External Examiner: _____

(Dr.)

Chairperson: _____

(Dr. Amina Zubari)

Dated:

ACKNOWLEDGMENT

In the Name of Allah the Most Gracious and the Most Merciful

*First of all, I would like to thank **Almighty Allah**, for giving me facilities, guidance and opportunities.*

*I would like to thank my supervisor, **Dr. Saeed ul Hassan Khan**, Assistant Professor Zoology, and **Dr Athar Abbas,** Senior Scientific officer NRLPD, NARC, for their kind support and guidance during my lab work and writing this dissertation.*

*I would like to thank my Seniors, Lab fellows, **Laraib, Rafia, Zohra and Aiman,** and Friends for their unconditional support and help.*

I would like to thank my family for always being there for me and for being a greatest support in every stage of my life.

KAINAT ABBAS

DEDICATION

This dissertation is dedicated to my beloved Parents, Mr.Ghulam Abbas and Shabnum Mehmood for their kind support, advice and love.

`LIST OF TABLES

Table 2.4. 1Reference values of AST results ……15
Table 2.6.1 PCR recipe for *oqxA, tetM, tetC, tetG, and tetL* genes**……**17
Table 2.6. 2 PCR recipe for *qnrA, qnrB, qnrS* Genes……18
Table 2.6.3 PCR recipe for *tetO, tetS genes*……...18
Table 2.6. 4Thermal Profiles of PCR……18
Table 2.6. 9 List of antibiotics, symbols and their concentration……..19
Table2.6.10 List of genes and primers……20
Table 3.1. 1Results of isolation of *Enterococcus* from fecal samples from different cities.....22
Table 3.5.1 Results of AST of 53 *E. faecalis* Isolates…….27
Table 3.5.2 Results of AST of 107 *E. faecium* Isolates……...…….28
Table 3.6. 1 Number and percentage of resistance genes detected in *Enterococcus* species……30
Table 3.6.2 Antimicrobial Resistance Pattern and Occurrence of Resistance Genes in *E. faecium*….30

LIST OF FIGURES

Fig 3.1.1. Distribution of *E. faecalis* and *E. faecium* in fecal samples from different cities... 23
Fig 3.1.2 Pie graph representing %age of positive or negative fecal samples. 23
Fig 3.1.3 Brain Heart Infusion broth before and after inoculation of bacterial culture..... 24
Fig 3.1. 4 Growth of *E. faecalis* and of *E. faecium* on SBA respectively..... 24
Fig 3.1. 5Growth of *Enterococcus* on Nutrient Agar..... 24

Fig 3.2.1. Catalase negative *Enterococcus* along with Catalase positive *Staphylococcus*..... 25
Fig 3.2. 2Non-Motile *Enterococcus*..... 25
Fig 3.2. 3Gram-positive *Enterococcus*..... 26

Fig 3.4. 1 Zone of inhibitions formed as result of different antibiotics..... 26

Fig 3.6.1. Agarose gel electrophoresis of PCR products for the detection of *tetL* gene (475bp), *tetO* gene (634bp) and *tetM* gene (657bp), L signifies DNA size marker (50bp DNA ladder, Thermo fisher Scientific) 29
Fig 3.6.2Agarose gel electrophoresis of PCR product for detection of *oqxA (671bp)*, *qnrA (516bp)* , *qnrB (469bp)*, *qnrS (417bp)* genes, L signifies DNA size marker (50bp DNA ladder, Thermo fisher Scientific) 29

TABLE OF CONTENT

DECLARATION ..... **IV**

CERTIFICATE OF APPROVAL ..... **V**

ACKNOWLEDGMENT ..... **VI**

DEDICATION ..... **VII**

`LIST OF TABLES ..... **VIII**

ABSTRACT ..... **XIII**

CHAPTER 1 ..... **1**

INTRODUCTION **1**

1.1 INTRODUCTION TO THE GENUS *ENTEROCOCCUS* 1

2.* DISEASES CAUSED BY *ENTEROCOCCUS 2

3. PREVALENCE OF *ENTEROCOCCUS* 4

4. ANTIBIOTIC RESISTANCE IN BACTERIA 6

5. Antibiotic Resistance in Enterococcus Species *8*

1.6 ISOLATION AND IDENTIFICATION OF *ENTEROCOCCUS* **11**

6. OBJECTIVES 12

CHAPTER 2 ..... **13**

METHODOLOGY **13**

2.1. STUDY AREA 13

2.2. Sample analysis: *13*

2.3.1 Grams' staining *14*

2.3.2 Catalase Test *14*

2.3.3 Motility *14*

2.4 Antibiotic Susceptibility Test *15*

2.5 MOLECULAR CHARACTERIZATION 16

2.5.1 Plasmid DNA Extraction *16*

2.6 POLYMERASE CHAIN REACTION (PCR) 17

2.6.2 GEL ELECTROPHORESIS **19**

RESULTS **22**

3.5 ANTIBIOTIC SUSCEPTIBILITY TESTING 26

3.5.1 Results of AST of E. faecalis *27*

3.5.2 Results of AST of E. faecium *28*

3.6.1 Results of PCRs for tetM, tetL, tetO genes *28*

3.6.2 Results of PCRs for oqxA, qnrA, qnrB, qnrS genes *29*

DISCUSSION **33**

CONCLUSION **36**

Abbreviations

E. faecium	*Enterococcus faecium*
E. faecalis	*Enterococcus faecalis*
AST	Antimicrobial Susceptibility Test
LSB	Lyallpur Silver Black
MRSA	Methicillin *Resistance* Staphylococcus aureus
VRE	vancomycin *Resistance Enterococcus*
Esp	Extracellular Surface Protein
UTI	Urinary tract Infections
PCR	Polymerase Chain Reaction
NARC	National Agriculture research Institute
NRLPD	National Reference Laboratory for Poultry Diseases
BPW	Buffered Peptone Water
BHI	Brain Heart Infusion Broth
SBA	Slanetz and Bartley Agar
NA	Nutrient Agar
SIM	Sulfur Indole Motility
MHA	Muller Hinton Agar
QC	Quality Control

ABSTRACT

Enterococci are Gram positive, catalase negative, facultative anaerobic cocci (single, paired or chained), and are found in intestinal tract of many animals including chicken. The present work was aimed at studying the prevalence of *Enterococcus* species *E. faecalis* and *E. faecium* in feces of commercial poultry. A total of 254 fecal samples were collected from apparently healthy broiler chicken sold in the market from random slaughter shops of different cities of Pakistan. From those 254 samples, 160 *Enterococcus* isolates were recovered. Further testing showed 53 (33%) of the isolates were *E. faecalis* and 107 (67%) were *E. faecium*. After isolation and confirmation, AST was done for 160 *Enterococcal* isolates. All *Enterococcus* isolates showed multidrug resistance. Among 53 *E. faecalis,* the drugs for which the sensitivity values were high were ampicillin (77%), nitrofurantoin (77%), fosfomycin (75%) and teicoplanin (60%) while the drugs to which the resistance values were greater than 50% were erythromycin (98%), chloramphenicol (96%), tetracycline (94%), levofloxacin (92%), minocycline (79%), doxycycline (79%), ciprofloxacin (77%), quinupristin (77%) and norfloxacin (64%). Among the 107 *E. faecium* isolates, the drugs which were highly effective towards *E. faecium* were fosfomycin (61%), teicoplanin (61%), and ampicillin (60%) while the antibiotics against which high resistance was observed were tetracycline (97%), erythromycin (94%), norfloxacin (90%), ciprofloxacin (86%), chloramphenicol (82%) and levofloxacin (82%), quinupristin (82%), doxycycline (80%), and minocycline (69%). Thus, both *Enterococcus* species showed highest resistance towards, erythromycin levofloxacin, tetracycline, and chloramphenicol. After AST, the most resistant 31 isolates were selected for detection of some selected antibiotic resistance genes. PCRs were done for antibiotic resistance genes against tetracyclines and quinolones. Among the six tetracycline resistance genes of *Enterococci*, two genes were detected from almost all the isolates. *tetM* was found in 57% and 42% of *E. faecalis* and *E. faecium* isolates respectively and *tetL* was noted in 40% and 60% of the *E. faecalis* and *E. faecium* respectively, while *tetO* was detected in only one isolate of *E. faecium*. While the quinolones resistance genes *oqxA qnrA, qnrB*, and *qnrS* were not observed in any of the isolate.

CHAPTER 1

INTRODUCTION

1.1 Introduction to the Genus *Enterococcus*

Enterococci are Gram positive cocci, catalase negative, facultative anaerobic bacteria found in gastrointestinal tract of many terrestrial animals. They appear as single, paired or chained cocci when observed under microscope. They are found in gut, genital tract of female and oral cavity of human and animals. It has the ability to survive in harsh conditions like 6.5% NaCl, pH 9.6 and 10-45°C temperature (Yilema *et al*, 2017) with optimum growth at 37°C. Because of these versatile characteristics it is considered as indicator for identifying the quality of food and water. It is a member of phylum *Firmicutes* and different species that have been detected from the Genus *Enterococcus* are*: Enterococcus faecalis (E. faecalis), E. faecium, E. durans, E. gallinarum, E. casseliflavus, E. avium, E. malodoratus, E. hirae, E. mundtii, E. pseudoavium, E. raffinosus, E. cecorum, E. columbae, E. saccharolyticus, E.dispar* (Devriese *et al*, 2006).

Basically, *Enterococcus* is not harmful but sometimes it can cause nosocomial infections in a community opportunistically. These bacteria are transmitted through blood (endogenous transmission) or through environment such as from hospitals, contaminated water etc. (exogenous transmission). It can cause opportunistic infections in many immmunocompromised patients and can easily get resistance against many antibiotics. That's why it is considered as indicators for assessment of antimicrobial drug resistance (Rojas *et al*, 2013).

A report of National Healthcare Safety Network on 2009-2010 revealed that *Enterococcus i*s the second leading cause of hospital borne diseases after *Staphylococcus aureus* and 3% of these are *Vancomycin Resistant Enterococcus* (Yilema *et al*, 2017). *Enterococcus* shows resistance against certain drugs such as vancomycin. Resistance may be produced by mutations and sometimes due to jumping genes or transposons (Masjost *et al*, 2015).

E. faecalis 62, isolated from a healthy Norwegian infant, its genomic sequencing disclosed that, it is made up of a single circular chromosome (2,988,673 bp; 37.2 % GC content) and 3 plasmids (EF62pA, EF62pB, and EF62pC) (Brede *et al*, 2011). A study based on the closed genome of the *E. faecium* endocarditis isolate TX16 (DO) from the United States, The genome of *E. faecium strain TX16* includes just a single chromosome with 2,698,137 bp and three plasmids, according to a comparison of this strain's genome with 21 other *E. faecium* strains genomes (Qin *et al* 2012).

2. Diseases Caused by *Enterococcus*

Extensive usage of antimicrobials has increased *Enterococcus* pathogenecity. *Enterococcus* causes many diseases in human including endocarditis, urinary tract infections and bacteraemia (Raza *et al* 2018).

Endocarditis is an infectious disease of heart endocardial surface and can be acute, sub acute or chronic that depends on severity and clinical signs of the disease and this disease can infect men more than women. Common symptoms of this disease are fever, anorexia, weight loss, night sweats, heart murmur and petechiae on skin (Mylenakis *et al* 2001).

E. faecalis is considered as the well known reason for causing infectious diseases like sepsis, abdominal infections and urinary tract infections worldwide and due to its resistance towards certain drugs, its treatment is being very difficult day by day (Xioayu Ma *et al* 2021).

The most common infectious disease that causes morbidity in humans is urinary tract infection (UTI). Trimethoprim sulfamethazole, ciprofloxacin, and ampicillin are the antibiotics used to treat UTI caused by *E. faecalis* (Mireles *et al* 2015).

A deadly infectious disease acute cholangitis (AC) is caused by gallstone, bile duct stone and bile duct stenosis. *Enterococcus* and other bacteria have also been isolated from AC patients. Its most common symptoms include fever, jaundice and abdominal pain (Karasawa *et al* 2020).

E.gallinarum infections of the musculoskeletal system are uncommon, but due to its multidrug resistance, the scientific community is paying closer attention. Patients who

have undergone invasive procedures or who are immmunocompromised are vulnerable (Amaro *et al* 2020).

The hazardous factors related to mortality in bloodstream infections (BSI) caused by *Enterococcus* species were determined and it was discovered that *E. faecalis* and *E. faecium*, have unique pathogenecity that is responsible for producing contrasting clinical factors in BSI (Zheng *et al* 2017).

Enterococcal spondylitis (ES), formerly known as *enterococcal* vertebral osteoarthritis (EVOA) in chickens, has been increasingly linked to *E. cecorum*. The majority of disease outbreaks were found in broiler chicken flocks grown in intensive production systems. Birds that have been clinically affected had locomotors issues as a result of spinal cord compression at the thoracic vertebrae caused by *E. cecorum-induced osteomyelitis*, as well as femoral head necrosis (FHN). Disease outbreaks can cause significant morbidity and mortality, as well as culling and carcass condemnation, as well as significant financial losses over a short time frame (Dolka *et al* 2016).

E. faecalis and *E. faecium* are dominating causes of opportunistic infections that may ranges from mild to severe infection. Many strains having resistance against antimicrobial drugs have been emerged as a leading cause of hospital-acquired infections in humans (O'Dea *et al,* 2019).

Enterococcus causes several diseases in poultry mainly in turkeys, chicken, ostrich, ducks and pigeon. The species that are associated with poultry diseases are *E. faecalis, E.cecorum, E.hirae, E. faecium, E.durans* and the diseases caused by these *Enterococcus* species are Omphalitis, endocarditis, meningitis, fibrinous arthritis, Pulmonary Hypertension Syndrome in broiler chicken, *enterococcal* spondylitis, femoral head necrosis, arthritis, First week Mortality Syndrome and Septicaemia in mature birds (Dolka *et al,* 2016).

The risks of increase in gut micro flora in poultry reared in open places or backyard farms is very high as compared to the poultry rearing in high biosecurity conventional farms. Backyard farming of poultry can yield high scale meat production but there is a need of proper management of those poultry farms by the government. Otherwise unprofessional personnel's will not be able to use antibiotics properly that will lead to

uncontrolled spread of infection and antimicrobial drug resistance and this will eventually effects human health (Ahmed *et al*, 2021). One of the fundamental reasons for MDR in bacteria like *Enterococcus* is the use of poultry litter as fertilizer. In such conditions bacteria make their way towards surface and ground water through different routes and make the water contaminated that's why ground and surface water quality should be monitored properly for MDR bacteria for improving the management practices in that particular area (Furtula *et al*, 2013).

3. Prevalence of *Enterococcus*

In Poland between2014-15 a research was done on poultry, to find out the prevalence of *Enterococcus* species which concluded that among all the isolates the most prevalent *Enterococcus* species was *E. faecalis 57% than E. cecorum 7% followed by E. faecium5.2%, E. hirae 3.6%, and E. gallinarum 2.5%, E. casseliflavus 0.7% and E. durans* 0.2% (Dolka *et al* 2016). A research in Turkey was conducted to determine the prevalence of *Enterococci* in poultry neck skin samples from Turkey's Ankara region, and the results concluded that the most abundant species was *E. faecium* 48% followed by *E. durans* 23% and *E. faecalis* 19%. While the species *E. gallinarum, E. hirae, E. mundtii and E. casseliflavus* were in very few amounts (Dogru *et al* 2010). A study was undertaken in Canada to determine the antibiotic resistance and virulence genes in *Enterococcus* species found in broiler chickens, which concluded that *E. faecium* was most prevalent 74%, followed by *E.hirae 13%, E. faecalis 10%*, and *E. gallinarum 3%* (Diarra *et al* 2010). In Zagazig University, Egypt to ascertain the prevalence of *Enterococcus* species in chicken meat 175 samples were collected from chicken breast, chicken thigh, chicken liver, chicken gizzard, chicken heart, cloacal skin. After bacteriological examination they concluded that chicken heart contains 60% *Enterococcus* species and 100% in cloacal skin (Abdalla *et al* 2019). A research was carried in University of North Carolina State to determine the AMR pattern of *Enterococcus* species in fresh food gathered from Southwest. 97 (52 %) of the 185 *Enterococcus* isolates were *E. faecium*, 38 (21 %) were *E. faecalis* (Johnston *et al* 2004). An exploration was directed to know the prevalence and AMR in *Enterococci* found in poultry and cattle farms. 390 samples were processed for *Enterococcus* species recovery: cloacal/rectal swabs (260) and manure (130).After isolation, identification, AST and Genotypic characterization it was revealed that *Enterococcus*

was found in 167 (42.8 %), with *E. faecium* being the most common (27.7%) (Ngbede *et al* 2016). There are very few reports on the prevalence of *Enterococcus* in Pakistan. It was investigated in University of Lahore to ascertain the diversity of pathogens in UTI; that *Enterococcus* was 8.3% prevalent after *E.coli* which was 66% (Bashir *et al* 2008). *E. faecalis* has been reported 70% in UTI patients in s recent study conducted in Chughtai Lab Lahore, Pakistan (Hussain *et al* 2016). At the species level, 162 *Enterococci,* 72 isolates of *E. faecalis*, 20 isolates of *E. gallinarum/casseliflavus,* 5 isolates of *E. faecium*, 4 isolates of *E. raffinosus*, and 2 isolates each of E. hirae and E. durans were detected at Mulago Hospital in Kampala, Uganda (Kateete *et al* 2019). According to AMR of five *enterococcal* species recovered from Australian meat chickens, *E. faecium* was the most often isolated species (37.6%), followed by *E. durans* (29.7%), *E. faecalis (20%), E. hirae (12.2%), and E. gallinarum* (0.5%) (O'Dea *et al* 2019). The most prevalent species detected in a research at the University of Maryland was *E. faecium (61%)* followed by *E. faecalis* (29%) and *E.hirae* (1%). In ground turkey (60 %), ground beef (65 %), and chicken breast (79 %) samples, *E. faecalis* was the most common species, whereas *E. faecalis* was the most common species in pork chops (54 %) (Hayes *et al* 2003). In s recent work to collect the data on *Enterococcus* strains isolated from chicken samples in Ankara, Turkey, with an emphasis on their prevalence, morphological and genotypic characteristics, and antibiotic resistance. 97 *enterococcal* isolates were found to be suspicious; *E. faecium* (61.85%) and *E. faecalis* (38.15%) were among the most frequently discovered *Enterococcus* species in the 16S rRNA sequencing analysis (Sanlibaba *et al* 2018). A study was done with the goal of determining the distribution of *Enterococcus* species, cloacal samples were taken from 179 healthy pigeons from 13 distinct flocks which revealed that *E. faecalis* and *E. faecium* were 11.7% prevalent (Dolka *et al* 2020). Prevalence of *Enterococcus* species in clinical specimens was determined in the microbiology laboratories of two Tabriz hospitals, with *E. faecalis* accounting for 90.5 %, *E. faecium* for 5.84 %, and other *Enterococcus* species accounting for 3.66 % of the isolates (Akhi *et al* 2009).

4. Antibiotic Resistance in Bacteria

The widespread usage of antibiotics plays a role in the emergence of novel antibiotic resistant bacterium strains. Because of the rapid rise in the number of novel bacterial strains, developed countries have set a strict prohibition on the overuse of antibiotics and are pushing the use of probiotics to treat enteric disorders (Apata, 2009).

Antibiotics have long been considered one of the twentieth century's most significant discoveries. Genuine, but the true miracle is the growing resistance to antibiotics connected with their usage in hospitals, communities, and the environment. Microorganisms' incredible genetic potential allows them to evolve different mechanisms of resistance to specific therapeutic, agricultural, or other practical antibiotics using any horizontal gene transfer technology and any source of resistance genes. To achieve complete recovery of antibiotic therapeutic use, further research on the function of environmental microflora in growing antibiotic resistance is required. A novel strategy for the development of new antibiotics, as well as their rapid and controlled introduction into treatment, is critical (Davies *et al* 2010).

According to a CDC (Center for Disease Control and Prevention) report, AMR is divided into four main categories, one of which is Serious Threats, which includes *Vancomycin-Resistant Enterococcus* (Saha and Sarkar 2021). Antimicrobial drug resistance is becoming a serious public health and economic concern. Bacteria may be immune to one or more of the antibiotics or more antimicrobial agents by nature, or they may develop resistance over time through various resistance mechanisms such as mutations in chromosome, plasmid, transposons and other biochemical pathways such as modification of target and interference with protein synthesis (Giedraitiene *et al* 2011).

Due to their high rates of consumption and horizontal gene transfer, antibiotic resistance has appeared across a variety of categories of antibiotics, including Beta-Lactamase and Aminoglycosides. MRSA (Methicillin-*Resistance* Staphylococcus aureus) and VRE (vancomycin *Resistance Enterococci*) are two bacteria that are highly resistant to antibiotics have been linked to pharmaceutical dose and resistance (Angela *et al*, 2010).

Resistance genes can be found on the chromosomes, plasmids, and transposons of bacteria. Drug inactivation/change, alterations in cell permeability, drug binding modification sites/targets lead to a reduction in Biofilm development and intracellular drug accumulation are all examples of mechanisms of resistance (Santajit *et al* 2016). Several enzymes found in bacteria can alter and break down antibiotics which are the reason for the rapid rise in AMR (Saha and Sarkar 2021).

Hydrolysis, group transfer, and redox processes are all chemical methods for antibiotic inactivation. Modification through acyl transfer, phosphorylation, glycosylation, nucleotide, ribosylation, and thiol transfer are among the most numerous types of group transfer, whereas hydrolysis is especially applicable clinically, especially when applied to beta-lactamase antibiotics. The ability of enzymes that physically convert antibiotics to actively reduce drug concentrations in the natural vicinity is a distinguishing property of these mechanisms. As a result, when evaluating innovative approaches to anti-infective therapy, they pose a unique challenge for researchers and physicians (Wright 2005).

Inhibition of cell wall building and folate metabolism, as well as interference with nucleic acid synthesis and ribosome binding are some of the other processes that produce bacterial resistance. To counter Bacteria have evolved genetic and pharmacological mechanisms in response to antimicrobial treatments. Mutation and the addition of new DNA are two genetic mechanisms. The resistance mechanisms of *enterococcal* and staphylococcal bacteria in therapeutic settings are very useful (McManus *et al* 1997).

Antibiotic-resistance microorganisms that are difficult or impossible to treat have spread to an increasing number of unexpected locations, posing a significant health risk. Many genes code for antibiotic resistance, with the majority of them able to switch between bacteria (Blair *et al* 2015).

In a study on antibiotic resistance concerns, it was discovered that the mass production of antibiotics in recent years has resulted in a lack of awareness of the threat of bacterial resistance. Because of the chromosomal alterations microscopic organisms have become resistant to antimicrobial treatment. Antibiotic resistance values have been fueled by the widespread use of antibiotics in local and emergency clinics,

suggesting that antibiotic program focus, greater sanitation, and the development of agents with improved antimicrobial activity should be used to combat bacterial resistance (Harlod Neu 1992).

In a study conducted in France to better understand the new system of antimicrobial obstruction in *Enterobacteriaceae* caused by porins, researchers discovered that multidrug resistance in *Enterobacter aerogenes* involves a decrease in external film penetrability linked to changes in uncharacterized porin. They suggested that the transformation is given the recognized role of this loop in defining the pore characteristics of porins; this clinical strain has acquired a unique resistance mechanism, with variations in porin channel function serving as another bacterial strategy for controlling b-lactam dispersion through porins (Emmanuelle *et al* 2001).

Some scientists based their evaluation on the executives of an antibiotic resistance database on the fact that the power of genetic change has not been completely realized in the field of antibiotic medication discovery and surveillance of new antibiotic-resistance bacteria. They have started to enhance such equipment using the Comprehensive Antibiotic Research Database (CARD). This platform will provide an informatics device that will help to remove the communication gaps between the health care, agriculture, and the environment in terms of antibiotic resistance (Andrew *et al* 2013).

5. Antibiotic Resistance in *Enterococcus* Species

When compared to other gram-positive bacteria, *Enterococcus* has a higher resistance to various antibiotics, although the reasons for this high resistance are unknown. However, their existence in the gastrointestinal tract may be the reason for them having to survive in a competent but potentially harmful habitat, and they may be more susceptible to antibiotics that travel through the gastrointestinal tract (Tannock *et al*, 2002).

Humans and many animals, including food-producing and companion animals, have *Enterococci* as natural occupants of the gut system. They have the ability to contaminate food, harm the environment, and infiltrate the food chain. In addition, *Enterococcus*, specifically *E. faecalis*, as well as *E. faecium* are the major

opportunistic pathogens that cause a variety of diseases. These microorganisms not only has built-in antimicrobial resistance mechanisms, but it also can acquire new ones (Torres *et al* 2018).

Enterococci do have potential to acquire resistance to practically most antibiotics used in medical care. The rise of the diseases as major nosocomial infections has been linked to an increase in antibiotic resistance among members of the genus. Antibiotic resistance mechanisms in *Enterococci* can be or horizontal transmission of resistance determinants-encoding genetic material (Hollenbeck *et al* 2012).

It is crucial to figure out whether newly discovered glycopeptide resistance from *Enterococci* can be therapeutically transmitted to Staphylococci. To prevent the spread of glycopeptide resistance, the use of vancomycin is strongly discouraged (McManus *et al* 1997).

The transferability of resistance genes was investigated in the 1970s. Later in the 1980s, transferable vancomycin resistance in *E. faecium* was described which opened a new door to research on transferable elements, their dissemination routes, and their environmental sustainability (Hegstad *et al*, 2010).

Enterococci have been found to have vancomycin-resistance Transposons with a unique collection of vancomycin resistance genes. *Enterococci* can replace the DDR of peptidoglycan with D-Ala-D-Lac when the vanA gene transposons Tn1546 are present, preventing vancomycin from attaching to their cell wall components. (Cui *et al* 2006). *Vancomycin-resistant Enterococcus* complicates the treatment and control of *Enterococcus*. *VRE* has developed resistance to all antibiotics used to treat vancomycin-susceptible *Enterococcus* (Osman *et al,* 2016).

Another cause of antimicrobial drug resistance is the widespread usage of antibiotic, can lead to the creation of resistance genes in pathogenic and commensal bacteria, as well as zoonotic bacteria (Lukasova.J. J *et al*, 2003). A recent study on poultry in Turkey revealed that approximately 25% of *E. faecalis* is MDR, i.e. resistance to erythromycin, tetracycline, and streptomycin (Kasimoglu-Dogru *et al*, 2010).

E. faecium and *E. faecalis* are the most frequent potentially pathogenic which induce hospital-acquired illnesses mostly in urinary and respiratory systems, according to

previous finding. In hospital isolates of *Enterococcus* species, antimicrobial resistance at multiple levels, including high levels, is common. Reserpine treatment reduces the MIC of ciprofloxacin, gatifloxacin, and levofloxacin in vitro by inhibiting the active efflux of these three Fluoroquinolones by *Enterococcus species*. Antibiotic resistance in *Enterococcus* species is connected towards the prevalence of the *enterococcal* multidrug - resistance efflux *ermA* gene. *Enterococcus* species prevalence and antibiotic resistance must be closely monitored to advice on *enterococcal* infection control and prevention (Jia *et al* 2014).

MDR *Enterococci* are a prevalent source of nosocomial infections and are a developing clinical concern. Through a variety of genetic processes, these organisms have gained resistance to nearly all antimicrobials currently employed in clinical treatment. Drug targ*et al*teration, therapeutic agent inactivation, efflux pump overexpression, and a complex cell envelope adaptive response all help MDR *Enterococci* survive in the human host and nosocomial environment (Miller *et al* 2014).

Another AMR study on broiler chicken in Canada discovered MDR in *E. faecalis* and *E. faecium* against antibiotics such as aminoglycosides, macrolides, tetracycline, streptogramins, bacitracin, and lincosamide (Diarra *et al*, 2010).

A study on AMR in poultry litter conducted in British Columbia (Canada) found that *Enterococcus* has varying levels of resistance to various antibiotics such as lincomycin (80.3 %), tetracycline (65.3 %), penicillin (61.1 %), ciprofloxacin (49.6 %), streptomycin (35.2 %), erythromycin (32.2 %), tylosin (31.4 %), and Synercid (quinupristin plus dalfopristin (0.8 %). Only 14% of the isolates tested positive for one antibiotic, but all other isolates tested positive for MDR (resistance to at least two antibiotics) (Furtula *et al*, 2013).

Extracellular Surface Proteins (Esp) play a key role in biofilm development and equip *Enterococcus* with resilience to harsh environmental conditions are one of many features in *Enterococcus* species that contribute to virulence. A recent study on samples taken from UTI patients at Tabriz University of Medical Sciences demonstrated the existence of a significant link between *Esp* pathogenicity and antibiotic-resistance genes in *Enterococcus* Species. *Esp* genes were found in 77.9% of

E. faecalis resistance to ampicillin, chloramphenicol, and tetracycline, and 76.1 % of *E. faecalis* resistance to vancomycin (Hossein and Ashraf, 2015).

According to a new study on the molecular epidemiology of antibiotic resistance, other resistance genes, and their resistance in *Enterococcus* species in South African poultry, *E. faecalis* exhibited 78% resistance to the *tetM* (tetracycline) gene and 80 % resistance to the *ermB* (erythromycin) gene, while *E. faecium* (take care of italics throughout the thesis) exhibited 86 % resistance to the *tetM* (Tetracycline) gene (Molechan *et al*, 2019).

According to a recent study on antibiotic resistance in *Enterococcus* and *Staphylococcus* conducted at the Department of Food Engineering at the University of Cukurova Adana in Turkey, *Enterococci* species showed 100% resistance to tetracycline, 75% erythromycin, vancomycin, and chloramphenicol resistance is 50%, and ciprofloxacin resistance is 50%. (Yurdakul *et al*, 2013). In a study conducted at the Hospital of Ningxia Medical University, Multidrug resistance *enterococcal* isolates were tested from clinical isolates of *Enterococcus* species. In *Enterococcus* species, the minimum inhibitory concentration (MIC) of three quinolones, ciprofloxacin, gatifloxacin, and levofloxacin, was discovered. Antimicrobial resistance in *E. faecalis* and *E. faecium* has been discovered to be substantially distinct. In *Enterococcus* species, reserpine therapy reduced resistance to ciprofloxacin, gatifloxacin, and levofloxacin. Aminoglycosides resistance genes *aac(6')-aph(2"), aph(3')-III, ant(6)-I and ant(2")-I* (77%), tetracycline resistance gene *tetM* (62%),erythromycin resistance gene *ermB*(26%), vancomycin resistance gene *vanA* (13%), and *enterococcal* multidrug resistance efflux gene *emeA* (36%) were detected in *Enterococcus* species isolated from clinical samples (Jia *et al* 2014).

According to recent review studies on antibiotic resistance in chicken, *Enterococci* showed 80% resistance to the antibiotic tetracycline, 59% resistance to erythromycin, and 34% resistance to nitrofurantoin (Apata, 2009).

1.6 Isolation and identification of *Enterococcus*

There are different methods used globally for the isolation and identification of *Enterococcus* species. Approximately 77% recovery of *E. faecalis and E. faecium* is

possible with the help of *API 20 STREP* (Devries *et al* 1995). Slanetz and Bartley Agar is differential media used for the isolation of most of the *Enterococcus* species including *E. faecium and E. faecalis* (Valenzuela *et al* 2010). *E. faecalis and E. faecium* can be detected by many biochemical tests including Gram's Staining (Gram positive), catalase test (catalase negative); grow in the media containing 6.5%NaCl and motility test (non-motile) (Domig *et al* 2003). *Enterococci* can be preserved in brain heart infusion broth with 20% glycerol at -70° C (Manero and Blanch 2020). Since *Enterococcus* is a pathogen of nosocomial significance, it is important to study antibiotic resistance in this pathogen. This will help suggest appropriate antibiotic therapy for infections produced by these bacteria.

6. Objectives

- To study the prevalence of *Enterococcus* species i.e. *E. faecalis* and *E. faecium* in the feces of commercial poultry.
- To investigate antibiotic resistance and resistance genes in *Enterococcus* species isolated from the feces of commercial poultry.

CHAPTER 2

METHODOLOGY

2.1. Study area

This study was conducted at the National Reference Laboratory for Poultry Diseases (NRLPD), National Agricultural Research Center (NARC) Islamabad and at the Department of Zoology, Quaid-i-Azam University Islamabad. In the current study fecal samples from the caeca of apparently healthy birds were collected from randomly selected chicken slaughtering shops in live bird markets of nine selected cities of Pakistan. The samples were collected by the provincial coordinating units and received at NRLPD from 16th-July- 2021 to 10th-Oct- 2021. A total of 254 samples were received and analyzed for presence of *Enterococcus*. The number of samples collected from different regions/cities were as following: Karachi (52), Islamabad (24), Rawalpindi (26), Mansehra (30), Muzaffarabad (30), Peshawar (30), Quetta (15), Gilgit (20) and Lahore (27).

For sample collection, the caecal material of 3-5 freshly slaughtered commercial broiler birds were collected aseptically using sterile scissors and forceps and pooled in pre-sterilized falcon tubes. The samples were stored at 4^{o}C in sampling boxes supplied with ice packs and shipped to NRLPD at refrigeration within 24 hours of the 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. 24233914, 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. After incubation, a loopful of samples was streaked on a differential medium that is Slanetz and Bartley Agar (Cat. No.ESLA051119058, BIOLAB) 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 sub-cultured on Nutrient Agar (Cat. No. 2429258,OXOID) and incubated for 24 hours at 37°C and stock solutions of the culture were made by using 70% Glycerol from Nutrient Agar. Then Grams' staining, motility and different biochemical tests were performed such as catalase were performed following standard protocols (FAO Regional Antimicrobial Resistance Monitoring and Surveillance Guideline, Vol 1).

2.3.1 Grams' staining

A drop of normal saline 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 neither too thin. Slide was heat fixed by passing over the flame thrice, and was stained by Grams' staining as follow: few drops of crystal violet were poured on the slide and were left for 1min and the slide was washed with distilled water. Grams iodine was added, slide was washed after 1min, decolorizer was added and after 30sec slide was washed at the end safranine was added and was washed after 2 min and the slides were observed under microscope at 100X lens under oil immersion.

2.3.2 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.

2.3.3 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.

2.4 Antibiotic Susceptibility Test

Antimicrobial susceptibility test was done against 17 antibiotics. For AST, the Disk Diffusion method was used, in which 90 mm Petri plate containing 25ml 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 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 the table 2.4.1.

Table 2.4. 1Reference values of AST results

Antibiotics	**Sensitivity (mm)**	**Intermediate (mm)**	**Resistance (mm)**
Ampicillin	**17**	**15**	**16**
Teicoplanin	**14**	**11-13**	**10**
Norfloxacin	**17**	**13-16**	**12**
Vancomycin	**17**	**15-16**	**14**
Chloramphenicol	**18**	**13-17**	**12**
Ciprofloxacin	**18**	**16-20**	**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 every city of Pakistan 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.

2.5 Molecular Characterization

2.5.1 Plasmid DNA Extraction

According to earlier study, the antibiotic resistance genes to be examined were plasmid-borne; hence plasmid DNA extraction was used. Most resistance thirty-one *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 Nutrient agar and incubated for 24 hours at 37° C. After incubation, 5ml distilled water was used 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 Thermo Scientific, GeneJET Plasmid Miniprep Kit (CAT. NO. K0502, Thermo Fisher 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 centre 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 next process.

2.6 Polymerase Chain Reaction (PCR)

After extraction plasmid DNA from 31 of the most resistance isolates, PCRs were performed for detection of antibiotic resistance genes. PCR recipe for *oqxA, tetM, tetC, tetG, and* genes are shown in table 2.6.1 while two multiplex PCR were done for *qnrA, qnrB, qnrS genes* table as shown in table 2.6.2, *tetO, tetS* as shown in table 2.6.3 and thermal cycles of all genes in table 2.6.4

Table 2.6.1 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 **(NH_4SO_4):**	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl
25mm MgCl2:	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 uM oqxA-F primer:	2.5 µl	2.5 µl	2.5 µl	1.0 µl	2.5 µl
10 uM oqxA-R 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. 2 PCR recipe for *qnrA, qnrB, qnrS* Genes

Reagents	*qnrA qnrB, qnrS* Genes
DNA:	5 µl
10X buffer **(NH_4SO_4)**:	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	**25 ul**

Table 2.6.3 PCR recipe for *tetO, tetS genes*

Reagents	***tetO, tetS***
DNA:	5 µl
10X buffer **(NH_4SO_4)**:	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 ul**

Table 2.6. 4Thermal Profiles of PCR

Genes	Initial denaturation	Denaturation	Annealing	Extension	Final Extension	Cycles	References
tetO,	95℃ for 5min	95℃ for 5	55℃ for	72℃	72℃ for	35	Aarestrup et

tetS		minutes,	60 sec	for 10 minutes	10min	cycles	al (2000).
tetL	95℃ for 5min	at 95℃ for 5 minutes	54℃ for 60 sec	72℃ for 3 minutes .	72℃ for 10min	35 cycles	Aarestrup et al (2000).
tetG	95℃ for 10 min	95℃ for 10 minutes	55℃ for 60 sec	72℃ for 2 minutes .	72℃ for 8min	35 cycles	Stanton and Humphrey (2003).
tetC	95℃ for 3min	at 95℃ for 3 minutes	68℃ for 30 sec	72℃ for 30 sec.	72℃ for 10min	40 cycles	Miranda et al (2003).
tetM	94℃ for 5min	at 94℃ for 3 minutes	45℃ for 60 sec	72℃ for 3 minutes	72℃ for 10min	35 cycles	Aarestrup et al (2000).
oqxA	94℃ for 5min	94℃ for 5 minutes	56℃ for 55 sec	72℃ for 10 minutes	72℃ for 10min	30 cycles	Qui et al (2019)
qnrA, qnrB, qnrS	Nil	at 94℃ for 45 sec	53℃ for 45 sec	72℃ for 60 sec	Nil	32 cycles	Robicsek et al (2006)

2.6.2 Gel Electrophoresis

Agarose gel electrophoresis of PCR products was performed on 1.2% agarose gel (Cat. No. AX-LE130, AXYGEN) using 50 bp DNA ladder (Cat.No.10416014, Thermo Fisher, Scientific) as a DNA size marker and visualized using a gel documentation system.

Table 2.6. 5 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
Fluoroquinolones	Norfloxacin	NOR	30
	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
Tetracyclines	Doxycycline	DO	30
	Minocycline	MH	30
	Tetracycline	TE	30
Nitrofurantoins	Nitrofurantoin	F	300

Table2.6.6 List of genes and primers

Gene	**Forward Primer**	**Product size (bp)**	**References**
***tetC*-F** ***tetC*-R**	CTTGAGAGCCTTCAACCCAG TGGTCGTCATCTACCTGCC	417	Miranda *et al* 2002
***tetG*-F** ***tetG*-R**	TTGTTTGAGAGCATTGCCTGC TTCAAGCCGGCTTGGAGAG	171	Stanton *et al* 2003
***tetL*-F** ***tetL*-R**	ATTACACTTCCGATTTCGG CATTTGGTCTTATTGGATCG	475	Aarestrup *et al* 2000
***tetM*-F**	GTTAAATAGTGTTCTTGGAG	657	,Aarestrup *et al* 2000

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

Chapter 3

Results

Results of the processed samples for *Enterococcus* are shown in table 3.1.1.

Table 3.1.1.Results of isolation of *Enterococcus* from fecal samples from different cities.

Cities	**No. of fecal samples received**	**Samples positive for *Enterococcus***	***E. faecium***	***E. faecalis***
Karachi	52	43	27	16
Rawalpindi	26	15	10	5
Islamabad	24	14	7	7
Lahore	27	17	17	0
Peshawar	30	20	20	0
Mansehra	30	25	15	10
Muzaffarabad	30	11	11	0
Quetta	15	8	0	8
Gilgit-Baltistan	20	7	0	7
Total	254	160	107 (67%)	53(33%)

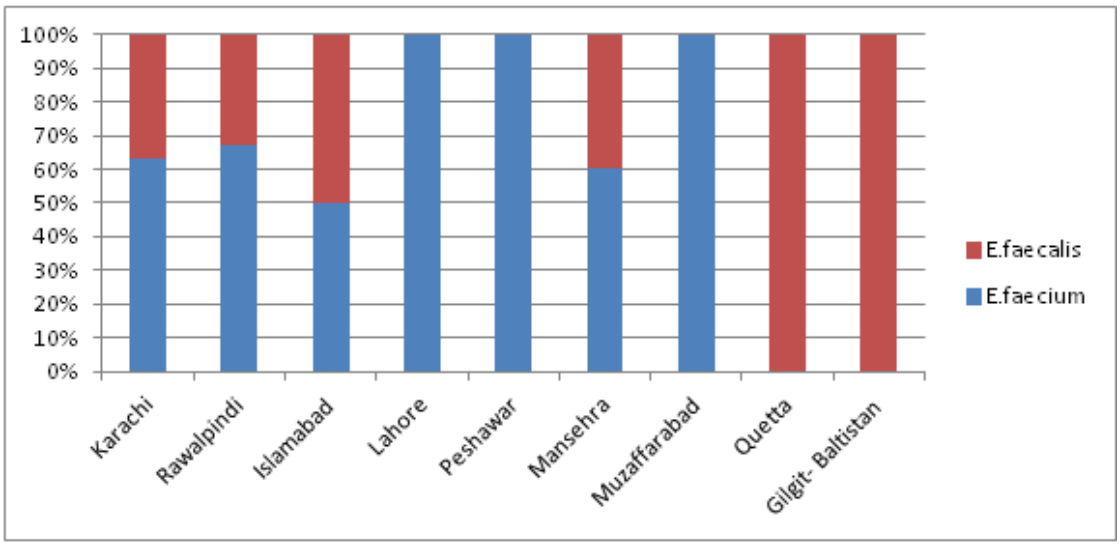

Fig 3.1.1. Distribution of *E. faecalis* and *E. faecium* in fecal samples from different cities.

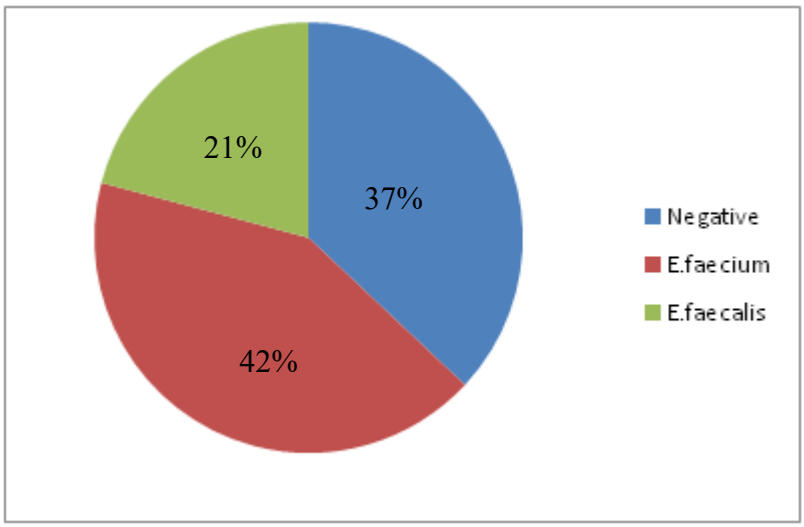

Fig 3.1.2 Pie graph representing %age of positive or negative fecal samples.

After inoculation of fecal samples, Brain heart infusion broth turned into a turbid and slightly dark brown color after incubation as shown in the Fig 3.1.3.

Fig 3.1.3 Brain Heart Infusion broth before and after inoculation of bacterial culture

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* as shown in Fig 3.1.4.

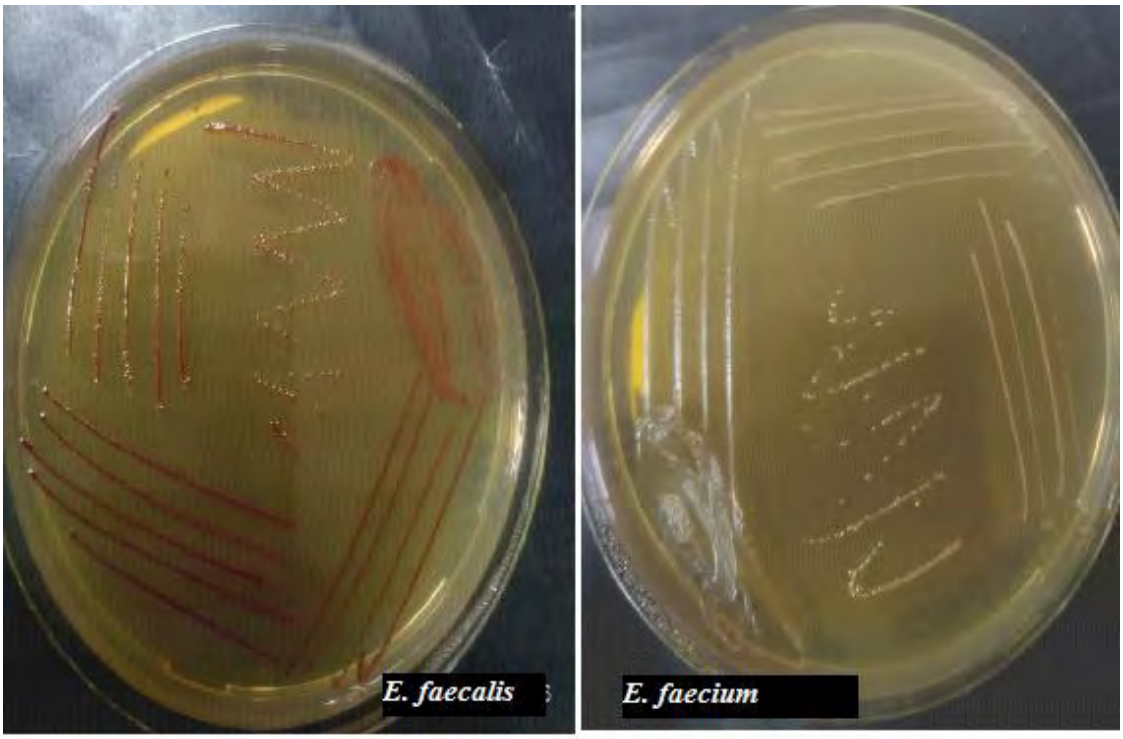

Fig 3.1. 4 Growth of *E. faecalis* and of *E. faecium* on SBA respectively

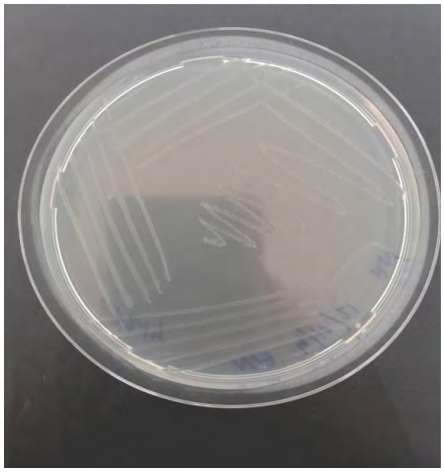

Fig 3.1. 5Growth of *Enterococcus* on Nutrient Agar

Then different tests were performed such as:

- Catalase that gave negative results for *Enterococcus* species as no bubbles were produced when a drop of H_2O_2 was put on slide containing colonies of *Enterococcus* as shown in Fig. 3.2.1

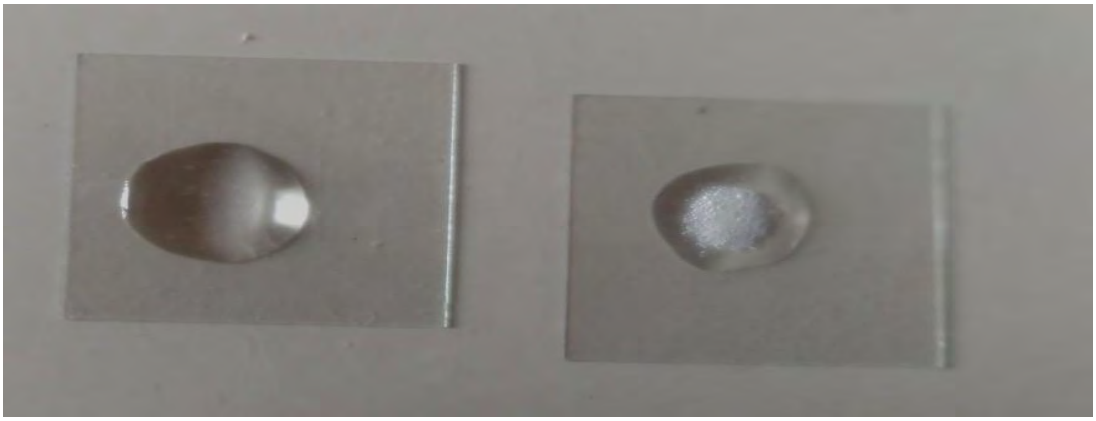

Fig 3.2.1. Catalase negative *Enterococcus* along with Catalase positive *Staphylococcus*

- Motility was analyzed by using SIM medium in which *Enterococcus* showed no motility while other bacterial species showed motility such as *Staphylococcus* that has also been used in the experiment as a QC as shown in the Fig. 3.2.2

Fig 3.2.2 Non-Motile *Enterococcus*

- Grams' staining: After Grams' staining purple colored Gram-positive Enterococci were clearly observed when slides were examined under microscope as shown in Fig. 3.2.3

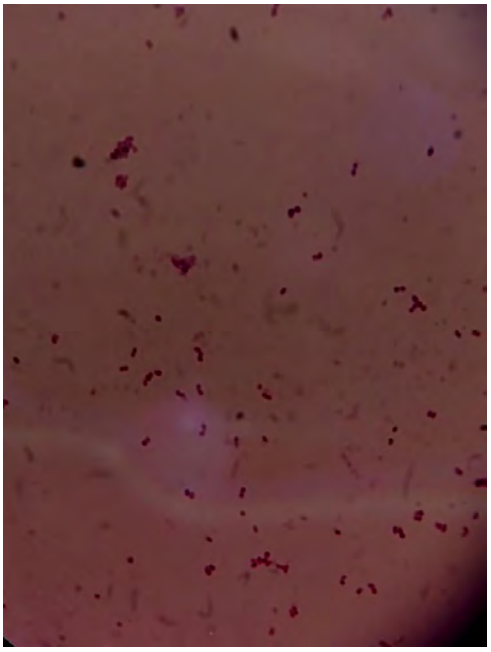

Fig 3.2. 3Gram-positive *Enterococcus*

Antimicrobial susceptibility test was done against 17 antibiotics that showed zone of inhibition in the pattern as shown in the Fig. 3.4;

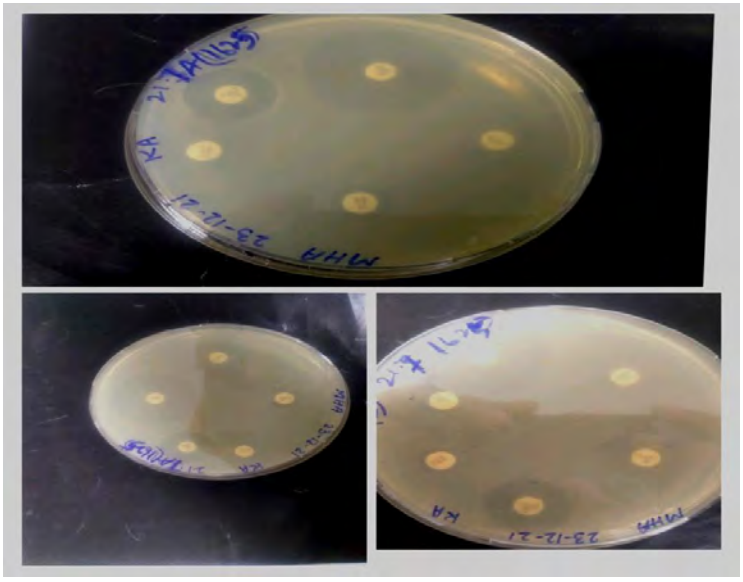

Fig 3.4. 1 Zone of inhibitions formed as result of different antibiotics.

3.5 Antibiotic Susceptibility Testing

Results of AST of *E. faecalis* and *E. faecium* are shown in tables 3.5.1 and table 3.5.2 respectively.

3.5.1 Results of AST of *E. faecalis*

All 53 *E. faecalis* isolates were resistant towards more than five antibiotics. The drugs for which the sensitivity rates were high were ampicillin (77%), nitrofurantoin (77%), fosfomycin (75%) and teicoplanin (60%) while the drugs to which the resistance profile were greater than 50% were erythromycin (98%), chloramphenicol (96%), tetracycline (94%), levofloxacin(92%), minocycline(79%), doxycycline (79%), ciprofloxacin (77%), quinupristin (77%) and norfloxacin (64%). Resistance, intermediate and sensitivity values of other antibiotics are shown below in table 3.5.1:

Table 3.5.1 Results of AST of 53 *E. faecalis* Isolates

Antibiotics	***Resistance* Isolates**	**Intermediate Isolates**	**Sensitive Isolates**
Ampicillin	11(21%)	1(2%)	41(77%)
Teicoplanin	2 (4%)	19(36%)	32(60%)
Norfloxacin	34(64%)	7(13%)	12(23%)
Vancomycin	12(22%)	22(42%)	19(36%)
Chloramphenicol	51(96%)	1(2%)	1(2%)
Ciprofloxacin	41(77%)	6(12%)	6(11%)
Rifampicin	18(34%)	30(57%)	5(9%)
Fosfomycin	7(13%)	6(12%)	40(75%)
Erythromycin	52(98%)	0(0%)	1(2%)
Linezolid	12(23%)	21(40%)	20(37%)
Levofloxacin	49(92%)	1(2%)	3(6%)
Doxycycline	42(79%)	7(13%)	4(8%)
Quinupristin	41(77%)	11(21%)	1(2%)
Penicillin	21(40%)	24(45%)	8(15%)
Minocycline	42(79%)	9(17%)	2(4%)
Tetracycline	50(94%)	0(0%)	3(6%)
Nitrofurantoin	6(11%)	6(12%)	41(77%)

3.5.2 Results of AST of *E. faecium*

All 107 *E. faecium* isolates were multidrug resistance. *E. faecium* was highly susceptible to fosfomycin (61%), teicoplanin (61%), and ampicillin (60%) while *E. faecium* has shown high resistance towards tetracycline (97%), erythromycin (94%), norfloxacin (90%), ciprofloxacin (86%), chloramphenicol (82%) and levofloxacin (82%), quinupristin (82%), doxycycline (80%), and minocycline (69%). Resistance, intermediate and sensitivity values of other antibiotics is shown below in table 3.5.2

Table 3.5.2 Results of AST of 107 *E. faecium* Isolates

Antibiotics	***Resistance* Isolates**	**Intermediate Isolates**	**Sensitive Isolates**
Ampicillin	28(26%)	15(14%)	64(60%)
Teicoplanin	10(9%)	32(30%)	65(61%)
Norfloxacin	96(90%)	3(3%)	8(7%)
Vancomycin	10(9%)	68(64%)	29(27%)
Chloramphenicol	88(82%)	10(9%)	9(8%)
Ciprofloxacin	92(86%)	9(8%)	6(6%)
Rifampicin	19(18%)	68(64%)	20(19%)
Fosfomycin	22(21%)	20(19%)	65(61%)
Erythromycin	101(94%)	3(3%)	3(3%)
Linezolid	26(24%)	28(26%)	53(50%)
Levofloxacin	88(82%)	9(8%)	10(9%)
Doxycycline	86(80%)	9(8%)	12(11%)
Quinupristin	88(82%)	9(8%)	10(9%)
Penicillin	52(49%)	29(27%)	26(24%)
Minocycline	74(69%)	25(23%)	8(7%)
Tetracycline	104(97%)	2(2%)	1(1%)
Nitrofurantoin	48(45%)	30(28%)	29(27%)

3.6.1 Results of PCRs for *tetM*, *tetL*, *tetO* genes

Out of 31 isolates *tetL* gene with 475bp was detected from 20 isolates, of which 12 resistance genes were detected from *E. faecium* while 8 were detected from *E. faecalis*.

Out of 31 isolates, *tetM* resistance genes with 657bp, were detected from 21 isolates of them 9 were detected from *E. faecium* while 12 were detected from *E. faecalis* and *tetO* resistance genes with 634bp was detected from only one *E. faecium* isolate and rest of tetracycline resistance genes were not detected after PCR as shown in fig. 3.6.1

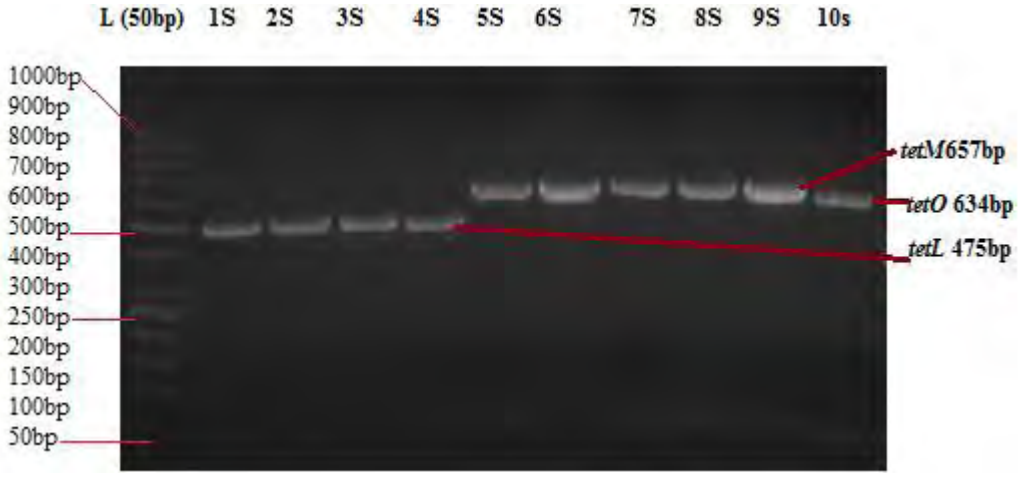

Fig 3.6.1. Agarose gel electrophoresis of PCR products for the detection of *tetL* gene (475bp), *tetO* gene (634bp) and *tetM* gene (657bp), L signifies DNA size marker (50bp DNA ladder, Thermo fisher Scientific)

3.6.2 Results of PCRs for *oqxA*, *qnrA*, *qnrB*, *qnrS* genes

Quinolones resistance genes were not detected from any isolate after PCR as shown in fig.3.6.2.

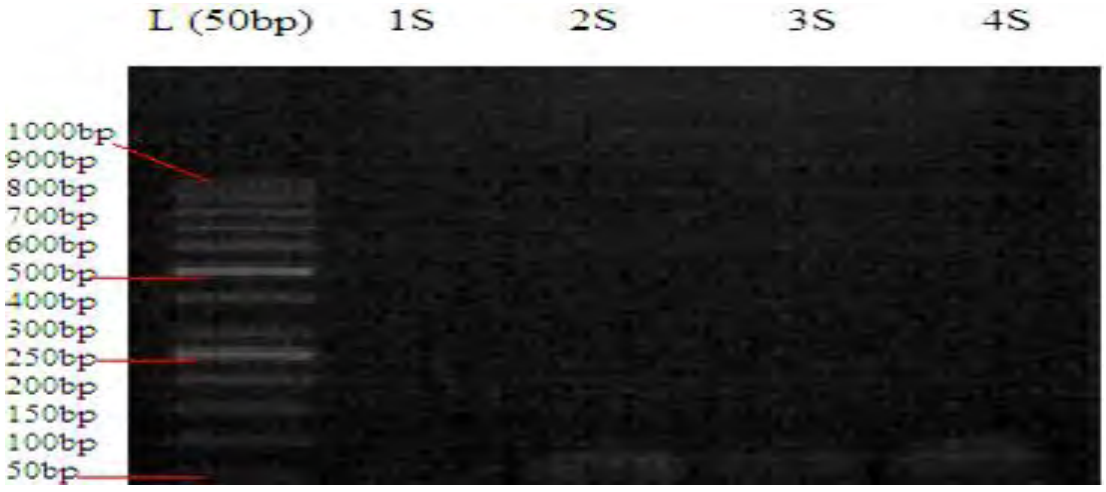

Fig 3.6.2Agarose gel electrophoresis of PCR product for detection of *oqxA (671bp)*, *qnrA (516bp)* , *qnrB (469bp)*, *qnrS (417bp)* genes, L signifies DNA size marker (50bp DNA ladder, Thermo fisher Scientific)

Table 3.6. 1 Number and percentage of resistance genes detected in *Enterococcus* species

S.No.	Gene	Positive *Enterococcus* Isolates Out of 31	Positive *E. faecium* Isolates Out of 31	Positive *E. faecalis* Isolates Out of 31
1	*tetC*	0	0	0
2	*tetG*	0	0	0
3	*tetL*	20(64.5%)	12(60%)	8(40%)
4	*tetM*	21(67.7%)	9(42.8%)	12(57%)
5	*tetO*	1(3.2%)	1(100%)	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 3.6.2 Antimicrobial Resistance Pattern and Occurrence of Resistance Genes in *E. faecium*

S.No.	Sample ID	Resistance pattern	No. of antibiotics	Genes detected
1.	21N-828	E, NOR, CIP, LEV,TE	5	*tetL,*
2.	21N-831	E, NOR, CIP, LEV, TE, DO, MH	7	*tetL, tetM,*
3.	21N-837	E, NOR, CIP, LEV, TE, DO, MH	7	*tetL, tetM,*

4.	21N-856	E, NOR, CIP, LEV, TE, DO, MH	7	*tetL, tetM, tetO*
5.	21N-864	E, CIP, LEV, TE, MH	5	*tetL, tetM,*
6.	21N-870	E, NOR, CIP, LEV, TE, MH	6	*tetL, tetM,*
7.	21N-983	E, NOR, CIP, TE, DO, MH	6	*tetL*
8.	21N-986	TE	1	*tetL, tetM*
9.	21N-987	E, NOR, CIP, LEV, TE, DO, MH	7	*tetM*
10.	21N-989	E, NOR, CIP, LEV, TE, DO, MH	7	
11.	21N-1013	E, NOR, CIP, LEV,TE, DO, MH	7	Nil
12.	21N-1017	E, NOR, CIP, LEV,TE, DO, MH	7	*tetL, tetM*
13.	21N-1018	E,NOR, CIP, LEV,TE, DO, MH	7	*tetL, tetM*
14.	21N-1076	E,CIP, LEV,TE, MH	5	Nil
15.	21N-1079	E,LEV,TE, DO, MH	5	*tetL*
16.	21N-1089	E,CIP, LEV,TE, DO, MH	6	*tetL*

Table 3.6.3 Antimicrobial Resistance Pattern and Occurrence of Resistance Genes in *E. faecalis*

S.No.	Sample ID	Resistance pattern	No. of antibiotics	Genes detected
1.	21N-999	TE, DO, MH	3	*tetL, tetM*
2.	21N-1002	E, NOR, CIP, LEV, TE, DO, MH	7	*tetL, tetM*
3.	21N-1042	E, NOR, CIP, LEV,TE, MH	6	*tetL, tetM*
4.	21N-1052	E, NOR, CIP, LEV,TE, MH	6	*tetM*
5.	21N-1060	E,NOR, CIP, LEV,TE, DO	6	*tetM*
6.	21N-1061	E,CIP, LEV,TE, MH		*tetM, tetL*
7.	21N-1062	E,CIP, LEV,TE, DO, MH	6	*tetM, tetL*
8.	21N-1068	E,NOR, CIP, LEV,TE, DO, MH	7	*tetM, tetL*
9.	21N-1091	E,NOR, CIP, LEV,TE, MH	6	*tetM*
10.	21N-1094	E,NOR, CIP, LEV,TE, DO, MH	7	*tetM*
11.	21N-1106	E,CIP, LEV,TE, DO, MH	6	*tetL, tetM*
12.	21N-1111	E, LEV,TE, DO, MH	5	*tetL, tetM*
13.	21N-1148	E,NOR, CIP, LEV,DO, MH	6	Nil
14.	21N-1149	E,LEV, DO	3	Nil
15.	21N-1153	E,NOR, CIP, LEV,TE, DO, MH	7	Nil

Chapter 4

Discussion

Enterococcus is a Gram's positive, catalase-deficient, facultative anaerobic cocci found in the guts of humans and other animals, including chickens (Fisher and Philips 2009). It was once thought to be a typical gut flora, but in recent years it has emerged as a major source of nosocomial infections and a variety of other illnesses, including endocarditis, urinary tract infections, *Enterococcal* bacteremia, CNS infections, and newborn infections (Murray 2000; Sood *et al* 2008; Drisoll and Crank 2015). 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, fecal samples (254) from nine different cities of Pakistan were analyzed to investigate the prevalence of *E. faecium* and *E. faecalis* in commercial poultry. Phenotypic characterization revealed that *E. faecium* was 67% prevalent while *E. faecalis* was least abundant with 33%, prevalence is similar as compared to previous studies i.e. prevalence of *E. faecium* is higher than *E.faecalis i.e. E. faecium* (76%) and *E. faecalis* (27%) in poultry environment has been reported in Karachi (Ali *et al* 2013*).* 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). But the percentage of prevalence of *E.faecium* is lower than the percentages of prevalence found in previous studies.

Extensive use of antibiotics and growth promoters for the excessive production of poultry and white meat, has led to increase in AMR (Nhung *et al* 2017). The present study was also aimed to investigate antibiotic sensitivity of *Enterococcus* isolates in poultry of different cities of Pakistan. In this study phenotypic and genotypic characterization was done with the help of AST and PCR respectively. AST was done against 17 antibiotics, which revealed that MDR exists in all *Enterococcus isolates*. *Enterococcus* has shown resistance against 9 out 17 antibiotics which were

erythromycin followed by chloramphenicol, norfloxacin, ciprofloxacin, levofloxacin, quinupristin, tetracycline, minocycline, doxycycline and quinupristin. Relatable work has also been done in which *Enterococcus* were isolated from commercial poultry meat, which revealed 27% resistance toward quinupristin while there was no resistance shown in poultry meat towards linezolid and vancomycin (Hayes *et al* 2004) as in our research *Enterococcus* has not shown any resistance against linezolid and vancomycin. Vancomycin susceptibility in *Enterococci* isolated from cloacal samples of poultry has also been observed in Nigeria by Ngbede *et al* (2017).

In current research antibiotics resistance against erythromycin very high, as highest profiles of resistance toward erythromycin and ampicillin in *Enterococci* isolated from poultry cloacal samples has also been shown in a study conducted in Nigeria (Ngbede *et al* 2016) but ampicillin resistance was not found in our research. Erythromycin resistance has also been observed in a recent research conducted at the University of Karachi on AMR in *Enterococcus* species isolated from poultry environment (Ali *et al* 2014). In our research *E. faecalis* and *E. faecium* have shown same resistance and sensitivity values towards all antibiotics while in a study conducted at the University of Maryland *E. faecalis* (42%) and of *E. faecium* (84%), isolated from litter, feed and water samples of conventional poultry have shown MDR (Sapkota *et al* 2011). Linezolid was one of those antibiotics for which *Enterococcus* species showed least resistance in our study and also in another research its resistance profile was 0.79% in *E. faecium* and 1.22% in *E. faecalis* isolated from chicken, pig and cattle litter and carcasses (Na *et al* 2020).

In the present study prevalence of resistance genes in *Enterococcus* species for tetracyclines and quinolones were detected by PCR. The results revealed that, out of six tetracycline resistance genes two were abundantly present i.e. *tetM* gene was present in 21 isolates while *tetL* gene was present in 20 isolates out of 31 and *tetO* gene was present in only one *E. faecium* isolate, while the rest of tetracycline resistance genes were not detected in PCR. Our results are consistent with previous studies in which, tetracycline resistance gene *tetM* has also been detected in *Enterococci* isolated from poultry droppings, caeca and cloacal samples from Lahore (Saleem *et al* 2018). In our study *tetM* was more prevalent than tetL same as found in previous studies in which, 95% of *tetM* genes were observed in *Enterococci* isolated

from poultry in Denmark while *tetL* genes were observed in 33% in *Enterococci*, isolated from retail meat, healthy and infected chicken samples, 38% *tetO* genes were detected (Aarestrup *et al.,* 2000) but in the current study *tetO* gene was detected in only one *E. faecium* (3.22%) isolate.

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 (Amin *et al* 1999).

Conclusion

Prevalence of *Enterococcus* species *E. faecium* and *E. faecalis* in feces of chicken from commercial poultry was studied. The prevalence of *Enterococcus* was found to be as follows: Karachi (82.8%), Mansehra (83.3%), Peshawar (66.6%), Lahore (62.9%), Islamabad (58.3%), Rawalpindi (57.7%), Quetta (53.3%), Muzaffarabad (36.6%), and Gilgit (35%). Amongst these *Enterococcus* isolates, prevalence rates of *E. faecalis* and *E. faecium* were 33% and 67% respectively. Results of AST showed that *E. faecalis* and *E. faecium* isolates were resistant to various groups of antibiotics, including tetracyclines, macrolides and quinolones. However, only tetracyclines resistance genes *tetM* (67.7%), *tetL* (64.5%) and *tetO* (3.22%) were detected by the PCRs. On the other hand, no quinolones resistance genes were found in any of the *Enterococcus* isolates. While prescribing treatment for infections caused by the *Enterococci* in humans, it is important to avoid those antibiotics against which both enterococcal species have shown high rates of resistance. Furthermore, since 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 in order to find out the mechanisms responsible for antibiotic resistance in the local enterococcal isolates.

REFERENCES

Aarestrup, F. M., Agerso, Y., Gerner–Smidt, P., Madsen, M., & Jensen, L. B. (2000). Comparison of antimicrobial resistance phenotypes and resistance genes in Enterococcus faecalis and Enterococcus faecium from humans in the community, broilers, and pigs in Denmark. *Diagnostic Microbiology and Infectious Disease*, *37*(2), 127-137.

Abdallah, M., & Al-Saafin, M. (2019). Overview of prevalence, characteristics, risk factors, resistance, and virulence of *Vancomycin- Resistant Enterococci* in Saudi Arabia. *Microbial Drug Resistance*, *25*(3), 350-358.

Akhi, M. T., Farzaneh, F., & Oskouei, M. (2009). Study of *enterococcal* susceptibility patterns isolated from clinical specimens in Tabriz, Iran. *Pakistan Journal of Medical Sciences*, *25*(2), 211-216.

Amaro, P., Ferreira, J., Viegas, R., Cardoso, A., Correia, J., & Maurício, H. (2020). Multifocal joint infection caused by *Enterococcus gallinarum*. *Modern Rheumatology Case Reports*, 1-4.

Apata, D. F. (2009). Antibiotic resistance in poultry. *International Journal of Poultry Science*, *8*(4), 404-408.

Bashir, M. F., Qazi, J. I., Ahmad, N., & Riaz, S. (2008). Diversity of urinary tract pathogens and drug resistance isolates of *Escherichia coli* in different age and gender groups of Pakistanis *Tropical Journal of Pharmaceutical Research*, *7*(3), 1025-1031

Blair, J. M., Webber, M. A., Baylay, A. J., Ogbolu, D. O., & Piddock, L. J. (2015). Molecular mechanisms of antibiotic resistance. *Nature Reviews Microbiology*, *13*(1), 42-51.

Brede, D. A., Snipen, L. G., Ussery, D. W., Nederbragt, A. J., & Nes, I. F. (2011). Complete genome sequence of the commensal *E. faecalis* 62, isolated from a healthy Norwegian infant. *Journal of Bacteriology*, 193(9)

Castillo-Rojas, G., Mazari-Hiríart, M., de León, S. P., Amieva-Fernández, R. I., Agis-Juárez, R. A., Huebner, J., & López-Vidal, Y. (2013). Comparison of *E. faecium* and *E. faecalis* strains isolated from water and clinical samples: antimicrobial susceptibility and genetic relationships. *Public Library of Science*, *8*(4), 459-491.

CLSI, Performance standards for antimicrobial susceptibility testing, 30th ed. CLSI supplement M100.Wayne, PA: *Clinical and Laboratory Standards Institute;* 2020.

Cui, L., Iwamoto, A., Lian, J. Q., Neoh, H. M., Maruyama, T., Horikawa, Y., & Hiramatsu, K. (2006). Novel mechanism of antibiotic resistance originating in vancomycin-intermediate Staphylococcus aureus. *Antimicrobial Agents and Chemotherapy*, *50*(2), 428-438.

Davies, J. (2006). Where have all the antibiotics gone?. *Canadian Journal of Infectious Diseases and Medical Microbiology*, *17*(5), 287-290.

Davies, J., & Davies, D. (2010). Origins and evolution of antibiotic resistance. *Microbiology and Molecular Biology Reviews*, *74*(3), 417-433.

Dé, E., Baslé, A., Jaquinod, M., Saint, N., Malléa, M., Molle, G., & Pagès, J. M. (2001). A new mechanism of antibiotic resistance in Enterobacteriaceae induced by a structural modification of the major porin. *Molecular Microbiology*, *41*(1), 189-198.

Devriese, L. A., Pot, B., Van Damme, L., Kersters, K., & Haesebrouck, F. (1995) Identification of *Enterococcus* species isolated from foods of animal origin *International Journal of Food Microbiology*, *26*(2), 187-197.

Devriese, L., Baele, M. A. R. G. O., & Butaye, P. A. T. R. I. C. K. (2006). The genus *Enterococcus*: taxonomy. *Prokaryotes*, *4*, 163-174.

Diarra, M. S., Rempel, H., Champagne, J., Masson, L., Pritchard, J., & Topp, E. (2010). Distribution of antimicrobial resistance and virulence genes in *Enterococcus spp.* and characterization of isolates from broiler chickens. *Applied and Environmental Microbiology*, *76*(24), 8033-8043.

Dolka, B., Chrobak-Chmiel, D., Makrai, L., & Szeleszczuk, P. (2016). Phenotypic and genotypic characterization of *Enterococcus* cecorum strains associated with infections in poultry. *BMC Veterinary Research*, *12*(1), 1-13.

Dolka, B., Czopowicz, M., Chrobak-Chmiel, D., Ledwoń, A., & Szeleszczuk, P. (2020). Prevalence, antibiotic susceptibility and virulence factors of *Enterococcus* species in racing pigeons (Columba livia f. domestica). *BMC Veterinary Research*, *16*(1), 1-14.

Dolka, B., Gołębiewska-Kosakowska, M. A. R. I. O. L. A., Krajewski, K., Kwieciński, P., Nowak, T., Szubstarski, J., ... & Szeleszczuk, P. (2017). Occurrence of

Enterococcus spp. in poultry in Poland based on 2014–2015. *Medycyna Weterynaryjna, 73*, 193-256.

Domig, K. J., Mayer, H. K., & Kneifel, W. (2003) Methods used for the isolation, enumeration, characterisation and identification of *Enterococcus spp.*: 2. Pheno- and genotypic criteria. *International Journal of Food Microbiology*, *88*(2-3), 165-188

El Amin, N., Jalal, S., & Wretlind, B. (1999) Alterations in GyrA and ParC associated with fluoroquinolone resistance in *Enterococcus faecium*. *Antimicrobial Agents and Chemotherapy*, *43*(4), 947-949.

FAO, Monitoring and surveillance of antimicrobial resistance in bacteria from healthy food animals intended for consumption, 85 pages, *Food and Agriculture Organization of the United Nations*. 2019

Flores-Mireles, A. L., Walker, J. N., Caparon, M., & Hultgren, S. J. (2015). Urinary tract infections: epidemiology, mechanisms of infection and treatment options. *Nature Reviews Microbiology*, *13*(5), 269-284.

Furtula, V., Jackson, C. R., Farrell, E. G., Barrett, J. B., Hiott, L. M., & Chambers, P. A. (2013). Antimicrobial resistance in *Enterococcus spp.* isolated from environmental samples in an area of intensive poultry production. *International Journal of Environmental Research and Public Health*, *10*(3), 1020-1036.

Giedraitienė, A., Vitkauskienė, A., Naginienė, R., & Pavilonis, A. (2011). Antibiotic resistance mechanisms of clinically important bacteria. *Medicina*, *47*(3), 19.

Hayes, J. R., English, L. L., Carr, L. E., Wagner, D. D., & Joseph, S. W. (2004). Multiple-antibiotic resistance of *Enterococcus spp.* isolated from commercial poultry production environments. *Applied and Environmental Microbiology*, *70*(10), 6005-6011

Hayes, J. R., English, L. L., Carter, P. J., Proescholdt, T., Lee, K. Y., Wagner, D. D., & White, D. G. (2003). Prevalence and antimicrobial resistance of *Enterococcus* species isolated from retail meats. *Applied and Environmental Microbiology*, *69*(12), 7153-7160.

Hegstad, K., Mikalsen, T., Coque, T. M., Werner, G., & Sundsfjord, A. (2010). Mobile genetic elements and their contribution to the emergence of antimicrobial resistant *E. faecalis* and *E. faecium*. *Clinical Microbiology and Infection*, *16*(6), 541-554

Hollenbeck, B. L., & Rice, L. B. (2012). Intrinsic and acquired resistance mechanisms in *Enterococcus*. *Virulence*, *3*(5), 421-569.

Hussain, A., M., & Abbas, Z. (2016) Prevalence of *Enterococcus faecalis* mediated UTI and its current antimicrobial susceptibility pattern in Lahore, Pakistan. *Journal of Pakistan Medical Association*, *66*(10), 1232-1236.

Hussain, J., Rabbani, I., Aslam, S., & Ahmad, H. A. (2015). An overview of poultry industry in Pakistan. *World's Poultry Science Journal*, *71*(4), 689-700.

Jia, W., Li, G., & Wang, W. (2014). Prevalence and antimicrobial resistance of *Enterococcus* species: a hospital-based study in China. *International Journal of Environmental Research and Public Health*, *11*(3), 3424-3442.

Kanematsu, E., Deguchi, T., Yasuda, M., Kawamura, T., Nishino, Y., & Kawada, Y. (1998) Alterations in the GyrA subunit of DNA gyrase and the ParC subunit of DNA topoisomerase IV associated with quinolone resistance in *Enterococcus faecalis*. *Antimicrobial Agents And Chemotherapy*, *42*(2), 433-435.

Karasawa, Y., Kato, J., Kawamura, S., Kojima, K., Ohki, T., Seki, M., ... & Toda, N. (2021). Risk factors for acute cholangitis caused by *E. faecalis* and *E. faecium*. *Gut and Liver*, *15*(4), 616.

Kasimoglu-Dogru, A., Gencay, Y. E., & Ayaz, N. D. (2010). Prevalence and antibiotic resistance values of *Enterococcus* species in chicken at slaughter level; absence of vanA and vanB genes in E. faecalis and E. faecium. *Research in Veterinary Science*, *89*(2), 153-158.

Kateete, D. P., Edolu, M., Kigozi, E., Kisukye, J., Baluku, H., Mwiine, F. N., & Najjuka, C. F. (2019). Species, antibiotic susceptibility profiles and van gene frequencies among *Enterococci* isolated from patients at Mulago National Referral Hospital in Kampala, Uganda. *BMC Infectious Diseases*, *19*(1), 1-9.

Ma, X., Zhang, F., Bai, B., Lin, Z., Xu, G., Chen, Z., & Yu, Z. (2021). Linezolid Resistance in *E. faecalis* Associated With Urinary Tract Infections of Patients in a Tertiary Hospitals in China: Resistance Mechanisms, Virulence, and Risk Factors. *Frontiers in Public Health*, *9*,50.

Maasjost, J., Mühldorfer, K., de Jäckel, S. C., & Hafez, H. M. (2015). Antimicrobial susceptibility patterns of *E. faecalis* and *E. faecium* isolated from poultry flocks in Germany. *Avian Diseases*, *59*(1), 143-148.

Manero, A., & Blanch, A. R. (1999). Identification of *Enterococcus spp.* with a biochemical key *Applied and Environmental Microbiology*, *65*(10), 4425-4430

McArthur, A. G., Waglechner, N., Nizam, F., Yan, A., Azad, M. A., Baylay, A. J., ... & Wright, G. D. (2013). The comprehensive antibiotic resistance database. *Antimicrobial Agents and Chemotherapy*, *57*(7), 3348-3357.

McManus, M. C. (1997). Mechanisms of bacterial resistance to antimicrobial agents. *American Journal of Health-System Pharmacy*, *54*(12), 1420-1433.

Miller, W. R., Munita, J. M., & Arias, C. A. (2014). Mechanisms of antibiotic resistance in *Enterococci* . *Expert Review of Anti-Infective Therapy*, *12*(10), 1221-1236.

Miranda, C. D., Kehrenberg, C., Ulep, C., Schwarz, S., & Roberts, M. C. (2003). Diversity of tetracycline resistance genes in bacteria from Chilean salmon farms. *Antimicrobial Agents and Chemotherapy*, *47*(3), 883-888.

Molechan, C., Amoako, D. G., Abia, A. L. K., Somboro, A. M., Bester, L. A., & Essack, S. Y. (2019). Molecular epidemiology of antibiotic-resistant *Enterococcus spp.* from the farm-to-fork continuum in intensive poultry production in KwaZulu-Natal, South Africa. *Science of the Total Environment*, *692*, 868-878.

Mylonakis, E., & Calderwood, S. B. (2001). Infective endocarditis in adults. *New England Journal of Medicine*, *345*(18), 1318-1330.

Na, S. H., Moon, D. C., Kim, M. H., Kang, H. Y., Kim, S. J., Choi, J. H., ... & Lim, S. K. (2020). Detection of the phenicol–oxazolidinone resistance gene poxta in *Enterococcus faecium* and *Enterococcus faecalis* from food-producing animals during 2008–2018 in Korea. *Microorganisms*, *8*(11), 1839

Neu, H. C. (1992). The crisis in antibiotic resistance. *Science*, *257*(5073), 1064-1073.

Ngbede, E. O., Raji, M. A., Kwanashie, C. N., & Kwaga, J. K. P. (2017). Antimicrobial resistance and virulence profile of *Enterococci* isolated from poultry and cattle sources in Nigeria. *Tropical Animal Health and Production*, *49*(3), 451-458.

O'Dea, M., Sahibzada, S., Jordan, D., Laird, T., Lee, T., Hewson, K., ... & Abraham, S. (2019). Genomic, antimicrobial resistance and public health insights into *Enterococcus spp.* from Australian chickens. *Journal of Clinical Microbiology*, *57*(8).

Ortiz, A., Banks, H. T., Castillo-Chavez, C., Chowell, G., & Wang, X. (2010). A discrete events delay differential system model for transmission of Vancomycin-resistant

Enterococcus (VRE) in hospitals. *Journal of Inverse and Ill-Posed Problems*, 787-821.

Qin, X., Galloway-Peña, J. R., Sillanpaa, J., Roh, J. H., Nallapareddy, S. R., Chowdhury, S., ... & Murray, B. E. (2012). Complete genome sequence of *E. faecium* strain TX16 and comparative genomic analysis of *E. faecium* genomes. *BMC Microbiology*, *12*(1), 1-20.

Qiu, Jianhua, Zhiyu Jiang, Zijing Ju, Xiaonan Zhao, Jie Yang, Huijun Guo, and Shuhong Sun. "Molecular and phenotypic characteristics of Escherichia coli isolates from farmed minks in Zhucheng, China." *Biomed Research International* 2019 (2019).

Raza, T., Ullah, S. R., Mehmood, K., & Andleeb, S. (2018). Vancomycin resistant *Enterococci* : A brief review. *Journal of Pakistan Medical Association*, *68*(5), 768-772.

Robicsek, A., Strahilevitz, J., Sahm, D. F., Jacoby, G. A., & Hooper, D. C. (2006). qnr prevalence in ceftazidime-resistant Enterobacteriaceae isolates from the United States. *Antimicrobial Agents and Chemotherapy*, *50*(8), 2872-2874.

Saha, M., & Sarkar, A. (2021). Review on Multiple Facets of Drug Resistance: A Rising Challenge in the 21st Century. *Journal of Xenobiotics*, *11*(4), 197-214.

Saleem, N., Nawaz, M., Ghafoor, A., Javeed, A., Mustafa, A., Yousuf, M. R., & Khan, I. (2018). Phenotypic and Molecular Analysis of Antibiotic Resistance in Lactobacilli of Poultry Origin from Lahore, Pakistan. *Pakistan Veterinary Journal*, *38*(4).

Sanlibaba, P., Tezel, B. U., & Senturk, E. (2018). Antimicrobial resistance of *Enterococcus* species isolated from chicken in Turkey. *Korean Journal for Food Science of Animal Resources*, *38*(2), 391.

Santajit, S., & Indrawattana, N. (2016). Mechanisms of antimicrobial resistance in ESKAPE pathogens. *Biomed Research International, 2016.*

Stanton, T. B., & Humphrey, S. B. (2003). Isolation of tetracycline-resistant Megasphaera elsdenii strains with novel mosaic gene combinations of tet (O) and tet (W) from swine. *Applied and Environmental Microbiology*, *69*(7), 3874-3882.

Stepien-Pysniak, D., Hauschild, T., Rozanski, P., & Marek, A. (2017). MALDI-TOF mass spectrometry as a useful tool for identification of *Enterococcus* spp. from wild birds and differentiation of closely related species. *Journal of Microbiology and Biotechnology*, *27*(6), 1128-1137.

Tannock, G. W., & Cook, G. (2002). *Enterococci* as members of the intestinal. microflora of humans. *The Enterococci : Pathogenesis, Molecular Biology, and Antibiotic Resistance*, 101-132.

Thu, W. P., Sinwat, N., Bitrus, A. A., Angkittitrakul, S., Prathan, R., & Chuanchuen, R. (2019). Prevalence, antimicrobial resistance, virulence gene, and class 1 integrons of *Enterococcus* faecium and *Enterococcus faecalis* from pigs, pork and humans in Thai-Laos border provinces. *Journal of Global Antimicrobial Resistance*, *18*, 130-138.

Torres, C., Alonso, C. A., Ruiz-Ripa, L., León-Sampedro, R., Del Campo, R., & Coque, T. M. (2018). Antimicrobial resistance in *Enterococcus spp.* of animal origin. *Microbiology Spectrum*, *6*(4), 6-4.

Valenzuela, A. S., Benomar, N., Abriouel, H., Cañamero, M. M., & Gálvez, A. (2010) Isolation and identification of *Enterococcus faecium* from seafoods: antimicrobial resistance and production of bacteriocin-like substances. *Food Microbiology*, *27*(7), 955-961.

Wright, G. D. (2005). Bacterial resistance to antibiotics: enzymatic degradation and modification. *Advanced Drug Delivery Reviews*, *57*(10), 1451-1470.

Yilema, A., Moges, F., Tadele, S., Endris, M., Kassu, A., Abebe, W., & Ayalew, G. (2017). Isolation of *Enterococci,* their antimicrobial susceptibility patterns and associated factors among patients attending at the University of Gondar Teaching Hospital. *BMC Infectious Diseases*, *17*(1), 1-8.

Zheng, J. X., Li, H., Pu, Z. Y., Wang, H. Y., Deng, X. B., Liu, X. J., & Yu, Z. J. (2017). Bloodstream infections caused by *Enterococcus* spp: A 10-year retrospective analysis at a tertiary hospital in China. *Journal of Huazhong University of Science and Technology (Medical Sciences)*, *37*(2), 257-263.