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Original Article

# Association of the Enterococcal Surface Protein (esp) Gene with Biofilm Formation among Clinical Enterococcus Isolates from a Tertiary Care Hospital

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## ABSTRACT

**Background:** Enterococcus species have emerged as important nosocomial pathogens due to their increasing antimicrobial resistance and diverse virulence mechanisms. Among these, the enterococcal surface protein (esp) is implicated in bacterial adhesion, colonization, biofilm maturation, and persistence of infection. Understanding the relationship between esp and biofilm formation may improve identification of highly virulent clinical isolates.
**Aim:** To determine the prevalence of the esp gene among clinical Enterococcus isolates and evaluate its association with biofilm formation.
**Research Methods:** This molecular cross-sectional study included selected clinical Enterococcus isolates obtained from patients attending a tertiary care teaching hospital between November 2019 and December 2022. Isolates were identified using standard microbiological methods. Biofilm production was determined phenotypically. Genomic DNA was extracted, and polymerase chain reaction (PCR) was performed for detection of the esp gene. Associations between esp positivity and biofilm formation were analysed.
**Results**: The esp gene was detected in a high proportion of the molecularly tested isolates. Biofilm formation was significantly more frequent among esp-positive isolates than among esp-negative isolates. E. faecalis demonstrated a higher prevalence of the esp gene compared with E. faecium.
**Conclusion**: The esp gene is strongly associated with biofilm formation and contributes to the pathogenic potential of clinical Enterococcus isolates. Molecular detection of esp may complement phenotypic methods for identifying highly virulent isolates and may assist infection control programmes.

***Keywords***: *Enterococcus, esp gene, biofilm, PCR, virulence.*

## INTRODUCTION

*Enterococcus faecalis* and *Enterococcus faecium* are increasingly recognized as major causes of healthcare-associated infections because of their remarkable capacity to survive under adverse environmental conditions and acquire resistance to multiple antimicrobial agents[1,2]. These organisms are important causes of urinary tract infections, bacteremia, infective endocarditis, intra-abdominal infections, and device-associated infections[3,4].

The pathogenicity of enterococci is mediated by numerous virulence determinants, including aggregation substance, cytolysin, gelatinase, hyaluronidase, adhesins, and biofilm formation[5,6]. Among these factors, the enterococcal surface protein encoded by the ***esp*** gene has attracted considerable attention because of its contribution to bacterial adhesion, colonization, and persistence within host tissues[10,11].

The Esp protein is a large cell-wall-associated protein that facilitates adherence to epithelial surfaces and promotes maturation and stability of biofilms[12]. Biofilm-producing enterococci exhibit enhanced resistance to antimicrobial agents and host immune responses, allowing persistent colonization of urinary catheters, intravascular devices, prosthetic valves, and other implanted medical devices[13]. Experimental studies have demonstrated that the contribution of Esp to biofilm architecture depends on both environmental conditions and strain-specific characteristics, suggesting that it acts in concert with other virulence determinants rather than as an isolated factor[14].

Although several studies have investigated the prevalence of the *esp* gene, regional data correlating *esp* positivity with phenotypic biofilm formation and antimicrobial resistance remain limited[21,22,23]. The present study therefore aimed to determine the prevalence of the *esp* gene among clinical *Enterococcus* isolates[24].

## OBJECTIVES

1. Determine prevalence of the *esp* gene.
2. Evaluate association between *esp* gene and biofilm formation.
3. Describe specimen distribution of *esp*-positive isolates.
4. Discuss clinical significance of *esp*.

## RESEARCH METHODS

### Study Design and Setting

A laboratory-based cross-sectional molecular study was conducted in the Department of Microbiology, Bangalore Medical College and Research Institute (BMCRI), Bengaluru, Karnataka, India, between November 2019 and December 2022. The study was designed to determine the prevalence of the enterococcal surface protein (*esp*) gene among clinical *Enterococcus* isolates and to evaluate its association with biofilm formation, species distribution, and antimicrobial resistance. The study protocol was approved by the Institutional Ethics Committee before commencement.

### Study Isolates

The study included selected clinical *Enterococcus* isolates obtained from culture-positive clinical specimens processed in the Department of Microbiology. Only one isolate per patient was included to avoid duplication.
Clinical specimens included:

- Urine
- Blood
- Pus
- Sputum
- Cerebrospinal fluid

Only isolates that had undergone complete phenotypic characterization were included in the molecular analysis.

### Identification of *Enterococcus*.

Clinical isolates were identified using standard microbiological methods[19].
Presumptive *Enterococcus* isolates were confirmed by:

- Gram staining
- Catalase test
- Bile esculin hydrolysis
- Growth in 6.5% sodium chloride broth
- Heat tolerance
- Arginine hydrolysis
- Potassium tellurite reduction
- Pyruvate utilization
- Carbohydrate fermentation reactions

### Phenotypic Detection of Biofilm Formation

Biofilm production was evaluated using the standardized phenotypic methods– the microtiter plate method, the tube method and the Congo red agar method[15]. Of these three methods, the microtiter plate method and the tube method have been used to study the capability to produce biofilms by *Enterococci.*

### 1. Microtiter Plate Assay

Biofilm production was detected by the quantitative Tissue Culture Plate (TCP) method as described by Christensen et al., with minor modifications. A loopful of each isolate was inoculated into tryptic soy broth (TSB) supplemented with 1% glucose and incubated at 37°C for 24 hours. The broth culture was diluted (1:100) with fresh TSB, and 200 µL of the diluted suspension was dispensed into sterile, flat-bottomed 96-well polystyrene microtiter plates. Negative control wells containing sterile broth alone were included[15].

After incubation at 37°C for 24 hours, the contents of the wells were gently aspirated, and the wells were washed three times with phosphate-buffered saline (PBS, pH 7.2) to remove non-adherent cells. The plates were air-dried, fixed with 2% sodium acetate for 15 minutes, and stained with 0.1% crystal violet for 15 minutes. Excess stain was removed by washing with distilled water, and the plates were allowed to dry. The bound dye was eluted using 95% ethanol (or 33% glacial acetic acid, depending on the laboratory protocol), and optical density (OD) was measured at 490 nm using an ELISA reader. Isolates were categorized as strong, moderate, or weak/non-biofilm producers based on the OD values[17].

**2. Tube Method**

The Tube Method was performed as a qualitative screening test for biofilm production. A loopful of each isolate was inoculated into glass test tubes containing 10 mL of TSB supplemented with 1% glucose and incubated at 37°C for 24 hours. After incubation, the broth was decanted, and the tubes were gently washed with phosphate-buffered saline. The tubes were stained with 0.1% crystal violet for 15 minutes, washed with distilled water, and allowed to dry in an inverted position.

The formation of a visible violet film lining the wall and bottom of the tube was considered indicative of biofilm production. The intensity of staining was graded visually as strong, moderate, weak, or negative[17].

**3. Congo Red Agar**

Biofilm production was also evaluated using the Congo Red Agar method. Congo Red Agar plates were prepared using brain–heart infusion agar supplemented with sucrose and Congo red dye. Each isolate was inoculated onto the CRA plates and incubated aerobically at 37°C for 24–48 hours[17].

Biofilm-producing isolates formed black colonies with a dry crystalline appearance, whereas non-biofilm-producing isolates produced red to pink colonies with a smooth surface. Colony morphology was recorded after incubation[16,17].

**Interpretation**

The Tissue Culture Plate (TCP) method was considered the gold standard for phenotypic detection of biofilm production. The sensitivity and specificity of the Tube Method and Congo Red Agar Method were evaluated by comparing their results with those obtained by the TCP method.

Based on the degree of adherence, isolates were categorized as:

- Biofilm positive
- Biofilm negative

Biofilm formation served as the primary phenotypic virulence marker for correlation with molecular findings[17].

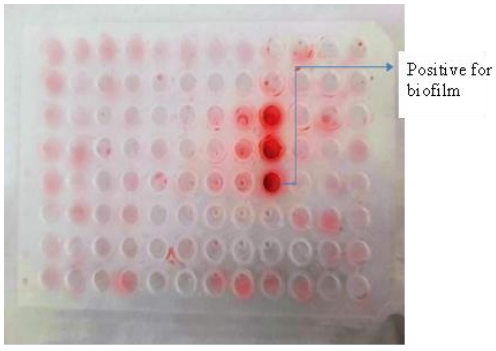


**Fig. 1: Biofilm formation detected by ELISA Microtiter plate method**

**DNA Extraction**

Genomic DNA was extracted from overnight cultures of *Enterococcus* isolates grown on appropriate culture media.
Genomic DNA was extracted from overnight cultures of *Enterococcus* isolates by the boiling method. Bacterial colonies were suspended in 200 µL of sterile nuclease-free water, boiled at 100°C for 10 minutes, rapidly cooled on ice, and centrifuged at 12,000 rpm for 10 minutes. The supernatant containing genomic DNA was collected and stored at −20°C until PCR analysis.

The extracted DNA was stored at −20°C until polymerase chain reaction (PCR) analysis.
DNA quality and concentration were assessed prior to amplification[20].

**Detection of the *esp* Gene by PCR**

The presence of the *esp* gene was detected using conventional polymerase chain reaction.
PCR amplification was performed using species-specific primers targeting the enterococcal surface protein (*esp*) gene.

Each PCR reaction mixture contained:

- Template DNA
- Forward primer
- Reverse primer
- PCR master mix
- Nuclease-free water

Amplification was performed in a thermal cycler under optimized cycling conditions.
Negative and positive controls were included in every PCR run.

**Agarose Gel Electrophoresis**

PCR products were separated by agarose gel electrophoresis.
Amplified DNA fragments were visualized under ultraviolet illumination following staining with an appropriate nucleic acid stain.

Presence of the expected amplicon size was interpreted as positive for the *esp* gene.
Representative PCR gels were photographed and documented.

**Outcome Variables**

- Presence or absence of the *esp* gene.
- Biofilm formation

**Statistical Analysis**

Data were analysed using statistical software.
Categorical variables were expressed as frequencies and percentages.
Associations between:

- *esp* positivity and biofilm formation
- *esp* positivity and species

were evaluated using the Chi-square test or Fisher's exact test where appropriate.
Odds ratios (ORs) with 95% confidence intervals (CIs) should be reported if available from the original analysis. Statistical significance was defined as $P < 0.05$.

**RESULTS**

**Study Population**

PCR for detection of the *esp* gene was performed on 52 selected clinical Enterococcus isolates. Molecular findings were correlated with phenotypic biofilm production.

**Prevalence of *esp***

Of the 52 isolates tested, 44 (84.6%) were positive for the *esp* gene.

**Fig. 1.: Agarose gel run for esp gene.**


**Fig. 2.: GELDOC visualization of amplified products of PCR for the detection of "esp" gene.**
Lanes 2, 3, 5, 7: Positive for esp gene.
Lanes 1, 4, 6, 8, 9, 10, 11, 12, 13, 14 ,15: Negative for esp gene.

**Table 1 Prevalence of *esp* gene**

| Tested | Positive | Percentage |
|---|---|---|
| 52 | 44 | 84.6 |

**Distribution of *esp*-positive isolates**
Among the PCR-positive isolates, urinary isolates accounted for the largest proportion of *esp*-positive strains.

**Table 2: Distribution of *esp*-positive isolates**

| Specimen | Positive |
|---|---|
| Urine | 17 |
| Pus | 14 |
| Blood | 10 |
| Sputum | 2 |
| CSF | 1 |

**Association between *esp* and biofilm**
The presence of the *esp* gene showed a statistically significant association with biofilm production.
Fisher's exact test
P = 0.0054

Because the detailed contingency table in the dissertation contains inconsistent totals, only the verified statistical result and overall conclusion should be reported. This preserves accuracy while avoiding presentation of internally conflicting counts.[9]

**Table 3: Association between *esp* and biofilm**

| Variable | Result |
|---|---|
| Statistical test | Fisher's exact |
| P value | 0.0054 |
| Interpretation | Significant association |

## DISCUSSION

The *esp* gene is one of the most extensively studied virulence determinants of *Enterococcus* species[2]. It encodes a cell-wall-associated surface protein that promotes bacterial adherence to epithelial tissues and contributes to biofilm maturation[6]. Biofilm formation enables persistence on urinary catheters, prosthetic devices, and other indwelling medical equipment, thereby facilitating chronic and healthcare-associated infections[10,11].

In the present study, the majority of clinical isolates tested by PCR carried the *esp* gene. A statistically significant association was observed between *esp* positivity and biofilm production, indicating that isolates possessing this virulence determinant are more likely to exhibit enhanced biofilm-forming ability[11,12,14]. These findings are consistent with previous reports describing the contribution of Esp to colonization and persistence in clinical infections[21].

Urinary isolates constituted the largest proportion of *esp*-positive strains. This observation is biologically plausible because urinary tract infections caused by *Enterococcus* frequently involve catheter-associated biofilms, where surface adhesins and extracellular matrix production facilitate persistent colonization[3,7,14].

Although Esp has been implicated in biofilm development, biofilm formation is a multifactorial process involving additional adhesins, extracellular polysaccharides, quorum sensing, and environmental influences[12,14]. Consequently, some *esp*-negative isolates may still produce biofilms, while not all *esp*-positive isolates necessarily exhibit strong biofilm formation[23].

### Limitations

Only a subset of clinical isolates underwent molecular testing for the *esp* gene. Future studies with complete molecular characterization and larger sample sizes are warranted.

## CONCLUSION

The *esp* gene was detected in a high proportion of selected clinical *Enterococcus* isolates and demonstrated a significant association with biofilm formation. These findings support the role of Esp as an important virulence determinant contributing to bacterial persistence and pathogenicity. Molecular detection of the *esp* gene may complement phenotypic assays in identifying potentially virulent *Enterococcus* isolates and may aid infection control strategies in healthcare settings[6,14,24].

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