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Genotypic and Phenotypic Characterization of Virulence Factors in Vancomycin- Resistant Enterococci Isolated from Urinary Tract Infections

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ABSTRACT

Background: Enterococci are a common cause of urinary tract infections. Particularly in vancomycin-resistant Enterococcus (VRE), the coexistence of virulence factors and antimicrobial resistance, presents significant therapeutic challenges. The study aimed to comprehensively evaluate the correlation between phenotypic and genotypic characterization of virulence factors in VRE isolated from urine samples.

Methods: A cross-sectional study was conducted from June 2022 to April 2024 in the Department of Microbiology, S.M.S. Medical College, and Jaipur. Out of 250 Enterococcal urinary isolates, 37 (14.8%) were identified as VRE using CLSI-guided antimicrobial susceptibility testing. Phenotypic detection of hemolysin, gelatinase, and slime-layer production was performed, and Polymerase chain reaction (P.C.R.) was used to detect *Asa1 (Aggregation substance)*, *CylA (Cytolysin gene)*, and *GelE (Gelatinase)* genes.

Results: Among VRE isolates, hemolysin, gelatinase, and slime-layer production were observed in 21.6%, 10.08%, and 29.72%, respectively, while *CylA*, *GelE*, and *Asa1* genes were detected in 16.2%, 24.3%, and 13.5%. Comparative analysis showed partial concordance, with higher phenotypic hemolysin expression than *CylA* detection and higher *GelE* gene prevalence than gelatinase activity. Slime-layer production was significantly associated with *Asa1* gene .Species distribution in V.R.E. isolates was8.1%, 83.78%, and 8.1% for *Enterococcus faecalis*, *E. faecium*, and other species.

Conclusion: The discrepancy between phenotypic and genotypic results indicates that both methods are needed for accurate virulence profiling in VRE. The phenotypic and genotypic association of slime-layer production with *Asa1 gene* highlights the role of VRE as a potential pathogen with implications for antimicrobial stewardship.

Key-words: *Asa1* gene, Enterococcus, VRE, UTI, PCR, Slime Layer, Virulence factors

INTRODUCTION

Enterococci are facultative anaerobic cocci, Gram-positive that occur in pairs or short chains[1] Although they are part of the normal intestinal microbiota but occasionally acts as opportunistic pathogens, particularly in immunocompromised individuals.[2]

They are increasingly implicated in both hospital and community -acquired infections, which includes, (UTIs) and bacteremia.[3] *E. faecalis* and *E. faecium* are most frequently associated with human infections among the clinically significant species. In healthcare settings *E. faecium* has emerged as a predominant pathogen, largely due to its enhanced resistance to antibiotics, including vancomycin resistance [4,5].

The pathogenicity of Enterococcus is mediated via virulence factors that facilitate host tissue damage, colonization, persistence. In the establishment of infection adhesion plays a major role, with aggregation

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substance (*Asa1*), a pheromone-inducible surface protein, promoting adherence to renal epithelial cells and facilitating exchange of plasmid .[6] In addition, the ability to form biofilms enables Enterococci to persist on host tissues and medical devices, contributing to chronic and recurrent infections.[7] Beyond adhesion, secreted virulence determinants like gelatinase (*GelE*),cytolysin, and serine protease (*SprE*) are involved in tissue invasion and immune evasion. Cytolysin acts as a hemolytic toxin, while *GelE* and *SprE* degrade host proteins, promoting bacterial dissemination and biofilm maturation. [8-10]

The correlation between phenotypic virulence expression and corresponding genes is unclear, with inconsistent findings reported. This phenotypic–genotypic discrepancy highlights a gap in understanding VRE pathogenicity. Moreover, no specific virulence markers have been defined for UTI-associated Enterococcal infections. Therefore, the present study evaluates key virulence factors in VRE isolates from UTIs and assesses their phenotypic–genotypic correlation to address this gap.

MATERIALS AND METHODS

Research Design- This study was undertaken to perform a comprehensive genotypic and phenotypic analysis of virulence factors in VRE urinary isolates. A laboratory-based cross-sectional study was conducted in the Bacteriology Laboratory, Department of Microbiology, SMS Medical College, Jaipur, from Jun 2022 to Apr 2024.

Study Population and Sample Size- A total of 250 Enterococcus isolates obtained in significant counts from urine samples were included.

Sample size- The sample size was determined based on an expected prevalence of VRE of approximately 20% (based on prev. regional studies), with a 95% confidence level and 5% absolute precision using the formula:

$$n = \frac{Z^2 \times p \times q}{d^2}$$

This yielded a minimum required sample size of ~246, which was rounded to 250 isolates for feasibility.

Isolation and Identification of Enterococcus Species- Presumptive identification of Enterococcus species was based on colony morphology and Gram staining. Confirmation was done using standard biochemical tests including: Catalase test, Bile esculin hydrolysis, Species Identification was done using Sugar Fermentation Test, Antimicrobial Susceptibility Testing (AST) was performed using the Kirby-Bauer disk diffusion method, and results were interpreted according to CLSI 2021 guidelines. [11]

Inclusion criteria- *Enterococcus* spp isolated in significant number from urine samples from OPD/IPD patients having symptoms of UTI.

Exclusion criteria- Urine samples from which bacteria other than *Enterococcus* was isolated.

Phenotypic Detection of Virulence Factors- VRE isolates were subjected to phenotypic screening for the following virulence factors:

Slime Layer Formation: Slime production was done using Congo Red Agar (C.R.A.), as described by Freeman et al and C.R.A. plates were incubated at 37°C. Observations were done at 24 and 48 hours. Colonies exhibiting a black, crystalline morphology were considered positive for slime production [12].

Hemolysin Production: Hemolytic activity was performed on 5% sheep blood agar plates. Plates were incubated at 37°C for 24 hours. A clear or partial zone of hemolysis around the colonies indicated hemolysin production, following the method by Furumura *et al.* [13].

Gelatinase Activity: Gelatinase production was determined by stab inoculation into nutrient gelatin broth containing 12% gelatin. After incubation at 37°C for 24 hours, the tubes were refrigerated at 4°C for 30 minutes. Liquefaction of the medium was interpreted as a positive result, as done by Cruz *et al.* [14]

Molecular Detection of Virulence Genes- The presence of virulence-associated genes (*GelE, Asa1*, *CylA*,) was evaluated using P.C.R. Bacterial DNA was extracted using the boiling method [15]. P.C.R. reactions were prepared in a final volume of 25 µL, comprising 12 µL of ready-to-use P.C.R. master mix, 1 µL each of forward and reverse primers specific to the target genes, 1 µL of DNA template (added in a separate extraction area), and 10 µL of molecular grade water. For the positive control *E. faecalis* ATCC 29212 was used, while a reaction tube containing all components except template DNA served as the negative control.

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Amplification was carried out in a Bio-Rad thermal cycler using the following thermal cycling conditions Initial denaturation at 95°C for 15 minutes,30 cycles of Denaturation at 95°C for 30 seconds, Annealing at 52°C for 60 seconds, Extension at 72°C for 1 minute. 1% agarose gel stained with ethidium bromide was used for electrophoresis of PCR. products. To determine the size of the amplified products corresponding to specific virulence genes a 100 base pair DNA. A ladder was included in each run. Results were documented using a gel documentation system (Fig. 1, Fig. 2), and oligonucleotide primer sequences used (Table 1).

Table 1: Oligonucleotide primers used for P.C.R. [22,29]

Target Product	Sequence of Oligonucleotide	Amplicon Size
Asa1 Gene	5'GCACGCTATTACGAACTATGA 3' 5'TAAGAAAGAACATCACCACGA 3'	375 Base Pair
Cyl A Gene	5'ACTCGGGGATTGATAGGC 3' 5'GCTGCTAAAGCTGCGCTT 3'	688 Base Pair
Gel E Gene	5' TATGACAATGCTTTTTGGGAT 3' 5'AGATGCACCCGAAATAATATA 3'	210 Base Pair

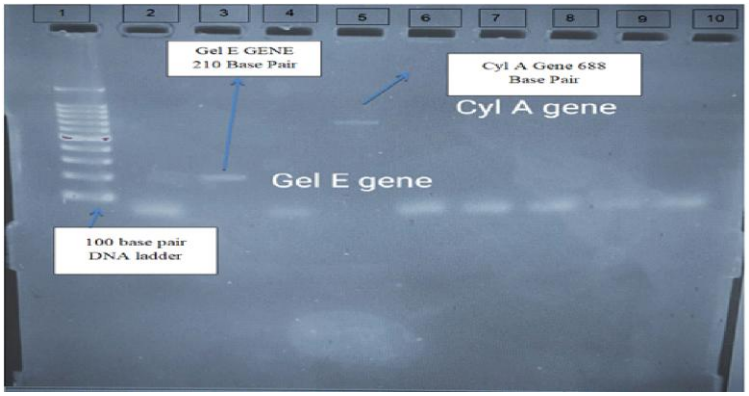

Fig. 1: Gel Documentation Image showing Detection of *GelE* Gene and *CylA* Gene in VRE Strains

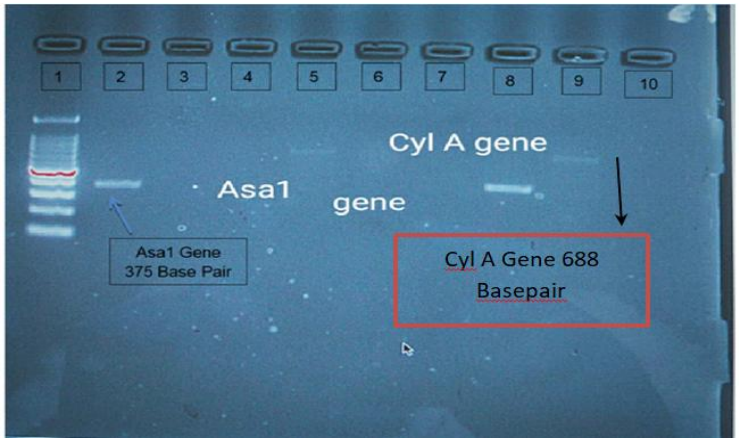

Fig. 2: Gel Documentation Image showing Detection of *Asa1* Gene and *CylA* Gene in VRE Strains

Statistical Analysis- Data were entered and analyzed using SPSS version 25. Descriptive statistics were expressed as frequencies and percentages, with results presented along with 95% confidence intervals (CI). Associations between phenotypic and genotypic virulence factors were assessed using the Chi-square test or Fisher's exact test, as appropriate. Fisher's exact test was applied when the sample size was small or when expected cell counts were less than 5. A p-value of <0.05 was considered statistically significant.

Ethical Considerations- Ethical approval was obtained from the Institutional Ethics Committee (Ethics no: MC/EC/ 2022/244).

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RESULTS

A total of 250 Enterococcus urinary isolates were analyzed during the study period. Among these, 37 isolates were identified as VRE yielding a prevalence of 14.8% (37/250; 95% CI: 10.6–19.9%).In V.R.E. total species distribution for *E. faecalis, E. faecium,* and other species was reported 3(8.1%), 31(83.78%), 3(8.1%).

Among the VRE isolates (n = 37), phenotypic expression of virulence factors was observed as follows: hemolysin production in 8/37 isolates (21.6%; 95% CI: 10.5–37.6%), gelatinase activity in 4/37 isolates (10.8%; 95% CI: 3.0–25.4%), and slime layer formation in 11/37 isolates (29.7%; 95% CI: 16.7–46.3%). In the present study, the predominant virulence gene in VRE isolates was *GelE* (24.3%) followed by Asa1 (13.5%) and *CylA* (16.2%). On Genotypic analysis lower detection rates of virulence-associated genes were observed (Table 2).

Table 2: Phenotypic and Genotypic Distribution of Virulence Factors among Vancomycin-Resistant *Enterococcus* Isolates (n=37)

Virulence Factor / Gene	Detection Method	Percentage (%)	95% Confidence Interval
Hemolysin	Phenotypic	8/37 (21.6%)	10.5–37.6
Gelatinase	Phenotypic	4/37 (10.8%)	3.0–25.4
Slime layer	Phenotypic	11/37 (29.7%)	16.7–46.3
Asa1 Gene	Genotypic	5/37 (13.5%)	4.5–28.8
GelE Gene	Genotypic	9/37 (24.3.%)	0.7–18.2
CylA Gene	Genotypic	6/37 (16.2%)	0–9.5

Among all 37 VRE isolates phenotypic and genotypic association was observed between slime layer and *Asa1* gene with phenotypic expression of slime layer (11/37; 29.7%) significantly higher than *Asa1* gene detection (3/37; 8.1%), and this difference was found to be statistically significant (p = 0.038). However for gelatinase, phenotypic detection (4/37; 10.8%) was higher than *GelE* gene detection (2/37; 5.4%); this difference was not statistically significant (p = 0.670).For hemolysin, phenotypic detection was observed in 8/37 isolates (21.6%), whereas no isolates were positive for the *CylA* gene (Table 3). Due to the absence of genotypic positivity (zero cell count), statistical analysis for this association could not be performed. Fisher's exact test was used to assess association between phenotypic and genotypic variables due to small sample size.

Table 3: Phenotypic and Genotypic association of Virulence Factors among Vancomycin-Resistant *Enterococcus* Isolates (n = 37)

Virulence Factor	Phenotypic Detection n (%) [95% CI]	Genotypic Detection n (%) [95% CI]	p-value
Hemolysin (*CylA gene*)	8/37 (21.6%) [10.5–37.6]	0/37 (0%) [0–9.5]	Not applicable*
Gelatinase (*GelE gene*)	4/37 (10.8%) [3.0–25.4]	2/37 (5.4%) [0.7–18.2]	0.670
Slime layer (*Asa1 gene*)	11/37 (29.7%) [16.7–46.3]	3/37 (8.1%) [1.7–21.9]	0.038

Overall, phenotypic methods demonstrated higher detection rates than genotypic methods, with a statistically significant difference observed only for slime layer formation. These findings highlight variability between phenotypic expression and genetic determinants of virulence in VRE isolates.

The species-wise distribution of virulence genes among VRE isolates is presented in Fig. 3.

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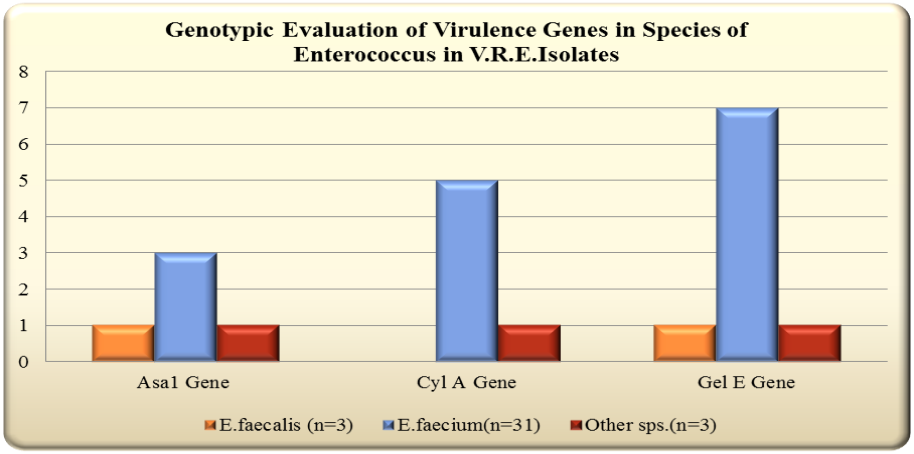

Fig. 3: Genotypic Evaluation of Virulence Genes in Species of Enterococcus in VRE

DISCUSSION

Phenotypes of V.R.E. are defined by the expression of virulence factors and vancomycin-related resistance, regardless of the genes expressed.In the present study, the predominant virulence gene identified was *GelE* (24.3%). This finding differs considerably from previous reports by Copur *et al.,* (3.2%) [16] and Marachi *et al*., who reported a much higher prevalence (71%). [28] Such discrepancies may be attributed to differences in study populations, geographic distribution of strains, sample size, and methodological variations, including differences in PCR sensitivity, gene targets, and criteria for isolate selection. Additionally, variation in species composition (e.g., *E. faecalis* vs *E. faecium*) across studies contributes to these inconsistencies.

Other virulence genes detected in this study included *Asa1* (13.5%) and *CylA* (16.2. %). The absence of *Asa1* reported by Copur *et al.,* [16] and the higher prevalence (>50%) observed by Backiam *et al.,* [17] highlight substantial inter-study variability. Similarly, the lack of *CylA* detection in Copur *et al.,* [16] and its high prevalence in Backiam *et al.,* [17] suggest that differences in isolate origin (clinical vs environmental), patient demographics, and laboratory detection techniques may significantly influence reported frequencies. These findings underscore the importance of interpreting virulence gene prevalence within the context of methodological and population-specific factors rather than as universal trends.

The present study demonstrated variable concordance between genotypic and phenotypic detection of virulence factors. Gelatinase activity was phenotypically observed in 10.08% of isolates, whereas the *GelE* gene was detected in only 5.4%.

This inverse relationship contrasts with earlier studies by Hashem *et al.,* [18], Roberts *et al.,* [19], and Mohamed *et al.,* [20], where gene prevalence exceeded phenotypic expression. Such discrepancies may arise due to differences in experimental conditions influencing gene expression, including environmental cues, growth phase, and regulatory pathways. Fsr quorum-sensing system mediates the expression of Gel E gene, particularly the *fsrA* and *fsrB* genes, with *fsrB* acting as a key activator of gelatinase production (Hashem *et al.*) [18]. The absence of investigation into these regulatory genes in the current study represents a limitation. Future studies should incorporate analysis of the fsr operon and related regulatory networks to better understand the molecular basis of discordance between genotype and phenotype.

Slime layer formation was phenotypically detected in 29.72% of VRE isolates, while the *Asa1* gene was identified in only 8.1%, with statistical significance ($p = 0.038$). However, studies by Kiruthiga *et al.,* [21] and Biswas *et al.,* [22] suggest that slime production is not solely dependent on *Asa1*. This indicates the possible involvement of alternative genes, regulatory mechanisms, or environmental triggers influencing biofilm formation. Additionally, the presence of silent genes or mutations affecting gene expression may contribute to this variation. These findings highlight the complexity of virulence regulation in VRE and the need for broader genomic and transcriptomic approaches in future research.

From a clinical perspective, slime layer (biofilm) formation is particularly significant, as it enhances bacterial adhesion to host tissues and medical devices, promotes persistence, and confers increased resistance to antimicrobial agents.[23] In hospital settings where VRE

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is associated with catheter-associated urinary tract infections Slime layer has direct role for infection control. Similarly, gelatinase contributes to tissue invasion and dissemination by degrading host proteins, thereby exacerbating disease severity. Therefore, comprehensive virulence profiling can aid in identifying high-risk strains, guiding targeted therapeutic strategies, and informing infection prevention protocols.

Hemolysin production was phenotypically observed in 21.6% of isolates; however,*CylA* was genotypically detected in 16.2% of isolates. In contrast, Hashem *et al.,* [18] reported higher rates of both gene presence (54%) and phenotypic activity (33%). Previous studies by Kiruthiga *et al.,* [21] and Hallgren *et al.,* [24] have also demonstrated inconsistencies between genotype and phenotype, including the presence of the *Cyl* gene without corresponding hemolytic activity. This further supports the role of regulatory mechanisms and environmental factors in modulating gene expression. The absence of *CylA* detection in the present study may reflect methodological limitations or genetic variation among isolates. These findings emphasize that reliance on phenotypic assays alone may lead to over- or underestimation of virulence potential, reinforcing the need for combined phenotypic and molecular approaches.

Additionally, the GelE gene in *E. faecium* observed in this study contrasts with findings by Suchi *et al.,* [25] and Samani *et al.,* [26], who reported higher prevalence in *E. faecalis*. This variation is due to differences in study population, design and sample size. The lack of statistical significance in species-wise comparison in the current study is likely due to the limited sample size, highlighting the need for larger, multicentric studies.

The observed variability between phenotypic and genotypic profiles, along with differences across studies, underscores the complex regulation of virulence in VRE. Future research should focus on regulatory gene networks such as the fsr system, employ larger sample sizes, and integrate molecular and functional analyses to better elucidate the pathogenic potential of these organisms. Moreover, incorporating virulence profiling into routine diagnostics may enhance clinical decision-making and improve infection control strategies.

E. faecium species has the ablity to thrive in hospital environments leading to its increased prevalence because of its persistence to build resistance to environmental stressors and antibiotics. [27]*E. faecium and E. faecalis* have now evolved as species which leads to serious health issues therefore the genotypic and phenotypic analysis of the virulence factors associated with antibiotic resistance is the one of the important studies among VRE.

CONCLUSIONS

In this study a correlation between virulence factors by phenotypic and genotypic methods in Enterococcus urinary isolates was observed. A significant association was observed between slime layer formation and the Asa1 gene, while no correlation was found between the GelE gene and gelatinase activity. These findings indicate that phenotypic expression does not always reflect the genetic profile. Slime layer production appears to be an important contributor to VRE pathogenicity. The variability may be due to complex regulatory mechanisms such as quorum sensing, two-component systems, and horizontal gene transfer. Larger molecular studies are needed to validate these findings. Overall, comprehensive virulence profiling may improve diagnosis and support the development of targeted therapies in healthcare settings.

CONTRIBUTION OF AUTHORS

Research concept- Dr Rajni Sharma
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Materials- Soumya Nigam
Data collection –Soumya Nigam
Data analysis and interpretation-Dr. Diksha Singh
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Article editing- Soumya Nigam, Dr. Diksha Singh, Dr Rekha Bachhiwal
Final approval- Dr Rajni Sharma

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