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RESEARCH ARTICLE

Comparative Assessment of Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry and Conventional Methods in the Identification of Clinically Important *Enterococcus* Species

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Abstract

Introduction: *Enterococcus* species, part of the normal gut flora, have emerged as significant nosocomial pathogens causing urinary tract infections, bacteremia, endocarditis, wound infections, and intra-abdominal infections. The rise of vancomycin-resistant enterococci (VRE), with a global prevalence of approximately 12.4%, poses a major therapeutic challenge. This study compared conventional identification methods with MALDI-TOF MS and evaluated antimicrobial susceptibility patterns. **Methods:** A cross-sectional study was conducted at PSG IMS & R from January to June 2025. A total of 200 *Enterococcus* isolates from blood, urine, and pus samples were identified using conventional biochemical tests (Gram stain, catalase, bile esculin, PYR, growth at pH 9.6, 6.5% NaCl tolerance, and carbohydrate fermentation) and MALDI-TOF MS. Antimicrobial susceptibility testing was performed using the VITEK 2 system, and MIC values were interpreted accordingly. **Results:** Conventional methods identified 97 *Enterococcus faecalis* and 94 *Enterococcus faecium* isolates but could not reliably identify other species. MALDI-TOF MS identified 97 *E. faecalis*, 94 *E. faecium*, 4 *E. gallinarum*, 2 *E. casseliflavus*, 2 *E. raffinosus*, and 1 *E. avium*. *E. faecium* demonstrated lower susceptibility to ampicillin (50%), ciprofloxacin (40%), high-level gentamicin (25%), and vancomycin (75%), but high susceptibility to linezolid (98%). *E. faecalis* showed higher susceptibility to vancomycin (99%), linezolid (99%), ampicillin (80%), and penicillin (79%), with lower susceptibility to ciprofloxacin (35%) and erythromycin (30%). **Conclusion:** MALDI-TOF MS demonstrated superior species-level identification compared to conventional methods and is a rapid, cost-effective tool for routine laboratory diagnosis. Higher rates of vancomycin and high-level gentamicin resistance were observed in *E. faecium* compared to *E. faecalis*, highlighting the need for continuous antimicrobial surveillance.

Keywords: Antimicrobial Resistance, Enterococcus, High Level Gentamicin, Mass Spectrometry, Minimum Inhibitory, Vancomycin Resistant Enterococci

1. Introduction

Enterococci, though naturally present in the human gut, have emerged as formidable pathogens in clinical environments posing significant challenges to patient care and infection control. These opportunistic bacteria are notorious for causing a wide array of infections, particularly in hospitalized patients and immunocompromised individuals. Among the most concerning infections linked to enterococci are nosocomial infections, Urinary Tract Infections (UTIs),

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bacteremia, endocarditis, wound infections and intra-abdominal infections[1].

Enterococcus is dangerous because of their dual weaponry—their resistance to antibiotics and their virulence factors. They possess both intrinsic and acquired resistance mechanisms rendering many conventional antibiotics ineffective. This Antimicrobial Resistance (AMR) complicates treatment and increases the risk of persistent and recurrent infections. Additionally, enterococcus employ a range of virulence factors, including Biofilm Formation and allows them to firmly adhere to medical devices such as catheters and prosthetic implants, shielding them from immune responses and antibiotic treatments[2,]. Production of Enzymes and Toxins substances enable them to invade host tissues, evade immune defences and cause significant damage. Patients with compromised immune systems, those undergoing invasive medical procedures, and individuals with prolonged hospital stays are particularly vulnerable to enterococcal infections.

This study aims to compare and evaluate the effectiveness of Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) versus conventional microbiological methods in identifying clinically relevant Enterococcus species.

2. Aim and Objectives

- To characterize Enterococcus species by conventional biochemical tests.
- To characterize Enterococcus species by using MALDI TOF MS.
- To compare identification of Enterococcus species by conventional methods and MALDI-TOF.
- To study antibiotic susceptibility profile of Enterococcus species.

3. Review of Literature

Enterococcus is one of the important causes of hospital acquired infection, identification of enterococcus species is crucial in treatment. In our study we compared the conventional method with MALDI-TOF identification.

Cheng *et al.,* demonstrated MALDI-TOF had a 98.5% accuracy in identifying Enterococcus species compared to 87.1% by conventional biochemical methods[2], Deng J *et al*., noted that misidentification by conventional methods often occurs due to overlapping biochemical profiles, a problem not encountered with MALDI-TOF due to its proteomic basis[3].

MALDI-TOF MS has revolutionized clinical microbiology with its ability to rapidly and reliably identify bacteria based on their unique protein spectral fingerprints, mainly ribosomal proteins[7]. Bacterial colonies are ionized via laser and analysed by MALDI-TOF mass spectrometry. The spectrum generated is matched against a reference database[7]. Identification within minutes after colony isolation[7]. Though the initial instrument cost is high, per-sample cost is low, making it economically viable[7]. The increasing prevalence of multidrug- resistant *Enterococcus* species has made their rapid and accurate identification crucial for effective clinical management and infection control[8]. Given the clinical significance of enterococci, it is imperative to employ accurate, rapid and reliable identification techniques for early diagnosis and appropriate antimicrobial therapy[8]. By assessing these techniques, this research seeks to highlight the advantages and limitations of each method, paving the way for faster, more precise diagnostics that could revolutionize enterococcal infection management. MALDI-TOF MS can be the game-changer in the battle against antibiotic-resistant enterococci[8].

Enterococcus is an important component of the normal intestinal flora, have become important nosocomial pathogens particularly in critically ill and immunocompromised individuals[9].

The most clinically significant species are *Enterococcus faecalis* and *Enterococcus faecium*. Both patient care and antimicrobial stewardship depend on accurate and timely species-level identification. Traditionally, species of the Enterococcus genus have been identified by phenotypic method like biochemical tests including Colony morphology, Gram stain, Catalase test, *Bile esculin* hydrolysis, Salt tolerance tests at 6.5% NaCl, Carbohydrate fermentation patterns like: arabinose, sorbitol, mannitol. These techniques are commonly used although they are Time-consuming[9].

Accurate and timely identification of *Enterococcus* species is crucial for effective antimicrobial therapy and infection control, especially with rising incidence of Vancomycin resistant Enterococci. Misidentification

can lead to inappropriate treatment, delayed diagnosis and poor patient outcomes[10]. In a study by Murray *et al.,* (1990), biochemical identification showed a sensitivity of only around 85% for common Enterococcal species, with a marked drop in accuracy for less common strains[11].

MALDI-TOF has emerged as a revolutionary tool in clinical microbiology and can deliver species level identification within minutes offering substantial improvements in turnaround time, accuracy, cost-efficiency in the long term. Clinical microbiology laboratories are a primary source of information in our fight against multidrug-resistant enterococcal infections[16]. Personalized medicine-based approaches like the development of precise testing algorithms to guide clinical laboratories to test susceptibility of pathogens to clinically relevant antimicrobials based on the precise clinical scenario would be a major advance in the field[16].

Patel *et al.,* showed that MALDI-TOF could accurately differentiate between *Enterococcus faecalis* and *Enterococcus faecium*, and even some rarer species, outperforming conventional method[17].

The rise of vancomycin-resistant *E. faecium* and, to a lesser extent, the increased prevalence of antimicrobial resistance determinants in *E. faecalis* often leaves clinicians with few to no treatment options. This situation creates an urgent need for novel tools that improve early detection, targeted treatment and global surveillance of these pathogens. Indeed, accurate, precise and accessible AST is critical for optimal patient care.

4. Material and Methods

4.1 Bacterial Isolation

A total of 200 *Enterococcus* species were isolated from blood, Urine, Pus and wound swab between January 2025 to June 2025 with inclusion criteria of all the *Enterococcus* species and exclusion criteria of all the other bacteria.

4.2 Conventional Method

All samples were cultured on Blood agar and MacConkey agar and incubated for 16-24 hrs @37°C . The suspected Enterococcal colonies were subjected to Gram stain, catalase test, Growth@ pH 9.6, salt tolerance test (6.5% Nacl), Bile esculin test, PYR test, Fermentation of Glucose, Maltose, Mannitol, Arabinose as per standard methods for species identification MALDI-TOF

Materials Required: MALDI-TOF MS(Bruker), MALDI-TOF Target plate, Matrix -α cyano-4-hydroxycinnamic acid (HCCA), Fresh inoculum, Sterile wooden toothpick, Sterile gloves

Procedure: We Smeared an isolated colony as a thin film directly onto an empty position on MALDI target plate and Overlayed the material with 1.0µL of HCCA solution within 30 min and allowed it to dry at room temperature. We Placed the MALDI target plate in the slide loading ionization chamber. Chemical compounds are ionized and mass to charge ratio is measured and the protein mass fingerprint is compared with system database and identification is made.

4.3 Vitek 2 Automated System-Biomerieux, Inc, USA

The SOP of microbiology laboratory PSGIMS and R was used as reference for performing the procedure. The Vitek 2 system (BioMerieux) uses plastic reagent cards 405/406 that contain microlitre quantities of antibiotics and test media in well.

Materials Required: Vitek 2 system-Automated instrument for microbial identification and susceptibility testing, Vitek 2 AST Cards-Specific cards for antimicrobial testing, Bacterial isolates, Saline solution.

Procedure: From a fresh (18-24 hrs) non-selective agar plate with pure colonies, inoculum was prepared by picking 3-5 isolated colonies and diluting them in4-5 ml of sterile saline and turbidity was adjusted to 0.5 McFarland solution which is equivalent to 107 CFU/ml. The bacterial suspension was transferred into Vitek 2 AST card using the integrated vacuum filling system. The inoculated AST card was placed into vitek 2 system and the system automatically incubates and reads the card. The Vitek 2 system measures the growth of bacteria and provides MIC (Minimum inhibitory Concentration) value. The Minimum inhibitory concentration is the lowest concentration that completely inhibits visible growth

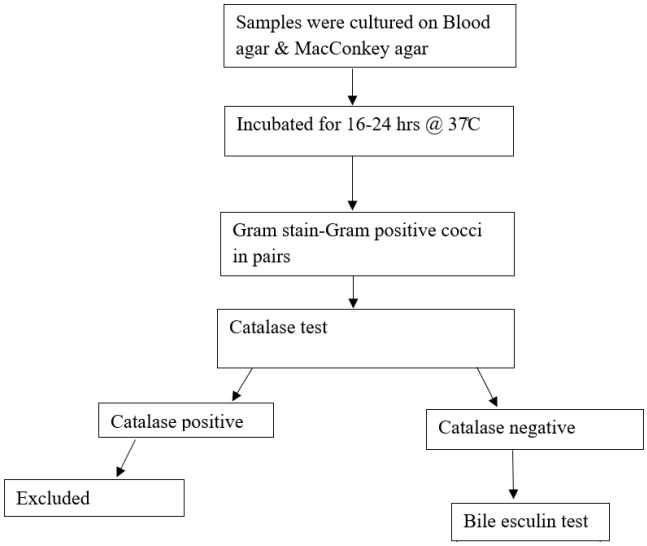

Chart 1. Methodology.

of the bacteria. Methodology has been explained in chart 1 and chart 2.

4.4 Statistical Analysis

Descriptive statistics like frequency and percentage was calculated. Graphical representation using bar graph was used.

5. Results (Including Observations)

In our study, 48 isolates were received from blood sample and 69 isolates from urine sample, 38 and 45 samples from pus and wound swab.

Isolates of Enterococcus species reported from different samples are tabulated in Table 1 and graphical representation of it were made in Figure 1.

5.1 Identification by Conventional Method

In conventional method, All Enterococcus faecium and Enterococcus faecalis showed positive for Bile aesculin test, PYR test, growth in media with PH 9.6 and tolerance with 6.5% NaCl. In sugar fermentation tests, Enterococcus faecalis and Enterococcus faecium both generally fermented Glucose, Mannitol and Maltose, but Enterococcus faecalis could not ferment Arabinose while Enterococcus faecium fermented Arabinose. Out of 200 isolates,97 Enterococcus faecalis were identified followed by 94 Enterococcus faecium but other Enterococcus species could not be identified by conventional method. Observation has been tabulated

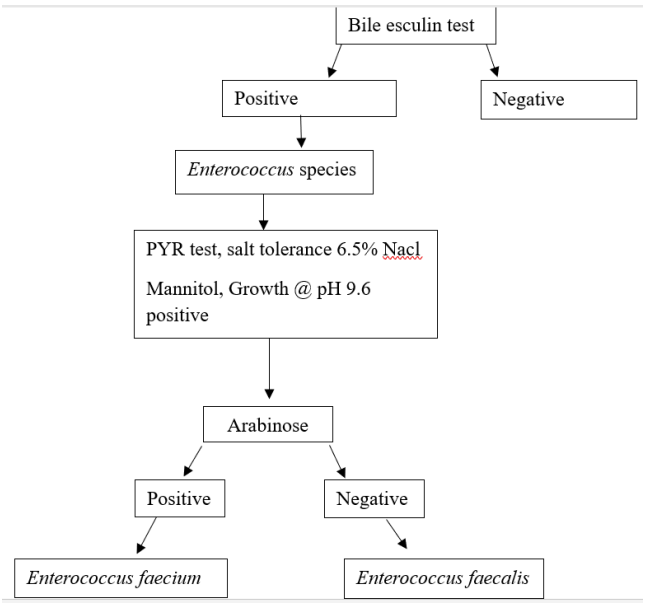

Chart 2. Methodology.

Table 1. Isolates of *enterococcus* species (N=200)

Samples	Number of isolates
Blood	48
Urine	69
Pus	38
Wound swab	45

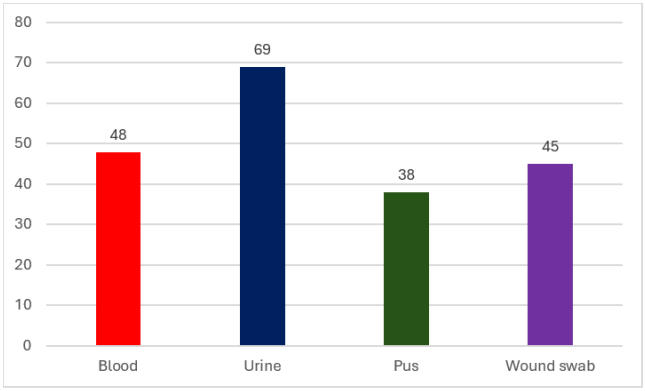

Figure 1. Isolates of *enterococcus* species (N=200).

in Table 2 and graphical representation is made in Figure 2.

5.2 Identification by MALDI-TOF

Out of 200 Enterococcus isolates, 97 *Enterococcus faecalis*, 94 *Enterococcus faecium*, 4 *Enterococcus gallinarum*, 2 *Enterococcus casseliflavus*, 2 *Enterococcus raffinosus*, and 1 *Enterococcus avium* isolates were

Table 2. Identification of *enterococcus* species by conventional methods

Biochemical methods	*Enterococcus faecium* (n=94)	*Enterococcus faecalis* (n=97)
Catalase test	-	-
PYR test	+	+
Bile esculin test	+	+
Mannitol	+	+
Arabinose	+	-

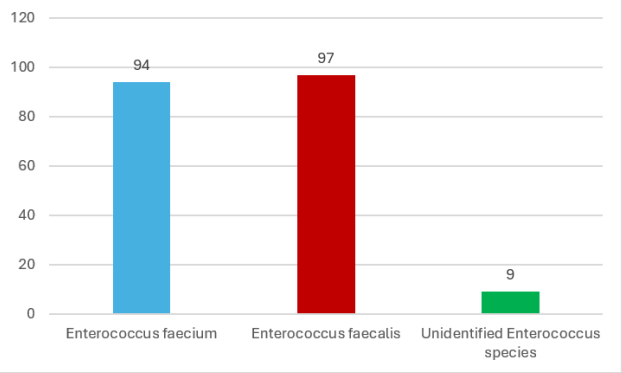

Figure 2. Identification of *enterococcus* species by conventional methods.

identified by MALDI-TOF MS. Tabulation and graphical representation were made in Table 3 and Figure 3.

5.3 Antimicrobial Susceptibility by Vitek 2 Biomerieux, Inc, USA

Enterococcus faecium showed 50% susceptibility to Ampicillin and Erythromycin, 40% susceptibility to Ciprofloxacin, 25% susceptibility to High level Gentamicin, 70% susceptibility to Penicillin, 98% susceptibility to Linezolid, 75% susceptibility to Vancomycin.

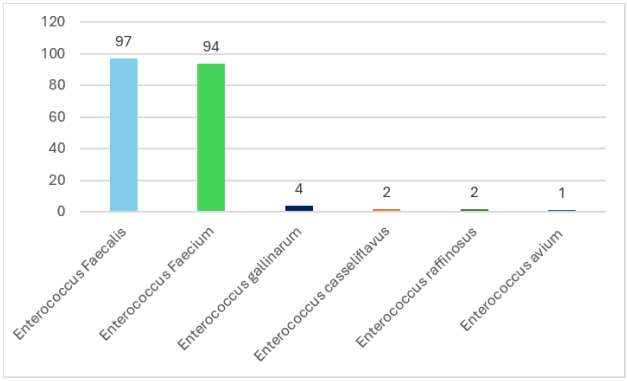

Figure 3. Identification of enterococcus species by MALDI-TOF (N=200).

Table 3. Identification of enterococcus species by MALDI-TOF

Species	Number of isolates
Enterococcus faecalis	97
Enterococcus faecium	94
Enterococcus gallinarum	4
Enterococcus casseliflavus	2
Enterococcus raffinosus	2
Enterococcus avium	1

Enterococcus faecalis shows 99% susceptibility to Vancomycin and Linezolid, 80% susceptibility to Ampicillin and 79% susceptibility to Penicillin, 56% susceptibility to High level gentamicin,35% susceptibility to Ciprofloxacin, 30% susceptibility to Erythromycin. Sensitivity of other *Enterococcus* species are summarized in Table 4. Vancomycin resistant rate and High-level Gentamicin resistant rate are more in *Enterococcus faecium* than *Enterococcus faecalis*.

Table 4. Antibiotic susceptibility pattern of different *enterococcus* species

	Enterococcus faecium	*Enterococcus faecalis*	*Enterococcus gallinarum*	*Enterococcus casseliflavus*	*Enterococcus raffinosus*	*Enterococcus avium*
Ampicillin	50	80	100	100	100	100
Penicillin	70	79	50	100	100	100
Linezolid	98	99	100	100	100	100
Vancomycin	75	99	R	R	100	100
High level gentamicin	25	56	87	100	100	100
Ciprofloxacin	40	35	100	100	50	100
Erythromycin	50	30	100	100	100	100

6. Discussion

All clinical isolates, including Enterococcus faecalis, *Enterococcus faecium*, and *Enterococcus gallinarum*, were accurately identified by MALDI-TOF MS. In addition to its high level of accuracy, MALDI-TOF MS was the most time- and cost-effective method among various other conventional biochemical methods. The genus Enterococcus comprises important nosocomial pathogens, most notably *Enterococcus faecalis* and *Enterococcus faecium*, which causes urinary tract infections, bacteremia, endocarditis, and surgical site infections[1].

This study compared the performance of MALDI-TOF MS (Matrix-Assisted Laser Desorption/ Ionization-Time of Flight Mass Spectrometry) with conventional phenotypic methods and found MALDI-TOF to be significantly superior in terms of speed, accuracy and species-level resolution. MALDI-TOF, when integrated with molecular techniques or used in tandem with selective media, can streamline workflows and improve laboratory efficiency (Angeletti *et al.,* 2017[7]).

Despite its high capital cost, the per-sample cost of MALDI-TOF MS is low, especially in high- throughput laboratories, making it cost-effective over time (Bizzini and Greub *et al.*, 2010[8]).

Phenotypic identification methods, including bile esculin hydrolysis, growth in 6.5% NaCl, and sugar fermentation profiles, are traditionally used in clinical microbiology laboratories. Although they are inexpensive and widely accessible, these tests can be labour-intensive, time-consuming (often requiring 24–72 hours) and prone to misidentification due to phenotypic overlap between species. Misidentification of these species using phenotypic methods can lead to inappropriate treatment decisions (Hollenbeck and Rice *et al.*, 2012[10]). Accurate and timely identification of Enterococcus species is essential, especially due to the increasing prevalence of multidrug-resistant strains such as Vancomycin-Resistant Enterococci (VRE[10]). Studies have shown that the conventional biochemical methods could misidentify uncommon Enterococcus species and may not reliably distinguish between Enterococcus faecalis and Enterococcus faecium, which differ significantly in their antimicrobial susceptibility patterns (Murray *et al.,* 1990; Facklam and Collins, 1989[9,11]).

In contrast, MALDI-TOF MS has revolutionized microbial identification by enabling rapid, accurate and cost-effective species-level identification within minutes. The technique analyses the protein composition of a bacterial colony and matches it to a reference spectral database. Numerous studies have demonstrated its high accuracy in identifying clinically important Enterococcus species. Similarly, Patel *et al.,* (2013[12]) highlighted the technique's ability to correctly identify Enterococcus faecalis and Enterococcus faecium with high sensitivity and specificity, outperforming conventional biochemical methods.

A key advantage of MALDI-TOF MS is its ability to identify rare Enterococcus species such as *Enterococcus gallinarum* and *Enterococcus casseliflavus*, which are intrinsically vancomycin- resistant due to the presence of the vanC gene. The ability of MALDI-TOF to detect such species accurately is contingent upon the comprehensiveness of the reference database, emphasizing the need for continual updates and inclusion of diverse clinical isolates (Schmitt and Cunningham, 2015[13]).

However, its adoption in resource-limited settings remains a challenge. In contrast, conventional methods remain more feasible in such environments due to lower infrastructure and training requirements, although they lack the precision and efficiency of modern technologies. Seng *et al.* (2009[14]) reported that MALDI-TOF MS correctly identified Enterococcus isolates to the species level in over 95% of cases, with results available in less than 30 minutes.

It is important to note that while MALDI-TOF MS excels in identification, it does not directly provide antimicrobial susceptibility information. Susceptibility testing must still be performed using disk diffusion, broth microdilution, or automated AST systems. Nonetheless, rapid species identification supports antimicrobial stewardship by narrowing empiric therapy and potentially reducing the use of broad-spectrum antibiotics (van Belkum *et al.,* 2013[15]).

Furthermore, early identification of VRE is crucial for infection control. It requires rigorous infection control measures to prevent the spread of VRE. While molecular methods such as PCR and whole- genome sequencing offer resistance gene detection, they are often more expensive and technically demanding

In our study, Vancomycin resistance rate was observed to be 25% in *Enterococcus faecium* and 1% in *Enterococcus faecalis*. Similarly, in a Multicentre study by Suraj Shrestha *et al*, vancomycin resistance rate was found to be 22.4% for *Enterococcus faecium* and 3.7% for *Enterococcus faecalis*[18]. In Abbas Moghimbeigi *et al* study done at Iran, the Vancomycin resistance was to be 33% in *Enterococcus faecium* and 3% in *Enterococcus faecalis*[19].

In our study, High level gentamicin resistance was observed to be 75% in *Enterococcus faecium* and 44% in *Enterococcus faecalis*. In Nita Gangurde et al study High Level Gentamicin Resistance (HLGR) was 44.4% in E. faecalis and 55.1% in E. faecium[20]. In study by Veljiko Mirovic *et al*. High level gentamicin resistance rate was observed to be 68.7% in *Enterococcus faecium* and 52.3% in *Enterococcus faecalis*[21].

Overall, this comparative assessment reinforces that MALDI-TOF MS is a superior diagnostic tool for the identification of clinically significant Enterococcus species. Its rapid turnaround, high accuracy, and potential for real-time identification contribute significantly to improving clinical outcomes and laboratory performance. Nevertheless, conventional methods remain valuable in certain settings and can serve as complementary tools when MALDI-TOF is unavailable. Future developments should focus on enhancing MALDI-TOF's capability for resistance profiling and expanding database coverage to include regionally relevant strains.

7. Summary and Conclusion

The results of this study indicate that MALDI-TOF MS can be used for routine identification of both usual and unusual Enterococcus, as it shows greater species-level discrimination than conventional methods. Further, MALDI-TOF MS is cost-effective and requires less time to identify the bacteria, making it a useful technique for diagnostic laboratories. It has rapid turnaround time, high accuracy and reliability in species level identification. It has decreased the labour use and reagent use. Although conventional methods remain useful in resource limited settings, they are often time consuming and prone to misidentification. Incorporating MALDI-TOF MS into routine clinical microbiology workflows can enhance patient care by faster diagnosis and timely initiation of antimicrobial therapy. Its adoption is beneficial in tertiary care hospital and high throughput laboratories, making it valuable asset for modern diagnostic microbiology. Enterococci exhibit significant resistance to a wide variety of antimicrobial agents. Vancomycin resistant rate and High-level Gentamicin resistant rate was found to be higher for Enterococcus faecium than Enterococcus faecalis.

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