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ORIGINAL ARTICLE

DOI: 10.69723/njms.04.01.0512

# MOLECULAR CHARACTERIZATION AND MUTATIONAL ANALYSIS OF ANTIBIOTIC RESISTANCE GENES ISOLATED IN ENTEROCOCCUS FAECALIS FROM URINARY TRACT INFECTION PATIENTS IN PESHAWAR

**Zahidullah Khan[1], Adnan Mumtaz[1], Sadaf Bilal[2], Maham Iqbal[2], Waseem Abbas[2], Muhammad Khalid[2*]**

**1.Medical Teaching Institute, Hayatabad Medical Complex Peshawar, Khyber Pakhtunkhwa.**

**2.Medical Teaching Institute, Khyber Teaching Hospital Peshawar, Khyber Pakhtunkhwa.**

**Correspondence Email: mkhalid92542@gmail.com**

**Received- 26th Dec 2024; Revisions received- 25th Jan 2025; Accepted- 3rd Feb 2025**

## ABSTRACT

**BACKGROUND:** Enterococcus faecalis (E. faecalis) is a leading causative agent of urinary tract infections (UTIs) and poses a significant public health concern due to its multidrug resistance. Understanding its resistance mechanisms is essential for improving treatment strategies.

**OBJECTIVE:** This study aimed to determine the prevalence, antibiotic susceptibility patterns, and molecular characteristics of antibiotic resistance genes in E. faecalis isolates from UTI patients in Peshawar.

**METHODOLOGY:** A cross-sectional study was conducted on 137 urine samples, collected from inpatients and outpatients diagnosed with UTIs at Hayatabad Medical Complex, Peshawar. Bacterial identification was performed using selective culture media, Gram staining, and biochemical tests. Antibiotic susceptibility testing was conducted using the Kirby-Bauer disk diffusion method following CLSI 2022 guidelines. Polymerase chain reaction (PCR) was employed to detect resistance genes (CTXM, AmpC, Tet A, Tet B), followed by Sanger sequencing for mutational analysis. Statistical analysis was performed using SPSS 23.0, with significance set at $p \leq 0.05$.

**RESULTS:** Enterococcus faecalis showed high resistance to erythromycin (92%), ciprofloxacin (92%), tetracycline (84%), and penicillin (94%) but remained susceptible to vancomycin, linezolid, and teicoplanin. Molecular analysis detected CTXM (73%), AmpC (78%), Tet A (71%), and Tet B (58%). Mutational analysis revealed non-synonymous mutations in CTXM and AmpC genes, potentially contributing to β-lactam antibiotic resistance.

**CONCLUSION:** The high prevalence of multidrug-resistant E. faecalis in Peshawar highlights the urgent need for enhanced surveillance and targeted antibiotic stewardship programs. This study provides crucial insights into resistance mechanisms, paving the way for molecular-based therapeutic interventions.

**KEY WORDS:** Enterococcus faecalis, urinary tract infections, antibiotic resistance, multidrug resistance, molecular characterization, mutational analysis

**HOW TO CITE THIS ARTICLE:** Khan Z, Mumtaz A, Bilal S, Iqbal M, Abbas W, Khalid M. Molecular Characterization and Mutational Analysis of Antibiotic Resistance Genes Isolated in Enterococcus faecalis from Urinary Tract Infection patients in Peshawar. Northwest J Med Sci. 2025; 4(1): 9-17

## INTRODUCTION:

Enterococcus faecalis (E. faecalis) is a Gram-positive species found in pairs or short chains, a facultatively anaerobic, non-spore-producing, and non-motile bacterium of Enterococcaceae.[1,2] Some strains of E. faecalis can cause a variety of infections, including nosocomial infections. However, UTIs are the most common infection caused by this species in humans, particularly in females. E. faecalis is one of the most common bacterial pathogens responsible for hospital-acquired infections, causing approximately 40% of cases, including UTIs.[3,4] E. faecalis is mainly reported in intensive care unit (ICU) patients and is considered one of the top three most pathogenic bacteria causing chronic and recurrent UTIs. E. faecalis could develop both intrinsic and acquired resistance to antibiotics, which can lead to the development of multiple drug resistance (MDR). Intrinsic resistance refers to the inherent properties of the bacterium, while acquired resistance results from the acquisition of antibiotic-resistance genes through horizontal gene transfer mechanisms such as transduction and conjugation. As a result, E. faecalis is considered notorious for its ability to acquire and spread antibiotic-resistant genes among other bacterial species.[5,6] Beta-lactamases are the enzymes responsible for bacterial resistance toward β-lactam antibiotics. These antibiotics contain a β-lactam ring that is broken down by the beta-lactamase enzyme, rendering the antibiotics ineffective against the bacteria. This allows the bacteria to survive and continue to multiply, leading to the development of antibiotic resistance.[7] Extended-spectrum β-lactamases (ESBLs) are a class of beta-lactamases that provide resistance to a broad range of β-lactam antibiotics, including penicillin, cephalosporins, monobactams, and aztreonam. Unlike other beta-lactamases, ESBLs can break down the extended-spectrum antibiotics used to treat serious infections, making them a significant threat in healthcare settings.[8] The β-lactamase enzyme is mainly plasmid-mediated in Enterococcus, Enterobacteriaceae, Pseudomonas species, and Neisseria gonorrhoeae.[9] According to a recent study, antibiotic

resistance is an alarming issue globally that leads to major health problems, found in both hospital and community-acquired infections.

The discovery of antibiotics has had crucial value in saving lives in different ways and has revolutionized medicine. Nowadays, advancements in different antibiotics are positive signs for human health. Unfortunately, the increasing emergence of bacterial strains is leading to antibiotic resistance. Recent studies have revealed more than 500 resistance genes in pathogenic bacterial strains.[10] The extended-spectrum β-lactamases (ESBLs) can be present on the chromosome or acquired by plasmid in some bacteria.[11] Due to the production of β-lactamases, the gram-negative and gram-positive bacteria are resistant to the β-Lactam antibiotics and are encoded by CTXM, SHV, AMP, and TEM genes respectively. Studies revealed that CTXM is a prominent and widespread ESBL in humans.[12, 13] The high prevalence of the (71%) CTXM resistance gene was reported in Saudi Arabia.[14] CTXM is reported in the Enterococcus species belonging to the class-

An enzyme, initiated from the ESBL chromosomal gene transfer; located in the Klebsiella species of the Enterobacteriaceae.[15,16] The high frequency of CTXM, SHV, and TEM was also reported in Lahore. In the last two decades, the ratios of infections related to ESBLs have increased.[17] Enterococcus resistance to tetracycline is due to exogenous genes carried either on the chromosome or acquired through plasmids. These genes mediate two functions: energy-dependent efflux of tetracycline from the bacterial cell, and ribosomal protection modifies the ribosome to prevent effective tetracycline binding.[18] According to the study, the first group is tetracycline efflux protein that includes TET K and TET L, which are more common in gram-positive bacteria; Staphylococcus and Enterococcus.[19, 20] The same study revealed that the second group, ribosomal protection proteins encoded by the Tet M, Tet O, and Tet S, were identified in Gram-positive bacteria.[21, 22] Tetracycline is not typically the first choice for treating infections caused by enterococcus, despite being a broad-spectrum antibiotic commonly used for most gram-positive bacteria.[23] It is crucial to control antibiotic-resistant infections and to determine the molecular characteristics of resistance genes in both hospital and community settings. The present study aims to determine the prevalence of Enterococcus faecalis in UTI patients, analyze its antibiotic resistance patterns, and conduct molecular characterization and mutational analysis of resistance genes in a Hayatabad Medical Complex, Peshawar

**METHODOLOGY**

This cross-sectional study was conducted at the Laboratory of Microbiology at Hayatabad Medical Complex Peshawar, from June 2024 to December 2024. Patients with UTI of both genders were included while the patients suffering from any other infection were excluded from the study. Upon proper consent and detailed medical history, a total of 137 urine samples were collected from both inpatients (IPD) and outpatients (OPD) diagnosed with urinary tract infections (UTIs). The samples were cultured on selective and differential media including Cysteine Lactose and Electrolyte Deficient (CLED), Esculin agar, and McConkey agar, followed by overnight incubation at 37°C. Subsequently, pure colonies were obtained and subjected to Gram staining and finally inoculation on Esculin agar media for species identification and characterization.

The ethical approval was obtained from the ethical review board of Hayatabad Medical Complex on 8th Jan 2024, approval No. 2164

**DNA Extraction**

The genomic DNA of *E. faecalis* was extracted from a 24-hour-old broth culture using the Thermo Scientific Gen Jet Genomic DNA Purification Kit. The purified DNA was subjected to electrophoresis on a 1% agarose gel in 1x Tris Acetate EDTA buffer, and the resulting bands were visualized using a Gel Documentation system for further analysis.

**Antibiotic susceptibility pattern**

The antibiotic susceptibility pattern of the clinical isolates against selected antibiotic discs was determined by inoculating them on Muller Hinton agar (MHA) followed by specific antibiotic discs and incubation at 37°C for 24 hours. The zone of inhibition was measured in millimetres (mm) according to Clinical and Laboratory Standards Institute (CLSI) 2022 guidelines. The resulting zones of inhibition were interpreted as susceptible (S), intermediate (I), or resistant (R).

**Amplification of Antibiotic Resistance Genes** By using the specific Primers of *CTXM, AmpC, Tet A,* and *Tet B* as shown in Figure II; the targeted DNA sequence was amplified by Thermal Cycler (PCR). The total 27 µl of Reaction for PCR were prepared by containing 12.5µl Taq Master Mix (Thermo Fischer Scientific), 0.5 µl each forward and reverse primers (Oligonucleotides, Macrogen Korea), 11.5 µl PCR grade, and 2 µl of DNA sample were added. To analyze the amplification of the antibiotic gene, the PCR products were loaded in the gel with a 100bp ladder and were further run on gel electrophoresis (BioRad). The gel was visualized with the help of the Gel Documentation system (BioRad DOC XR+).

## METHODOLOGY:

This comparative cross-sectional validation study was conducted at CMH, Rawalpindi, from 1st July 2023 to 31st Dec 2023 after obtaining approval from the institutional ethical review board. Appropriate sample size was calculated using the WHO sample size calculator for single population proportions with specified absolute precision using the following formula:

For the calculations, the following assumptions were made: a confidence level of 95%, an absolute precision of 4.5%, and an anticipated sensitivity of 96.7% for the RIPASA score in diagnosing acute appendicitis.[12]This gave us a sample size of 61.

All patients who were aged 14 years or above, both male and female gender, who presented with classical pain in RUQ with tenderness and rebound tenderness were included in this study. Patients who were unfit to undergo surgery already had an

appendectomy, those requiring emergency appendectomy due to appendiceal perforation, and those for which an alternative diagnosis was made for abdominal pain were excluded from the study.

Patients were selected by using a non-probability consecutive sampling technique. Once selected, all the baseline characteristics, including age (in years), gender, and duration of symptoms (in hours), were documented. All the patients were subjected to standard pre-operative assessment protocol through a set of blood tests and basic radiology. After explaining the study's purpose, informed consent was presented to patients (parents in case of minors) to get signatures for assuring their official consent of participation in the study and for surgery. After those two, researchers assessed patients separately for each score. ALVARADO score is a 10-point score having parameters including left shift (> 75% neutrophils), > 10,000/mm3 white cells, pain migration to right lower quadrant (RLQ), nausea/vomiting, anorexia, rebound tenderness, elevated temperature and RLQ tenderness. RIPASA score is a 15-point score, and its parameters include "age, gender, right iliac fossa (RIF) pain, pain migration to RIF, nausea/vomiting, anorexia, duration of symptoms, RIF tenderness, RIF rebound tenderness, RIF guarding, Rovsing's sign, fever, raised white count and negative urinalysis. One researcher assessed the patient and calculated the ALVARADO score, and a score of ≥ 7 was considered diagnostic of acute appendicitis. [13] Once the first researcher finished their assessment, the second researcher assessed the same patient and calculated the RIPASA score and a score of ≥ 7.5 was considered diagnostic of acute appendicitis. [14] After this, all these patients underwent laparoscopic removal of the appendix by the same surgical team as per the standard 3-port technique led by a consultant surgeon with a minimum of two years of experience. The removed appendix was sent for histopathological assessment to the pathology department, where a consultant pathologist made the histopathological diagnosis of acute appendicitis. Based on these results, a 2x2 contingency table was drawn for each score, which was then used to calculate sensitivity (SN), specificity (SP), positive predictive value (PPV), negative predictive value (NPV), and accuracy of ALVARADO score and RIPASA score in the diagnosis of acute appendicitis with histopathological finding as reference standard.

Data was analyzed by using Statistical Package for Social Sciences (SPSS) 20.00. The normality of data was checked by the Shapiro-Wilk test. Quantitative data (age and duration of symptoms) were presented as mean ± standard deviation (SD). Quantitative data (gender, presence of ALVARADO score ≥ 7, presence of RIPASA score ≥ 7.5, and histopathological diagnosis of acute appendicitis) was represented by using percentage and frequency. Based on standard formulas, sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of both scores were calculated.

Ethical approval with reference no.565 was obtained from the Hospital Research and Ethical Committee (IREB) of Combined Military Hospital, Rawalpindi on 1/07/23.

**Table 1:** PCR Condition for Specific Primer-Designed Antibiotic Resistance Gene

| Gene Name | Sequence (bp) | Product size (bp) | Condition for PCR |
|---|---|---|---|
| ***AmpC*** | FP: CCCCGCTTATAGAGCAACAA<br>RP: TCAATGGTCGACTTCACACC | 634 | Annealing 58 °C for 30 seconds (35 cycles) |
| ***Tet A*** | FP: GGTTCACTCGAACGACGTCA<br>RP: CTGTCCGACAAGTTGCATGA | 577 | Annealing 57 °C for 30 seconds (35 cycles) |
| ***Tet B*** | FP: CCTCAGCTTCTCAACGCGTG<br>RP: GCACCTTGCTCATGACTCTT | 634 | Annealing 54 °C for 30 seconds (35 cycles) |
| ***CTXM*** | FP: TGTGCAGCACCAGTAAAGT<br>RP: TGATGTAACACGGATTGACC | 545 | Annealing 54 °C for 30 seconds (35 cycles) |

**DNA Sequencing and Data Analysis**

PCR products amplified from the antibiotic resistance genes were randomly selected and purified using the Thermo Scientific Gen Jet PCR Purification Kit, according to the manufacturer's protocol. The purified products were then sent to Macrogen Korea for Sanger sequencing. The FASTA sequences of amplified PCR products were analysed by comparing and aligning with reference sequences obtained from the GenBank National Centre for Biotechnology Information (NCBI) database. The nucleotide sequences were analysed by conducting a basic local alignment search tool (BLAST) search in BioEdit version 7.1 software.

**Statistical analysis**

SPSS 23.0 was used to conduct Statistical analysis, including the chi-square and one-way variance analysis (ANOVA). The expected frequencies were compared against the observed frequencies to determine whether significant associations existed. A p-value of ≤ 0.05 was set for statistical significance. The total number of samples (n) analyzed in this study was 137, and the degrees of freedom were calculated as n - 1.

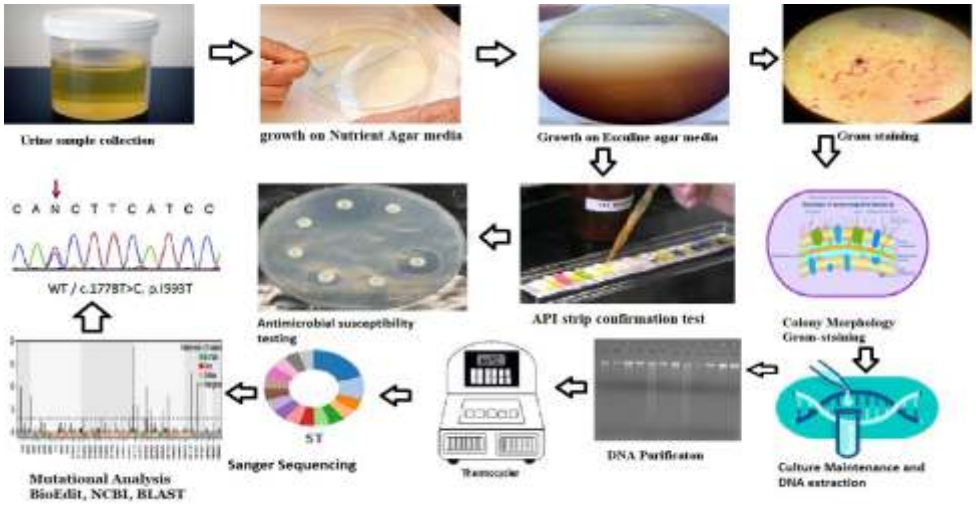


**Figure 1:** Overall Flowchart of Methodology

## RESULTS

**Sample Collection and Identification**

Clinical samples were collected from suspected UTI patients and identified based on the appearance of red-purple colonies on MacConkey agar and yellowish colonies on CLED agar. Gram staining revealed that the clinical samples retained a purple color, indicating the Gram-positive status of *E. faecalis*. Catalase, oxidase, citrate, and urease tests were performed, and all gave negative results, which further confirmed the identification of *E. faecalis*. For final confirmation, the clinical samples were inoculated onto Esculin agar media containing esculin and bile. A color change in the media was used to differentiate *E. faecalis* from other Gram-positive species. A total of 137 positive species of *E. faecalis* were collected, including 89 (64.96%) from females and 48 (35.04%) from males. The frequency distribution shows, the highest prevalence was recorded above than age of 60 years of 51 (37.2%) followed by 41-60 years 41 (29.9%), 21-40 years 27 (19.8%), and the least prevalence were recoded below than 20 years 18 (13.2%) (Table 2).

**Table 2:** Different parameters for *E. faecalis,* including gender-wise distribution and age groups

| Gender | Frequency (%) | p-valve |
|---|---|---|
| **Male** | 89(64.96) | 0.0005 |
| **Female** | 48(35.04) | |
| **Age-wise groups** | | |
| **0-20 years** | 18 (13.2) | |
| **21-40 years** | 27 (19.8) | |
| **41-60 years** | 41 (29.9) | |
| **>60 years** | 51 (37.2) | |

Antibiotic profiling of E. faecalis

According to the CLSI 2022 guidelines, all the E. faecalis species were tested against 17 antibiotics by the disc diffusion method. Some species showed resistance, and some showed sensitivity to the antibiotics. E. faecalis determined the highest resistance to Erythromycin and Ciprofloxacin, with 126 cases (92%) each, as shown in Table 3.

Table 3: Antibiogram of E. faecalis isolates, showing resistance and sensitivity patterns with the number (n) and percentage (%) for each antibiotic tested.

**Table 3:** Antibiogram of E. faecalis isolates, showing resistance and sensitivity patterns with the number (n) and percentage (%) for each antibiotic tested.

| Antibiotics (Symbol) | Sensitive %) | Resistance (%) | p-valve |
|---|---|---|---|
| **Tetracycline (TGC)** | 22 (16) | 115 (83) | <0.0001 |
| **Doxycycline (DO)** | 94 (69) | 43 (31) | |
| **Erythromycin (E)** | 11 (8) | 126 (92) | |
| **Chloramphenicol (C)** | 54 (39) | 83 (61) | |
| **Norfloxacin (NOR)** | 43 (31) | 94 (69) | |
| **Minocycline (MIN)** | 22 (16) | 115 (84) | |
| **Levofloxacin (LEV)** | 13 (9) | 124 (91) | |
| **Ciprofloxacin (CIP)** | 11 (8) | 126 (92) | |
| **Vancomycin (V)** | 137 (100) | 0 (0) | |
| **Teicoplanin (TEI)** | 94 (69) | 43 (31) | |
| **Rifamycin (RIF)** | 42 (30) | 95 (70) | |
| **Penicillin (P)** | 43 (31) | 94 (69) | |
| **Nitrofurantoin (N)** | 74 (54) | 63 (46) | |
| **Linezolid (LZD)** | 106 (77) | 31 (23) | |
| **Gentamicin (GN)** | 37 (54) | 63 (46) | |
| **Fosfomycin (FOS)** | 117 (85) | 20 (15) | |
| **Ampicillin (AMP)** | 33 (23) | 104 (77) | |

Molecular Characterization of Antibiotic Resistance Genes

The overall antibiotic resistance gene reported in clinical isolates of E. feacalis conducted in the current research study revealed that AmpC has 107 (78%), followed by ctxM at 100 (73%), Tet A was detected at 97 (71%), and the lowest was recorded in Tet B at 79 (58%) shown in Table 4. The amplified PCR products were analyzed with a 100bp ladder and on gel electrophoresis (BioRad). The visualized gel-on-gel documentation system is shown in Figure 2.

**Table 4:** Molecular characterization of antibiotic resistance genes in *E. faecalis* clinical isolates, showing the frequency (n) and percentage (%) of detected genes.

| Antibiotic resistance gene | Frequency (n) | Percentage (%) | p-valve |
|---|---|---|---|
| ***AmpC*** | 107 | 78 | 0.22 |
| ***CTX-M*** | 100 | 73 | |
| ***Tet-A*** | 97 | 71 | |
| ***Tet-B*** | 79 | 58 | |

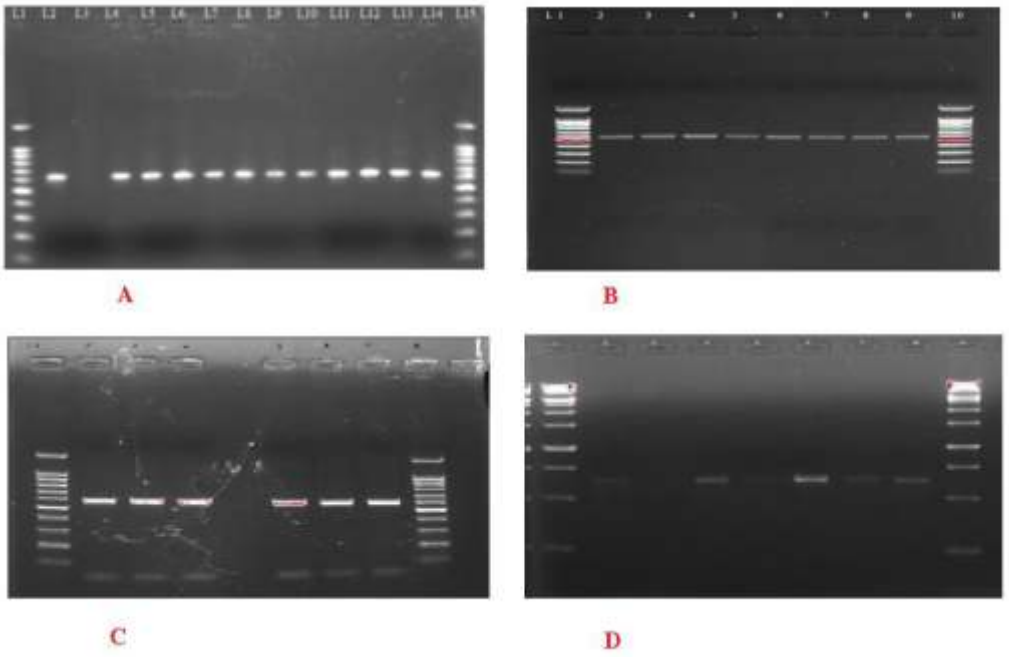


**Figure 2:** L: DNA ladder 100bp, **(A)** Amplified products of AmpC (634bp). **(B)** amplified PCR of *ctx*M (454bp). **(C)** Amplified PCR products of *Tet-A* (577 bp). **(D)** Amplified PCR products of *Tet-B* (634 bp)

Analysis of Mutations predicted in the Antibiotic Resistance gene

The amplified products obtained from PCR were sequenced with the help of Sanger sequencing to determine mutations associated with resistance in tetracycline and cefotaxime clinical isolates. Two PCR products were randomly selected for molecular characterization for each resistance gene—CTX-M, AmpC, Tet A, and Tet B. Sequencing analysis of the CTX-M gene revealed a nucleotide mutation at position 379 bp, where adenine was replaced by guanine, resulting in an alteration of the amino acid sequence. Among the 137 clinical isolates, Tet A and Tet B were detected in 97 and 79 isolates, respectively. Sequencing of two randomly selected samples for each gene showed no mutations in their nucleotide sequences. For the AmpC resistance gene, detected in 107 (78%) of the samples, the mutational analysis identified a nucleotide change at position 106 bp, where cytosine was replaced by thymine, leading to an altered amino acid sequence.

## DISCUSSION

Enterococcus is found in the normal flora of humans, including the gastrointestinal and genitourinary tract, and is an opportunistic pathogen of both hospitals and community-acquired infections.[24, 25] Urinary tract infections are life-threatening infections caused by E. faecalis present in hospitals and community-acquired settings.[26] Baghaw et al, reported E. faecalis is the third most uropathogenic-causing UTIs in intensive care units after E. coli.[27] The ratio of UTIs caused by Enterococcus is more common in the above than 60 years due to frequent abstractive uropathy.[28] In the current research study, A total of 137 positive species of E. faecalis were collected, including 89 (64.96%) from females and 48 (35.04%) from males. The results reveal the highest ratio in females and older ages. The frequency distribution shows, that the highest prevalence was recorded above than age of 60 years of 51 (37.2%) followed by 41-60 years 41 (29.9%), 21-40 years 27 (19.8%), and the least prevalence were recoded below than 20 years 18 (13.2%). Similarly, Madrazo et al. reported a study, in a cohort of 659 patients to identify predictive factors of UTIs caused by E. faecalis. Out of the total samples, 87 patients (13.2%) had E. faecalis as the causative agent. The mean age of the patients with E. faecalis UTI was 82.3 years, and a significant proportion of these patients had multiple comorbidities. The study found that risk factors for E. faecalis UTI in older patients included the presence of an indwelling urinary catheter and previous urinary tract instrumentation.[29] All the species of Enterococcus were identified, but Enterococcus faecalis is the most dominant species for UTIs and is a good concern with other clinical diseases.[24] This research study has great concern with previously reported data that E. faecalis is more resistant to tetracycline, penicillin, ampicillin, cefotaxime, and ciprofloxacin.[30] In the current research study, E. faecalis species were tested against 17 antibiotics by disc diffusion method. Some species showed resistance, and some showed a sensitive nature against the antibiotics. E. faecalis determined the

highest resistance to Erythromycin and Ciprofloxacin, with 126 cases (92%). Similarly, Miskeen et al. reported the study, perfectly aligned with the current research study. The reported study analyzed the antimicrobial susceptibility patterns of Enterococcus species isolated from urinary tract infections (UTIs) in 147 patients. Among the isolates, Enterococcus faecalis was the most prevalent species, accounting for 87.07% of the cases, followed by E. faecium (10.88%) and E. durans (2.05%). The isolates were tested for susceptibility to various antibiotics using disk diffusion, agar dilution, and E-test methods. Resistance to penicillin and ampicillin was observed in 23.13% of the isolates, while ciprofloxacin resistance was higher at 55.78%. Resistance to nitrofurantoin, co-Amoxil-clavulanate, and amp-sulbactam was low at 0.78%, 8.16%, and 2.72%, respectively. High-level aminoglycoside resistance was observed in 33.84% of the strains for streptomycin and 36.92% for gentamicin. Importantly, all Enterococcus strains were found to be susceptible to glycopeptide antibiotics, including vancomycin and teicoplanin.[26] Mediratta et al. reported that Enterococcus is the uropathogenic cause of bacteriuria in elderly male patients and revealed an isolated bacteria ratio of 22.5%. The AmpC resistance genes study was reported in Bangalore, India, the same study was conducted in Tehran with low prevalence.[31] The high prevalence of the AmpC resistance gene (2-10%) was reported worldwide [26]. The current study revealed that molecular characterization detected the highest prevalence (78%) in the urine samples, subjected to Sanger sequencing for mutational analysis. Through BioEdit software, the results were analysed with the reference sequence in GenBank. The ctxM resistance gene revealed the presence of the mutation in the nucleotide sequence at 379 Bp in which the Adenine is replaced by Guanine and did not alter the amino acid sequence. The results of the AmpC resistance gene revealed the mutational nucleotide change at position 106; cytosine was replaced with Thymine but did not alter the amino acid sequence. Same as the mutational study of the Tet-A and Tet-B studies revealed no change in nucleotide sequence.

## CONCLUSION

Enterococcus faecalis is identified as a predominant etiological agent of Urinary Tract Infections (UTIs), with a significant presence in both nosocomial and community settings, representing a critical public health challenge. The findings of a current research study revealed the antibiogram profiles of multidrug-resistant (MDR) E. faecalis isolates from District Peshawar, providing essential data for the development of evidence-based infection control strategies. These measures are crucial for the identification of the spreading of MDR pathogens within healthcare and community environments. Furthermore, the study offers a foundation for the identification and sequencing of antibiotic resistance genes, enabling a further study of the molecular mechanisms leading to resistance. The understanding generated will support the optimization of targeted antibiotic therapies, enhancing clinical outcomes in UTI management.

## CONFLICT OF INTEREST

The authors declare no conflict of interest related to this publication

## FINANCIAL DISCLOSURE STATEMENT

No dedicated financial support or external funding was received for the completion of this work

RIPASA scoring system is a better clinical scoring system with a relatively higher accuracy of 91.80% as compared to the ALVARADO score with an accuracy of 85.25% in diagnosing acute appendicitis when the histopathological assessment of removed appendix is kept as a reference standard. Given its higher accuracy, the RIPASA scoring system should be considered the preferred tool for evaluating patients with suspected acute appendicitis, particularly in settings with limited access to advanced imaging. It is recommended that healthcare professionals be trained in the application of RIPASA to ensure consistent use across clinical settings, and further research with larger, diverse populations is needed to validate its broader applicability. While RIPASA alone offers high diagnostic reliability, its use in combination with imaging techniques may still be beneficial in uncertain cases. Lastly, clinical guidelines should be updated to incorporate RIPASA as a primary diagnostic tool in the management of acute appendicitis.

### Conflict of Interest Statement

The authors declare no conflict of interest related to this publication.

### Financial Disclosure Statement

No dedicated financial support or external funding was received for the completion of this work.

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| **Key Contributions of the Authors** | |
|---|---|
| **Author Names** | **Author Contributions** |
| Zahidullah Khan | A, C, D |
| Adnan Mumtaz | A, B, D |
| Sadaf Bilal | A, C, D |
| Maham Iqbal | B, C, D |
| Waseem Abbas | C, D |
| Muhammad Khalid | D |

**Key for Author Contributions:**
A.Conception, Planning of the Research
B.Initial Review of Data Collection, Research Proposal of Article
C.Writing of manuscript of Article
D.Critical Review of Article
E.Data collection and interpretation of Article
All the authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved