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A comparative evaluation of conventional and automated (VITEK 2) methods for antibiotic susceptibility testing of enterococcus species isolated from various clinical samples

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Abstract

Enterococcus species, once regarded as commensals of the human gut, have now emerged as significant pathogens responsible for both nosocomial and community-acquired infections. Their ability to develop resistance to most commonly used antibiotics, through innate or acquired mechanisms, poses a major therapeutic challenge. The present study aimed to identify *Enterococcus* species and compare their antimicrobial susceptibility patterns using conventional and automated (VITEK 2 Compact, bioMérieux) methods. A total of 64 *Enterococcus* isolates were examined and speciated. Antibiotic susceptibility testing was performed according to CLSI guidelines using the Kirby–Bauer disc diffusion method and the VITEK 2 system. Among the 64 isolates, 42 (65.6%) were from male patients and 22 (34.4%) from female patients. The predominant species identified were *E. faecalis* (39.1%) and *E. faecium* (33.1%), followed by *E. avium* (10.9%) and *E. durans* (7.8%). *E. casseliflavus*, *E. dispar*, and *E. gallinarum* were isolated in equal proportions (3.1%), while *E. raffinosus* was the least common (1.6%). All isolates were sensitive to linezolid. The highest resistance was observed to penicillin (59.4%), followed by tetracycline and high-level gentamicin (31.2%), with minimal resistance to vancomycin (1.6%). Multidrug resistance was detected in 54.7% (35/64) of isolates. The conventional method for antimicrobial susceptibility testing (AST) was less efficient in differentiating bactericidal from bacteriostatic activity and involved delayed reporting. The VITEK 2 system, however, provided faster and more reliable results, aiding clinicians in timely and appropriate antibiotic selection.

Keywords: *Enterococcus*; antimicrobial resistance; conventional method; Kirby–Bauer disc diffusion; VITEK 2 compact system

Introduction

Enterococci are Gram-positive, facultative anaerobic bacteria that occur singly, in pairs, or in short chains. Lancefield classified them as Group D streptococci [1]. Over the past few decades, *Enterococcus* species have evolved from being common intestinal commensals in humans to major nosocomial pathogens, causing significant morbidity and mortality [2]. They are associated with a wide range of infections, including urinary tract infections (UTIs), surgical site infections (SSIs), bacteremia, intra-abdominal infections, and endocarditis [3]. Understanding their epidemiological distribution and antimicrobial resistance patterns is crucial in the clinical setting [4].

Enterococcus species are identified as Gram-positive cocci, catalase-negative, positive for L-pyrrolidonyl-β-naphthylamide (PYR) and leucine-β-naphthylamide (LAP) tests, and positive for bile esculin hydrolysis (Figure 1), arginine hydrolysis, and mannitol fermentation [5]. Among clinical isolates, *E. faecalis*

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(85–90%) and *E. faecium* (5–10%) are the predominant species, while others are less frequently encountered [6]. Historically, enterococcal infections were treated with cell wall-active agents such as penicillin or ampicillin, in combination with aminoglycosides like streptomycin or gentamicin. However, the failure of combination therapy to produce synergistic effects has been attributed to the emergence of resistance to high-level aminoglycosides (HLAR) and β-lactam antibiotics [7].

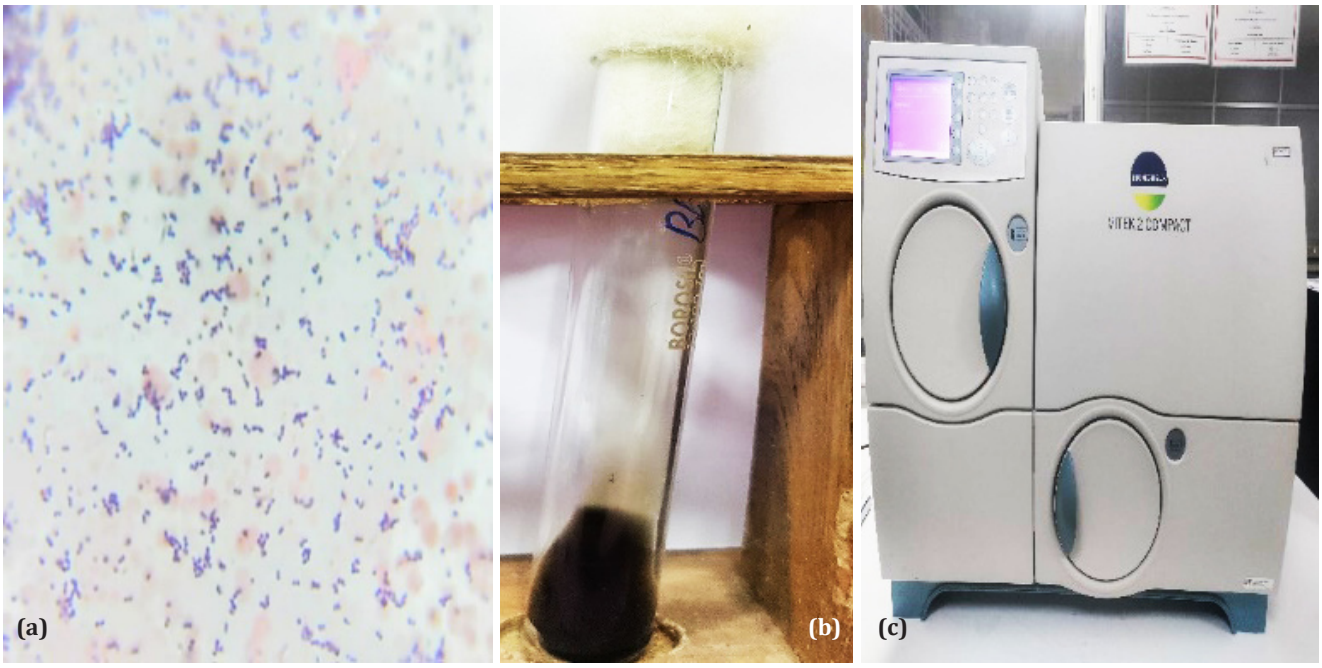

Figure 1a-c: Key Morphological and biochemical features used for the identification of *Enterococcus* species. (a) Gram stain: Arranged in pairs & short chains, (b) Bile esculin Test: Positive, (c) VITEK 2 system: Automated ABST method.

Vancomycin subsequently became the first-line drug for treating enterococcal infections; however, in recent years, the incidence of infections caused by vancomycin-resistant enterococci (VRE) has risen markedly. Resistance to ampicillin and vancomycin is more frequently associated with *E. faecium* than with *E. faecalis*. This growing resistance has significantly reduced available treatment options and is associated with increased mortality, prolonged hospital stays, ICU admissions, surgical interventions, and higher healthcare costs [8–10].

Traditionally, most clinical microbiology laboratories have relied on conventional culture and biochemical methods for species identification, and the Kirby–Bauer disc diffusion method for antimicrobial susceptibility testing (AST). However, these conventional procedures are time-consuming and may not always yield reliable results, leading to possible misidentification of organisms due to atypical biochemical traits or misinterpretation of zone diameters [11].

To address these limitations, a new automated technology—the VITEK 2 Compact system (bioMérieux)—has been developed for rapid and accurate identification and antimicrobial susceptibility testing of clinical isolates, including *Enterococcus* species. Identification is based on biochemical profiling, while minimum inhibitory concentration (MIC) values are determined using growth kinetics analyzed through built-in algorithms [12].

Although enterococcal infections are treatable with appropriate antibiotics, multidrug resistance (MDR) remains a serious global health concern. Therefore, it is essential for hospitals to rapidly identify, isolate, and speciate *Enterococcus* isolates to better understand their pathogenic potential and resistance mechanisms. Monitoring antibiotic resistance among *Enterococcus* isolates provides valuable insights into HLGR, VRE, and MDR patterns. Considering the limitations of conventional methods, automated systems such as the VITEK 2 Compact can help overcome these challenges. Only a few studies have evaluated this recently developed automated technology; hence, the present study was undertaken to assess the performance of the VITEK 2 Compact system compared to conventional methods.

The present study aimed to detect and classify various species of *Enterococcus* isolated from different clinical samples and to compare their antibiotic susceptibility patterns using both conventional and automated (VITEK 2 Compact, bioMérieux) methods. Specifically, the study sought to isolate, identify, and speciate *Enterococcus*

species in the diagnostic laboratory through the Kirby–Bauer disc diffusion method and the VITEK 2 Compact system. Furthermore, it aimed to compare and determine the antibiogram patterns obtained by these two methods to evaluate their reliability and diagnostic efficiency.

Materials and methods

A prospective cross-sectional study was conducted in the Central Laboratory of the Melmaruvathur Adhiparasakthi Institute of Medical Sciences and Research from February 2023 to July 2023. Ethical clearance for the study was obtained from the Institutional Ethics Committee (Human Studies) of Melmaruvathur Adhiparasakthi Institute of Medical Sciences and Research (Approval No. MAPIMS/IEC/52/2023, January 2023). A total of 64 *Enterococcus* isolates were obtained from various clinical specimens received in the microbiology laboratory. All samples were collected and processed following standard microbiological procedures using both conventional and automated techniques.

All culture samples, including urine, pus, blood, cerebrospinal fluid (CSF), pericardial fluid, peritoneal fluid, and high vaginal swabs, irrespective of the patient's age and sex, were included in the study for the isolation of *Enterococcus* species. Repeated isolates from the same patient and samples obtained from sputum, stool, throat swabs, and other gastrointestinal specimens were excluded from the study.

For the conventional method, each clinical specimen was inoculated onto nutrient agar, blood agar, and MacConkey agar plates. Urine samples were inoculated on cystine lactose electrolyte-deficient (CLED) agar. The inoculated plates were incubated at 35°C for 24–48 hours, and visible growth was observed after incubation. Colonies on blood agar appeared grey, 0.5–1 mm in diameter, and showed variable hemolysis (alpha, beta, or gamma). On MacConkey agar, small, magenta-colored, smooth, and convex colonies were observed. Preliminary identification of *Enterococcus* was done using Gram staining, which revealed Gram-positive cocci arranged in pairs. The isolates were catalase- and oxidase-negative, and positive for bile esculin hydrolysis. For species identification, isolates were further inoculated into 1% sugar fermentation media containing bromothymol blue as an indicator in peptone water. The sugars tested included glucose, mannitol, raffinose, sorbitol, lactose, sucrose, and arabinose [13–14].

Antimicrobial susceptibility testing (AST) was performed using the Kirby–Bauer disc diffusion method as per Clinical and Laboratory Standards Institute (CLSI) guidelines. Three to four well-isolated colonies were inoculated into peptone water and incubated for approximately three hours until the turbidity matched the 0.5 McFarland standard. A sterile swab was used to prepare a lawn culture of the standardized inoculum on Mueller–Hinton agar plates, and antibiotic discs were placed at an appropriate distance (approximately 24 mm apart), with six discs per plate. The antibiotics tested included penicillin (10 U), erythromycin (15 µg), vancomycin (30 µg), high-level gentamicin (120 µg), linezolid (30 µg), nitrofurantoin (300 µg), norfloxacin (10 µg), and tetracycline (30 µg). The plates were incubated at 35°C for 16–18 hours, and the results were interpreted according to CLSI standards. All antibiotic discs used were commercially available [13–14].

VITEK 2 compact system (bioMérieux)

For identification and antimicrobial susceptibility testing, inoculum preparation and processing were carried out according to the manufacturer's instructions. Two reagent inoculation (RIA) vials, each with a capacity of 5 mL, were filled with 3 mL of sterile saline. The first vial was inoculated with approximately 3–4 freshly isolated colonies from a 24-hour blood agar culture for identification of *Enterococcus* species. The turbidity of the suspension was adjusted to 0.5–0.63 McFarland units using a digital densitometer. For antimicrobial susceptibility testing (AST), 280 µL of saline was added to the second RIA vial. The Gram-positive identification card (ID-GPC) and AST card were then inserted into their respective RIA vials. The loaded plastic cassette was placed into the VITEK 2 Compact system, where the cards were automatically filled by vacuum and subsequently transferred into the incubator module maintained at 35.5°C. The system measured kinetic fluorescence readings every 15 minutes to monitor bacterial growth and biochemical reactions. After completion of the procedure, all used cards were automatically discarded into the waste container. Subculturing was performed from AST vials incubated at 37°C for 24–48 hours to confirm the purity and viability of the isolates [15].

Analysis of the report

The results for bacterial identification and antimicrobial susceptibility testing were interpreted using the VITEK 2 software (version 9.02). The ID-GPC database was employed for species identification, and the outcomes were categorized as follows: (i) correct identification, when isolates were accurately identified to the species level; (ii) low discrimination, when two or more possible species were proposed by the system, one of which matched the reference method; and (iii) no identification, when no species was detected.

Antimicrobial susceptibility interpretation was performed in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines. The minimum inhibitory concentration (MIC) values generated by the system were used to classify isolates as susceptible, susceptible dose-dependent, intermediate, or resistant. The average time required for obtaining results was also recorded for both identification and AST procedures [15].

Statistical analysis

All collected data were entered into a master chart using Microsoft Excel 2021 (Microsoft Corporation, Redmond, WA, USA). Statistical analyses were performed using SPSS software version 20.0 (IBM Corp., Armonk, NY, USA). Descriptive statistics, including frequency, rate, ratio, and percentage, were calculated and summarized. A p-value of ≤ 0.05 was considered statistically significant.

Results

In this study, a total of 64 clinical samples were collected from patients attending the hospital. Among these, 42 (65.6%) isolates were obtained from male patients and 22 (34.4%) from female patients, giving an estimated male-to-female ratio of approximately 2:1. The highest number of positive cases, 22 (34.4%), was observed in the age group of 51–60 years, whereas the lowest number of cases occurred in patients below 20 years of age. Table 1 summarizes the distribution of isolates between inpatient and outpatient samples.

Table 1: Demographic and clinical details of patients with *Enterococcus* infection.

Category	*Data*	*Number of Enterococcus identified*	*Percentage*
Gender-Wise	Male	42	65.6
	Female	22	34.4
Hospital status	Inpatient	41	64.6
	Outpatient	23	35.9
	0-10 years	1	1.6
	11-20 years	3	4.7
	21-30 years	5	7.8
	31-40 years	6	9.4
	41-50 years	8	12.5
	51-60 years	22	34.4
	61-70 years	17	26.6
	71-80 years	2	3.1

Urine specimens constituted the majority of samples, with 31 (48.4%) isolates, most of which were obtained from catheterized patients. This was followed by pus samples, accounting for 16 (25.0%) isolates, mainly from cases of cellulitis, surgical site infections, abscesses, and diabetic foot ulcers. Blood samples contributed 9 (14.1%) isolates, while both high vaginal swabs and peritoneal fluid samples yielded 4 (6.3%) isolates each. No *Enterococcus* isolates were recovered from pericardial fluid or cerebrospinal fluid (CSF). Both conventional and automated methods were used to identify *Enterococcus* species, as presented in Table 2.

Table 2: Distribution of *Enterococcus* isolates according to sample type.

Specimen	*Total*	*Percentage*	*Isolates*
Urine	31	48.4	E.faecalis, E.faecium, and E.raffinosus
Pus	16	25.0	E.faecalis, E.faecium, E.avium, E.casseliflavus, and E.dispar
Blood	9	14.1	E. faecalis, E. faecium, and E. gallinarum.
High vaginal swab	4	6.3	E.faecalis, E.faecium
Peritoneal Fluids	4	6.3	E.durans
Cerebrospinal fluid	0	0	-
Pericardial	0	0	-
Total	64	100	

The predominant species isolated was *E. faecalis* (39.1%), followed by *E. faecium* (31.3%). The remaining species isolated are detailed in Table 3. Using the VITEK 2 Compact system, two *E. durans* isolates showed low discrimination and were misidentified as *E. hirae*.

Table 3: Speciation of *Enterococcus* isolates using conventional and VITEK 2 compact methods.

Species	*Number of isolates*	*Percentage*
E. faecalis	25	39.1
E. faecium	20	31.3
E. avium	7	10.9
E. durans	5	7.8
E. casseliflavus	2	3.1
E. dispar	2	3.1
E. gallinarum	2	3.1
E. raffinosus	1	1.6
Total	64	100

The system also failed to differentiate between *E. casseliflavus* and *E. gallinarum*. Additionally, five isolates (three *E. faecium* and two *E. faecalis*) were unidentifiable by the automated system.

In the conventional method, all 64 isolates were found to be susceptible to vancomycin and linezolid. However, in the VITEK 2 Compact system, one isolate of *E. faecium* (1.6%) demonstrated resistance to vancomycin. The rates of resistance to other antibiotics, as determined by both methods, are presented in Tables 4 and 5. Among the 20 isolates that exhibited high-level gentamicin (HLG) resistance, the majority belonged to *E. faecalis* and *E. faecium*. Multidrug resistance (MDR) was detected in 35 of the 64 isolates, representing a prevalence of 54.7%. Statistical analysis of the antibiotic susceptibility patterns obtained by both methods revealed a *p*-value of <0.05, indicating a significant difference between the two testing approaches.

Table 4: Antimicrobial susceptibility pattern of *Enterococcus* isolates determined by the Kirby–Bauer disc diffusion method.

Antimicrobial agent and disc content	*Conventional method*			*P value (<0.05 significant)*
	S(%)	*I(%)*	*R(%)*	
Penicillin (10 U)	26 (40.6)	0	38 (59.4)	0.027
Erythromycin (15µg)	42 (65.6)	4 (6.3)	18 (28.1)	0.008
Vancomycin (30µg)	62 (96.8)	2 (3.2)	0	0.038
High-level gentamycin (120µg)	44 (68.8)	0 (0)	20 (31.2)	0.035
Linezolid (30µg)	64 (100)	0 (0)	0 (0)	0.043
Nitrofurantoin (300µg)	59 (92.2)	0 (0)	5 (7.8)	0.026
Norfloxacin (10µg)	47 (73.4)	2 (3.2)	15 (23.4)	0.022
Tetracycline (30µg)	41 (64.1)	3 (4.7)	20 (31.2)	0.031

Table 5: Antimicrobial susceptibility of *Enterococcus* isolates determined using the VITEK 2 compact system.

Antimicrobial agent and disc content	*Automated method*			*P value (<0.05 significant)*
	S (%)	*I (%)*	*R (%)*	
Penicillin (10 U)	26 (40.6)	0	38 (59.4)	0.027
Erythromycin (15µg)	42 (65.6)	4 (6.3)	18 (28.1)	0.008
Vancomycin (30µg)	62 (96.8)	1 (1.6)	1(1.6)	0.038
High level gentamycin (120µg)	44 (68.8)	0 (0)	20 (31.2)	0.035
Linezolid (30µg)	64 (100)	0 (0)	0 (0)	0.043
Nitrofurantoin (300µg)	59 (92.2)	0 (0)	5 (7.8)	0.026
Norfloxacin (10µg)	47 (73.4)	2 (3.2)	15 (23.4)	0.022
Tetracycline (30µg)	41 (64.1)	3 (4.7)	20 (31.2)	0.031

Discussion

The emergence of *Enterococcus* species as significant nosocomial pathogens in recent years is attributed to their intrinsic resistance to antibiotics such as cephalosporins, their ability to adhere to indwelling medical devices, and their capacity to survive in harsh environments. Their increasing antimicrobial resistance, particularly among hospitalized patients, poses a serious global health threat [16].

In this study, male patients (65.6%) were more frequently affected than female patients (34.4%), which may be associated with higher exposure to trauma, co-morbidities, certain health behaviors, and outdoor activities among men [18,19]. The highest number of isolates was observed in the 51–60 years age group (34.4%), followed by the 61–70 years group (26.6%), which is consistent with previous studies by Saraswathy et al., who reported the most affected age group as 40–60 years, and Soraj G et al., who observed higher prevalence in patients over 61 years [20,21].

Among the isolates, 41 (64.6%) were from hospitalized patients, while 23 (35.9%) were from outpatients. The majority of isolates were recovered from urine specimens (48.4%), followed by pus (25%), blood (14.1%), high vaginal swabs (6.3%), and peritoneal fluid (6.3%). No isolates were recovered from CSF or

pericardial fluid. These findings are similar to those reported by Sinha S. et al., who found 56% of isolates from urine, 33% from pus, and 5% from blood, with 6% from other body fluids such as ascitic fluid (2%) and vaginal swabs (4%) [17]. In contrast, Tripathi A et al. reported a higher proportion of pus samples (51%), followed by urine (24.4%), blood (19.2%), and other body fluids (5.4%) [22]. The elevated urine isolate levels in hospitalized patients may be due to the higher prevalence of catheterization in orthopedic and surgical patients, as well as the proximity of the anus to the urethra, since enterococci are commensals in the gastrointestinal tract [23].

Consistent with other studies by Gangurde N et al. in Chennai and Haritsa KB et al. in Bangalore, *E. faecalis* was the most frequently isolated species in this study (39.1%), followed by *E. faecium*, *E. avium*, *E. durans*, *E. casseliflavus*, *E. dispar*, *E. gallinarum*, and *E. raffinosus* [24,25]. This differs from the findings of Telkar et al. in South India, where *E. faecium* predominated over *E. faecalis* [26].

The VITEK 2 system demonstrated low discrimination in two *E. durans* and *E. faecalis* isolates, as well as three *E. faecium* isolates. Additionally, some isolates of *E. casseliflavus* and *E. gallinarum* could not be differentiated from one another. Ligozzi M. et al. reported similar discrepancies with automated systems [15]. The reasons for these misidentifications in our study remain unclear.

Beta-lactams and aminoglycosides are commonly preferred for treating enterococcal infections; therefore, resistance to these drugs has significant therapeutic implications. In this study, penicillin resistance was observed in 59.4% of isolates, comparable to the 53% reported by Jaiswal S et al., potentially due to poor affinity of penicillin-binding proteins or the production of β-lactamases [3]. Tetracycline and high-level gentamicin (HLG) resistance were observed in 31.2% of the 20 isolates, mainly in *E. faecalis* and *E. faecium*, which is consistent with previous studies [4,27]. Resistance to commonly used antibiotics, such as norfloxacin, was also observed, in agreement with Shivani S et al. [17].

Among the isolates tested by the automated method, only one *E. faecium* isolate (1.6%) exhibited vancomycin resistance. Other studies have reported vancomycin resistance rates ranging from 1% to 21% [8,28]. In contrast, Kanthishree B et al. observed vancomycin resistance in two *E. faecalis* isolates, although resistance is generally more frequent in *E. faecium* [8].

Multidrug resistance (MDR), defined as resistance to at least one agent in three or more antimicrobial classes, was observed in 35 out of 64 isolates (54.7%), which is comparable to the findings of Deshpande et al. [29]. This highlights a growing concern for clinicians worldwide. Statistical analysis of antibiotic susceptibility patterns yielded a p-value of <0.05, indicating significant differences. Notably, all isolates in this study were susceptible to linezolid, consistent with other reports [5,30].

The search for effective therapies against vancomycin-resistant enterococci (VRE) remains crucial. Linezolid and quinupristin/dalfopristin are considered the preferred treatment options; however, linezolid resistance has been reported in some studies [3,31]. Quinupristin/dalfopristin resistance is also rising, underscoring the need for cautious and judicious use [32,33].

Limitations: The main limitations of the present study include the small sample size and the absence of molecular identification of resistant *Enterococcus* species. These limitations could be addressed in future studies with larger sample sizes and the incorporation of molecular techniques to provide more precise characterization of resistant strains.

Conclusion

This study highlights that *E. faecalis* is the predominant species in clinical infections, while *E. faecium* is more frequently associated with multidrug resistance. The prevalence of high-level gentamicin resistance (HLGR), vancomycin resistance (VRE), and multidrug resistance (MDR) was 31.2%, 1.6%, and 54.7%, respectively. Accurate identification of enterococcal species and their antibiotic susceptibility patterns is essential for guiding effective treatment. While conventional methods are less sensitive and slower, the VITEK 2 system provides rapid, reliable detection of species and resistance profiles, enabling timely clinical decisions. Routine surveillance, strict antibiotic stewardship, and infection control strategies are critical to prevent hospital-acquired infections and reduce morbidity and mortality associated with multidrug-resistant enterococci.

Conflicts of interest

Authors declare no conflict of interest.

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