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Original article

Molecular detection and the frequency of a pore-forming toxin in *Enterococcus faecalis* isolated from urinary tract infections

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ABSTRACT

Background: *Enterococcus faecalis (E. faecalis)* is a causative agent for urinary tract infections (UTIs) in Iraq and worldwide, even though it is a commensal bacterium in human and animal intestines. It can cause different illnesses due to its ability to produce various virulent factors. A pore-forming toxin (cytolysin) is the most virulent factor in this bacterium. **Objective:** This study aims to molecularly investigate the frequency of cytolysin toxin among *E. faecalis* isolated from UTIs. **Methods:** A hundred and eighty urine specimens were collected from females diagnosed with UTIs. Traditional laboratory and molecular methods were used for bacterial identification and toxin detection using a modified DNA extraction method. **Results:** The findings revealed that 27.7% (50\180) of causative agents in UTIs were *E. faecalis* based on the molecular technique that targeted a housekeeping gene (*ddI*) with specific primers using polymerase chain reaction (PCR). Most of the isolates harbored the cytolysin toxin gene (*cylL*$_L$) with a frequency rate of 92% (46\50). **Conclusions:** A considerable prevalence of cytolysin-positive isolates in UTIs is worrying and indicates the extensive spread of a toxic strain in UTIs. The modified method for DNA extraction in gene detection was successfully used to amplify a housekeeping gene (*ddI*) and a virulence gene (*cylL*$_L$) for cytolysin toxin detection, and this approach can be utilized for rapid bacterial identification and gene detection in medical and research purposes with a large sample size in an inexpensive manner within a short time.

Introduction

Enterococcus faecalis, which was initially named *Streptococcus faecalis* in 1906 and then reclassified as Enterococcus in 1984 [1], has been identified as a pathogen causing endocarditis from its first publication in English by **Andrewes et al.** in 1906 [2]. *Enterococcus faecalis* is a prevalent bacterium in the gastrointestinal systems of humans and animals. It is commonly transmitted by water, soil, food, and plant materials [3]. Even though it occurs naturally in the gastrointestinal tract of both humans and animals, it is also found in other locations, such as the mouth, urine, blood, dental root canals, and vagina [4,5]. In addition, it is considered one of the most opportunistic pathogens for UTIs, peritonitis, surgical-sit-infection, burn infection, cholecystitis, bacteremia, and neonatal meningitis [6,7].

The most common virulence factors identified in *Enterococci* include adhesin to collagen, enterococcal surface protein, gelatinase, serine protease, hemolysin, cytolysin, and

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hyaluronidase [8]. Moreover, *Enterococci* can create biofilms, enhancing their pathogenicity and antibiotic resistance [9]. Toxins are important virulence factors that help pathogens invade and survive in host cells [10]. *Enterococcus faecalis* produces cytolysin toxin, a pore-forming toxin controlled by the cytolysin operon and quorum-sensing systems [11]; it is a unique cytolysin toxin that involves several activities controlled by a single system [12]. This toxin has been classified as a member of the two components that are modified post-translationally, which are a mature large subunit *cylL$_L$* (comprised of 38 residues) and a mature small subunit *cylL$_S$* (comprised of 21 residues) encoded by two genes on the cytolysin operon, which is located on the bacterial chromosome or the pAD1 plasmid [13]. Cytolysin has been found to lysis eukaryotic cells. In addition, it is active against several Gram-positive bacteria, such as *Staphylococci*, *Clostridia*, and *Lactobacillus*, and as a result, it has lantibiotic activity [11, 14].

The cytolysin operon consists of eight genes, two of them coded for the regulatory proteins (*cylR1* and *cylR2*), which are transcribed by a separate promoter, while the others for the functional and structural proteins, including (*cylA*, *cylB*, *cylL$_L$*, *cylL$_S$*, *cylI*, and *cylM*) [15], *cylL$_L$* and *cylL$_S$* are encoded for the large and small subunits of cytotoxic peptide toxins, respectively, both subunits are required for full toxicity [16].The *cylM is* encoded for lanthionine synthetase (CylM), which is responsible for post-translational modification. The *cylB* is encoded for (CylB), which facilitates the transport of modified toxins into the environment using an ATP-binding cassette transporter system. The *cylA* is encoded for a serine protease known as cytolysin activator (CylA), which activates the secreted modified CylL$_L$ and CylL$_S$ on the cell surface by removing six residues from the N-terminal to produce an active toxin [11]. Finally, *cylI* is encoded for immunity protein (CylI); this protein has transmembrane domains and a motif for zinc binding metalloprotease indicated by bioinformatic analysis, and it is the mode of action of protection against cytolysin toxicity has not been determined yet [12]. This study aimed to investigate the frequency of the cytolysin toxin in *E. faecalis* isolated from urinary tract infections in Iraq by targeting the large subunit gene (*cylL$_L$*) using PCR technique.

Methods

Collection of specimens and bacterial identification

Mid-stream urine samples (180) from in-patient females who were diagnosed with UTIs aged between 15 and 50 years old were collected from different hospitals in Baghdad: The Medical City Hospital in Baghdad, Al-Kadhimiya Hospital, and AL-Yarmook Hospital. Sterile screw-cap containers (4 - 5 ml) were used for urine collection from patients. Urine samples were centrifuged first, and then the precipitated was cultured on a selective medium and incubated at 37°C for 24 hours. The primary bacterial identification at the genus level was performed using cultural characteristics on a Pfizer medium (Oxoid, England) and microscopic examination [5,17]. The identification of the suspected Enterococcus isolates to the species level was confirmed using a molecular method by targeting a segment of the *ddI* gene, which encoded for D-alanine-D-alanine ligase in *E. faecalis* [18, 19]. Ethical approval from the ethics committees of the Department of Biology, College of Science, University of Baghdad, under the reference (CSEC\1024\0066) was obtained for this study.

Design and preparation of oligonucleotide primers

The nucleotide sequence of *cylL$_L$* for cytolysin toxin detection and *ddI* for bacterial identification were downloaded from the National Center for Biotechnology Information (NCBI); the accession numbers were (L37110.1) for *cylL$_L$*, and (XCL82967.1) for the *ddI*. Serial cloner and Amplifx software were used to design the oligonucleotide primers that were manufactured by Macrogen Company; the primers pair for *cylL$_L$* was CylL-F: 5`ATGGAAAATTTAAGTGTAGTTCC TA3`; CylL-R: 5`TTAACAATGTTTTAAAGACA CAACTAC3` with an amplicon size of (207 bp), for *ddI* was ddI-F: 5`CTAGTGTCGGAATTAGC AAAGCGG3`; ddI-R; 5`CGATTCAAAGCTAAC TGAATCAGTTCC3` with an amplicon size of (484 bp). The primer annealing temperatures were determined using Thermo Fisher Scientific's Tm calculator [20]. A primers stock solution of (100 µM/ µl) from Lyophilised primers were prepared according to the manufacturing company using nuclease-free water, and then a 10 µM/ µl of working solution was prepared from the stock solution.

Bacterial DNA extraction

This study used a novel procedure for whole bacterial genomic extraction by combining and modifying the PCR colony method [21] and boiling method [22]. Bacterial cells were collected from 0.5 ml of 24-hour bacterial culture by centrifuged for 15 minutes at 13000 rpm and washed twice with distilled water by resuspension and centrifugation. After that, the washed bacterial cells were suspended in 0.5 ml of Tris-EDTA buffer (TE) and subjected to boiling at 100 ºC for 10 minutes using a water bath (Memmert, Germany). To remove the denatured proteins and cell debris, the boiling lysate was centrifuged for 15 minutes at 13000 rpm, and the supernatant-containing DNA was added directly to the PCR mixture as a DNA template without the need for DNA purification, as illustrated in **figure (1).**

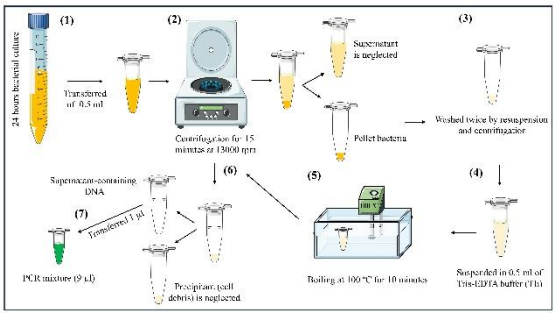
Amplification of target genes by PCR technique

The PCR technique was utilised to amplify a fragment of the target genes (*cylL$_L$* and *ddI*) in a total reaction mixture of (10 µl). First a stock PCR mixture for each gene was prepared in a total volume of 100 µl, which composed of 50 µl Go Taq G2 Green Master Mix (2X, Promega/USA), 5 µl of each forward and reverse primer (10 µM/µl), and 40 µl of nuclease-free water, then the stock PCR mixture divided into 10 PCR tubes (9 µl in each tube) and 1 µl of supernatant-containing DNA was added as a DNA template. The amplification of genes was performed in a thermocycler (Applied Biosystems, England) using the following PCR conditions: 5 min of initial denaturation at 95^{o}C followed by 35 cycles of (denaturation for 30 sec at 95^{o}C, annealing for 30 sec at 58^{o}C, and extension for 40 sec at 72 ^{o}C), then 5 min of extension at 72 ^{o}C. Five µl of PCR products were run against 4 µl of DNA marker (100 bp, Promega, USA) in electrophoresis using 2 % agarose gel supplemented with 4 µl of ethidium bromide (Promega, USA) at 100 V for 50 min. The DNA bands were visualised using a UV viewer illuminator (Fisher Scientific, England).

Statistical analysis

Pearson-Chi-square test was used to reveal significant differences in percentages. *P≤0.05* was measured as significant. The data were analyzed using SPSS v. 22.0 statistical software and Excel 2016.

Figure 1. A representative methodology of a modified method for whole DNA extraction is used in gene detection by PCR. Images from 'Smart Servier Medical Art' (https://smart.servier.com).



Results

Isolation and identification of bacterial isolates

From 180 urine specimens of infected females with UTIs, 56 isolates of *Enterococcus* species (spp.) (31.11%, 56/180) were identified compared to 68.88% (124/180) of other causative agents. The identification of *Enterococcus* spp. isolates was based on cultural characteristics on Pfizer medium and microscopic examination. Results of microscopic examination showed that all isolates were gram-positive cocci, and the growth on Pfizer medium colonies appeared as pin-point colonies with a black point in the center, as shown in **figure (2).**

The results of the molecular detection that targeted a segment of *ddI* gene showed that 50 bacterial isolates gave a distinct DNA band of 484 bp, confirming the identification of bacterial isolates as *E. faecalis*, which were subjected for *cylL$_L$* gene detection, while other bacterial isolates (isolates number 2,7,8,11,12 and 40) were neglected, which could be belong to other *Enterococcus spp*. as depicted in **figure (3).** Based on the molecular results, the isolation percentage of *E. faecalis* from urine was 27.7%, as demonstrated in **figure (4).**

Molecular detection of pore-forming toxin genes

The results of large subunit gene (*cylL$_L$*) detection revealed that 46 of bacterial isolates harbour the *cylL$_L$* gene (92 %, 46\50), with a significant difference (P<0.01) compared with the isolates lacking the *cylL$_L$* gene, which were 4 bacterial isolates (8%, 4\50), as shown in **table (1)** and depicted in **figure (5).**

Table 1. Frequency and percentage of large subunit gene (*cylL*$_L$) in *E. faecalis*.

E. faecalis	**Frequency**	**Percentage (%)**	***P*-value**
cylL$_L$ positive isolates	46	92	*P*<0.01
cylL$_L$ negative isolates	4	8	
Total	50	100%	

Figure 2. Growth of *Enterococcus Spp* on Pfizer medium after incubation for 24 hours at 37ºC.

Figure 3. Gel electrophoresis (2% agarose) of a housekeeping gene (*ddI*) amplification. Numbers correspond to the bacterial isolates; M corresponds to the DNA marker (100pb). Electrophoresis was performed for 50 minutes at 100 volts.

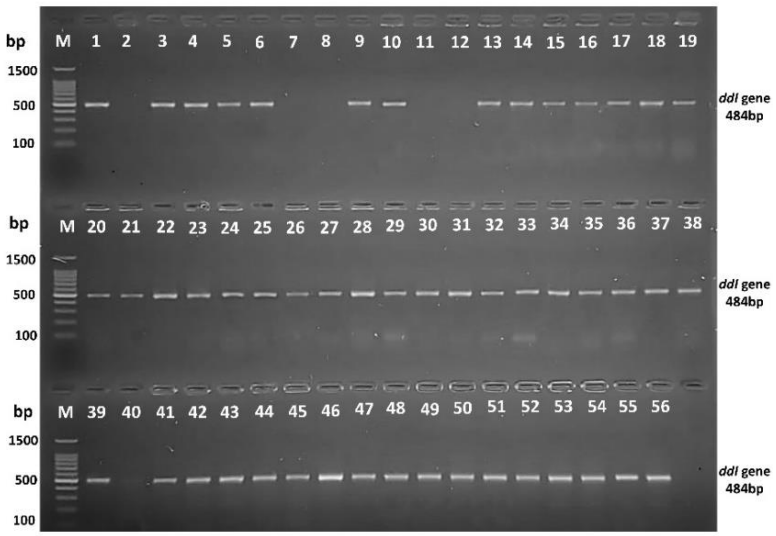


Figure 4. Percentage of *Enterococcus* isolated from urine specimens. **A.** Percentage of *Enterococcus spp*. compared with other causative agents. **B.** Percentage of *Enterococcus faecalis* compared with other causative agents.

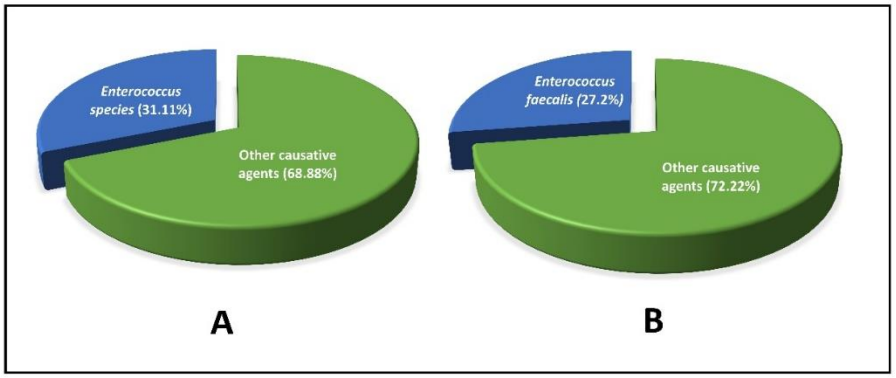
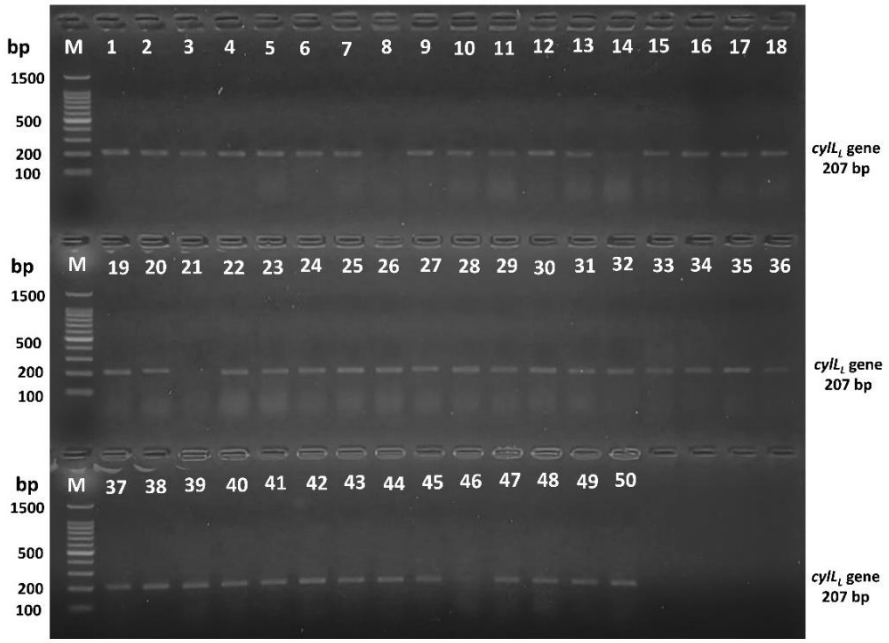


Figure 5. Gel electrophoresis (2% agarose) of a large subunit gene (*cylLL*) of cytolysin toxin amplification. Numbers 1 to 50 correspond to the bacterial isolates; M corresponds to the DNA marker (100pb). Electrophoresis was performed for 50 minutes at 100 volts.



Discussion

Cultural characteristics and biochemical tests are essential for primary bacterial isolation and identification. It helps to neglect other not targeting bacteria under investigation, such as *Pseudomonas aeruginosa*, *E. coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*, and others, which may be involved in UTIs [23], leading to saving time and material. In the current study, *Enterococcus spp* were successfully isolated and distinguished from other causative agents in UTIs using Pfizer medium, which is a selective medium for *Enterococcus spp*. [5, 24]. However, molecular identification of bacteria based on the detection of a specific gene or a fragment of the gene is a reliable bacterial identification method that allows the identification of the bacteria at the species level [25,26]. 89.28% (50 \ 56) of bacterial isolates were identified as *E. faecalis* based on *ddI* gene detection, while the other (10.7%) represented the other *Enterococcus spp. Enterococcus faecalis* is a high-frequency species in UTIs than other *Enterococcus spps.* as reported by another researcher, **Jalal et al.** found that *E. faecalis* represented 74% of *Enterococcus spp* [27]. The chromosomal *ddI* gene encoded for the D-alanine D-alanine ligase known as a gene marker for *E. faecalis* [18, 28], which is different than the *ddI* gene in other *Enterococcus spp.* in terms of sequences. Therefore, this gene was used for *Enterococcus spp.* identification at the species level [19].

Urinary tract infections are a common health problem in communities. They are known as inflammatory diseases as a result of microorganisms invading the urinary tract. Several bacterial species are responsible for UTIs. However, *E. coli* is the main causative agent for UTIs, followed by other bacteria such as *E. faecalis*, *Klebsiella pneumoniae*, *Staphylococcus saprophyticus*, *Proteus mirabilis* and *Pseudomonas aeruginosa* [23]. *Enterococcus faecalis* has been widely reported as an important causative agent of UTIs. Locally, different isolation percentages of *E. faecalis* from UTIs were reported, which were lower than the current study: 22% [29], 21.3% [30],16.1% [31], 16% [32], 12.3% [33], and 10% [34]. On the other hand, other local studies reported slightly higher percentages, which were 30% [28], and 33.92 % [24]. Different factors that affect the isolation percentage of bacteria from patients include medical conditions, hygiene standards, region, cultural traditions, awareness levels, the time and volume of specimens' collection, the use of antibiotics, and other treatments prior to the collection of specimens [35]. The ability of bacteria to cause disease depends on their ability to produce several virulence factors that facilitate their colonisation, proliferation, and spreading in the host cells [36]. Pathogenic strains

of *E. faecalis* possess different virulence factors, which could explain their high frequency in UTIs.

Toxins are the most detrimental virulence factors secreted by pathogenic microbes due to their irreversible damage to the host. Cytolysin is a peptide toxin that can lyse the target cells by forming pores in the cytoplasmic membrane [37]. However, it is crucial to distinguish between cytolysin A (ClyA, also known as HlyE or SheA) released by *Escherichia coli*, which is an alpha pore-forming toxin that has a molecular weight of 34-kDa [38], and the cytolysin produced by *E. faecalis*, even though one enzyme produced by cytolysin operon known as ClyA, which is a cytolysin activator. Despite they belong to the pore-forming toxin family but, they have distinct molecular structures and functions. Cytolysin produced by *E. faecalis* is composed of two peptides with lantibiotic activities [39]. In the current study, the prevalence of cytolysin toxin in *E. faecalis* was high, and most isolates contain the *cylL*$_L$ gene of the large subunit toxin, which indicates the presence of the cytolysin operon in high frequency. This could be explained by the ability of bacteria to acquire the cytolysin operon by genetic mobile elements or horizontal gene transfer [40]. Moreover, the transmissible pAD1 plasmid carries the cytolysin operon in *E. faecalis* [13], which facilitates the cytolysin operon transfer among *E. faecalis*.

Bacterial genomic extraction is a critical step in many molecular techniques. In some experiments, pure DNA is required for accurate results, and for further analysis and studies, many commercial bacteria DNA extraction kits are available for different purposes. However, these kits are expensive and time-consuming, including several essential steps, and missing one leads to false results. In the current study, an inexpensive method was designed for gene detection using conventional PCR. This method was a combination of two methods: the colony PCR method used in genetic engineering for gene insert detection after the transformation step [21], and a boiling method used for extraction of DNA from bacteria by boiling the bacterial culture and subsequently treated with different materials to separate and purify the DNA from other cell components [22].This method was successfully used for the detection of a housekeeping gene and virulence gene, whatever the gene is in bacterial chromosomes or the plasmid, without required DNA isolation and purification; this, in turn, saves time and materials.

Conclusion

Enterococcus faecalis represented a predominant *Enterococcus spp*. isolated from UTIs, and most of the isolates carried a cytolysin operon with the possibility to express cytolysin toxin under appropriate conditions. The high frequency of cytolysin toxin is an alarming sign of the extensive spreading of toxic bacteria in UTIs, which requires attention to eliminate these isolates to avoid the potential determinantal effect of the toxin. The technique for gene detection in the current study can be utilised for bacterial identification and gene detection in medical and research purposes with a large sample size in an inexpensive manner within a short time.

Declaration of interest

The authors report no conflicts of interest.

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Contributors

All authors contributed to the paper's the design, writing and data analysis. H.S.A. undertook molecular experiments and N.H.O. undertook microbiology work.

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