

Original Article

Investigating the presence and virulence potential of *Enterococcus faecalis*, with a focus on the hyaluronidase factor, in environmental samples: Insights from a pilot study

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ABSTRACT

Objectives: *Enterococcus faecalis* is implicated in various human diseases, including urinary tract infections, dental conditions such as periodontitis, dental caries, systemic disorders such as infective endocarditis, meningitis, intra-abdominal infections, and wound infections. The virulence genes associated with these diseases include adhesins (*ace*, *efaA*, *efaB*, *ebp*), biofilm-forming factors (*esp*, *gelE*, *fsr*), cytotoxin (*cylA*, *cylB*, *cylM*, *cylL*), hyaluronidase (*hyl*), aggregation substance (*asp*), gelatinase (*gelE*), enterococcal surface protein (*esp*), among others. In a prior investigation, we observed a higher prevalence of the *hyl* gene in medical isolates, prompting an exploration of its occurrence in the environment.

Material and Methods: 32 samples from diverse locations in and around Chennai were collected. *Enterococcus* spp. isolation was achieved using a partially selective medium (Ethyl Violet Azide medium). Subsequently, the isolates were identified as *E. faecalis* through polymerase chain reaction targeting the 16S ribosomal RNA (rRNA) gene. Positive 16S rRNA samples were further scrutinized for the presence of the *hyl* gene using Sequence Specific Oligonucleotide Primers.

Results: Our findings indicated an *E. faecalis* incidence of 41%. However, none of the isolates tested positive for the *hyl* virulence gene.

Conclusion: This study suggests that the *hyl* gene may play a pivotal role in pathogenesis, as previous investigations in our laboratory involving medical isolates reported a 71% incidence of the *hyl* gene. In addition, our results highlight the opportunistic nature of the organism.

Keywords: *Enterococcus faecalis*, Virulence genes, Environmental prevalence, 16S ribosomal ribonucleic acid gene, Opportunistic pathogen

INTRODUCTION

Enterococcus faecalis, a Gram-positive bacterium, is a ubiquitous inhabitant of various ecological niches, including the gastrointestinal tracts of both humans and animals, as well as soil, water, and food sources. While typically regarded as a commensal microorganism, *E. faecalis* can transition into an opportunistic pathogen by acquiring specific virulence genes. This bacterial species has been identified as a significant contributor to nosocomial infections, constituting approximately 90% of all enterococcal infections in humans, with a notable predilection for individuals with compromised immune systems.^[1,2] The genomic landscape of *E. faecalis*, encompassing a circular genome of 3 million base pairs and 3000 genes, provides a comprehensive understanding of its biological intricacies and evolutionary pathways. Notably, *E. faecalis* has developed intricate mechanisms to counteract antibiotics, including the alteration of targets, modification

of enzymes, and even autonomous antibiotic production. These mechanisms collectively contribute to the resilience of *E. faecalis* strains against conventional antibiotic treatments, posing challenges in the clinical management of infections caused by this bacterium.^[3] At the heart of *E. faecalis*' pathogenicity lies an array of virulence factors, each playing a crucial role in host colonization and the establishment of infection. One such factor of particular interest is hyaluronidase (*Hyl*), encoded by the *hyl* gene. *Hyl* is a degradative enzyme known for its involvement in tissue damage. Functionally, it depolymerizes the mucopolysaccharide components of connective tissues, enhancing the invasiveness of bacteria. This enzyme, identified in various organisms, acts as a potent spreading factor, facilitating the movement of bacteria and their toxins through host tissues.^[4] *Hyl* activity facilitates the spread of infection by breaking the extracellular matrix potentially impacting the stability and resistance that influence

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the severity and persistence of oral related disease. The detection of the *hyl* gene in oral microbiota can serve as a biomarker for the presence of pathogenic bacteria, which can be useful in diagnosing oral infections. The focus of the present research extends beyond clinical settings to explore the prevalence of *E. faecalis* in diverse environmental samples, including soil, water, bird feces, and animal feces in the vicinity of Chennai. Furthermore, this study seeks to unravel the distribution and implications of the *hyl* gene in environmental isolates of *E. faecalis*. By examining these factors in a broader context, the research aims to contribute valuable insights into the ecological dynamics and potential public health ramifications associated with this pathogenic bacterium and its virulence factors in various environmental reservoirs.

MATERIAL AND METHODS

Ethics approval

The collection of environmental samples for this study was carried out in strict adherence to ethical guidelines. The Institutional Ethics Committee (IEC) granted the necessary approval for the conduct of the study (CSP/23/JUN/130/540).

Sample collection

A total of 32 environmental samples were procured from various locations in and around Chennai. The collection process employed sterile 15 mL Falcon tubes to ensure the integrity and uncontaminated nature of the samples. 10 mL of water sample was collected using sterile falcon tubes. For solid samples, 5 g was collected from different environments. As this is a pilot study, no specific criteria were applied for sampling except that the samples should be of different types such as water and soil. The details of the sample collection are given in Table 1.

Sample processing

To prepare the samples, 4.0 mL of distilled water was added to 1.0 g of solid samples in Falcon tubes. Subsequently, 2.5 mL of the resulting diluted sample was mixed with an equal volume of Ethyl Violet Azide broth (EVA broth). For water samples, 2.5 mL directly combined with 2.5 mL of EVA broth. The tubes were then subjected to a 48-h incubation period.

Bacteriological isolation and biochemical identification

Bacterial isolation was conducted using EVA agar, a partially selective medium specifically inhibiting the growth of non-enterococcal Gram-positive and Gram-negative bacteria due to the inclusion of sodium azide. After 24–48 h of incubation at 37°C, gray colonies indicative of enterococcal species were observed. These gray colonies were subcultured onto Brain Heart Infusion agar. In addition, colonies were streaked onto Bile esculin agar plates to confirm the presence of *Enterococcus* species through esculin hydrolysis, resulting in

Table 1: Details of sample collection.

S. No.	Sample type	Location
S1	Soil	Redhills
S2	Soil	Guduvancheri
S3	Bird Feces	Kattupakkam
L4	liquid	Redhills
S5	Soil	Nungambakkam
S6	Soil	Pattinapakkam
S7	Soil	Aminjikai
S8	Soil	Lake
S9	Soil	Park
S10	Soil	Guduvanchery Bus Stand
S11	Soil	Iyyappanthangal Depot
S12	Soil	Anna Nagar East
L13	Water	Aminjikai
S14	Soil	Mogappair
S15	Soil	Sinthamani
S16	Soil	Iyyappanthangal
S17	Soil	Porur
L18	Liquid	Porur
S19	Soil	Karayanchavadi
S20	Soil	Kumananchavadi
S21	Soil	Poonamallee
L22	Liquid	Mangadu
S23	Soil	Lake2
S24	Soil	Pond Guduvancheri
L25	Liquid	Guduvancheri Water
S26	Soil	Guduvancheri Garden
S27	Cow Feces	Shenoy Nagar
S28	Goat Feces	Shenoy Nagar
S29	Crow Feces	Shenoy Nagar
S30	Cow Feces	Cuddalore
L31	Cow Shed	Cuddalore
S32	Hen Feces	Cuddalore

brownish-black precipitate colonies. All dehydrated culture media were procured from (HiMedia™ Laboratories Pvt Ltd).

DNA extraction

DNA extraction from all samples utilized the boiling lysis method as described by Dashti *et al.* and Yost and Nattress.^[5,6] Following centrifugation of 1.0 mL of the broth culture for 5 min at 10,000 rpm, the supernatant was discarded, and 200 µL of sterile distilled water was added. The mixture was then subjected to a dry bath at 95°C. Two microliters of the resulting supernatant served as the template in polymerase chain reaction (PCR), as per the method outlined by Mullis *et al.*^[7] Extracted DNA was stored at –20°C for subsequent analysis.

Molecular identification using 16S ribosomal RNA (rRNA)

Primer designing

Enterococcus isolates were further identified as *E. faecalis* using Sequence-Specific Oligonucleotide Primers (SSOPs)

in PCR. Primers were designed with the assistance of the online primer designing tool, Primer3.^[8] The 16S rRNA gene sequence, retrieved from the Nucleotide database of National Center for Biotechnology Information (accession id: AB036835.1), was submitted to the Primer3 tool. Default parameters were slightly modified to ensure the product size of the amplicon fell between 500 and 1000 nucleotides. The pick primer option was employed, and the results are illustrated in Figure 1. The melting temperatures of the forward and reverse primers were 59.03°C and 59.01°C, respectively.

Molecular detection

For the molecular identification of all isolates in the study, PCR amplification of the *E. faecalis* 16S rRNA gene was performed and the primers were as follows: Forward primer 5’CTGGGAGACTTGAGTGCAGA3’ and Reverse 5’ATTTGACGTCATCCCCACCT3’ (Sigma Aldrich, USA). The primer details are given in Table 2. A primer concentration of 0.4 µM was employed. PCR was executed using Taq DNA Polymerase 2x Master Mix RED (Ampliqon, Denmark) in a

20 µl reaction maintaining a pH of 8.5 with Tris-HCl pH 8.5, ammonium sulfate. It also has 3 mM magnesium chloride, 0.2% Tween[®]20, 0.4 mM of each deoxynucleotide triphosphate, and 0.2 units/µL Taq DNA polymerase. Moreover, the TaqMix is added with an inert red dye for tracking in the agarose gel. The cycling parameters for amplification were as follows: Initial denaturation - 95°C; 5’, denaturation - 95°C; 45”, annealing - 60°C; 45”, extension - 72°C; 1’, repeated for 30 cycles. Subsequent to an 8-min final extension at 72°C, the reaction mixtures were cooled to 4°C. Gel electrophoresis of the PCR products was carried out on 1.2% agarose gel and visualized using an ultraviolet (UV) Transilluminator (White/UV transilluminator UVP). The 2 Kbp DNA ladder (Thermo Fisher Scientific, US) was used in all agarose gel electrophoresis experiments.

Detection of the virulence factor, *hly*

***hly* primers**

The design of *hly* primers followed the procedure outlined earlier for the 16S rRNA primers. Primers were designed and synthesized based on the sequences presented in [Table 3]. Subsequently, PCR was conducted utilizing these primers to amplify the region of interest associated with the *hly* virulence factor.

PCR-cycling parameters

For the 16S rRNA PCR of the *hly* gene, the cycling conditions are as follows: Initial denaturation - 95°C; 5’, denaturation - 95°C; 45”, annealing - 60°C; 45”, extension - 72°C; 1’ with 30 cycles. Following an 8-min final extension at 72°C, the reaction mixtures were then cooled to 4°C.

Visualization and documentation

PCR products were run on a 1.2% agarose gel that has ethidium bromide at a working concentration of 0.2 µg/mL (16 µL/80 mL). Electrophoresis was conducted in a 1× TBE buffer for 30 min at 70V. Subsequently, bands were visualized under a UV transilluminator (UVP Upland, USA) and documented using a Gel Documentation System (Vilber Lourmat, USA). Molecular Marker 16S rRNA PCR was conducted for all isolates, with primers designed specifically for detection, yielding an amplicon size of 557bp. *E. faecalis* ATCC 29212 served as the positive control, and a 2 Kbp DNA ladder (Thermo Fisher Scientific, US) was used as a marker with 500 bp as the lowest band. DNA bands were observed in all wells except the negative control, indicating the absence of

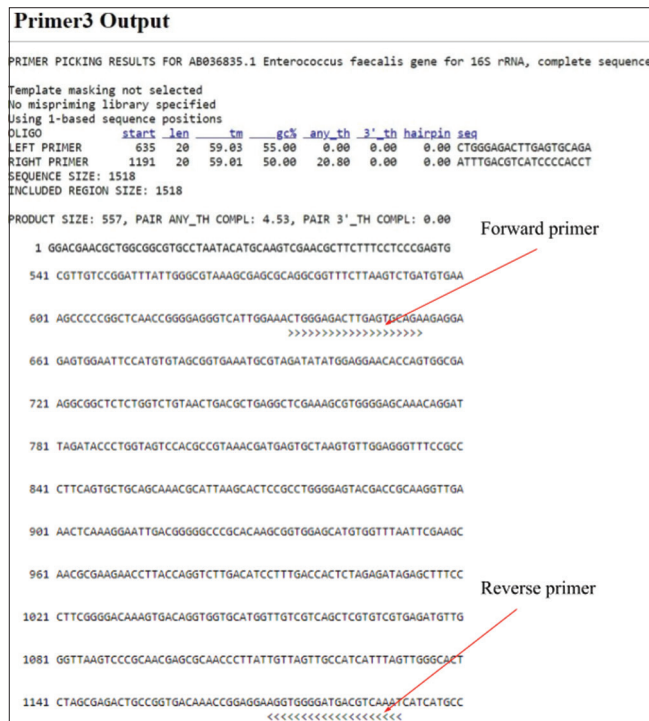


Figure 1: Primer designing using primer 3.

Table 2: 16S rRNA primer sequence.

Primers	Nucleotide Sequence (5’-3’)	Expected product size	Reference
Forward	5’-CTGGGAGACTTGAGTGCAGA-3’	557 bp	Designed in this study
Reverse	5’-ATTTGACGTCATCCCCACCT-3’		

16S rRNA: 16S Ribosomal RNA

Table 3: *Hyl* primer sequence.

Primer (<i>hyl</i>)	Nucleotide Sequence (5'- 3')	Expected product size	Reference
Forward	5'CCCTGGACACATGAAATGCG 3'	501 bp	Designed in this study
Reverse	5'AGCATCGGCCGTTGATAGAC 3'		

Hyl: Hyaluronidase

contamination. The results were documented using the Gel Documentation instrument and UV illuminator (White/UV Transilluminator UVP).

RESULTS

Isolation and biochemical identification

For the biochemical identification of *Enterococcus* species, EVA plates were employed to culture the isolates. Following an overnight incubation period, colony morphology on EVA plates were scrutinized. The isolates inoculated onto EVA plates and incubated at 37°C showed the formation of typical opaque, entire, and pinpoint colonies while reading. Subsequent subculturing of the 32 isolates on EVA agar plates revealed 30 isolates showing grayish pinpointed colonies characteristic of *E. faecalis*. Notably, isolates S16 and L4 were exceptions, yielding negative results in this biochemical identification process. The results are shown in Figures 2 and 3.

Bile esculin test

Colonies sourced from the EVA plates were streaked onto bile esculin agar plates, and the outcomes revealed a color change from dark brown to black. This transformation indicated the hydrolysis of bile salts within the medium.

Molecular detection of *E. faecalis*

The isolates underwent PCR analysis using SSOPs for the 16S rRNA gene. The anticipated amplicon was consistently observed across all isolates. Documentation of results was carried out using the gel documentation system. Both the positive and negative controls demonstrated satisfactory outcomes, with ATCC 29212 serving as the positive control. A 2 Kbp DNA ladder was employed as a marker, with the lowest band at 500 bp. The expected amplicon size of 557 bp was evident on the 1.2% agarose gel for 11 isolates (41%), refer to Figure 4 for a visual representation of the results.

Detection of *hyl* gene in *E. faecalis* isolates

PCR targeting the *hyl* gene was conducted for all 11 isolates using SSOPs. Notably, the results indicated that none of the 11 isolates tested positive for the presence of the *hyl* gene. The results are shown in Figure 5.

DISCUSSION

E. faecalis, a versatile pathogen implicated in various human diseases, including urinary tract infections, bacteremia, infective endocarditis, meningitis, intra-abdominal infections,

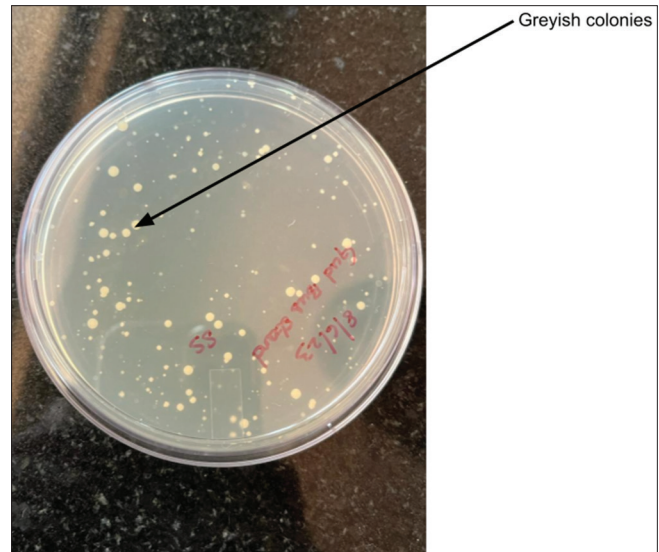


Figure 2: Ethyl Violet Azide plate with greyish white colonies.

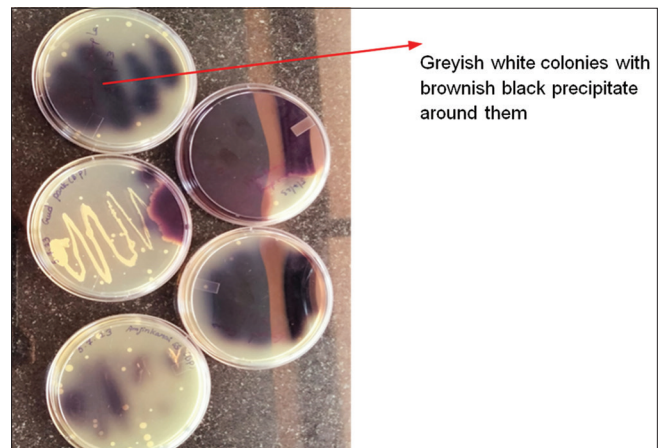


Figure 3: Bile Esculin plate with brownish-black precipitate colonies.

and wound infections, poses a significant public health concern. This study aimed to investigate the prevalence of *E. faecalis* in environmental samples collected in and around Chennai, shedding light on its distribution and the occurrence of one of its key virulence factors, the *hyl* gene. The comprehensive environmental sampling strategy, involving 32 diverse locations, revealed a substantial 41% incidence of *E. faecalis*. This prevalence underscores the widespread presence of the organism in the sampled areas. However, the intriguing finding was the absence of the

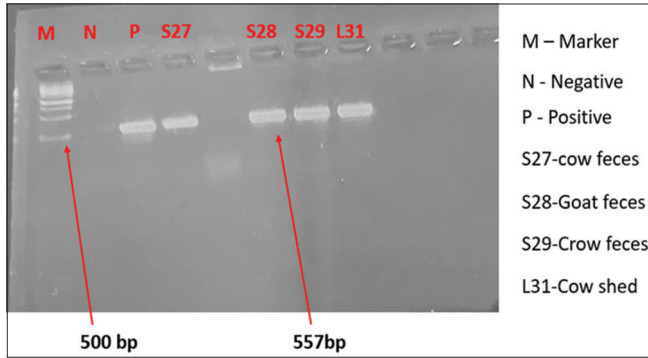


Figure 4: 16S Ribosomal ribonucleic acid polymerase chain reaction.



Figure 5: Hyaluronidase gene polymerase chain reaction.

hyl gene in any of the isolates from these environmental samples. This emphasizes a clear distinction between the environmental isolates and those associated with various diseases, particularly dental conditions. Contrastingly, in disease-related isolates, especially from dental caries, a high incidence of the *hyl* gene (72.4%) was observed. This stark difference in *hyl* gene prevalence between environmental and disease-related isolates points toward the pathogenicity and virulence factor specificity of *E. faecalis* in different contexts. Our findings align with previous studies that have reported the absence of the *hyl* gene in environmental samples. Studies conducted in regions such as Southwestern Iran, California, Puerto Rico, and poultry environments have reported varying occurrences of the *hyl* gene in *E. faecalis* isolates.^[9-12] The current study, in harmony with these reports, strengthens the notion that the *hyl* gene is more commonly associated with clinical isolates, particularly those linked to oral health conditions. Moreover, the investigation of biofilm formation and vancomycin-resistant genes in *Enterococcus faecium* from clinical and environmental specimens has revealed a connection between virulence genes and biofilm formation. However, our study distinctively focuses on the absence of the *hyl* gene in environmental samples, contributing to the understanding of its distribution and significance in different settings. While our study provides valuable insights into the prevalence of *E. faecalis* and the

absence of the *hyl* gene in environmental samples, the limitation lies in the relatively small number of isolates investigated. Future studies could expand on this research by including a larger sample size and exploring other potential virulence factors associated with *E. faecalis* in environmental settings.

In summary, our study contributes to the growing body of knowledge on *E. faecalis*, emphasizing the distinctive prevalence patterns of the *hyl* gene in environmental versus disease-related isolates. This understanding not only aids in the development of effective screening methods for environmental isolates but also underscores the potential health implications associated with the presence or absence of the *hyl* gene in different contexts.

CONCLUSION

Although our study identified a substantial prevalence of *E. faecalis* in environmental samples in and around Chennai, the incidence of *hyl* gene is low, necessitating further investigation with a higher number of samples. The absence of the *hyl* gene in these isolates challenges its conventional ubiquity, suggesting a potential divergence in pathogenicity between clinical and environmental strains. These findings emphasize the need for ongoing research to comprehend the specific dynamics of *E. faecalis* in diverse settings, informing targeted public health strategies.

Authors' contributions

DR and AS: Sample collection, analysis, manuscript preparation; SV: Ethical clearance, manuscript preparation; B PC: Work plan, design, manuscript preparation.

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Ethical approval

The research/study was approved by the Institutional Review Board at Sri Ramachandra Institute of Higher Education and Research, number CSP/23/JUN/130/540, dated 30th January 2023.

Declaration of patient consent

Patient's consent not required as there are no patients in this study.

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Nil.

Conflicts of interest

There are no conflicts of interest.

Use of artificial intelligence (AI)-assisted technology for manuscript preparation

The authors confirm that there was no use of artificial intelligence (AI)-assisted technology for assisting in the

writing or editing of the manuscript, and no images were manipulated using AI.

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