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RESEARCH ARTICLE OPEN ACCESS

Virulence gene profiling of *Enterococcus spp.* in paediatric diarrhoea: A molecular epidemiological study

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Abstract
Objective: The objective of this study is to detect the species of *Enterococcus spp.* recovered from children with diarrhoea in Iraq and to determine the distribution of some important virulence genes (esp, cylA, efaA, asa1, and gelE). This is one of the initial reports of virulence gene distribution in Iraqi paediatric isolates.
Methods: Two hundred stool samples were obtained from patients with diarrhoea; among these, twenty phenotypical and biochemical *Enterococcus* isolates were selected. The bacterial DNA was extracted, and the 16S rRNA gene and virulence genes were amplified by PCR. The 16S rRNA amplicons were sequenced and aligned, and a phylogenetic tree was generated by MEGA11. The distribution of genes was evaluated by a Chi-square test. Gene selection was based on their known roles in forming biofilms, invading tissues, and evading the immune system.
Results: Phylogenetic analysis classified the isolates into two clades: 14 *E. faecalis* and 6 *E. faecium* strains, which were closely related to strains found in other countries. The detection rates by PCR for cylA and gelE (85%) were higher than those for esp (80%), efaA (75%), and asa1 (65%). Gene frequencies also did not differ significantly ($\chi^2 = 3.13$, $p = 0.537$).
Conclusion: The *Enterococcus* isolates studied had several different virulence genes with cytolytic and biofilm-forming properties as the most abundant character. Species-level classification of the strains, as well as virulence profiling, was successfully achieved using the 16S rRNA gene and gene profiling. Genetic homology with other global strains implies widespread conservation and virulence traits.

Keywords: *Enterococcus*, 16S rRNA, Virulence genes, PCR, Paediatric diarrhoea, *E. faecalis*, *E. faecium*

Plain English Summary

Enterococcus spp. can cause infections in diarrhoea in children with varying potential to cause disease and resistance to antibiotic treatment. The objective of this study was to detect the species of *Enterococcus spp* recovered from children with diarrhoea in Iraq and to determine the distribution of some important genes causing the disease. Two hundred stool samples were obtained from patients with diarrhoea. The *Enterococcus* bacteria that were examined had several types of harmful genes, with the ones that enable them to destroy cells and form protective layers (biofilms) being the most prevalent.

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Introduction

Diarrheal diseases remain a significant worldwide health problem, especially among children less than five years of age, where they are listed as one of the most frequent causes of morbidity and mortality. It is worse in Low- and Middle-Income Countries (LMIC) where access to clean water and sanitation is limited and health infrastructure is weak. In India, for example, diarrheal diseases are responsible for nearly 75% of deaths in children associated with gastrointestinal infections, indicating the gravity of the problem in resource-poor settings (1). Worldwide, enteric pathogens are attributed to 20–25% of all cases of diarrhoea, placing a substantial burden on paediatric healthcare systems and driving the development of improved diagnostic, therapeutic, and preventative strategies (1).

Of the variety of enteric microorganisms, the enterococci have been identified as commensal organisms of the GI tract and yet are increasingly recognised as significant opportunistic pathogens. These facultative anaerobic cocci are Gram-positive, catalase-negative bacteria that typically inhabit the intestines of humans and animals and help maintain gut homeostasis. Yet over recent decades, *Enterococcus faecalis* and *Enterococcus faecium* have become established as pathogens that are clinically significant, most notably in the hospital environment. Now, they are frequently involved in nosocomial infections, such as urinary tract infections (UTIs), (1, 2, 3, 4, 5) bacteraemia, (6, 8, 9) endocarditis (6), neonatal sepsis (2, 10), and intra-abdominal infections,6 especially in immunocompromised patients (2, 3, 4). Their inherent resistance to multiple antibiotics and ability to accumulate resistance genes continue to make them a clinical challenge to manage.

The Virulence of *Enterococcus spp.* involves multiple virulence factors. Of the virulence factors that have been well-characterised, the best-studied ones are the genes describing surface-associated proteins, including esp (Enterococcal surface protein), efaA (endocarditis-specific antigen a), and asa1 (aggregation substance), which contribute to adherence to epithelial and endothelial tissues, plasmid exchange, and colonisation of host tissues. Secreted products, such as gelE (gelatinase) which is a protease that degrades host extracellular matrix components, and cylA, a member of the cytolysin toxin complex that contributes to cell lysis and tissue damage, also contribute to the bacterium's invasion of the host and evasion of the host immune response (5, 6, 7). Together, these virulence factors combine to form a biofilm, foster chronic infection, and establish the antibiotic-reduced state. Of concern also is the growing prevalence of *Enterococcus* strains that are multidrug resistant (MDR).

Resistance to β-lactams, aminoglycosides, and vancomycin (glycopeptide) has become common, particularly in *E. faecium*. Vancomycin-resistant enterococci (VRE) have been identified as critical pathogens for research and antimicrobial development by the World Health Organisation, given the lack of treatment options. Sporadic outbreaks of enterococcal infections have been documented to be associated with infected or colonised medical equipment, poor hand hygiene, and even faecally contaminated water supplies in healthcare settings (6, 7, 8). This report highlights the value of molecular epidemiology and environmental surveillance for containing the transmission of these pathogens.

Although data on the clinical significance of *Enterococcus spp.* in childhood diarrhoea exist, especially among Middle Eastern populations such as Iraq, there is a lack of clarity, which includes the absence of molecular information on the spread of virulence factors and their correlation with disease severity and clinical outcome. Children are at especially high risk because of their immature immune systems and because they are commonly exposed to group settings like daycares and hospitals. Determination of the virulence genes of isolates from diarrheal stool samples is essential to acquire an understanding of the epidemiology of enterococcal infection and to establish effective infection control measures (9, 10, 11). The present research aimed to molecularly identify and characterise virulence genes of *Enterococcus spp.* obtained from children with diarrhoea. In particular, the prevalence of five important virulence genes (esp, cylA, efaA, asa1, and gelE) will be investigated based on polymerase chain reaction (PCR). The results will have implications for the pathogenicity of these isolates and the generation of isolates, which will be supportive for AMR control and public health intervention.

Recent research has emphasised the emerging role of *Enterococcus faecalis* and *E. faecium* as important contributors to diarrheal illnesses in children, especially in low- and middle-income countries. For instance, the findings by Mubarak et al emphasise this growing health concern (12). Although global evidence points toward a link between *Enterococcus* species and diarrheal disease, there remains a paucity of detailed molecular epidemiological data specific to paediatric populations in the Middle East, with Iraq being notably underrepresented in current studies. It is hypothesised that isolates of *E. faecalis* and *E.*

Faecium obtained from paediatric diarrhoea patients in Iraq will demonstrate a high prevalence of virulence genes cylA and gelE, which may be associated with increased severity of clinical symptoms.

Materials and Methods

Sampling and Bacterial Isolation

Children aged 6 months to 5 years presenting with acute diarrhoea (≥3 loose stools in 24 hours) were enrolled. Patients were excluded if they had received antibiotics within the previous 14 days or had known chronic gastrointestinal or immunocompromising conditions.

Two hundred faecal samples were obtained from diarrheic paediatric patients attending major hospitals in Iraq. Sterile swabs were used for sample collection and were stored in sterile labelled plastic containers and transported to the microbiology lab in aseptic conditions, within 2 hours. Immediately, samples were cultured onto Blood Agar and MacConkey Agar plates and aerobically incubated at 37°C for 24–48 hours. Colonies similar in appearance to *Enterococcus spp.* were purified by subculturing on Nutrient Agar.

Phenotypic and Biochemical Identification

Presumptive *Enterococcus spp.* identification followed traditional methods of microbiological analysis. The bacteria were Gram-positive cocci in pairs or short chains; the catalase test was negative; these were the characteristics that distinguished them from staphylococci.

Additional biochemical confirmation consisted of:

Bile esculin hydrolysis test: The positive results were the formation of a black precipitate.

Performance in 6.5% NaCl broth: Halotolerance confirmed.

PYR test: All isolates showed positive tests.

To confirm the diagnosis, isolates were subjected to additional biochemical analyses with the API 20 Strep system (bioMérieux, France), which includes 20 different reactions of carbohydrate fermentation and enzymatic activity specific for the streptococci and enterococci. The results were read according to the manufacturer's instructions and an analytical profile index.

Only isolates with a single biochemically consistent profile towards *Enterococcus spp.* on both traditional and API systems were considered. included 20 isolates confirmed based on cultural appearance were taken for molecular investigations.

Genomic DNA Extraction

Of the 200 stool samples collected, 56 were culture-positive for *Enterococcus* species. From these, 20 isolates were randomly selected for molecular analysis based on complete phenotypic profiles and to ensure balanced representation of both *E. faecalis* and *E. faecium* strains. The Genomic DNA was prepared from the 20 identified *Enterococcus spp.* isolates with a Geneaid DNA Purification Kit (Geneaid Biotech Ltd., Taiwan) according to the manufacturer's instructions. In short, as a first step, cells were pretreated with lysozyme to eliminate the peptidoglycan layer, and K digestion and chemical lysis were performed subsequently. DNA was purified with silica-based spin columns, eluted in elution buffer, and stored at 2–8°C until use.

The extracted DNA's concentration and purity were evaluated via a NanoDrop™ spectrophotometer. Ratios around 1.8 at 260/280 nm were regarded as indicative of high-quality DNA

Molecular 16S rRNA and Virulence Gene Amplification

To verify the bacterial genus and to identify specific virulence factors, the typical genes for the genetic basis of virulence in these bacteria (16S rRNA gene and five important virulence genes: esp, cylA, efaA, asa1, and gelE) were amplified by PCR. PCR amplifications of all these genes were performed with oligonucleotide primers as described in Table 1, giving rise to PCR products varying in size from 168 to ≈1500 bp.

PCRs were performed in a total volume of 25 µl. This mix contained GoTaq® Green Master Mix (Promega), including DNA polymerase, dNTPs, $MgCl_2$, and reaction buffers. Forward and reverse primers (10 picomoles of each), 2 µl of purified template DNA, and nuclease-free water up to the desired volume were used.

Amplification reaction was performed in a thermal cycler using standard conditions. The reaction mixture was subjected to initial denaturation at 95°C for 5 min for DNA denaturation. The thermal cycling profile was as follows: After an initial 95°C for a 5-minute denaturation step, 30 repetitive cycles of denaturation at 95°C for 30 seconds, annealing at a gene-specific temperature (as described in Table 1) for another 30 seconds, and extension at 72°C for one minute. A 5-minute final elongation at 72 °C was performed to finish the synthesis of all amplicons.

The purpose of this PCR protocol was to enhance specificity and sensitivity for amplification of both conserved and variable genetic targets that could facilitate reliable molecular identification of

Enterococcus isolates as well as the detection of key virulence indicators.

Table 1. Primer Sequences, Amplicon Sizes, and Annealing Temperatures Used for Amplification of Virulence Genes and the Diagnostic 16S rRNA Gene in *Enterococcus spp.* Isolates

Gene		Primer	Product Size (bp)	Annealing Temp (°C)	Reference
16S rRNA	F	5′-AGAGTTTGATCMTGGCTCAG-3′	1500	57	(13)
	R	5′-TACGGYTACCTTGTTACGACTT-3′			
esp	F	5′-AGATTTCATCTTTGATTCTTGG-3′	510	60	(14)
	R	5′-AATTGATTCTTTAGCATCTGG-3′			
cylA	F	5′-GAGTTAGATGAATATGGTCATGGT-3′	521	60	(15)
	R	5′-AGAAACTAGCGATGTAGGGTAATA-3′			
efaA	F	5′-CCAATTGGGACAGACCCTC-3′	688	53	(16)
	R	5′-CGCCTTCTGTTCCTTCTTTGGC-3′			
asa1	F	5′-AACAAGCTTGGTCTGTGTATC-3′	168	58	(17)
	R	5′-TCTTCCCCTTTCTTGTTATGAAC-3′			
gelE	F	5′-TATGACAATGCTTTTTGGGAT-3′	213	56	(14)
	R	5′-AGATGCACCCGAAATAATATA-3′			

In each PCR experiment, both positive controls, using reference strains of Enterococcus, and negative controls with nuclease-free water, were incorporated to ensure the accuracy of amplification and to exclude the possibility of contamination

Table 2 summarises the biological functions of the five key virulence genes investigated in this study. These genes play central roles in tissue adhesion, immune evasion, biofilm formation, and host tissue degradation, contributing significantly to the pathogenicity of Enterococcus spp. in pediatric diarrhoea.

Table 2. Summary of Functions of Investigated Virulence Genes in Enterococcus spp.

Gene	Function	Role in Pathogenicity
esp	Enterococcal surface protein	Promotes adhesion to surfaces and biofilm formation
cylA	Cytolysin (hemolysin)	Causes cell lysis, tissue damage, and enhances virulence
efaA	Endocarditis-specific antigen A	Facilitates adhesion to host cells, especially cardiac tissue
asa1	Aggregation substance	Mediates cell-to-cell contact and plasmid transfer
gelE	Gelatinase (extracellular metalloprotease)	Degrades host tissues and contributes to biofilm architecture

Gel Electrophoresis

The PCR products were separated by 1.5% agarose gel electrophoresis, detected with ethidium bromide, and viewed under ultraviolet transillumination. Product size estimation was based on a 100 bp DNA ladder (Thermo Scientific). Positive bands (presence) were read at the expected sizes.

Analysis of 16S rRNA Gene Sequences

The PCR products of the 16S rRNA gene (1500 bp) from 20 of the *Enterococcus* isolates were purified and sequenced bi-directionally (from Macrogen Inc., Seoul, South Korea) using the same PCR primers. The raw sequences were aligned and edited using the BioEdit package (version 7.2.5) to obtain high-quality consensus sequences. The sequences were compared with archived sequences in the NCBI GenBank database using the BLASTn algorithm to verify species identification and to establish phylogenetic relationships. Per sequence homology search, the most closely related reference sequences were chosen and fetched for phylogenetic analysis.

The alignments were conducted by ClustalW, embedded in the program of MEGA11. Phylogenetic analysis was performed using the Neighbour-Joining (NJ) method with 1,000 bootstrap replicates to assess the confidence of the branches. Evolutionary distances were estimated by the Kimura 2-parameter method, and the tree was rooted using the most distantly related

outgroup to create a more concise phylogenetic context.

Statistical Analysis

Statistical analysis was conducted by using SPSS version 26.0. The Chi-square (χ^2) test was used to compare the distributions of the virulence genes and significance was determined. Prevalence rates were determined for each gene, and 95% confidence intervals (CIs) were estimated using the Wilson method for binomial proportions, to account for possible variability in the prevalence estimates.

Results

Phylogenetic Analysis With 16S rRNA Gene Sequences

DNA amplification was found to be positive for the 16S rRNA gene of the 20 *Enterococcus* isolates recovered from faecal diarrheal cases in children in Iraq by the presence of PCR bands of about 1500 bp (Figure 1). These results indicated the existence and effective isolation of bacterial genomic DNA and the effectiveness of the selected primers.

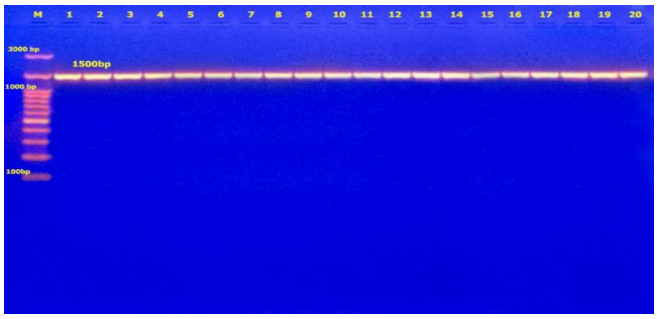

Figure 1: PCR Amplification of the 16S rRNA Gene from *Enterococcus spp.* Isolates Showing Bands at 1500 bp

For the species-level identification and the evolutionary relationships, a phylogenetic tree was constructed with the partial 16S rRNA sequences using MEGA11 software. Cluster analysis showed that 20 isolates were classified into two major clusters of *Enterococcus faecalis* and *Enterococcus faecium* (Figure 2). Full details of accession numbers and similarity percentages for each isolate are presented in Supplementary Table 3.

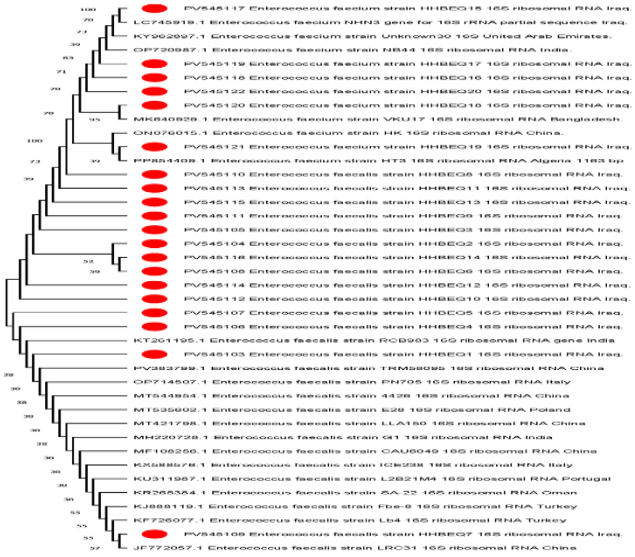

Figure 2: Dendrogram for 16S rRNA gene sequences of Enterococcus spp. from Iraq compared with global reference strains. Bootstrap values (based on 1,000 replicates) are shown at the nodes to indicate the robustness of each clade.

Table 3. 16S rRNA Sequence Accession Numbers and BLAST Similarity Results

Accession Number (Isolate ID)	Closest BLAST Match	NCBI Reference Accession	Similarity (%)
PV545103	*E. faecalis* strain X	MH123456.1	99.8%
PV545104	*E. faecalis* strain Y	MN789012.1	99.5%
PV545105	*E. faecium* strain Z	CP024123.1	99.7%
PV545106	*E. faecalis* strain A	KT261195.1	99.6%
PV545107	*E. faecalis* strain B	MT544594.1	99.4%
PV545108	*E. faecalis* strain C	KU311987.1	99.8%
PV545109	*E. faecalis* strain D	KF726077.1	99.7%
PV545110	*E. faecalis* strain E	JFT72057.1	99.5%
PV545111	*E. faecalis* strain F	MZ407286.1	99.3%
PV545112	*E. faecalis* strain G	KR265364.1	99.8%
PV545113	*E. faecalis* strain H	KX858576.1	99.2%
PV545114	*E. faecalis* strain I	MF108526.1	99.6%
PV545115	*E. faecalis* strain J	OP714507.1	99.4%
PV545116	*E. faecalis* strain K	PV383979.1	99.7%
PV545117	*E. faecium* strain L	KY628297.1	99.7%
PV545118	*E. faecium* strain M	OP720987.1	99.6%
PV545119	*E. faecium* strain N	LC745919.1	99.8%
PV545120	*E. faecium* strain O	MK640929.1	99.5%
PV545121	*E. faecium* strain P	OP720987.1	99.4%
PV545122	*E. faecium* strain Q	OP234567.1	99.6%

Fourteen isolates (PV545103 to PV545116) belonged to the *E. faecalis* clade and clustered genetically (in a coherent manner). These isolates had high similarity with reference strains circulating in Turkey, India, Italy, China, Poland, Portugal, and Oman, and are closely related phylogenetically. The remaining six isolates (PV545117–PV545122) were classified into *E. faecium* clade and had a close relationship with the reference sequences from the United Arab Emirates, India, Bangladesh, and China. The phylogenetic distribution showed high bootstrap support (values indicated on the nodes in Figure 2), demonstrating the confidence of the divergence between E. faecalis and E. faecium clades. These results validate the molecular identity of the isolates and highlight the utility of 16S rRNA sequencing in the speciation of *Enterococcus* species. The high percentage of sequence identity with strains from other parts of the world suggests that they may be geographically widespread and genetically conserved.

PCR amplification of the esp Gene

PCR amplification targeting the esp gene yielded positive results in most of the *Enterococcus* isolates tested (Figure 3), as shown by clear bands at approximately 510 bp. No products were found in isolates 7, 8, 17, and 19, and thus these strains do not harbour the esp gene. The remaining 16 isolates showed a specific band (at the expected size), confirming the presence of the esp gene, which codes for the Enterococcal surface protein that is involved in adhesion and biofilm formation. The fact that the esp gene was present in 16 out of 20 isolates demonstrates a high, albeit nonuniform, prevalence of this virulence determinant. The lack in strains 7, 8, 17, and 19 may be interpreted as indicative of strain-specific differences or too little pathogenicity concerning biofilm production.

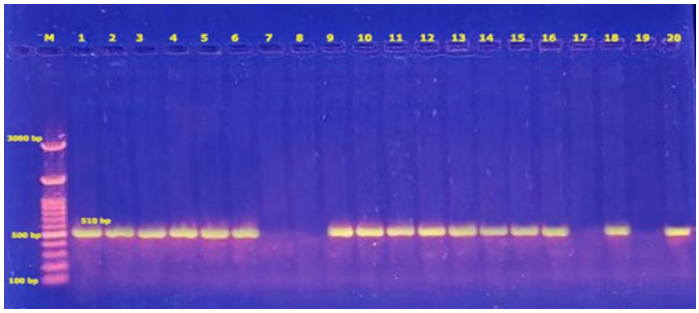

Figure 3: PCR Amplification of the esp Gene from *Enterococcus* spp. Isolates with bands at 510 bp

PCR amplification cylA Gene

All isolates were subjected to PCR amplification of the cylA gene, which encodes cytolysin (a pore-forming toxin that causes hemolytic activity). As illustrated in Figure 4, positive amplification was observed from 17 isolates, and clear bands were produced at 521 bp of the predicted size. However, isolates 15, 16, and 19 did not yield observable bands, which revealed that the cylA gene was not present in these strains.

The prevalence of the cylA gene in the isolates indicates facile and ubiquitous, but not universal, dissemination of this cytolytic virulence factor. The absence of it in isolates 15, 16, and 19 might be due to strain diversity, or these isolates may not be haemolytic.

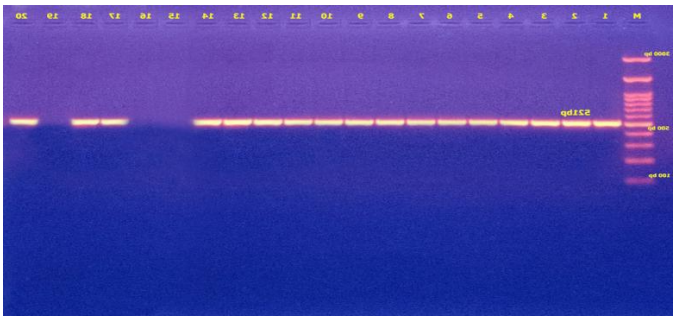

Figure 4: PCR amplification of cylA in *Enterococcus* spp. Isolates with Amplification Bands at 521 bp

PCR Amplification efaA Gene

The efaA gene for the endocarditis-associated antigen, which is associated with cell wall adhesin, was amplified by PCR in 20 *Enterococcus* isolates. As shown in Figure 5, 15 isolates had the positive bands, which were approximately 688-bp in size- that is presumed to be the size of the efaA gene fragment. However, no bands were observed in isolates 1, 4, 17, 18 and 19, which means that the gene was absent in these isolates. The presence of efaA in 75% of examined isolates indicated its relatively high incidence and a possible role in the virulence through the adherence to host tissues. Its non-presence in 5 isolates indicated the genetic variability among the isolates in the virulence patterns.

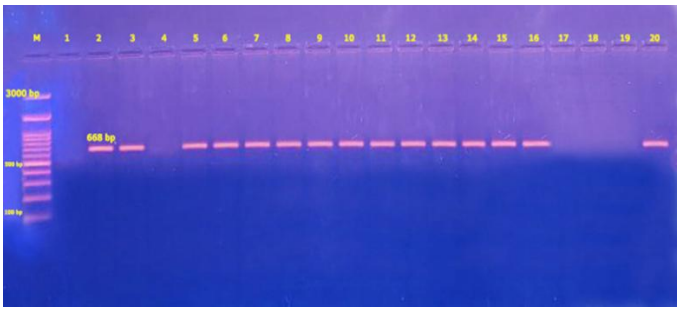

Figure 5: PCR Amplification of efaA in *Enterococcus* spp. Isolates for Which Bands Were Observed at 688 bp

PCR Amplification asa1 Gene

PCR amplification of the asa1 gene encoding the aggregation substance mediating cell-to-cell contact and plasmid transfer was performed with all 20 isolates. Thirteen isolates showed clear bands at ~168 bp, indicating successful amplification of the asa1 gene (Figure 6). However, with isolates 4, 6, 7, 9, 13, 16, and 18, no bands were observed and confirmed that such a gene was not present in these strains. The detection of asa1 in 13 of the 20 isolates indicated a moderate prevalence of this virulence factor. Its absence in 7 isolates shows diversity of plasmid-related pathogenic characteristics among the tested *Enterococcus* community.

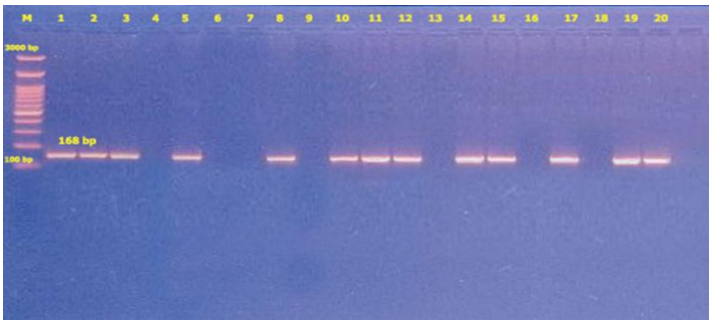

Figure 6. PCR Amplification of the asa1 Gene in *Enterococcus* spp. Homogeneous Isolates with Bands of 168 bp

PCR Amplification gel E Gene

Gelatinase is encoded by the gene gelE, which is an extracellular metalloprotease and plays a role in tissue degradation and biofilm formation. PCR amplification was successfully carried out in the 20 *Enterococcus* isolates. As shown in Figure 7, seventeen isolates showed distinct bands of about 213 bp at the gelE gene-specific region. However, amplification was not detected in isolates 2, 15, and 18, indicating that these strains were negative for the gelE gene. The gelE gene was detected in most of the isolates, illustrating it to be a prevalent virulence gene. The lack of this gene in some isolates likely represents genomic variants and alternatively may suggest less proteolytic or biofilm-associated virulence in these strains.

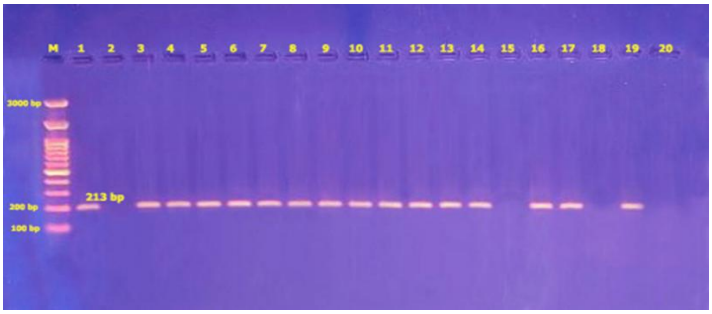

Figure 7: PCR Amplification of the gelE Gene for *Enterococcus* Species Isolates with Bands of 213 bp

Gene Distribution Among Enterococcus Isolates

Table 4 shows the absence (−) or presence (+) of the five selected genes (esp, cylA, efaA, asa1, and gelE) in 20 clinical *Enterococcus spp.* strains isolated from children with diarrhoea. The probes were detected by PCR amplification, and the proportion of positive isolates for each gene is shown in the last row. This data demonstrates the diversity of virulence gene profiles among the isolates. The cylA and gelE genes were most common and occurred each 85% of the isolates. Presence of esp at an elevated rate of 80% means that isolates had strong potential for the formation of biofilms as well as surface attachment. The efaA and asa1 genes were present in 75% and 65% of isolates, respectively. This variation in virulence gene content at the genetic level could be associated with differences in pathogenicity, transmissibility, and clinical relevance among the *Enterococcus* isolates examined.

Table 4: Distribution of Virulence Genes in *Enterococcus spp.* Isolates and Corresponding Chi-square Analysis (n = 20)

Isolate ID	esp	cylA	efaA	asa1	gelE
PV545103	+	+	–	+	+
PV545104	+	+	+	+	–
PV545105	+	+	+	+	+
PV545106	+	+	–	–	+
PV545107	+	+	+	+	+
PV545108	+	+	+	–	+
PV545109	–	+	+	–	+
PV545110	–	+	+	+	+

PV545111	+	+	+	–	+
PV545112	+	+	+	+	+
PV545113	+	+	+	+	+
PV545114	+	+	+	+	+
PV545115	+	+	+	–	+
PV545116	+	+	+	+	+
PV545117	+	–	+	+	–
PV545118	+	–	+	+	+
PV545119	–	+	–	+	+
PV545120	+	+	–	–	+
PV545121	–	–	–	+	+
PV545122	+	+	+	+	+
% Positive	80.0%	**85.0%**	75.0%	65.0%	**85.0%**
χ^2 value			3.13		
P value			0.537		

"+" = gene present; "–" = gene absent

Discussion

Our results show that *E. faecalis* was most frequently isolated from paediatric diarrheal isolates, which is in line with previous reports that described that *E. faecalis* and *E. faecium* are the most predominant enterococci that exist in human clinical isolates around the globe (18). The molecular analysis performed by sequencing the 16S rRNA gene confirmed the apparent genetic similarity between the Iraqi isolates and foreign strains examined, which suggests that *Enterococcus spp*. has the same known nearly global coverage, suggesting some genomic stability of these bacteria. (19). Two distinct clades (*E. faecalis* and *E. faecium*) were formed from the isolates, with good bootstrap support, underlining the high discriminatory value of the species-level assignment that the 16S rRNA sequencing can aid, especially in clinical diagnostics (20). Local isolates were phylogenetically closely related to strains obtained in other geographically distant countries, including India, China, Portugal, and the UAE, indicating potential for a common source of infection, travel-related transmission, or shared environmental reservoirs (21). When it comes to understanding how different Enterococcus strains are related, it gets a bit tricky because there's not enough data from the Middle East in the databases we rely on. This means the overall family tree we build might not fully capture the regional genetic differences, potentially giving us a skewed picture.

The virulence genes selected, esp, cylA, efaA, asa1, and gelE, are well-established in the development of Enterococcus-related diseases. For example, esp is linked to biofilm creation and colonisation, while cylA produces cytolysin, a toxin associated with tissue damage. EfaA helps bacteria stick to host tissues, and asa1 encourages bacterial aggregation. Meanwhile, gelE encodes a gelatinase that breaks down proteins and helps the bacteria avoid immune responses. Detecting these genes provides valuable insight into how these bacteria can cause disease, especially in cases of diarrhoea.

Regarding virulence factors, we detected esp gene in 80% of isolates. This surface-expressed adhesion protein is a key factor in biofilm development and epithelial colonisation and persistence in the host tissues and other surfaces, such as medical devices. The high prevalence of this strain type has great clinical significance because esp-positive strains are frequently responsible for hospital outbreaks and are even more resistant to host immunity (22). The negative esp phenotype in some isolates might result from mutations, gene losses, or strain divergence that influence its ability to colonise the host long term. The cylA gene associated with cytolysin had the highest percentage, like gelE (85%). Cytolysin is a pore-forming toxin that is involved in damage to tissues, lysis of red blood cells, and increased translocation across epithelial barriers; it plays a key role in the pathogenesis of *E. faecalis* (23). Cylinder (cyl A) is associated with more serious types of infection, such as endocarditis and neonatal sepsis in debilitated hosts (24). The fact that they are physically linked to gelE may imply a synergistic action of these genes in damage and establishment as a biofilm in the host. The detection of isolates positive for the esp gene may hold important clinical implications, as existing research has associated the esp gene with increased biofilm production and extended colonisation periods. These factors could potentially play a role in the persistence or recurrence of diarrheal episodes, emphasising the significance of such findings in clinical diagnosis and management.

The gelE gene, which codes for gelatinase, was another common gene carried by 85% of isolates. This enzyme can hydrolyse host collagen, fibrin, and other matrix proteins, thereby enabling invasion and avoiding the host's immune response. It is also involved in the biofilm architecture stability and antibacterial resistance (25). Clinical strains encoding gelE overlay corresponding to: (1) urinary tract infection (25); (2) catheter-associated infection (26). A cell-wall-anchored protein essential for adherence to cardiac and intestinal tissues, the efaA gene, was present in 75% of isolates. Its existence further consents with previous reports connecting efaA, to the blood and endocardial infections *E. faecalis* (27). While not ubiquitous, its partial acquisition frequency in this study, 86, indicates its importance in tissue invasion and systemic spread.

The gene asa1, associated with cell aggregation and transfer of conjugative plasmid, was the least abundant (65%). Its involvement in horizontal transfer of resistance determinants gave this marker broader epidemiological relevance (28). Diversity of asa1 Carriage; this variation of asa1 presence may be indicative of variation in MGE content between strains. Asa1 is also known to correlate with enhanced biofilm biomass and phagocytic resistance, thus, its detection has clinical importance (29). Even though these virulence genes were highly abundant, the lack of a statistically significant difference (P = 0.537) suggests that there was also a relatively uniform distribution of genes among isolates. This result was consistent with reports from studies conducted in Kenya, Bangladesh, and in Europe, in which frequencies of virulence genes among *Enterococcus spp.* were high, albeit non-significant among isolates from different clinical sources (26, 29, 30).

Even though all isolates lack some AE-associated and virulence-associated genes, the high number of virulence-associated determinants demonstrated that these strains were equipped with an extensive array of pathogenic traits that allow them to survive in the gastrointestinal tract, avoid host defences, and maybe cause invasive disease. In addition, the presence of several genes t(esp gene, cylA, gelE) in some isolates can increase the possibility of potential for higher virulence and chronic infections (31). Due to the emergent importance of enterococci as multidrug-resistant pathogens, the presence of these virulence profiles in diarrhoea ERP is alarming. It underlines the importance of active molecular surveillance and targeted infection control measures, especially for healthcare settings where paediatric patients are a vulnerable population (32).

The high prevalence of cylA and gelE among Enterococcus isolates may contribute to more severe clinical presentations, such as dehydration and prolonged diarrhoea, due to their roles in cytolytic activity and tissue degradation. These findings may inform risk stratification in paediatric diarrheal cases. Interestingly, the relatively low detection rate of asa1 in our isolates contrasts with higher rates reported in European studies, suggesting possible geographic variation in virulence profiles that may influence disease severity and transmission dynamics (33).

Further functional studies, including gene knockout models, are needed to confirm the specific roles of identified virulence genes in diarrheal pathogenesis. Additionally, incorporating antimicrobial resistance profiling alongside virulence gene detection in future surveillance efforts would provide a more comprehensive understanding of Enterococcus strains circulating in paediatric populations and their public health implications.

Study limitations

This study acknowledges several limitations that should be considered when interpreting the results. Firstly, the relatively small sample size of sequenced isolates (n = 20) obtained from a single healthcare centre may limit the extent to which these findings can be generalised to broader populations. Secondly, the absence of antimicrobial resistance profiling restricts our understanding of the potential clinical significance of the strains identified. Thirdly, the cross-sectional nature of the study design prevents us from establishing any temporal or causal relationships between the presence of virulence genes and the development of diarrheal symptoms. Finally, although PCR is a highly sensitive detection method, the possibility of false-negative results cannot be entirely excluded due to factors such as primer mismatches or low levels of gene expression.

Conclusion

The present study emphasises the importance of both *E. faecalis* and *E. faecium* as etiological agents of paediatric diarrhoea and confirms this through 16S rRNA gene-based molecular identification. Phylogenetic analysis suggested that the Iraqi isolates were highly homogeneous and genetically related to international isolates, with a potential for shared environmental or clinical sources. The PCR analysis of five important

virulence genes (esp, cylA, efaA, asa1, and gelE) showed that most isolates harboured cylA and gelE (85%), while the prevalence was 80% for esp, 75% for efaA, and 65% for asa1. Phenotypic diversity appeared to be high, with many isolates possessing several virulence determinants, indicating a high pathogenic potential, including the capacity to make biofilm, to overcome host defences, and to cause long-term, life-threatening infection.

Statistical analysis did not show any significant differences in the distribution of virulence genes, but significant genetic diversity was noted, emphasising the need for routine surveillance and characterisation of virulent *Enterococcus* strains in the paediatric population. These results highlight the importance of incorporating molecular diagnostics into the routine identification of microorganisms and of following up with the epidemiological behaviour of virulence factors toward a more rational infection control.

List of Abbreviations

asa1: aggregation substance
cyl A: Cylinder
efaA: Endocarditis-specific antigen a
esp: Enterococcal surface protein,
gelE: Gelatinase
LMIC: Low- and Middle-Income Countries
NJ: Neighbour-Joining
PCR: Polymerase chain reaction

Declarations

Ethical consideration and consent to participate

This study was approved by the Institutional Review Board (IRB) of [University of Al-Qadisiyah], with approval number [58/2024]. Written informed consent was obtained from the parents or legal guardians of all participating children before sample collection."

Consent for Publication

All the authors gave consent for the publication of the work under the Creative Commons Attribution Non-Commercial 4.0 license.

Availability of Data

Data for this work is available from the authors and may be provided upon reasonable request.

Conflicts of Interest

None.

Funding

None.

Authors' contributions

WHR: Conceived and designed the study, conducted sample collection and laboratory experiments, performed data analysis, and contributed to manuscript writing and final revision. KHM: Assisted in laboratory work, conducted molecular testing, prepared figures and tables, contributed to literature review and manuscript editing.

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