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Original Article

Comparative Analysis of Two Consecutive Genome Sequencing Results of *Enterococcus Faecium* Strain Entfacye

Maryam Yazdanizad[1], Ali Akbar Saboor Yaraghi[2], Golshid Javdani Shahedin[3], Mahla Asadian[4], Ramin Mazaheri Nezhad Fard[2, 4*]

1. Department of Medical Biotechnology, TMS.C., Islamic Azad University, Tehran, Iran.
2. Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran.
3. Department of Research and Development, Pasteur Institute, Tehran, Iran.
4. Food Microbiology Research Center, Tehran University of Medical Sciences, Tehran, Iran.

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ABSTRACT

Introduction: The overuse of antimicrobials in healthcare has driven the emergence, persistence, and rapid spread of antimicrobial-resistant pathogens. Vancomycin-resistant enterococci (VRE), mainly *Enterococcus faecium*, have recently emerged as multi drug-resistant (MDR) bacteria worldwide. Consequently, enterococcal infections have become more challenging to treat due to their increased multiple-drug resistance. Studying the genome of an enterococcal isolate and investigating genome changes over time help researchers better understand the development of antimicrobial resistance in bacterial isolates.

Materials & Methods: In the present study, *E. faecium* EntfacYE isolate from a human biological sample was assessed. After phenotypic, biochemical,and molecular verifications of the bacterial isolate, the bacterial genome was completely sequenced.

Results: In total, the EntfacYE genomic subsystems contained 23 various categories with 46 antimicrobial resistance genes. In the current study, 31 antimicrobial resistance genes were reported in the subsystems, and 15 genes had no subsystems. Genes conferring tetracycline resistance were reported in this study. Increases in the number and type of antimicrobial resistance genes were recorded, indicating that bacteria are becoming rapidly resistant to available antimicrobials.

Conclusion: In general, the study of antimicrobial resistance genes in bacteria can be effective in better understanding resistance patterns and mechanisms, which can lead to find novel protocols for limiting spread of antimicrobial resistance in bacteria.

*** Corresponding Author:**
Ramin Mazaheri Nezhad Fard, Associate Professor.
Address: *Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran.*
Tel: *+98 (21) 42933208*
E-mail: *r-mazaherinf@sina.tums.ac.ir*

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1. Introduction

Enterococci are a part of the natural intestinal flora of mammals, birds and humans. Of the various *Enterococcus* species, *Enterococcus faecalis* and *Enterococcus faecium* are the most common human species, while *E. gallinarum* and *E. casseliflavus* are less common. *Enterococcus* spp. can opportunistically cause fatal endocarditis and urinary tract (UT) infections in humans [1]. Excessive use of antimicrobials in human healthcare has resulted in the emergence- and rapid spread of antimicrobial resistance, whereby microorganisms demonstrate resistance to a range of common antimicrobials (multiple-drug resistance) [2]. Antimicrobial-resistant bacteria are major causes of healthcare-associated infections (HAIs) worldwide. Infections caused by multi drug-resistant (MDR) microorganisms significantly increase morbidity, mortality and treatment costs. Recently, the World Health Organization (WHO) has enlisted antimicrobial-resistant priority pathogens that pose major threats to human health, including *E. faecium* [3, 4]. Enterococci are potential bacteria in expression of resistance genes [1]. While vancomycin-resistant enterococci (VRE) pose threats to public health, MDR enterococci (MDR) act as repositories for the horizontal gene transfer (HGT) of antimicrobial resistance determinants to other pathogenic microorganisms since transmission of vanA from *Enterococcus* spp. to *Staphylococcus aureus* has frequently been reported [5]. In this study, *E. faecium* EntfacYE was re-cultured and exposed to bacteriophages. This bacterial strain was previously isolated from a human biological sample and was wholly sequenced. The genome of the isolate was re-sequenced, and changes in its antimicrobial resistance determinants, virulence factors, mobile genetic elements (MGEs), and multi-source sequencing patterns within a three-month time period were investigated.

2. Materials and Methods

2.1. Isolation of *E. faecium* EntfacYE and phenotypic identification

The *E. faecium* EntfacYE isolate was previously obtained from a patient's blood at Imam Khomeini Hospital, Tehran, Iran, using conventional microbiological methods [6]. The bacterial isolate was identified using routine methods such as Gram staining, as well as oxidase, catalase, NaCl tolerance, PYR hydrolysis and bile esculin tests. Disc diffusion (Kirby) method was used to assess antimicrobial resistance of the bacterial isolate against erythromycin, clindamycin, linezolid, ceftriaxone, cefoxitin and vancomycin.

2.2. Molecular verification of the bacterial isolate

Sanger sequencing was used for molecular verification of bacterial isolate. First,the bacterial genome was extracted using heating method. Polymerase chain reaction (PCR) was then carried out on the genome using specific primers designed for the elongation factor Tu (EF-Tu) encoding gene. Primer sequences included Ent1: 5'-TACTGACAAACCATTCATGATG-3' and Ent2: 5'-AACTTCGTCACCAACGCGAAC-3' [7]. Additionally, PCR products were detected in 1% agarose gels with TBE buffer (0.5%) and further investigated under UV. The PCR products were subsequently used for partial sequencing using Sanger method.

2.3. Complete genome sequencing

First, genome of *E. faecium* EntfacYE was manually extracted using ethanol and propanol methods and then wholly sequenced using the Illumina Hiseq platform (Novogen, China). Sequencing results were assembled using de novo technology and the SPAdes algorithm. In addition,the reference assembly method was applied for the analysis of raw data. The bacterial genome was generally analyzed using Rapid Annotation using Subsystem Technology (RAST) [8], and results were annotated in DNA Data Bank of Japan (DDBJ)[9].

3. Results

3.1. Phenotypic and molecular verification results

After phenotypic and biochemical assessments on the bacterial isolate, the isolate was verified as *E. faecium*. Sanger sequencing of *tuf* gene confirmed the initial characteristics of the isolated bacteria (DDBJ accession Nos. LC580430 and LC580431).

3.2. Complete genome sequencing results

In this study, the complete bacterial genome was analyzed and its structure was studied. The *E. faecium* EntfacYE genome included 3,056,624 bp; of which 37.5% were GC content. Furthermore, genome included 160 contigs. In total, the number of subsystems in the bacterial genome was 231 (Table 1)

Results were annotated in DDBJ (accession Nos. BPUK01000001–BPUK01000160). In total, the *E. faecium* EntfacYE genome subsystems contained 23 categories. Among these, carbohydrates, protein me-

Table 1. General genomic information of the *E. faecium* EntfacYE

Genome	*E. Faecium* EntfacYE
DDBJ accession nos.	BPUK01000001–BPUK01000160
Isolation source	Patient blood sample
Size (bp)	3,056,624
GC content (%)	37.5
Contigs	160
Subsystems	231
Coding sequences	3155
RNAs	60

tabolism, amino acids, and derivatives represented the highest frequencies, whereas metabolism of aromatic compounds, sulfur metabolism and cell division and cell cycle showed the lowest frequencies (Figure 1).

3.3. Antimicrobial resistance assessment

Results of the antimicrobial resistance assessments revealed that the bacterial isolate was resistant to common antimicrobials, includingvancomycin, clindamycin, erythromycin, ceftriaxone, and cefoxitin. The bacterial resistance genes were classified into two main groups: Those with and those without subsystems. The subsystem group included 24% and the non-subsystem group included 74% of the bacterial genome. In total, 31 antimicrobial resistance genes were located within specific subsystems, and 15 genes were located in no specific subsystems (Tables 2 and 3). In addition, resistance genes of cadmium, cobalt, copper, zinc and mercury were identified in this study.

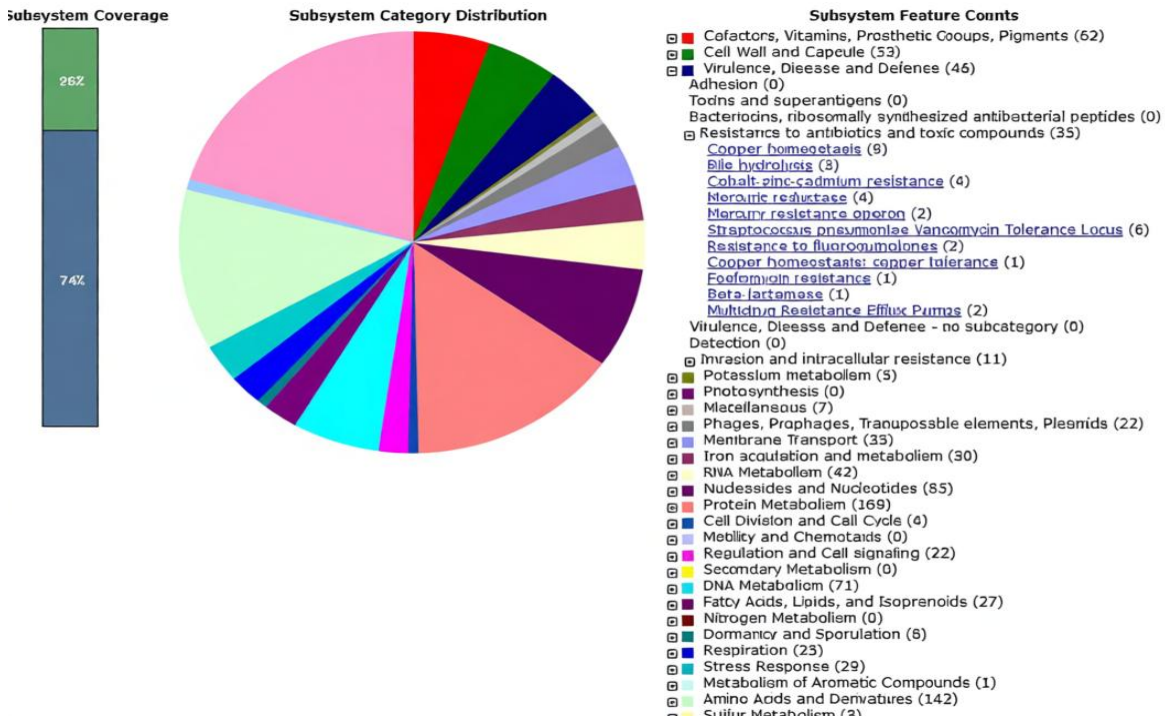

Figure 1. Genome subsystem of the *E. faecium* EntfacYE analyzed through rapid annotation using subsystem technology

Table 2. Antimicrobial-resistance subsystems from genomic analysis of *E. faecium* EntfacYE

No.	Subsystem	Feature
1	Copper homeostasis	Negative transcriptional regulator-copper transport operon
1	Copper homeostasis	Copper-translocating P-type ATPase (EC 3.6.3.4)
1	Copper homeostasis	Copper chaperone
1	Bile hydrolysis	Choloylglycine hydrolase (EC 3.5.1.24)
1	Cobalt-zinc-cadmium resistance	Cobalt-zinc-cadmium resistance protein
1	Cobalt-zinc-cadmium resistance	Probable cadmium-transporting ATPase (EC 3.6.3.3)
1	Cobalt-zinc-cadmium resistance	Transcriptional regulator, MerR family
1	Mercuric reductase	PF00070 family, FAD-dependent NAD(P)-disulfide oxidoreductase
1	Mercuric reductase	Mercuric ion reductase (EC 1.16.1.1)
1	Mercury resistance operon	Mercuric ion reductase (EC 1.16.1.1)
1	*S. pneumoniae* vancomycin tolerance locus	Sensor histidine kinase VncS
1	*S. pneumoniae* vancomycin tolerance locus	ABC transporter, ATP-binding protein Vex2
1	*S. pneumoniae* vancomycin tolerance locus	Two-component response regulator VncR
1	*S. pneumoniae* vancomycin tolerance locus	ABC transporter membrane-spanning permease, Pep export, Vex1
1	*S. pneumoniae* vancomycin tolerance locus	ABC transporter membrane-spanning permease, Pep export, Vex3
1	Resistance to fluoroquinolones	DNA gyrase subunit B (EC 5.99.1.3)
1	Resistance to fluoroquinolones	DNA gyrase subunit A (EC 5.99.1.3)
1	Copper homeostasis: Copper tolerance	Cytoplasmic copper homeostasis protein CutC
1	Fosfomycin resistance	Fosfomycin resistance protein FosX
1	Beta-lactamase	Metal-dependent hydrolases of the beta-lactamase superfamily I
1	MDR efflux pumps	MDR efflux pump PmrA
1	MDR efflux pumps	Multi antimicrobial extrusion protein (Na(+)/drug antiporter), MATE family of MDR efflux pumps
1	*Mycobacterium* virulence operon involved in protein synthesis (SSU ribosomal proteins)	SSU ribosomal protein S7p (S5e)
1	*Mycobacterium* virulence operon involved in protein synthesis (SSU ribosomal proteins)	Translation elongation factor G
1	*Mycobacterium* virulence operon involved in protein synthesis (SSU ribosomal proteins)	Translation elongation factor Tu
1	*Mycobacterium* virulence operon involved in protein synthesis (SSU ribosomal proteins)	SSU ribosomal protein S12p (S23e)
2	*Mycobacterium* virulence operon involved in DNA transcription	DNA-directed RNA polymerase beta subunit (EC 2.7.7.6)
1	*Mycobacterium* virulence operon involved in protein synthesis (LSU ribosomal proteins)	LSU ribosomal protein L35p
1	*Mycobacterium* virulence operon involved in protein synthesis (LSU ribosomal proteins)	Translation initiation factor 3
1	*Mycobacterium* virulence operon involved in protein synthesis (LSU ribosomal proteins)	LSU ribosomal protein L20p

Table 3. Non-subsystem antimicrobial resistance genes from genomic analysis of *E. faecium* EntfacYE

Type	Length (bp)	Subsystem	Function
CDS	399	Uncharacterized	Tetracycline resistance, MFS efflux pump => TetA(P)
CDS	1170	Uncharacterized	Tetracycline resistance, ribosomal protection type => TetB(P)
CDS	402	Uncharacterized	Mercuric resistance operon regulatory protein MerR
CDS	531	Uncharacterized	Uncharacterized protein YacP, similar to C-terminal domain of ribosome protection-type Tc-resistance proteins
CDS	321	Uncharacterized	Small MDR family (SMR) protein
CDS	1713	Uncharacterized	Heterodimeric efflux ABC transporter, MDR => LmrC subunit of LmrCD
CDS	1773	Uncharacterized	Heterodimeric efflux ABC transporter, MDR => LmrD subunit of LmrCD
CDS	1920	Uncharacterized	Tetracycline resistance, ribosomal protection type => Tet(M)
CDS	900	Uncharacterized	Cobalt/zinc/cadmium resistance protein CzcD
CDS	372	Uncharacterized	Glyoxalase/bleomycin resistance protein/dioxygenase superfamily protein
CDS	486	Uncharacterized	Teicoplanin resistance protein VanZ
CDS	1170	Uncharacterized	Tetracycline resistance, ribosomal protection type => TetB(P)
CDS	939	Uncharacterized	Tetracycline resistance, MFS efflux pump => TetA(P)
CDS	969	Uncharacterized	D-lactate dehydrogenase VanH, associated with vancomycin resistance (EC 1.1.1.28)
CDS	609	Uncharacterized	D-alanyl-D-alanine dipeptidase (EC 3.4.13.22) of vancomycin resistance => VanX

3.4. Comparative analysis of the two whole-genome sequencing sets

In a study by Elahi et al. (2021), whole-genome sequencing of *E. faecium* EntfacYE was carried out. They reported a genome size of 3,624,552 bp, which was 567,928 bp longer than that obtained in the present study [6]. Naturally, differences in the chromosome size and presence or absence of plasmids, as well as other MGEs, could cause changes in genome size of a bacterial species. In the current study, GC content decreased by 1.5%, compared to that reported previously [6]. Elahi et al. (2021) [6] described one contig, compared to 160 contigs of the current study [6]. Technically, repetitive and inserted genetic elements complicate assembly, resulting in changes in the number of contigs. The number of the subsystems reported in *E. faecium* EntfacYE genome by Elahi et al. (2021) was 242, which was 11 subsystems greater than that reported the isolate genome by the present study [6].

In both sequencing sets, the genomes totally included 23 various categories. Among these, carbohydrates, amino acids and protein metabolism categories contained the most-frequent subsystems. In the two studies, sulfur metabolism, cell division, and cell cycle were the least frequent categories. A notable difference was that metabolism of aromatic compounds was reported in the current study, while phosphorus metabolism was reported in the previous study [6]. Elahi et al. (2021) reported similar results in bacterial resistance to vancomycin, erythromycin, clindamycin, cefoxitin and ceftriaxone [6]. They also reported that subsystem group included 23% of the bacterial genome [6], which was 3% smaller than that of the present study. Conversely, non-subsystem group contained 77% of the bacterial genome, reflecting a 3% increase compared to the current findings. In the current study, 31 antimicrobial resistance genes were located within specific subsystems, while 15 genes without specific subsystems were reported. These categories were reported as 49 and 10, respectively, in the previous study [6]. In addition, resistance genes of cobalt, cadmium, zinc, copper and mercury were reported in both sequenced E. faecium EntfacYE genomes (Table 4) [6].

4. Discussion

Antimicrobial resistance is a serious concern in both human and veterinary medicines, limiting treatment options and complicating infection controls. Selective

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Table 4. Comparison of the whole genome sequencing results

Ref.	Various Categories	The Most-frequent Subsystems	The Least-frequent Subsystems	Resistance Drug	Antibiotic Resistance Genes with Specific Subsystems	Genes without Specific Subsystems	Resistance Genes
Elahi et al. [6]	23	Carbohydrates, amino acids and protein metabolism	Sulfur metabolism, cell division and cell cycle and phosphorus metabolism	Erythromycin vancomycin, clindamycin, ceftriaxone and cefoxitin	49	10	Cobalt, cadmium, zinc, copper and mercury
Current study	23	Carbohydrates, amino acids and protein metabolism	Metabolism of aromatic compounds, sulfur metabolism and cell division and cell cycle	Erythromycin, vancomycin, tetracycline, clindamycin, ceftriaxone and cefoxitin	31	15	Cobalt, cadmium, zinc, copper and mercury

pressures from antimicrobial uses have resulted in rapid prevalence of resistant bacterial strains. Investigating the genetic basis of antimicrobial resistance is crucial for a better understanding its transmission and persistence. Extensive and continuous application of antimicrobials in medicine, and specifically in animal breeding, has been a critical factor in the evolutionary development of the antimicrobial resistance in bacteria [10]. Enterococci are inherently resistant to several antimicrobial classes. Over the past few decades, significant increases have been reported in the level of acquired antimicrobial resistance capability of enterococci, especially *E. faecium*. Recently, WHO has published a list of the preferred bacterial pathogens requiring novel antimicrobials and treatment protocols, with Vancomycin-resistant *E. faecium* included in this high-priority category [11, 12]. In this study, 26% of the genes were associated with various subsystems (Figure 1), representing a 3% increasecompared to the previous study [6]. Antimicrobial susceptibility assessments showed that the bacterial isolate was resistant to vancomycin, tetracycline, erythromycin, ceftriaxone, cefoxitin, and clindamycin.

Similar findings were reported by Sun et al. (2020), who observed resistance to various antimicrobials, including vancomycin, clindamycin and erythromycin [13]. Genomic studies are essential for identifying antimicrobial resistance determinants and assessing their potential spreads. Analyzing bacterial genomes allows researchers to track acquisition of resistance genes and investigate their distribution across bacterial populations. These insights enhance the current understanding of the mechanisms driving resistance development. Naturally, three specific mechanisms are complicated in resistance to tetracycline: 1) antimicrobial efflux pumps, 2) target modification through ribosomal protection protein (RPP), and 3) antimicrobial inactivation [14]. Elahi et al. reported one type of Tet(M) encoding gene, while no evidence of tetracycline resistance was seen in the bacterial isolate [6]. In this study, two types of efflux pump genes of Tet(A), two types of efflux pump genes of Tet(B) and one type of RPP gene of Tet(M) were detected and tetracycline resistance was reported as well. Since Tet encoding genes can horizontally be transferred between the bacteria by the plasmids [15], it is plausible that Tet encoding genes might be transferred to the bacterial isolate over time. Enterococci are commonly resistant to various drugs, including vancomycin [16]. Between 2014 and 2017, vancomycin resistance in *E. faecium* isolates increased from 11.2 to 26.1% [17]. Nearly 30% of healthcare-related infections by enterococci are resistant to vancomycin and these VRE are usually resistant to other antimicrobials as well [14]. In this study, resistance of the isolated EntfacYE to vancomycin was due to the Van (VanZ, VanH and VanX) encoding genes. Since Elahi et al. (2021) also reported Van encoding genes [6], no changes in resistance of the isolate to vancomycin was reported in the present study. Sanderson et al. (2020) reported vancomycin resistance in nine out of 11 *E. faecium* isolates [18]. In the present study, results of the antimicrobial susceptibility assessments showed that EntfacYE was resistant to erythromycin. Since active drug efflux mechanism is common for the development of bacterial resistance to macrolides (e.g. erythromycin), genes that encoded efflux proteins might be responsible for the development of this resistance in EntfacYE. Because number and type of the genes encoding efflux proteins were similar in both studies on *E. faecium* EntfacYE [6], no change in erythromycin resistance was observed. In 2020, Sanderson et al. similarly reported resistance of their isolates to erythromycin [18]. In another study by Amachawadi et al., most

strains were resistant to erythromycin [19]. Clindamycin resistance was seen in the present study. Bozdogan et al. showed that resistance to clindamycin was due to the encoded ribosomal methylase [20]. In the present study, resistances to ceftriaxone and cefoxitin were recorded because of beta-lactamase genes; as also reported by Elahi et al. [6]. In a similar study by Edirmanasinghe et al., all isolates were resistant to cefoxitin and ceftriaxone [21]. Fosfomycin-resistance genes were detected in the present study as well as a previously published study by Elahi et al. [6]. Fosfomycin is an active antimicrobial used against MDR and extensively drug-resistant (XDR) gram-positive and gram-negative bacteria [22]. Due to the spread of bacteria in the environment, releases of toxic and heavy metals in various forms may lead to increases in antimicrobial resistance of the bacteria. It has been suggested that heavy metals in the environment can develop antimicrobial-resistance bacteria, since the resistance genes to both classes of antimicrobials are mostly carried on the same MGEs such as integrons [23]. Naturally, bacteria may become resistant to copper, which can be encoded by the plasmids or bacterial chromosomes [24]. In this study, a copper homeostasis subsystem was reported; as reported by Elahi et al. [6]. Festa et al. reported presence of copper homeostasis in *Streptococcus pneumoniae*, *S. aureus* and *Mycobacterium tuberculosis* [25]. In the current study, bile salt hydrolysis gene was investigated in *E. faecium* EntfacYE. Similarly, Elahi et al. detected this gene in their study [6]. Enterococcus antimicrobial-resistance genes are genetically transferred by transposons and plasmids [26]; therefore, presence of genes that are responsible for the resistance to heavy metals such as copper, mercury and cadmium in the genome of bacteria, might be seen because of gene transfer by these MGEs. Investigation of heavy metal resistance genes in enterococci can be addressed as an effective way to identify potential antimicrobial-resistant enterococci [27]. Overall, genome analysis of antimicrobial-resistant enterococci, combined with studies of heavy-metal resistance, may be effective in identifying resistant enterococci and providing appropriate methods for their treatment. Increased prevalence of antimicrobial-resistant pathogens highlights the great importance of continuous monitoring and research in this field. Without appropriate surveillance schemes, resistance may continuously evolve, further complicating treatment strategies. Whole-genome sequencing and comparative analyses provide essential data for understanding resistance dynamics and developing effective control measures.

5. Conclusion

In this study, *E. faecium* EntfacYE genome was sequenced and genes encoding antimicrobial resistance were analyzed and results were compared with the results of a similar study by Elahi et al. three months earlier. In most cases, antimicrobial resistance gene schemes were similar in the two studies. Regarding tetracycline resistance genes, number of the genes increased in the present study compared to that number of the genes did in the previous study, which possibly occurred due to the activity of plasmids. Despite the short time interval between the current and previous studies, this increase has revealed that the bacteria become more rapid resistant to antimicrobials than that it was previously thought. The current ability to treat bacterial infections has been challenged by the emergence and spread of antimicrobial-resistant bacteria. Complete sequencing of the bacterial genomes and study of their resistance genes can lead to important insights into effective treatments for the severe infections caused by antimicrobial resistant bacteria.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Research Ethics Committee of Tehran University of Medical Sciences, Tehran, Iran (Code: IR.TUMS.SPH.REC.1397.139).

Data availability

Data availability is verified by the corresponding author.

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Authors' contributions

Conceptualization, supervision: Ramin Mazaheri Nezhad Fard; Experiments and writing the original draft: Maryam Yazdanizad; Data analysis: Maryam Yazdanizad, Golshid Javdani Shahedin, and Mahla Asadian; Review and editing: Golshid Javdani Shahedin, Ali Akbar Saboor Yaraghi, Ramin Mazaheri Nezhad Fard, and Mahla Asadian; Project consultant: Ali Akbar Saboor Yaraghi.

Conflict of interest

The authors declared no conflict of interest.

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