Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2022.035

RESEARCH ARTICLE

# Molecular Characterization of Antibiotic Resistance in Poultry Gut Origin *Enterococci* and Horizontal Gene Transfer of Antibiotic Resistance to *Staphylococcus aureus*

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ARTICLE HISTORY (22-010)

Received: January 03, 2022
Revised: March 31, 2022
Accepted: May 07, 2022
Published online: May 28, 2022

**Key words:**
Antibiotic resistance
transferability
Ciprofloxacin
*Enterococcus faecalis*
*Enterococcus faecium*
Vancomycin Resistant
Enterococci

**A B S T R A C T**

*Enterococci*, the normal inhabitant of gastrointestinal tract of humans and animals, have emerged as significant antibiotic resistant nosocomial pathogens. The current study was designed to determine the antibiotic resistance profile and genes harbored by isolated strains of *Enterococci* along with study of antibiotic resistance transfer potential from resistant *Enterococci* to susceptible pathogenic *Staphylococcus aureus in vitro*. The PCR based prevalence of *Enterococcus faecalis* and *Enterococcus faecium* from 118 broiler cloacal swabs was 60.46 and 30.23%, respectively, indicating that *E. faecalis* is the predominant species in broilers followed by *E. faecium. Enterococci* (n=86) were examined for the phenotypic resistance against eleven antibiotics which showed higher level of resistance to lincomycin (96.51%), erythromycin (90.69%), tetracycline (86.04%) and streptomycin (75.58%), intermediate level of resistance to ciprofloxacin (54.65%) and doxycycline (48.83%), and low resistance level to penicillin (26.74%), chloramphenicol (26.74%), amoxicillin (17.44%), augmentin (11.62%) and vancomycin (10.46%). Over 80% Enterococcal isolates were found multidrug resistant (MDR). On the basis of PCR analysis, *erm* (B) and *tet* (M) genes were identified in all phenotypically erythromycin and tetracycline resistant strains while *van* (B) was identified in only 4/9 (44.4%) of vancomycin resistant enterococci (VRE) with no detection of *van* (A) gene in any VRE. One strain *E. faecalis* (FME-41) was able to transfer the erythromycin resistance to pathogenic *Staphylococcus aureus* (M-11) in broth mating assay. MDR *Enterococci* pose therapeutic threat to human community and control on the spread of such MDR Enterococci from poultry to human food chain is crucial.

**To Cite This Article:** Malik F, Nawaz M, Anjum AA, Firyal S, Shahid MA, Irfan S, Ahmed F and Bhatti AA 2022. Molecular characterization of antibiotic resistance in poultry gut origin *Enterococci* and horizontal gene transfer of antibiotic resistance to *Staphylococcus aureus*. Pak Vet J, 42(3): 383-389. http://dx.doi.org/10.29261/pakvetj/2022.035

## INTRODUCTION

*Enterococci* are Gram positive, non-spore forming, and facultative anaerobes that are found as normal commensals and intestinal pathogens of humans and animals (Lee *et al*., 2021). *Enterococci* are used as indicator of environmental fecal contamination and are opportunistic pathogens causing different infections annually including septicemia, endocarditis, meningitis, paranasal sinuses and urinary tract infections (Fiore *et al*., 2019; Lee *et al*., 2021). *Enterococcus faecalis* and *Enterococcus faecium* are clinically more important among all other species of this genus. *E. faecium* is recognized as most prevalent nosocomial pathogen in clinically ill and immunocompromised patients worldwide, posing public health problem (Hughes *et al*., 2019; Zhou *et al*., 2020).

The contemporary increase and spread of antibiotic resistance in human pathogens have become a worldwide issue, largely owing to misuse or overuse of antibiotics in humans. However, many other sources of selective pressure are known to be the drivers of antimicrobial

resistance such as antibiotics use in agriculture and in animal production for therapy and control of infectious diseases (Manson *et al*., 2019). *Enterococci* have ability to resist more than two classes of antibiotics that are clinically beneficial. Due to restricted therapeutic choices for the treatment of vancomycin resistant *Enterococci*, they are considered as a significant problem (Manson *et al.,* 2019). The pressure of antibiotic resistant bacterial selection is very high in poultry and there is continuously a large quantity of resistant bacteria in poultry feces. So, human can be infected directly or indirectly via food by the antibiotic resistant poultry fecal *Enterococci* (Hughes *et al*., 2019; Manson *et al*., 2019).

The mobile genetic elements, such as transposons and plasmids carrying antimicrobial resistance and virulence determinants, has led to the development of multi-resistant *Enterococci* (Osei Sekyere and Mensah 2020; Fatoba *et al*., 2022). As donors and recipients of genes for antimicrobial resistance, *Enterococcus* species play a significant role in the spread of antibiotic resistant genes via horizontal gene transfer (Iannelli *et al*., 2021). Conjugative plasmids are self-transmissible and contain genes for the transfer machinery. Antibiotic resistance genes present on mobile genetic elements are transmitted from *Enterococci* of animal food origin to *Enterococci* of human intestinal origin, thus becomes challenging for various antimicrobial treatments in humans (Conwell *et al*., 2017; McInnes *et al*., 2020).

The current study was conducted to evaluate antibiotic resistance pattern of *Enterococcus* isolates especially *E. faecalis* and *E. faecium* of poultry gut origin in Lahore, Pakistan. Further, we examined the prevalence of antibiotic resistant genes in MDR *Enterococci* using PCR. This study also aimed to determine whether antibiotic resistance from poultry origin *Enterococcus* can be transmitted to human pathogenic *Staphylococcus aureus* or not.

## MATERIALS AND METHODS

**Sample collection:** A total number of 118 cloacal swabs were obtained from 39 poultry shops of Lahore, Pakistan, and transported under appropriate conditions to Institute of Microbiology, UVAS, Lahore.

**Isolation and identification of *Enterococci:*** Samples were soaked in normal saline, vortexed, and 100µl was plated on Bile Esculin Azide agar. Plates were incubated at 37ºC for 24 hours. Smooth, dark brown to black colored, pinpoint, shiny colonies were sub-cultured for purification. Identification of isolates was performed by microscopic analysis, colony characteristics, and biochemical tests.

**DNA extraction and PCR based identification of *Enterococci:*** Genomic DNA was extracted with the help of DNA extraction kit (GeneAll). PCRs, as described in Table 1, were used and the PCRs mixtures (25µl) were prepared. The mixture included 7.5µl water (nuclease free), 12.5µl nTaq Master mix (Wizbio solutions), 1.5µl of forward & reverse primers each, and the template DNA (2 µl). PCR reaction mixtures were subjected to a thermal cycler (Bio-Rad) using previously described programmes (Jackson *et al*., 2004). Electrophoresis of amplified products was done using agarose gel (1.5%) at 120 V for 40 min. A gel documentation system (Cleaver Scientific, UK) was used to observe and record the gel results.

**Antibiotic susceptibility analysis:** Analysis of antibiotic susceptibility was done using Kirby-Bauer disc diffusion. Briefly, a bacterial lawn was prepared, comparing with 0.5 McFarland standard, by swabbing Mueller Hinton agar plates. Oxoid antibiotic discs were positioned on surface of media at suitable distances. Zones of inhibition (mm) against all antibiotics after 24 hours of incubation were recorded.

**Amplification of antibiotic resistant genes:** The PCRs of antibiotic resistance genes, erythromycin (*erm*-B), tetracycline (*tet*-M) and vancomycin (*van*-A and *van*-B), were performed using 40 amplification cycles employing annealing temperatures presented in Table 2. Agarose gel electrophoresis analysis of PCR amplicons was performed using TAE buffer and gels were ethidium bromide (5 µg/mL) stained. Visualization of products was done using gel documentation system (Nawaz *et al*., 2011).

**DNA sequencing and analysis:** PCR amplicons of 16S rRNA gene of three enterococci (FME-13, FME-14 and FME-15), amplicons of *erm* (B) of isolate (FME-89 and FME-222) and amplicon of *tet* (M) of isolate FME-54 were sequenced by Advance Biosciences International (ABI) company. Sequences were analyzed using Chromas Software version 2.6.5. Genes were identified using

**Table 1:** Primers used for identification of *Enterococcus spp.*

| Target Taxon | Primers Sequence (5´-3´) | | Target gene | $T_a$˚C | Amplicon Length | Reference |
|---|---|---|---|---|---|---|
| *Enterococcus* | F | TCAACCGGGGAGGGT | *16SrRNA* | 55˚C | 733 | (Jackson *et al*., 2004) |
| | R | ATTACTAGCGATTCCGG | | | | |
| *E. faecalis* | F | ACTTATGTGACTAACTTAACC | *sodA* | 55˚C | 360 | (Jackson *et al*., 2004) |
| | R | TAATGGTGAATCTTGGTTTGG | | | | |
| *E. faecium* | F | ACAATAGAAGAATTATTATCTG | *sodA* | 55˚C | 214 | (Jackson *et al*., 2004) |
| | R | CGGCTGCTTTTTTGAATTCTTCT | | | | |

**Table 2:** Primers used for detection of antibiotic resistant genes

| Gene | Primer's sequence (5´-3´) | | $T_a$ ˚C | Amplicon Length (bp) | Reference |
|---|---|---|---|---|---|
| *erm* (B) | F | GAAAAGRTACTCAACCAAATA | 52˚C | 642 | (Nawaz *et al*., 2011) |
| | R | AGTAACGGTACTTAAATTGTTTAC | | | |
| *tet* (M) | F | GTTAAATAGTGTTCTTGGAG | 55˚C | 576 | (Nawaz *et al*., 2011) |
| | R | CTAAGATATGGCTCTAACAA | | | |
| *van* (B) | F | GTGACAAACCGGAGGCGAGGA | 58 ˚C | 413 | (Saadat *et al*., 2014) |
| | R | CCGCCATCCTCCTGCAAAAAA | | | |
| *van* (A) | F | ATGAATAGAATAAAAGTTGCAATAC | 62 ˚C | 1029 | (Miele *et al*., 1995) |
| | R | CCCCTTTAACGCTAATACGAT | | | |

BLAST. Nucleotide sequences were also submitted to GenBank to obtain Accession numbers. The nucleotide sequences were aligned using BioEdit, and MEGA 6.0 was used to make maximum likelihood phylogenetic trees.

**Antibiotic resistance transferability of *E. faecalis:*** The potential of *Enterococcus faecalis* to transfer antibiotic resistance to the recipient strains was analyzed by broth mating and filter mating experiments (Table 4). Four strains (MSF-1, MSF-2, M-11, and M-49) of vancomycin, erythromycin and tetracycline sensitive *Staphylococcus aureus* were used as recipients to acquire antibiotic resistant genes. The *van* (B), *tet* (M), and *erm* (B) positive *E. faecalis* (FME-41, FME-222, FME-444) strains were used as donors. Broth mating experiment was performed as described previously (Tomita *et al*., 2002) with a donor/recipient ratio of 1:4. Overnight 50µl culture of donor and 200 µl of recipient were added to 5 ml of fresh nutrient broth and the mixtures were incubated at 37 ºC in shaking incubator at 100 rpm for 24 hours. One milliliter of this mixed culture was inoculated in 5ml of fresh nutrient broth having 15µg/ml of erythromycin and 12.5 µg/mL of vancomycin, separately, then incubated at 37ºC in shaking incubator for 24 hours at 100 rpm. Portions of mixed cultures were then transferred to solid media plates with appropriate selective antibiotics. After overnight growth, Gram's staining and catalase tests were performed for suspected transconjugant colonies. Identified *Staphylococcus aureus* transconjugants were further purified on mannitol salt agar. Disc diffusion method was used to phenotypically confirm the transfer of antibiotic resistance. During filter mating assay, the mating mixture of donor and recipient was prepared as described above and 2ml of this mating mixture was filtered through 0.22µm nitrocellulose membrane filter (Millipore) and filter placed on agar plates. Bacterial cells trapped on filter paper were inoculated in normal saline followed by 10-fold serial dilutions. Appropriate dilutions were transferred to plates of solid media containing selective antibiotics. Following incubation at 37ºC for 48 hours, suspected transconjugants were selected, identified, and analyzed by antimicrobial susceptibility testing.

## RESULTS

**Identification of Enterococcal isolates based on cultural and molecular techniques:** Out of 118 samples, 72.88% (86/118) were positive for *Enterococci* based on cultural, morphological and biochemical identification methods. All positive *Enterococci* were further confirmed by genus and species-specific PCRs. Out of 86 Enterococcal isolates, 52 were *E. faecalis*, 26 were *E. faecium* and 8 were unidentified *Enterococci*. Representative PCR amplicons of genus *Enterococcus, E. faecalis* and *E. faecium* resolved on 1.5% agarose gel are shown in Fig. 1A, 1B and 1C.

**Antibiotic resistance profiling of *Enterococci* species:** *E. faecalis* showed higher level of resistance to lincomycin (94.23%), erythromycin (92.30%), tetracycline (86.53%), streptomycin (75%) followed by intermediate level of resistance to ciprofloxacin (59.61%), doxycycline (50%) and low level of resistance to penicillin (32.69%), chloramphenicol (30.76%), amoxicillin (13.46%), vancomycin (11.53%) and augmentin (9.61%) (Table 3). *E. faecium* indicated higher level of resistance to lincomycin (100%), erythromycin (88.46%) and tetracycline (84.61%) followed by intermediate level of resistance to streptomycin (73.07%), ciprofloxacin (53.84%) and doxycycline (46.15%) and low resistance level to amoxicillin (23.07%), chloramphenicol (23.07%), penicillin (19.23%), augmentin (15.38%) and vancomycin (3.84%) (Table 3). Unidentified *Enterococci* (n=8) had higher level of resistance to lincomycin (100%), erythromycin (87.5%), tetracycline (87.5%) and streptomycin (87.5%) followed by intermediate level of resistance to doxycycline (50%) and low resistance level to ciprofloxacin (25%), amoxicillin (25%), vancomycin (25%), chloramphenicol (12.5%), augmentin (12.5%) and penicillin (12.5%) (Table 3).

**PCR based detection of antibiotic resistant genes in *Enterococci:*** *E. faecalis* and *E. faecium* were screened by PCR for known resistant genes (Fig. 1D, 1E and 1F). Approximately 86.04, 90.69 and 10.46% isolates were phenotypically resistant to tetracycline, erythromycin and vancomycin respectively. Prevalence of *tet* (M) and *erm* (B) in tetracycline and erythromycin resistant *Enterococcus* respectively was 100%. Molecular detection of 9 VRE (*E. faecalis*) was conducted by PCR for *van* (A) and *van* (B) genes. It was found that *van* (B) gene was only 4/9 (44%) prevalent in vancomycin resistant *E. faecalis* and no *van* (A) detected. Two *van* (B), *erm* (B), and *tet* (M) positive strains (FME-222 and FME-444) were used in this study for the transferability of resistant genes.

**DNA sequence analysis of 16S rRNA, *erm*-B and *tet*-M partial gene sequences from selected *Enterococci:*** Amplified 16S rRNA gene products of *Enterococcus faecium* strains FME-13, FME-14 and FME-15 were sequenced (accession numbers MN153563, MN153564 and MN153565 respectively) and the sequence were compared with other sequences in the GenBank through BLAST. All these three sequences showed similarity with *Enterococcus faecium*. Phylogenetic tree showed that all samples had close resemblance with *Enterococcus faecium* (Fig. 2A). Amplified antibiotic resistant genes *erm* (B) of *E. faecalis* (FME-89 and FME-222), sequenced and subjected to the sequence similarity index through BLAST against the deposited sequences in GenBank database, showed similarity with *E. faecalis* strains. Maximum likelihood phylogenetic tree, based on *erm* (B) gene, showed that FME-89 gene had close resemblance with *erm* (B) gene of *Lactobacillus* strain PDL40 and FME-222 gene had close resemblance with *erm* (B) gene of *Enterococcus faecium* isolate F9631160 (Fig. 2B). Amplified antibiotic resistant gene *tet* (M) of *E. faecium* (FME-54), sequenced and searched against sequences in GenBank database via BLAST, showed similarity with *Enterococcus*. Maximum likelihood phylogenetic tree showed that FME-54 has close resemblance with *tet* (M) gene of *Lactobacillus* strain PDL28 (2C).

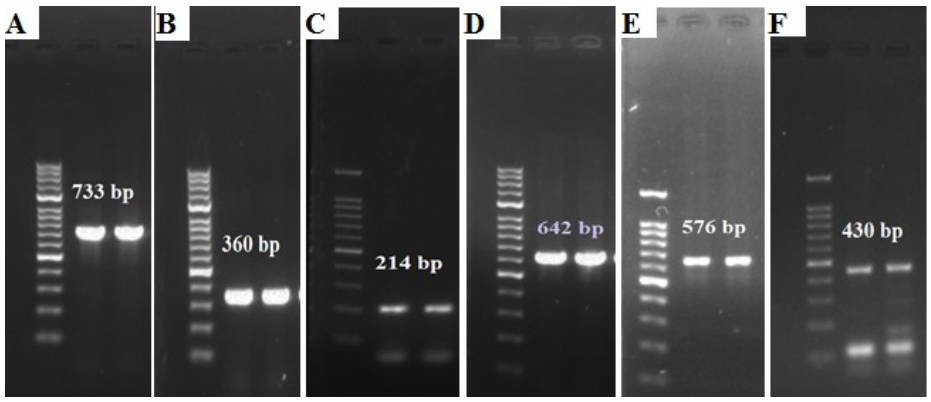


**Fig. 1:** PCR-based identification of (A) *Enterococcus* genus, (B) *E. faecalis*, (C) *E. faecium*, (D) *erm* B gene, (E) *tet* M and (F) *van* B gene. Each figure has 100 base pair (bp) ladder on left side.

**Table 3:** Antibiotic resistance profiles of *Enterococcus* isolates recovered in current study

| Antibiotic resistance profile | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *E. faecalis* (n=52) | | | *E. faecium* (n=26) | | | *Enterococcus spp.* (n=8) | | | Total (n=86) | | |
| Antibiotic | Disc (µg) | S | I | R | S | I | R | S | I | R | S | I | R |
| | | n(%) | n(%) | n(%) | n(%) | n(%) | n(%) | n(%) | n(%) | n(%) | n(%) | n(%) | n(%) |
| P | 10 units | 35 (67.30) | 0 (0) | 17 (32.69) | 21 (80.76) | 0 (0) | 5 (19.23) | 7 (87.50) | 0 (0) | 1 (12.50) | 63 (73.25) | 0 (0) | 23 (26.74) |
| AMX | 30 | 45 (86.53) | 0 (0) | 7 (13.46) | 20 (76.92) | 0 (0) | 6 (23.07) | 6 (75) | 0 (0) | 2 (25) | 71 (82.55) | 0 (0) | 15 (17.44) |
| VA | 30 | 27 (51.92) | 19 (36.53) | 6 (11.53) | 15 (57.69) | 10 (34.60) | 1 (3.84) | 3 (37.5) | 3 (37.50) | 2 (25) | 45 (52.32) | 32 (37.20) | 9 (10.46) |
| AMC | 30 | 47 (90.38) | 0 (0) | 5 (9.61) | 22 (84.61) | 0 (0) | 4 (15.38) | 7 (87.50) | 0 (0) | 1 (12.50) | 76 (88.37) | 0 (0) | 10 (11.62) |
| E | 15 | 3 (5.76) | 1 (1.92) | 48 (92.30) | 2 (7.69) | 1 (3.84) | 23 (88.46) | 1 (12.50) | 0 (0) | 7 (87.50) | 6 (6.97) | 2 (2.32) | 78 (90.69) |
| TE | 30 | 6 (11.53) | 1 (1.92) | 45 (86.53) | 4 (15.38) | 0 (0) | 22 (84.61) | 1 (12.50) | 0 (0) | 7 (87.50) | 11 (12.79) | 1 (1.16) | 74 (86.04) |
| DO | 5 | 19 (36.53) | 7 (13.46) | 26 (50) | 10 (38.46) | 4 (15.38) | 12 (46.15) | 3 (37.50) | 1 (12.50) | 4 (50) | 32 (37.20) | 12 (13.95) | 42 (48.83) |
| CIP | 30 | 6 (11.53) | 15 (28.84) | 31 (59.61) | 5 (19.23) | 7 (26.92) | 14 (53.84) | 2 (25) | 4 (50) | 2 (25) | 13 (15.11) | 26 (30.23) | 47 (54.65) |
| C | 30 | 28 (53.84) | 8 (15.38) | 16 (30.76) | 13 (50) | 7 (36.92) | 6 (23.07) | 4 (50) | 3 (37.50) | 1 (12.50) | 45 (52.32) | 18 (20.93) | 23 (26.74) |
| L | 2 | 3 (5.76) | 0 (0) | 49 (94.23) | 0 (0) | 0 (0) | 26 (100) | 0 (0) | 0 (0) | 8 (100) | 3 (3.48) | 0 (0) | 83 (96.51) |
| S | 10 | 6 (11.53) | 7 (13.46) | 39 (75) | 4 (15.38) | 3 (11.53) | 19 (73.07) | 1 (12.50) | 0 (0) | 7 (87.50) | 11 (12.79) | 10 (11.62) | 65 (75.58) |

P=Penicillin; AMX= Amoxicillin; VA=Vancomycin; AMC= Augmentin; E=Erythromycin; TE=Tetracycline; DO=Doxycycline; CIP=Ciprofloxacin; C=Chloramphenicol; L=Lincomycin; S=Streptomycin.

**Antibiotic resistance transferability of *Enterococci* to *S. aureus:*** Different techniques of transferring resistant genes from *E. faecalis* to *S. aureus*, including filter mating and broth mating, were used (Table 4). No transconjugants were detected during filter mating (1:1 & 1:4). Only one strain *E. faecalis* (FME-41) was able to transfer erythromycin resistance to the recipient *S. aureus* (M-11) in broth mating using 1:4 donor/recipient ratio. After plating donor/recipient mixture (1:4 ratio) on nutrient agar containing erythromycin (15 µg/ml), growth of *S. aureus* was observed which was further confirmed by catalase test. Suspected colonies of transconjugant were further purified on nutrient agar containing erythromycin and also by streaking on Mannitol Salt Agar (Fig. 3E and F). Disc diffusion test for antibiotic susceptibility of the transconjugant revealed that *E. faecalis* FME-41 has transferred erythromycin resistance to the recipient *S. aureus* M-11 which was sensitive to erythromycin, tetracycline and vancomycin before broth mating (Fig. 3B). This transconjugant is now erythromycin resistant (Zone of inhibition less than 13 mm is considered resistant as per CLSI standards), but still tetracycline and vancomycin sensitive (Fig. 3C). This horizontal transfer of resistance, confirmed by disc diffusion method, indicates that *E. faecalis* FME-41 harbors erythromycin resistant conjugative plasmid which was most probably transferred from *E. faecalis* FME-41 to *S. aureus* M-11 strain during conjugation (Fig. 3A-F).

## DISCUSSION

The gastrointestinal tract (GIT) of poultry harbors a very complicated microbiota with more than 600 distinct bacterial species from over 100 different genera. *Enterococci* are commensal inhabitant of GIT of humans and animals. *Enterococcus spp.* have been frequently isolated from poultry (Ngbede *et al*., 2017; de Jong *et al*., 2018). The present study employed *Enterococcus* genus, *E. faecalis* and *E. faecium* specific PCRs for speedy detection. Genus and species level detection, based on PCR, of *Enterococcus* have been used in several previous studies (Lee *et al*., 2021; Fatoba *et al*., 2022). The present

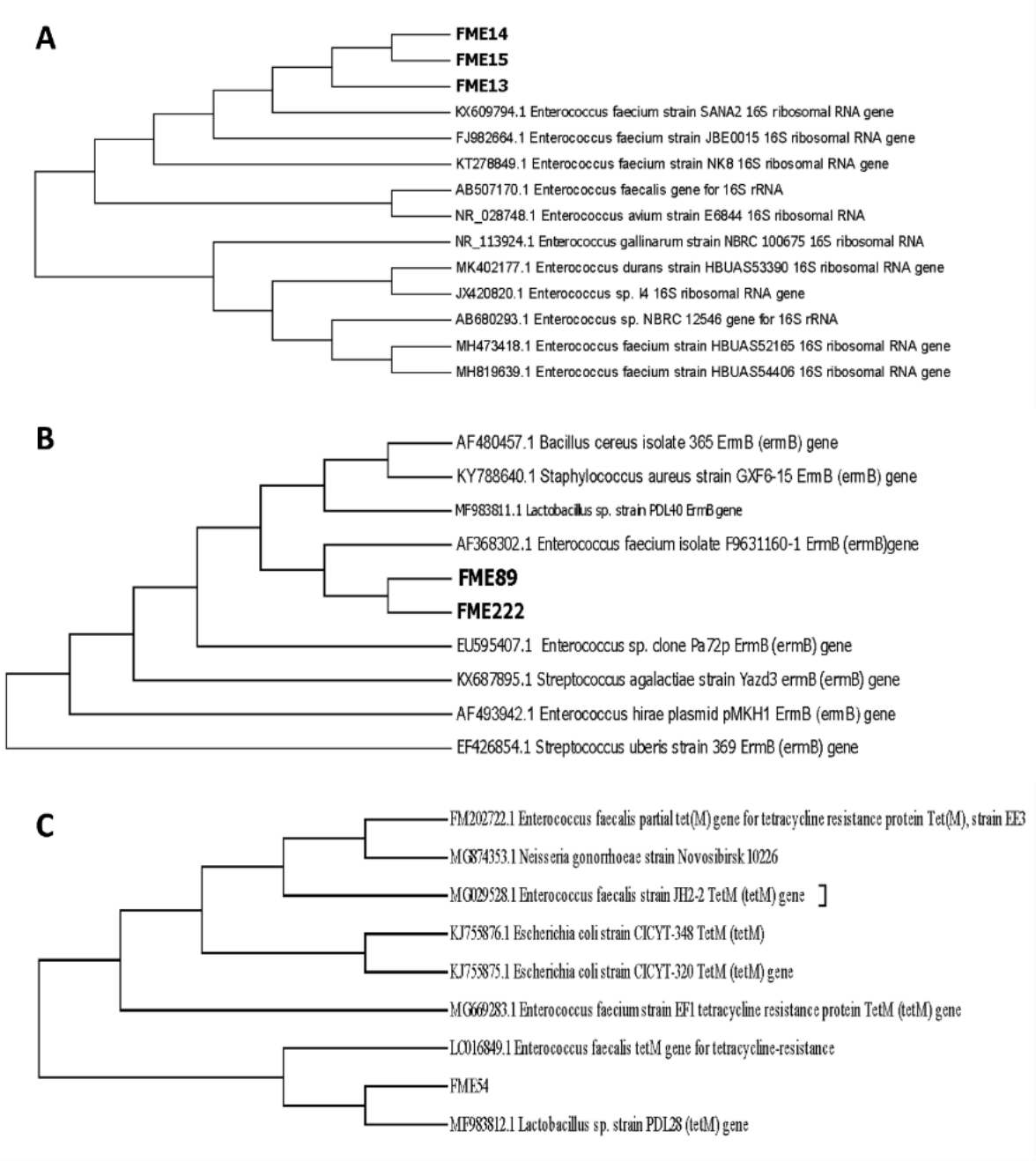


**Fig. 2:** Phylogenetic analysis of selected Enterococci strains based on (A) 16S rRNA, (B) erm (B), and (C) tet (M) gene partial DNA sequences. MEGA 6.0 was used to prepare maximum likelihood phylogenetic trees.

**Table 4:** Filter and broth mating assays for transfer of antibiotic resistance genes from *Enterococcus faecalis* to *Staphylococcus aureus*.

| Sr. No. | Experiment Type | Donor strains (*E. faecalis* genotype) | Recipient strains (Vancomycin, tetracycline and erythromycin sensitive *S. aureus*) | Donor: recipient (Ratio) | Trans-conjugants detection |
|---|---|---|---|---|---|
| 1 | Filter mating | FME-222 and FME-444 (van-B, tet-M and erm-B positive) | MSF-1 and MSF-2 | FME-222:MSF-1 FME-444:MSF-2 (1:1) | None |
| 2 | Broth mating | FME-222 (van-B, tet-M and erm-B positive) FME-41 (tet-M and erm-B positive) | M-11 and M-49 | FME-222:M-49 FME-41:M-11 (1:10) | None |
| 3 | Filter mating | FME-222 and FME-444 (van-B, tet-M and erm-B positive) | M-11 and MSF-2 | FME-222:M-11 FME-444:MSF-2 (1:4) | None |
| 4 | Broth mating | FME-222 (van-B, tet-M and erm-B positive) & FME-41 (tet-M and erm-B positive) | M-11 and M-49 | FME-222:M-49 FME-41:M-11 (1:4) | Detected |

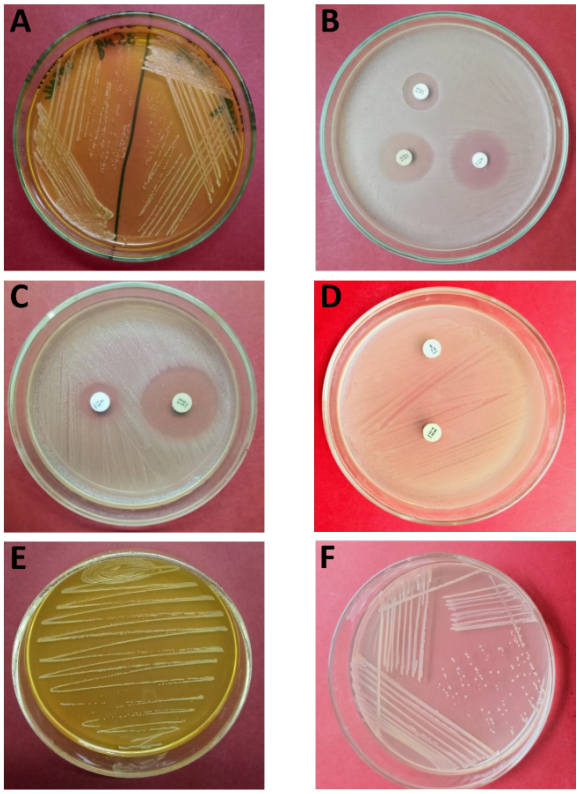


**Fig. 3:** (A) Growth of 2 different colonies of *Staphylococcus aureus* on mannitol salt agar. (B) Zones of inhibition of *S. aureus* against erythromycin (22 mm), tetracycline (24 mm) and vancomycin (17 mm) before mating experiments. (C) *S. aureus* transconjugant resistant to erythromycin (Zone of inhibition less than 13 mm is considered resistant as per CLSI standards) and sensitive to tetracycline (Zone of inhibition 26 mm) after broth mating experiment. (D) Donor strain of *Enterococcus faecalis* FME-41, erythromycin and tetracycline resistant, with no zone of inhibition against erythromycin and tetracycline. (E) Purified culture of *S. aureus* transconjugant on mannitol salt agar. (F) Purified culture of *S. aureus* transconjugant on nutrient agar containing 15 µg/mL of erythromycin.

study reported antibiotic susceptibility profiles of *E. faecalis, E. faecium* and unidentified *Enterococci* isolated directly from the poultry gut. This study reported the isolation rate of *E. faecalis* and *E. faecium* as 60.46% and 30.23% respectively. Only 9.30% isolates were unidentified Enterococcal species. It was found that *E. faecalis* and *E. faecium* are the predominant species in poultry which is in agreement with the previous findings (Ngbede *et al*., 2017; Stępień-Pyśniak *et al*., 2016).

Antimicrobial resistance pattern of *Enterococci* has been reported quite frequently establishing that these microbes are antibiotic resistant (Labibzadeh *et al*., 2018). Present study revealed that only 26.74% and 17.44% isolates were resistant to penicillin and amoxicillin, respectively. *Enterococci* rarely exhibit high level of resistance against this class of antibiotics (de Jong *et al*., 2018). A higher level of resistance against lincomycin has been observed in current study and it is in accordance with previous studies (Różańska *et al*., 2019; Gołaś-Prądzyńska and Rola 2021). All studied isolates showed resistance to at least one of the eleven antimicrobial agents tested. Only 9/86 (10.4%) isolates showed resistance to vancomycin in current study. Similar resistance patterns were observed for *Enterococci* against vancomycin in previous studies (Miller *et al*., 2020) while another study reported no vancomycin resistance in *Enterococci* (Ruzauskas *et al.,* 2010). So, it can be concluded that the resistance pattern against vancomycin is quite variable. Overall, the data of current study revealed that 81% of *E. faecalis*, 69% of *E. faecium* and 75% of unidentified *Enterococci* are multidrug resistant (MDR).

The rapid emergence and rise of antimicrobial resistance to various drugs in *Enterococci* has been attributed to the irrational and misuse of antibiotics in poultry industry. The increase in *Enterococci* resistance to frequently used antibacterial drugs in veterinary and public health is of great concern to medical care worldwide (McInnes *et al*., 2020). To the best of our knowledge, this is the first study assessing prevalence of antibiotic resistance genes in poultry gut origin *Enterococci* from Pakistan, although few studies are available on the prevalence of antimicrobial resistance in *Enterococci* from poultry environment. In this study, Enterococcal isolates were screened for known resistant genes of tet (M), erm (B), van (A), and van (B). Approximately 86.04%, 90.69% and 10.46% isolates were phenotypically resistant to tetracycline, erythromycin and vancomycin, respectively, and almost 100% prevalence of *tet* (M) and *erm* (B) was detected in tetracycline and erythromycin resistant *Enterococci*. *Among all erythromycin resistant genes in Enterococci, erm* (B) gene was the most prevalent in erythromycin resistant isolates, *and* 63% of the tetracycline resistant enterococcal strains carried *tet* (M) (Graham *et al*., 2009). Another study reported that *erm (*B) gene was present in 81.8% of the erythromycin-resistant enterococcal strains (Hummel *et al*., 2007). This study reported molecular detection of 9 Vancomycin Resistant *Enterococci* (VRE) (*E. faecalis*) and found that only 4/9 (44%) VRE *E. faecalis* harbored the *van* (B) gene while *van* (A) was not amplified in any VRE. It might be due to unavailability of positive control or absence of this gene in studied strains. The VRE gene operons exhibit discrete genetic variation and continuous evolution results in different antimicrobial susceptibility phenotypes and environmental and livestock reservoirs of the common vancomycin resistant genes (Ahmed and Baptiste 2018; Miller *et al*., 2020).

The microbial flora of intestinal origin exchanges their resistance genes with each other. Bacteria residing in human intestine are considered as pools for various genes related to antibiotic resistance (Kim *et al*., 2019). A poultry gut origin VRE strain FME41, phenotypically resistant to vancomycin, tetracycline and erythromycin, found to be *tet* (M) and *erm* (B) positive but *van* (B) and *van* (A) negative, was used as donor in broth mating experiment to transfer antibiotic resistance to *Staphylococcus aureus* M-11 strain (vancomycin, erythromycin and tetracycline sensitive). After adopting different antibiotic resistance transfer techniques, FME-41 was able to transfer erythromycin resistance to the recipient M-11 strain in a broth mating experiment. This transconjugant was found erythromycin resistant, but still tetracycline and vancomycin sensitive, as confirmed by disc diffusion method. It is concluded that VRE strain FME-41 harbors transferable erythromycin resistant conjugative plasmid. Similarly, *erm* (B) and *tet* (M) antibiotic resistant genes from *Lactobacilluss spp.* were

effectively shifted to *E. faecalis* in filter mating experiment (Nawaz *et al*., 2011). Tetracycline resistance was transferred from 50% of *Lactobacillus* isolates to susceptible *E. faecalis* while no conjugal transfer from *Lactobacilluss spp.* to *S. aureus* was detected (Gevers *et al*., 2003). Another study reported successful transfer of resistance from *Enterococcus faecalis* to *Staphylococcus aureus* (Noble *et al*., 1992).

Horizontal gene transfer mediated by conjugative plasmids is quite common in human and animal gut microbiome (McInnes *et al*., 2020; Brito 2021). During present study, plasmids bearing erythromycin, tetracycline and vancomycin resistant genes were also detected in *Enterococci*. Erythromycin resistance transfer from *Enterococcus faecalis* to *Staphylococcus aureus* in current study is also thought to be mediated by such conjugative plasmid; however, future studies should be focused on further characterization of transconjugants and donor/recipients strains to determine the molecular basis of erythromycin resistance in *S. aureus* transconjugants.

**Conclusion:** Current study reported antibiotic resistance profiling of poultry gut origin *Enterococci* and is also the first study reporting PCR-based detection of multiple antibiotic resistance genes in *Enterococci* in Pakistan. Furthermore, the potential of antibiotic resistance transferability of *E. faecalis* to human pathogenic *S. aureus*, by horizontal gene transfer, is being reported for the first time.

**Acknowledgement:** This study was funded in part by Higher Education Commission/ NPRU Project No. 7069/Punjab/NPRU/R&D/HEC/2017.

**Conflict of Interest:** All authors declare that there is no conflict of interest.

**Authors contribution:** MN, AAA and SF conceived and designed the study. FM carried out the experiments and evaluated the study results. MN, MAS and FA are involved in results interpretation. FM, SI and MAS prepared the manuscript. MAS and AAB critically revised the manuscript. All authors are involved in final approval of this manuscript.

## REFERENCES

Ahmed MO and Baptiste KE, 2018. Vancomycin-Resistant enterococci: a review of antimicrobial resistance mechanisms and perspectives of human and animal health. Microb Drug Resist 24:590-606.

Brito IL, 2021. Examining horizontal gene transfer in microbial communities. Nat Rev Microbiol 19:442-53.

Conwell M, Daniels V, Naughton PJ, *et al.,* 2017. Interspecies transfer of vancomycin, erythromycin and tetracycline resistance among Enterococcus species recovered from agrarian sources. BMC Microbiol 17:19.

de Jong A, Simjee S, Garch FE, *et al.,* 2018. Antimicrobial susceptibility of enterococci recovered from healthy cattle, pigs and chickens in nine EU countries (EASSA Study) to critically important antibiotics. Vet Microbiol 216:168-75.

Fatoba DO, Amoako DG, Akebe ALK, *et al.,* 2022. Genomic analysis of antibiotic-resistant Enterococcus spp. reveals novel enterococci strains and the spread of plasmid-borne Tet(M), Tet(L) and Erm(B) genes from chicken litter to agricultural soil in South Africa. J Environ Manage. 302:114101.

Fiore E, Van Tyne D and Gilmore MS, 2019. Pathogenicity of Enterococci. Microbiol Spectr 7.

Gevers D, Huys G and Swings J, 2003. In vitro conjugal transfer of tetracycline resistance from Lactobacillus isolates to other Gram-positive bacteria. FEMS Microbiol Lett 225:125-30.

Gołaś-Prądzyńska M and Rola JG, 2021. Occurrence and Antimicrobial resistance of enterococci isolated from goat's milk. J Vet Res 65:449-55.

Graham JP, Price LB, Evans SL, *et al.,* 2009. Antibiotic resistant enterococci and staphylococci isolated from flies collected near confined poultry feeding operations. Sci Total Environ. 407:2701-10.

Hughes A, Ballard S, Sullivan S, *et al.,* 2019. An outbreak of vanA vancomycin-resistant Enterococcus faecium in a hospital with endemic vanB VRE. Infect Dis Health 24:82-91.

Hummel A, Holzapfel WH and Franz CM, 2007. Characterisation and transfer of antibiotic resistance genes from enterococci isolated from food. Syst Appl Microbiol 30:1-7.

Iannelli F, Santoro F, Fox V, *et al.,* 2021. A Mating Procedure for Genetic Transfer of Integrative and Conjugative Elements (ICEs) of Streptococci and Enterococci. Methods Protocols. 4:59.

Jackson CR, Fedorka-Cray PJ and Barrett JB, 2004. Use of a genus- and species-specific multiplex PCR for identification of enterococci. J Clin Microbiol 42:3558-65.

Kim E, Park S, Cho S, *et al.,* 2019. Comparative Genomics Approaches to Understanding Virulence and Antimicrobial Resistance of Salmonella Typhimurium ST1539 Isolated from a Poultry Slaughterhouse in Korea. J Microbiol Biotechnol 29:962-72.

Labibzadeh M, Kaydani GA, Savari M, *et al.,* 2018. Emergence of High-level Gentamicin Resistance among Enterococci Clinical Isolates from Burn Patients in South-west of Iran: Vancomycin Still Working. Pol J Microbiol 67:401-6.

Lee T, Jordan D, Sahibzada S, *et al.,* 2021. Antimicrobial Resistance in Porcine Enterococci in Australia and the Ramifications for Human Health. Appl Environ Microbiol 87.

Manson AL, Van Tyne D, Straub TJ, *et al.,* 2019. Chicken Meat-Associated Enterococci: Influence of Agricultural Antibiotic Use and Connection to the Clinic. Appl Environ Microbiol 85.

McInnes RS, McCallum GE, Lamberte LE, *et al.,* 2020. Horizontal transfer of antibiotic resistance genes in the human gut microbiome. Curr Opin Microbiol 53:35-43.

Miele A, Bandera M and Goldstein BP, 1995. Use of primers selective for vancomycin resistance genes to determine van genotype in enterococci and to study gene organization in VanA isolates. Antimicrob Agents Chemother 39:1772-8.

Miller WR, Murray BE, Rice LB, *et al.,* 2020. Resistance in Vancomycin-Resistant Enterococci. Infect Dis Clin North Am. 34:751-71.

Nawaz M, Wang J, Zhou A, *et al.,* 2011. Characterization and transfer of antibiotic resistance in lactic acid bacteria from fermented food products. Curr Microbiol 62:1081-9.

Ngbede EO, Raji MA, Kwanashie CN, *et al.,* 2017. Antimicrobial resistance and virulence profile of enterococci isolated from poultry and cattle sources in Nigeria. Trop Anim Health Prod 49:451-8.

Noble WC, Virani Z and Cree RG, 1992. Co-transfer of vancomycin and other resistance genes from Enterococcus faecalis NCTC 12201 to Staphylococcus aureus. FEMS Microbiol Lett 72:195-8.

Osei Sekyere J and Mensah E, 2020. Molecular epidemiology and mechanisms of antibiotic resistance in Enterococcus spp., Staphylococcus spp., and Streptococcus spp. in Africa: a systematic review from a One Health perspective. Ann N Y Acad Sci 1465:29-58.

Różańska H, Lewtak-Piłat A, Kubajka M, *et al.,* 2019. Occurrence of Enterococci in Mastitic Cow's Milk and their Antimicrobial Resistance. J Vet Res 63:93-7.

Ruzauskas M, Siugzdiniene R, Seputiene V, *et al.,* 2010. Antimicrobial resistance of enterococcus SPP. spread in poultry products in Lithuania.

Saadat S, Solhjoo K, Norooz-Nejad MJ, *et al.,* 2014. VanA and VanB Positive Vancomycin-resistant Staphylococcus aureus Among Clinical Isolates in Shiraz, South of Iran. Oman Med J 29:335-9.

Stępień-Pyśniak D, Marek A, Banach T, *et al.,* 2016. Prevalence and antibiotic resistance of Enterococcus strains isolated from poultry. Acta Vet Hung 64:148-63.

Tomita H, Pierson C, Lim SK, *et al.,* 2002. Possible connection between a widely disseminated conjugative gentamicin resistance (pMG1-like) plasmid and the emergence of vancomycin resistance in Enterococcus faecium. J Clin Microbiol 40:3326-33.

Zhou X, Willems RJL, Friedrich AW, *et al.,* 2020. Enterococcus faecium: from microbiological insights to practical recommendations for infection control and diagnostics. Antimicrob Resist Infect Control 9:130.