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PREVALENCE AND PHENOTYPIC CHARACTERISTICS OF HIGH-LEVEL GENTAMICIN-RESISTANT ENTEROCOCCUS ISOLATES FROM CLINICAL SPECIMENS

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ABSTRACT

Background: Enterococcus faecalis and Enterococcus faecium are important opportunistic pathogens with a notable capacity to acquire antimicrobial resistance. High-level gentamicin resistance (HLGR) is clinically significant because it abolishes aminoglycoside synergy with appropriate cell-wall-active agents. Virulence-associated phenotypes such as biofilm formation, gelatinase production, hemolysin production, and adhesion may further contribute to persistence and pathogenicity. the objective is to determine the prevalence of HLGR among clinical isolates of E. faecalis and E. faecium and to characterize their antimicrobial susceptibility and phenotypic virulence-associated characteristics.

Materials and Methods: This observational cross-sectional laboratory-based study was conducted at a tertiary-care teaching hospital in Central India from January 2024 to December 2025. A total of 370 non-duplicate clinical isolates of E. faecalis and E. faecium were included. Isolates were identified using standard microbiological methods, and antimicrobial susceptibility testing was performed according to the applicable CLSI criteria. HLGR was screened using a 120-µg high-content gentamicin disk. The 141 HLGR isolates were further evaluated for hemolysin production, gelatinase production, biofilm formation, and adhesion. Categorical variables were compared using chi-square or Fisher exact tests, with $p<0.05$ considered statistically significant.

Results: Among 370 clinical Enterococcus isolates, 141 were HLGR, giving an overall prevalence of 38.1%. Of the 276 E. faecalis and 94 E. faecium isolates, HLGR was detected in 88 (31.9%) and 53 (56.4%), respectively, with a significantly higher prevalence among E. faecium ($p<0.001$). Urine was the most frequent source of HLGR isolates (47.5%), followed by blood (27.0%); specimen type was not significantly associated with HLGR status ($p=0.706$). Resistance among HLGR isolates was highest to ciprofloxacin (71.6%), followed by levofloxacin (66.7%), penicillin (59.6%), high-level streptomycin (57.4%), and ampicillin (52.5%). Adhesion was detected in 75.2%, biofilm formation in 70.9%, gelatinase production in 48.9%, and hemolysin production in 32.6%. Gelatinase production was significantly more frequent among E. faecalis than E. faecium (61.4% vs 28.3%; $p<0.001$).

Conclusion: HLGR was common among clinical enterococcal isolates and was significantly more frequent in E. faecium. HLGR isolates also demonstrated considerable co-resistance and frequent expression of persistence-associated phenotypes, particularly adhesion and biofilm formation. These findings support routine high-level aminoglycoside-resistance screening, species-level identification, and continued institution-specific antimicrobial-resistance surveillance.

Keywords: Enterococcus faecalis; Enterococcus faecium; high-level gentamicin resistance; HLGR; biofilm; gelatinase; hemolysin; adhesion; antimicrobial resistance.

INTRODUCTION

Enterococci are Gram-positive, facultatively anaerobic cocci that normally colonize the gastrointestinal tract but have emerged as important opportunistic and healthcare-associated pathogens.[1–4] Enterococcus faecalis and Enterococcus faecium account for most clinically significant infections and are associated with urinary tract infections, bloodstream infections, infective endocarditis, wound infections, intra-abdominal infections, and device-associated infections.[1–4]

The management of enterococcal infections is complicated by intrinsic resistance and the ability of these organisms to acquire additional antimicrobial-resistance determinants.[2–5] Enterococci exhibit intrinsic low-level resistance to aminoglycosides because of limited drug uptake. In selected serious enterococcal infections, an aminoglycoside combined with an active cell-wall agent may produce synergistic bactericidal activity; however, this effect is lost when high-level aminoglycoside resistance develops.[5,6]

High-level gentamicin resistance is therefore clinically important because it predicts loss of gentamicin-associated synergistic killing.[5,6] One important mechanism is production of the bifunctional AAC (6′)-Ie-APH (2″)-Ia enzyme, which may be associated with transferable genetic elements and hospital-adapted enterococcal lineages.[7,8]

Considerable institutional and geographic variation in high-level aminoglycoside resistance has been reported. Indian studies have documented HLGR or HLAR prevalence ranging from approximately 39% to more than 70%, depending on the study population, specimen distribution, and laboratory methodology.[9–14]

In addition to antimicrobial resistance, enterococci possess several virulence-associated characteristics. Biofilm formation facilitates adherence to host tissues and medical devices, whereas gelatinase, hemolysin /cytolysin, and adhesion-related mechanisms may contribute to colonization, tissue injury, and persistence.[15–20]

The present study was undertaken to determine the prevalence of HLGR among clinical E. faecalis and E. faecium isolates and to characterize their antimicrobial susceptibility and phenotypic virulence-associated characteristics.

MATERIALS AND METHODS

Study design and setting: This observational cross-sectional laboratory-based study was conducted in the Department of Microbiology, Index Medical College Hospital & Research Centre, Malwanchal University, Indore, Madhya Pradesh, India, from January 2024 to December 2025.

Sample Size: Sample size was calculated using an expected prevalence of 39%, a 95% confidence level, and 5% absolute precision. The minimum calculated sample size was approximately 366 isolates. A total of 370 clinical Enterococcus isolates were included in the final analysis.

Inclusion Criteria

The study included:

- clinically significant isolates identified as E. faecalis or E. faecium;
- isolates recovered from eligible clinical specimens;
- isolates for which species identification and antimicrobial susceptibility testing were satisfactorily completed; and
- one clinically relevant isolate per patient/infection episode, where applicable.

Exclusion Criteria

The following were excluded:

- Enterococcus species other than E. faecalis and E. faecium;
- duplicate isolates representing the same organism from the same patient and clinical episode;
- inadequately collected, contaminated, or insufficient specimens; and
- isolates for which complete laboratory characterization could not be performed.

Gentamicin-susceptible isolates were retained in the study population because they are required for calculation of HLGR prevalence.

Study Workflow

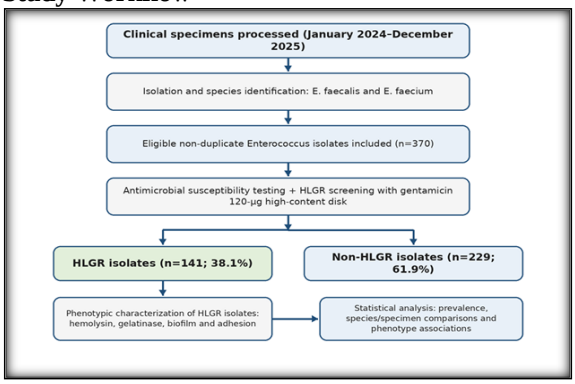

Figure 1: Study methodology and analytical workflow.

Isolation and Identification of Enterococcus Species

Clinical specimens were processed according to standard microbiological procedures. Primary isolation was performed on culture media appropriate for the respective specimen type. Suspected enterococcal colonies were evaluated on the basis of colony morphology and Gram-stain characteristics. Enterococci were identified as Gram-positive cocci occurring singly, in pairs, or in short chains.

Identification included: catalase test, Pyrrolidonyl arylamidase (PYR) test, bile-esculin hydrolysis, tolerance to 6.5% sodium chloride, and appropriate carbohydrate-fermentation tests, including arabinose fermentation for species differentiation where required.

The laboratory protocol also lists blood agar, MacConkey agar, bile-esculin azide agar, Brain Heart

Infusion media, Mueller-Hinton agar, and other media used for isolation and phenotypic characterization. For the 6.5% NaCl tolerance test, one to two colonies from an 18–24-hour culture were inoculated into Brain Heart Infusion broth containing 6.5% NaCl and incubated at 35°C for 24 hours. Visible turbidity with a colour change to pink was considered positive. Enterococcus faecalis ATCC 29212 was used as a positive control.

Antimicrobial Susceptibility Testing: Antimicrobial susceptibility testing was performed on Mueller-Hinton agar using a bacterial suspension equivalent to 0.5 McFarland turbidity.

The following antimicrobial agents were evaluated: Ampicillin, Penicillin, Vancomycin, Teicoplanin; Linezolid, Ciprofloxacin, levofloxacin, Tetracycline, high-level gentamicin and high-level streptomycin. Nitrofurantoin and fosfomycin were tested only for appropriate urinary isolates where applicable.

Susceptibility results were categorized as susceptible, intermediate, or resistant according to CLSI criteria applicable during the study period.[21]

Detection of High-Level Gentamicin Resistance: High-level gentamicin resistance was screened phenotypically using a gentamicin 120-µg high-content disk, according to the CLSI criteria followed by the laboratory.[21] An isolate fulfilling the relevant high-level gentamicin resistance screening criteria was categorized as HLGR. The study protocol also defines HLGR as a gentamicin MIC ≥500 µg/mL when an MIC-based screening method is used.

Detection of High-Level Streptomycin Resistance: High-level streptomycin resistance was evaluated separately from HLGR because gentamicin high-level resistance does not reliably predict high-level streptomycin resistance.[4,5]

Phenotypic Detection of Virulence-Associated Characteristics: The 141 HLGR isolates were further evaluated for phenotypic virulence-associated characteristics, including hemolysin production, gelatinase production, biofilm formation, and adhesion. The study protocol specifically identifies hemolysin, biofilm formation, gelatinase, and related phenotypic virulence traits as study objectives.

Hemolysin Production: Hemolysin production was assessed on Brain Heart Infusion agar supplemented with 5% human blood. Plates were incubated aerobically at 37°C for 24 hours. The presence of a clear zone of β-hemolysis surrounding the colonies was interpreted as a positive hemolysin phenotype.[2]

Gelatinase Production: Gelatinase production was evaluated on Todd-Hewitt agar supplemented with 3% gelatin. Following incubation at 37°C for 48 hours, the plates were examined after application of the gelatin-precipitating reagent. A clear zone around the bacterial colonies was interpreted as gelatinase positive.[2,3]

Biofilm Formation: A semiquantitative microtiter-plate adherence assay is an established and reproducible method for phenotypic assessment of biofilm production among clinical enterococci.[2,4]

A fresh overnight culture of each isolate was prepared in Brain Heart Infusion broth. The culture was diluted in glucose-supplemented broth, and approximately 200 µL of the bacterial suspension was transferred to sterile flat-bottomed polystyrene microtiter wells.

The plates were incubated aerobically at 37°C for 24 hours.[2]

After incubation:

1. The contents of each well were removed.
2. Wells were washed carefully to remove non-adherent organisms.
3. The plate was allowed to dry.
4. Adherent cells were fixed with 95% ethanol.
5. The wells were stained with 1% crystal violet for approximately 15 minutes.
6. Excess stain was removed by washing.
7. Biofilm-associated optical density was determined using a microplate reader.

Sterile broth without bacterial inoculum was used as the negative control, while Staphylococcus epidermidis ATCC 35984 has been used as a positive biofilm control in published enterococcal biofilm studies.[2] In the method described by Banerjee and Anupurba, isolates with optical density >0.5 were categorized as strong biofilm producers and those with values >0.2 but <0.5 as moderate producers.[2]

Adhesion Phenotype: Adhesion-associated activity was evaluated by a haemagglutination assay. A loopful of bacterial growth was mixed with 25 µL of a 3% human erythrocyte suspension prepared in phosphate-buffered saline (pH 7.4) in a 96-well microtiter plate. Following gentle rotation for 5 minutes and incubation at room temperature for 30 minutes, visible haemagglutination was interpreted as a positive adhesion phenotype.[2]

Statistical Analysis: Categorical variables were expressed as frequencies and percentages. Continuous variables were summarized using mean and standard deviation where appropriate. Overall HLGR prevalence was calculated using all eligible Enterococcus isolates as the denominator. Species-specific prevalence was calculated using the total number of isolates belonging to each species.

Associations between categorical variables were evaluated using Pearson's chi-square test. Yates continuity correction was used for appropriate 2×2 contingency tables, and Fisher exact test was considered when expected cell frequencies were small. A two-sided p value <0.05 was considered statistically significant.

RESULTS

A total of 370 non-duplicate clinical isolates of Enterococcus were included. Of these, 276 (74.6%) were identified as E. faecalis and 94 (25.4%) as E. faecium. High-level gentamicin resistance was detected in 141 isolates, giving an overall prevalence of 38.1% (141/370).

Species-Specific Prevalence of HLGR: Among 276 E. faecalis isolates, 88 demonstrated HLGR, corresponding to a species-specific prevalence of

31.9%. Among 94 E. faecium isolates, 53 were HLGR, giving a prevalence of 56.4%. Although E. faecalis accounted for a greater absolute number of HLGR isolates, the proportion of resistant isolates within the species was significantly greater among E. faecium.

Table 1: Species-wise prevalence of high-level gentamicin resistance

Species	Total isolates	HLGR n (%)	HLG susceptible n (%)
E. faecalis	276	88 (31.9)	188 (68.1)
E. faecium	94	53 (56.4)	41 (43.6)
Total	370	141 (38.1)	229 (61.9)

χ^2=16.82, df=1, p<0.001

Thus, there was a statistically significant association between Enterococcus species and HLGR status.

Demographic and Patient-Setting Characteristics

The mean age of patients from whom HLGR isolates were recovered was 50.3 ± 15.2 years, with a range of 18–88 years and a median age of 52 years. Among the 141 HLGR isolates, 77 (54.6%) were recovered from male patients and 64 (45.4%) from female patients. Most isolates were obtained from inpatients, accounting for 117 (83.0%), while 24 (17.0%) were obtained from outpatients.

Table 2: Demographic and patient-setting characteristics of HLGR isolates

Characteristic	Category	n	%
Sex	Male	77	54.6
	Female	64	45.4
Patient setting	Inpatient	117	83.0
	Outpatient	24	17.0

Specimen-Wise Distribution and Prevalence of HLGR: Urine was the most frequent source of HLGR isolates, accounting for 67 (47.5%), followed by blood in 38 (27.0%), wound swabs in 14 (9.9%), pus in 13 (9.2%), and other body fluids in 9 (6.4%). When HLGR prevalence was calculated within each specimen category, it was 41.4% among urinary isolates, 40.9% among other body-fluid isolates, 37.8% among wound-swab isolates, 35.8% among blood isolates, and 30.2% among pus isolates.

Table 3: Specimen-wise distribution and prevalence of HLGR

Specimen	Total Enterococcus isolates	HLGR isolates	Distribution among HLGR n (%)	HLGR prevalence within specimen (%)
Urine	162	67	67 (47.5)	41.4
Blood	106	38	38 (27.0)	35.8
Wound swab	37	14	14 (9.9)	37.8
Pus	43	13	13 (9.2)	30.2
Other body fluids	22	9	9 (6.4)	40.9
Total	370	141	141 (100.0)	38.1

There was no statistically significant association between specimen type and HLGR status (χ^2=2.16, df=4, p=0.706).

Department-Wise Distribution of HLGR Isolates

The largest number of HLGR isolates was recovered from patients under Medicine, 47 (33.3%), followed by ICU, 39 (27.7%), Surgery, 19 (13.5%), and Urology, 17 (12.1%).

Table 4: Department-wise distribution of HLGR isolates

Department/Unit	HLGR isolates n (%)
Medicine	47 (33.3)
ICU	39 (27.7)
Surgery	19 (13.5)
Urology	17 (12.1)
Nephrology	10 (7.1)
Orthopaedics	7 (5.0)
Cardiology	2 (1.4)
Total	141 (100.0)

These values represent the distribution of HLGR isolates and not department-specific prevalence.

Antimicrobial Susceptibility Profile of HLGR Isolates

Among the 141 HLGR isolates, the highest resistance was observed to ciprofloxacin (71.6%), followed by levofloxacin (66.7%), penicillin (59.6%), high-level streptomycin (57.4%), and ampicillin (52.5%). Resistance to tetracycline was present in 44.7%, while resistance to teicoplanin and vancomycin was observed in 19.9% and 17.0%, respectively. No linezolid-resistant isolate was identified.

Table 5: Antimicrobial susceptibility profile of HLGR isolates

Antimicrobial agent	Susceptible n (%)	Intermediate n (%)	Resistant n (%)
Ampicillin	63 (44.7)	4 (2.8)	74 (52.5)
Penicillin	54 (38.3)	3 (2.1)	84 (59.6)
Vancomycin	117 (83.0)	0	24 (17.0)
Teicoplanin	113 (80.1)	0	28 (19.9)
Linezolid	138 (97.9)	3 (2.1)	0
Ciprofloxacin	31 (22.0)	9 (6.4)	101 (71.6)
Levofloxacin	43 (30.5)	4 (2.8)	94 (66.7)
Tetracycline	71 (50.4)	7 (5.0)	63 (44.7)
High-level streptomycin	60 (42.6)	—	81 (57.4)

Species-Wise Antimicrobial Resistance

Among HLGR E. faecium, particularly high resistance was observed to penicillin (92.5%), ciprofloxacin (88.7%), and ampicillin (79.2%). The corresponding resistance among HLGR E. faecalis isolates was 39.8%, 61.4%, and 36.4%, respectively.

Table 6: Species-wise antimicrobial resistance among HLGR isolates

Antimicrobial	E. faecalis n=88	E. faecium n=53
Ampicillin resistant	32 (36.4%)	42 (79.2%)
Penicillin resistant	35 (39.8%)	49 (92.5%)
Vancomycin resistant	14 (15.9%)	10 (18.9%)
Teicoplanin resistant	15 (17.0%)	13 (24.5%)
Linezolid resistant	0	0
Ciprofloxacin resistant	54 (61.4%)	47 (88.7%)
Levofloxacin resistant	57 (64.8%)	37 (69.8%)
Tetracycline resistant	39 (44.3%)	24 (45.3%)
High-level streptomycin resistant	51 (58.0%)	30 (56.6%)

Phenotypic Characteristics of HLGR Isolates

Phenotypic characteristics associated with bacterial persistence were frequently detected. Adhesion was the most common phenotype and was demonstrated by 106/141 (75.2%) isolates. Biofilm production was identified in 100 (70.9%), gelatinase production in 69 (48.9%), and hemolysin production in 46 (32.6%).

Table 7: Phenotypic characteristics of HLGR isolates

Phenotypic characteristic	Positive n (%)	Negative n (%)
Adhesion	106 (75.2)	35 (24.8)
Biofilm formation	100 (70.9)	41 (29.1)
Gelatinase production	69 (48.9)	72 (51.1)
Hemolysin production	46 (32.6)	95 (67.4)

Species-Wise Comparison of Phenotypic Characteristics

Among HLGR E. faecalis, adhesion, biofilm formation, gelatinase production, and hemolysin production were detected in 77.3%, 72.7%, 61.4%, and 38.6% of isolates, respectively. The corresponding frequencies among HLGR E. faecium were 71.7%, 67.9%, 28.3%, and 22.6%.

Table 8: Comparison of phenotypic characteristics between HLGR E. faecalis and E. faecium

Phenotypic characteristic	E. faecalis n=88	E. faecium n=53	p value
Adhesion positive	68 (77.3%)	38 (71.7%)	0.589
Biofilm positive	64 (72.7%)	36 (67.9%)	0.677
Gelatinase positive	54 (61.4%)	15 (28.3%)	<0.001
Hemolysin positive	34 (38.6%)	12 (22.6%)	0.076

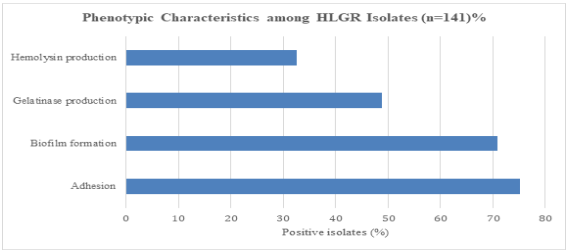

Figure 2: Frequency of phenotypic characteristics among HLGR isolates (n=141).

Gelatinase production was significantly more frequent among HLGR E. faecalis than E. faecium (61.4% vs 28.3%; p<0.001). Although hemolysin production was numerically more frequent among E. faecalis, the difference did not reach statistical significance. No significant species-wise differences were observed for biofilm formation or adhesion.

DISCUSSION

The present study demonstrated a 38.1% prevalence of high-level gentamicin resistance (HLGR) among 370 clinical Enterococcus isolates. This is clinically important because high-level aminoglycoside resistance abolishes the synergistic bactericidal activity of gentamicin with appropriate cell-wall-active agents.[5]

The observed prevalence was comparable with Bhatt et al,[12] who reported high-level aminoglycoside resistance in approximately 39% of clinical enterococci, and Arundathi et al,[9] who reported HLGR in 42%. Mendiratta et al,[10] and Banerjee et

al,[15] also documented substantial high-level aminoglycoside resistance, with the latter reporting HLGR in 47.4% of clinical isolates. These differences likely reflect geographic variation, antimicrobial exposure, patient population, and institutional resistance patterns.

E. faecalis was the predominant species in the present study, accounting for 74.6%, while E. faecium accounted for 25.4%. Similar predominance of E. faecalis was reported by Arundathi et al,[9] and Parameswarappa et al,[13] whereas Mohanty et al,[14] reported a relatively greater contribution of E. faecium.

Despite the predominance of E. faecalis, the species-specific prevalence of HLGR was significantly higher in E. faecium than E. faecalis (56.4% vs 31.9%; p<0.001). Arundathi et al,[9] similarly reported greater HLGR among E. faecium. This is consistent with the recognized ability of hospital-associated E. faecium to acquire and maintain multiple antimicrobial-resistance determinants.[2,4] Hasani et al,[22] also reported a high HLGR prevalence of 60.45%, confirming the substantial resistance burden among clinical enterococci.

Urine was the most frequent source of HLGR isolates (47.5%), followed by blood (27.0%). Similar predominance of urinary isolates has been reported by Parameswarappa et al,[13] and Banerjee et al.[15] However, specimen type was not significantly associated with HLGR status in the present study (p=0.706), indicating that resistance was distributed across different clinical specimen categories.

Considerable co-resistance was observed among HLGR isolates, particularly to ciprofloxacin (71.6%), levofloxacin (66.7%), penicillin (59.6%), high-level streptomycin (57.4%), and ampicillin (52.5%). Bhatt et al,[12] and Fernandes et al,[17] similarly reported substantial multidrug resistance among clinical enterococci. Species-wise analysis showed markedly greater resistance to penicillin, ciprofloxacin, and ampicillin among E. faecium, consistent with the multidrug-resistant phenotype commonly associated with this species.[2,4]

High-level streptomycin resistance was present in 57.4% of HLGR isolates. Because high-level gentamicin and streptomycin resistance may arise through different mechanisms, both should be evaluated separately when clinically relevant.[5]

Phenotypic characterization revealed that adhesion (75.2%) and biofilm formation (70.9%) were the most frequent characteristics, followed by gelatinase production (48.9%) and hemolysin production (32.6%). Biofilm formation in the present study was higher than the 32.5% reported by Upadhyaya et al,[16] and the lower prevalence reported by Banerjee et al,[15] but was comparable with the approximately 77% reported by Toru et al.[18] Differences may be related to study population, species distribution, and laboratory methodology. Mohamed et al,[23] emphasized the importance of biofilm formation in adherence to biotic and abiotic surfaces and persistence in device-associated infections.

Gelatinase production was observed in 48.9% of HLGR isolates, comparable with the 39% reported by Upadhyaya et al,[16] and approximately 40.6% reported by Fernandes et al.[17] Importantly, gelatinase production was significantly higher among E. faecalis than E. faecium (61.4% vs 28.3%; p<0.001). Similar species-related differences in virulence determinants have been reported by Hasani et al,[22] and Comerlato et al.[24]

Hemolysin production was detected in 32.6% of isolates, closely resembling the approximately 31.6% reported by Banerjee et al.[15] Upadhyaya et al,[16] reported a lower prevalence, whereas Fernandes et al,[17] reported a higher frequency. In the present study, hemolysin was more frequent among E. faecalis than E. faecium (38.6% vs 22.6%), although the difference was not statistically significant (p=0.076).

Adhesion was the most commonly detected phenotype, occurring in 75.2% of HLGR isolates. Banerjee et al,[15] also evaluated haemagglutination as a phenotypic indicator of adhesion, although a lower prevalence was reported. Differences may reflect variation in assay methods and the selected HLGR population.

Overall, the present study demonstrates that HLGR frequently coexists with resistance to additional antimicrobial agents and phenotypic traits associated with persistence. The significantly higher HLGR prevalence among E. faecium and greater gelatinase production among E. faecalis represent important species-specific findings. These results support routine high-level aminoglycoside-resistance screening, accurate species-level identification, and continued institution-specific surveillance of resistant enterococci.

Limitations: This study was limited by its single-centre design, which may restrict generalizability. Phenotypic virulence traits may vary with laboratory conditions and may not fully reflect in-vivo pathogenicity. Molecular mechanisms, clonal relatedness, and detailed patient-level risk factors or clinical outcomes were not evaluated. Therefore, the findings should be interpreted primarily as institution-specific microbiological and phenotypic surveillance data.

CONCLUSION

High-level gentamicin resistance was detected in a substantial proportion of clinical Enterococcus isolates, with a significantly higher species-specific prevalence among E. faecium than E. faecalis. HLGR isolates also demonstrated considerable resistance to several additional antimicrobial agents, particularly fluoroquinolones, penicillin, ampicillin, and high-level streptomycin. Phenotypic characteristics associated with bacterial persistence were common among HLGR isolates, with adhesion and biofilm formation being the most frequent. Gelatinase production was significantly more common among E.

faecalis than E. faecium, while differences in hemolysin production, biofilm formation, and adhesion between the two species were not statistically significant. These findings highlight the importance of routine detection of high-level aminoglycoside resistance, accurate species-level identification, and continued institution-specific antimicrobial-resistance surveillance. Further multicentre studies incorporating molecular characterization, clonal analysis, and clinical outcome data would help clarify the epidemiological and clinical significance of HLGR enterococci.

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