Molecular Detection of sprE, fsrA and cpd Genes among E. faecalis Isolates

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Abstract

Patients who were hospitalized and examined at Baghdad's Al-Karama Hospital and Medical City Hospital over the course of a three-month period (May to July 2022) supplied urine and vaginal specimens to the study's 200 participants as part of the research. At a temperature of 37 degrees Celsius, the samples were grown in a variety of media for 18–24 hours. After that, they were incubated at a temperature of 37 degrees Celsius for 18 to 24 hours on a variety of selective medium (Chromo Agar Medium). When we originally set out to determine who *E. faecalis*, researchers used colony morphology, microscopic investigations, and biochemical tests as their primary methods of investigation. However, only 44 (22%) of the isolates were related with E. coli, despite the fact that all 200 clinical samples cultured positive. faecalis. All previously identified isolates' DNA was taken and used in conventional PCR to amplify the *ddl* gene, the results of gel electrophoresis, which demonstrated that, each of the 44 isolates (100%) had, in fact, generated the same 941bp DNA fragment when run alongside a ladder. All *E. faecalis* isolates were subjected to *cpd* gene molecular investigations utilizing targeted PCR markers. 36(81.8%) of the 44 *E. faecalis* isolates tested positive for the presence of a *cpd* gene with a long length (782bp). For gene identification of *sprE*, a specific PCR primer was utilized. Only 12 (27.2%) of the 44 *E. faecalis* isolates tested positive for the *sprE* gene, with a long length of 591 base pairs (bp). Among 44 *E. faecalis* isolates tested for the presence of the *fsrA* gene, only 10 (22.7%) were found to be positive, when the (474 bp) band was compared to the allelic ladder, positive findings were obtained for the *fsrA* virulence gene.

Keywords: PCR, Enterococcus faecalis, sprE, fsrA and cpd, Genes.

1. Introduction

Enterococcus is a genus of Gram-positive, catalase-negative, non-spore-forming, facultatively anaerobic bacteria that may exist alone or in chains. The lactic acid bacteria (LAB) that generate bacteriocins include the enterococci. Not only do these organisms not produce endospores, but their little flagellum may also provide them with some degree of motility. They cluster together in a white, creamy color (Giannakopoulos *et al.*, 2019).While the majority of enterococci are anaerobes, there are a few species that need oxygen to survive. Pectin and cellulose are not digested by enterococci, and nitrate reduction is not a typical metabolic process for these bacteria. They are a common, possibly dangerous species that has evolved a resistance to or phenotypic tolerance for various types of disinfectants and physical agents (Deng *et al.*, 2022). The genomic size of an average *Enterococcus* organism is 3.20 Mb, and its average GC content is 37.99%. There are 605 genes in the core-genome, the vast majority of which are involved in cellular processes including glucose, protein, nucleic acid, and lipid metabolism. Comparative genome research of several *Enterococcus* strains from various origins demonstrates that environmental factors play a significant role in the development of *Enterococcus* species, with genetic similarities being more strongly shared across strains that originated in the same environment (Zhong *et al.*, 2017). The topology of the time tree also revealed that humans and animals could have been the first hosts of enterococci, and that afterwards, other species of enterococci migrated from their former hosts to plants, birds, food, and other places (Dennehy, 2017). Enterococci may be more reliably and accurately identified to the genus and species level by molecular characterisation utilizing polymerase chain reaction (PCR) and sequencing. In place of phenotypic identification, multiplex PCR has been developed as a faster method for determining which species of enterococci are present. Currently, the most reliable method for identifying enterococci is by *16S rRNA* gene sequencing (Ganda *et al.*, 2016). Better discriminative methods for separating enterococci into species may be found in their corresponding *16S rDNA*, RNA polymerase subunit beta (rpoB), and superoxide dismutase (sodA) genes (Savas *et al.*, 2019). The fsr quorum-sensing system positively regulates serinase and gelatinase via a gene called sprE that is situated next to the fsr genes and shares a promoter with them (Deepika & Bramhachari, 2018). The fsr locus, which is very comparable to the agr regulatory locus in *S. aureus*, positively regulates the transcription of the sprE- gelE operon at different stages of growth. Located upstream of the sprE operon is the fsr locus, which contains the three regulatory genes *fsrA*, *fsrB*, and *fsrC*. Sequence similarity between *agrA* and *agrC* suggests that *fsrA* and *fsrC* make up a conventional two-component system, with fsrC serving as a histidine kinase sensor and fsrA as a response regulator. A glutamyl

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endopeptidase I of 25 kDa, encoded by the *sprE* gene, has been identified as a virulence factor of *Enterococcus faecalis*. This protease has a role in pathogenesis in a wide range of organismal models (including humans, animals, and plants) (Graham, 2017). Multiple plasmids in *E. faecalis* are sensitive to sex pheromones and encode bacteriocins, aggregation chemicals, and a wide variety of antibiotic resistance determinants (Rodrigues *et al.*, 2019). Peptide pheromones (auto inducers) are carried through the ATP-binding cassette transport mechanism. These auto inducers accumulate in the extracellular environment, where their receivers may detect them and use this information to control mating and conjugation processes (Jaafar *et al.*, 2022). There was a significant amount of intricacy and a double role for pheromone response in plasmid conjugation and in the control of enterococcal virulence that was uncovered by studying its regulation and function. The stabilization/partition mechanisms, along with other functional modules found in pheromone plasmids, are essential for the plasmid molecule to be maintained reliably inside the host bacterium (Ferchichi *et al.*, 2021). The enterococcal phenotype is a major vector of antibiotic resistance in this species, and pheromone-responsive plasmids play a role in this. It was discovered that these plasmids included not just vanA and vanB, but also several additional resistant phenotypes. Both aggregation substance (AS) and cytolysin, two fundamental agents of enterococcal pathogenicity, are encoded by these genes. AS not only helps enterococci cling to human tissues during infection, but it also plays a role in mating-pair formation during conjugation. Cytolysin, the second protein, may cleave red blood cells and aid in the invasion of eukaryotic cells (Erickson *et al.*, 2020). The *cpd* gene encodes a peptide pheromone that is produced by recipient enterococcal cells to activate the conjugative apparatus of donor enterococcal cells. The pheromone-responsive plasmids they mediate may include virulence genes that aid in biofilm formation or regulate biofilm development (Ferchichi *et al.*, 2021). Because the particular mechanism or processes by which a plasmid limits synthesis of its pheromone remain unclear, the event has been dubbed "pheromone shutdown." However, the elaboration of pheromones specific for other plasmids continues unabated. Another peptide, dubbed inhibitor, is secreted by plasmid-containing cells; it acts as a competitive inhibitor of the related pheromone (Lassinantti *et al.*, 2021).

Aim of study

Molecular detection of *sprE*, *fsrA* and *cpd* genes among *E. faecalis* isolates.

2. Materials and methods

In this research, 200 patients were enrolled, and urine and vaginal specimens were collected from patients who were hospitalized to and visited Al-Karama Hospital and Medical City Hospital in the Iraqi capital from May until the end of July 2022.

Ethical Approval

The appropriate ethical permission must be acquired from the ethical committees of hospitals, patients, and their followers. In addition, all participants in this study are verbally informed, and the requisite consent for conducting experiments and publishing this study is acquired prior to sample collection.

Clinical specimens

Urine samples and vaginal swabs for bacteriological examination are detailed in the next section. These samples were gathered in an appropriate manner to prevent any potential contamination. then the urine samples and vaginal swabs were inoculated on selective media (Chromo agar) and incubated aerobically at 37°C for 24h (Ali, 2017).

DNA Extraction

This procedure was developed in accordance with the genomic DNA purification Kit provided by the manufacturer Geneaid (Korea).

Detection of d-Ala:d-Ala ligases (ddl) E. faecalis gene

DNA (extract from bacterial cells) was used as a template in specific PCR for the detection of *d-Ala:d-Ala ligases E. faecalis* gene. DNA was purified from bacterial cells by using the Geneaid DNA extraction Kit. The primers used for the amplification of a fragment gene were listed in Table (1).

Table (1): Primers sequences and PCR conditions of *E. faecalis*

Genes	Primer sequence (5'.3')	Size of product bp	PCR condition	Reference
ddl of *E. faecalis*	F:ATCAAGTACAGTTAGTCTTTATTAG R: ACGATTCAAAGCTAACTGAATCAGT	941	95°C, 5 min. 95°C, 1 min. 56°C, 1 min. 72°C, 1 min. 72°C, 10 min.	Fines *et al.*, (1999)

Detection of some of Enterococcus faecalis virulence genes

Some of the virulence genes were detected by using DNA (extracted from bacterial cells) as a template in targeted polymerase chain reactions (PCRs). The Geneaid DNA extraction Kit was used to isolate DNA from bacterial cells. Table 1 shows the primers that were used to amplify a gene fragment (2).

Table (2): the primers, sequences, and PCR conditions				
Gene name	Primer sequence (5´- 3´)	Size of Bp	Conditions	References
Cpd	F: TGGTGGGTTATTTTTCAATTC R: TACGGCTCTGGCTTACTA	782	95°c, 5 min. 95°c, 1 min. 56°c, 1 min. 72°c, 1 min. 72°c, 10 min.	Poeta *et al.*, (2005)
Spre	F: TTGAGCTCCGTTCCTGCCGAAAGTCATTC R:TTGGTACCGATTGGGGAACCAGATTGACC	591	95°c, 5 min. 95°c, 1 min. 58°c, 1 min. 72°c, 1 min. 72°c, 10 min.	Poeta *et al.*, (2005)
Fsra	F:CGTTCCGTCTCTCCATAGTTA R:GCAGGATTTGAGGTTGCTAA	474	95°c, 5 min. 95°c, 30 sec. 58°c, 30 sec. 72°c, 30.0 sec. 72°c, 10 min.	Aghdam *et al.*, (2017)

3. Results and discussion

Patients who were hospitalized and examined at Baghdad's Al-Karama Hospital and Medical City Hospital over the course of a three-month period (May to July 2022) supplied urine and vaginal specimens to the study's 200 participants as part of the research. At a temperature of 37 degrees Celsius, the samples were grown in a variety of media for 18–24 hours. After that, they were incubated at a temperature of 37 degrees Celsius for 18 to 24 hours on a variety of selective medium (Chromo Agar Medium). When we originally set out to determine who E. faecalis, researchers used colony morphology, microscopic investigations, and biochemical tests as their primary methods of investigation. However, only 44 (22%) of the isolates were related with E. coli, despite the fact that all 200 clinical samples cultured positive. faecalis.

All previously identified isolates' DNA was taken and used in conventional PCR to amplify the *ddl* gene using the sequences and program in Table (1). Figure (1) displays the results of gel electrophoresis, which demonstrated that, each of the 44 isolates (100%) had, in fact, generated the same 941bp DNA fragment when run alongside a ladder.

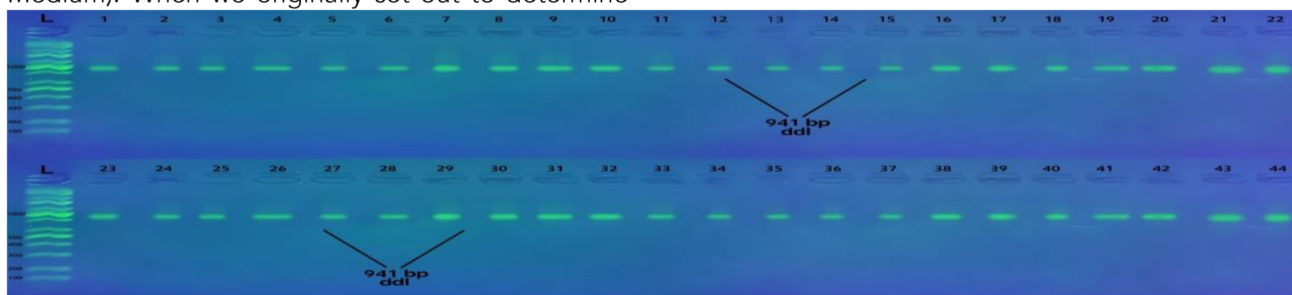

Figure (1): 1% Agarose gel electrophoresis image at 75 V, (20 mA) for 1 hour that showed the PCR product analysis of ddl gene in Enterococcus faecalis isolated from clinical samples. Where L: marker (100-1500bp) and Lane (1-44) showed some positive Enterococcus faecalis isolates were showed (1-32 urine, 33-44 vagina) at (941bp) PCR product.

Results from this investigation corroborated those by Lim, (2018), who utilized species-specific primers to confirm the presence of *E. faecalis* in 28 urine samples. Results from this study conflict with those from studies by Supotngarmkul *et al.*, (2020) who found that, of 100 clinical isolates, only 34 were found to be *E. faecalis* when tested with a specific primer, and by Zaheer *et al.*, (2020), who found that, of clinical isolates related to *E. faecalis*, 90% were detected. The results of this research show that, conventional PCR using a selected specific target is faster, easier, and more accurate than earlier approaches for identifying *E. faecalis*. The use of PCR showed that the comparative genomics technique correctly identified the target. When identifying species that are difficult to discriminate using phenotypic methods, PCR using species-specific primers is a helpful tool that may substitute sophisticated molecular clustering techniques and standard microbiological testing (Rubin et *al.*, 2022). The identification of *E. faecalis* by molecular means involves the use of PCR primers based on particular gene sequences. Ghalavand *et al.*, (2020) observed amplification of a particular *ddl* sequence of the *E. faecalis* genome in urine sample from individuals with UTIs. All *E. faecalis* strains could be reliably identified by a single, highly unique marker: the *ddl* gene (Almahdawy *et al.*, 2019). Because *ddl* genes are the most widely shared housekeeping genetic markers, researchers have utilized them to examine the identification, taxonomy, and phylogeny of bacteria. Their ubiquitous existence in bacteria, where they often occur as multigene families or operons, and the fact that their function has not

altered through time imply that random sequence changes are a more accurate indicator of temporal evolution. Because of their size, these genes are suitable for use in informatics (Kim *et al.*, 2020). Occasionally, it might be difficult or impossible to phenotypically identify a certain *Enterococcus* species because that species lacks defining traits. Ideally, labs that study microorganisms would have access to more efficient and reliable techniques (Rajapaksha *et al.*, 2019). In contrast to the culture technique, PCR is the most reliable method for identifying *E. faecalis* infections because of its superior accuracy, sensitivity, specificity, uniqueness, and cost-effectiveness. Results from a molecular approach may be obtained in 24 hours, but those from a convective method, such as normal culture followed by a biochemical test, take between 24 and 48 hours. PCR has enabled the rapid, culture-independent identification of enterococci in a wide range of clinical specimens. Results may be obtained in as little as a few hours. However, all existing PCR-based tests are exclusively species-specific for enterococci, which are of clinical importance (Vasala *et al.*, 2020).

All *E. faecalis* isolates were subjected to *cpd* gene molecular investigations utilizing targeted PCR markers. As can be seen in Figure (2), 36(81.8%) of the 44 *E. faecalis* isolates tested positive for the presence of a *cpd* gene with a long length (782bp).

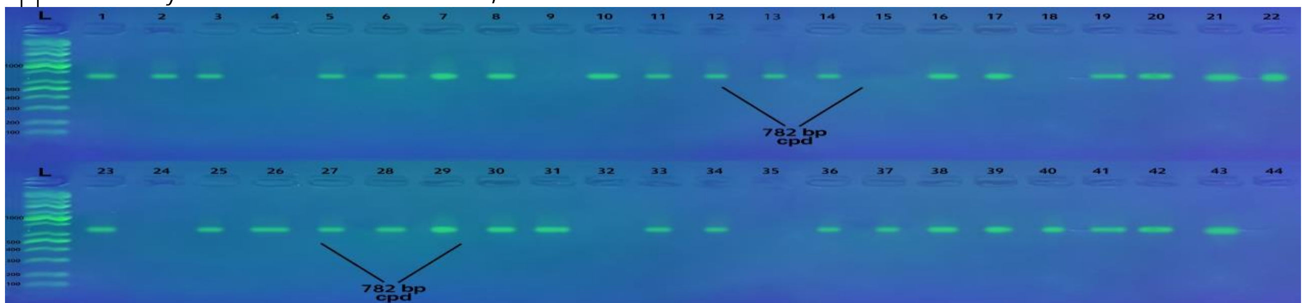

Figure (2): Agarose gel electrophoresis (1.5%) of PCR amplified of cpd gene at (782bp) of E. faecalis for (55) min at 5 volt/cm2. 1x TBE buffer for 1:30 hours. L: DNA ladder (100).

Twenty four (96%) of 25 *E. faecalis* isolates from urine samples were confirmed to be linked to the *cpd* gene, as reported by Ferchichi *et al.*, (2021). Gene *cpd* encoding for sex pheromone peptides was more common among *E. faecalis*, according to a research by Stpie-Pyniak *et al.*, (2021). This means that, strains of *Enterococcus* that had and produced virulence components were more likely to result in a severe illness. The *cpd* gene has been shown to be more prevalent in clinical *E. faecalis* isolates, according to another investigation (Jahansepas *et al.*, 2020). Aggregation substance, a sex pheromone plasmid-encoded surface protein, was shown to have the highest frequency of detection among *E. faecalis* strains by Venkateswaran *et al.*, (2022). All *E. faecalis* strains have the *cpd* genes. Further, *E. faecalis* production of sex pheromones may facilitate the acquisition of antibiotic resistance and virulence from other enterococci, leading to enhanced virulence. One of the most effective conjugative plasmid transfer mechanisms in bacteria is found in *E. faecalis* sex pheromone plasmids, as described by Hirt *et al.*, (2018). Peptide pheromones (such as those encoded by *cpd*) are released by recipient enterococcal cells and act as a signal to activate the conjugative apparatus of donor enterococcal cells. Thus, they serve as a mediator for the transfer of pheromone-responsive plasmids, which may have virulence genes that increase biofilm development or regulation. Key virulence factors in Enterococcus spp. are induced in response to higher cell density (Ferchichi *et al.*, 2021). Multiple short peptide sex pheromones are secreted by plasmid-free *Enterococcus faecalis* strains, and these pheromones trigger a mating response in plasmid-containing bacteria. When it comes to plasmids, each pheromone has its own target (Mouton, 2019). Plasmid-containing cells react to the particular pheromone by producing a proteinaceous "aggregation material," localized to the cell surface, which aids in the formation of mating aggregates; other processes necessary for transfer of plasmid DNA are also activated. When a plasmid is acquired, the pheromone activity that was formerly observable no longer is (Bandyopadhyay, 2018).

For gene identification of *sprE*, a specific PCR primer was utilized. Only 12 (27.2%) of the 44 *E. faecalis* isolates tested positive for the *sprE* gene, with a long length of 591 base pairs (bp), as shown in Figure (3).

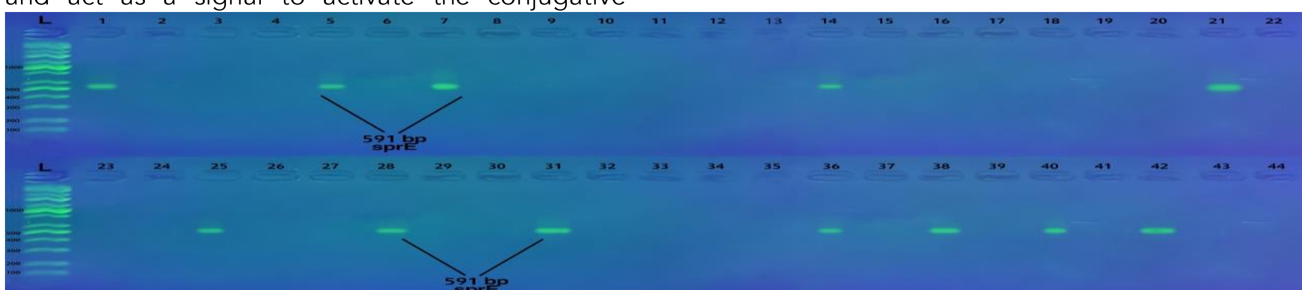

Figure (3): Agarose gel electrophoresis (1.5%) of PCR amplified of sprE gene at (591bp) of E. faecalis for (55) min at 5 volt/cm2. 1x TBE buffer for 1:30 hours. L: DNA ladder (100).

In contrast to the findings obtained by Hasan *et al.*, (2018), who reported that, downstream *sprE* gene were identified in all *E. faecalis* isolates (100%) that were discovered by PCR, the results of this investigation showed no such consistency. Based on these findings, *sprE* may have synergistic effects on virulence. Yet another interpretation of the data is that the *sprE* system controls virulence genes. The quorum-sensing fsr locus controls the transcription of the gelatinase (*gelE*) and serine protease (*sprE*) genes. It has the ability to cleave sex pheromones, which are strong chemo-attractants and may alter the host's reaction (Ali *et al.*, 2022). *GelE* and *sprE* genes were present in *E. faecalis* strains having gelatinase activity, correlating with previous findings by Hashem *et al* (2021). Kao and Kline, (2019) showed that the proteases separately contribute to *E. faecalis* pathogenicity in distinct infections. As shown by Venkateswaran *et al.*, (2022), sprE promotes and represses autolysis in *E. faecalis*. The fsr quorum-sensing system positively regulates the expression of sprE, a serine protease. The *gelE* and *sprE* genes are close to the *fsr* genes and are controlled by the same promoter. Together, the fsr quorum-sensing system and these proteases play a role in pathogenicity, host-tissue destruction, and biofilm development (Willett *et al.*, 2022).

Among 44 *E. faecalis* isolates tested for the presence of the *fsrA* gene, only 10 (22.7%) were found to be positive. As can be seen in Figure (4), when the (474 bp) band was compared to the allelic ladder, positive findings were obtained for the *fsrA* virulence gene.

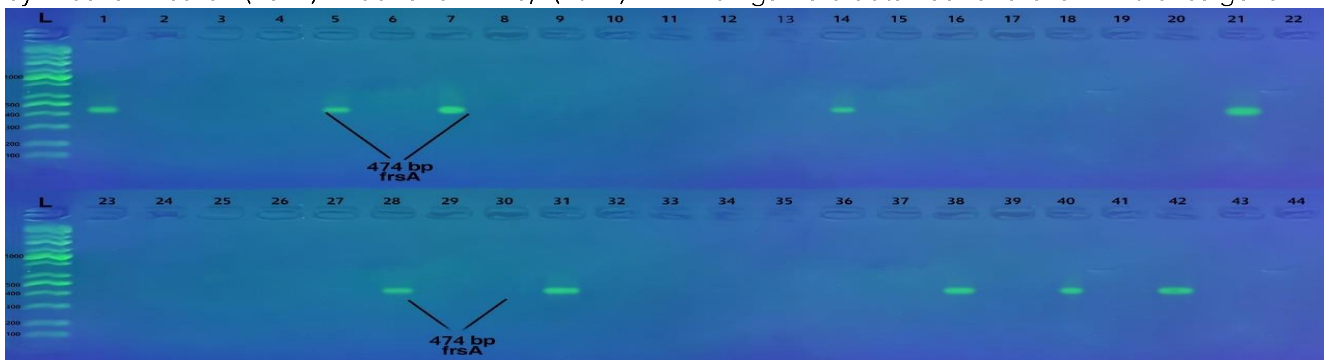

Figure (4): Agarose gel electrophoresis (1.5%) of PCR amplified of fsrA gene at (591bp) of E. faecalis for (55) min at 5 volt/cm2. 1x TBE buffer for 1:30 hours. M: DNA ladder (100).

Current findings are in accordance with the fsrA gene was linked to an *E. faecalis* infection in a study by Najafi *et al.* (2020). The fsr locus of *E. faecalis* was discovered to encode a two-component regulatory mechanism that detects the cell density and modulates virulence by Patil *et al.*, (2021). There are four genes that make up the 2.8 kb fsr locus: fsrA, fsrB, fsrD, and fsrC. *Enterococcus faecalis*, a bacterium that causes nosocomial infections, relies on quorum sensing mechanisms to regulate key virulence characteristics. The cytolysin operon, which encodes the cytolysin toxin, is a component of the *E. faecalis* quorum-sensing system (Ali *et al.*, 2022). In addition, the expression of gelatinase, serine protease, and enterocin O16 is regulated by the *E. faecalis* Fsr regulator system (Willett *et al.*, 2022). Human and animal models of enterococcal illness have been connected to the cytolysin and Fsr virulence factor systems (Bin-Asif & Ali, 2019). Thus, there is a strong need to learn about and manipulate these regulatory mechanisms in order to create new treatments for the prevention and treatment of enterococcal infections (Venkateswaran *et al.*, 2022). Therapeutic drugs that decrease quorum sensing may be useful in reducing the harmful effects of *E. faecalis*. Therapeutic strategies for preventing *E. faecalis* infection are discussed, along with the control of cytolysin, the LuxS system, and the Fsr system, and their roles in *E. faecalis*-mediated infections (Desouky *et al.*, 2022). As Krishnamoorthy *et al.*, (2020) report, the Fsr system is required for the expression of four genes: *gelE*, *sprE*, *ef1097*, and *ef1097b*. The FsrA protein is encoded by the *fsrA* gene and contains a DNA-binding domain of the LytTR family. *FsrA* is likely a response regulator of the Fsr system since phosphorylated FsrA binds to LytTR-binding sites in the upstream region of ef1097, fsrB, and gelE. In particular, fsrA transcription is not reliant on the Fsr quorum-sensing system since it is controlled by a constitutive promoter. FsrA, a member of the AgrA family of accessory gene regulator proteins, is encoded by the *fsrA* gene (Deepika & Bramhachari, 2018). Wu and Luo (2021) revealed that the *E. faecalis* fsr system is the second example of a quorum-sensing system that modulates virulence gene expression during bacterial infection of both simple model organisms and mammalian hosts. Many pathogenic prokaryotes may employ quorum sensing as a key method for adjusting to novel settings. In addition to gelE and sprE, our findings suggest that, the fsr system in *E. faecalis* controls other virulence genes (McBrayer *et al.*, 2020).

4. Conclusion

Rare infectious diseases (RIDs) are often seen in the medical field. Not only did researchers identify some interesting things regarding *E. faecalis* infections, but they also provided evidence that various virulence factor genes had been created in different isolates.

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