Infection and Drug Resistance

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ORIGINAL RESEARCH

Molecular Characterization Of Vancomycin-Resistant *Enterococcus faecalis* Among Inpatients At Iranian University Hospitals: Clonal Dissemination Of ST6 And ST422

This article was published in the following Dove Press journal:
Infection and Drug Resistance

Infection and Drug Resistance downloaded from https://www.dovepress.com/
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Purpose: Over the past two decades, enterococci have emerged as an important opportunistic pathogen causing life-threatening infections in hospitals. The purpose of the present study was to examine the prevalence of genes encoding virulence factor and molecular characterization of vancomycin-resistant *E. faecalis* strains isolated from hospitalized patients in Isfahan, the central city of Iran.

Patients and methods: A total of 53 vancomycin-resistant *E. faecalis* isolates (VRE) obtained from clinical samples of hospitalized patients were characterized by phenotypic and genotypic methods, and 25 selected VRE isolates from internal and ICU wards were typed by multilocus sequence typing.

Results: The *efa* was the most prevalent virulence gene (100%) among isolates, followed by *gelE* (92.45%), *asa1* (90.56%), *ace* (86.79%), *esp* (75.47%), *cylA* (39.62%), and *hyl* (18.86%). More than 80% of the isolates were HLGR. Multilocus sequence typing showed eight different sequence types including ST6, ST422, ST28, ST448, ST531, ST328, ST421, and ST495. STs were grouped into two clonal complex (CC) including CCA (ST6, ST422, ST448, ST531) and CCF (ST28, ST421) and two singletons (ST328, ST495).

Conclusion: Our data indicated a high prevalence of virulence genes among STs described in this study. In addition, the molecular analysis demonstrated a relatively high genetic diversity among selected VRE strains from the ICU in comparison with the internal ward. Therefore, in order to prevent the colonization of virulent strains in the hospital environment, infection control procedures should be performed.

Keywords: *Enterococcus faecalis*, virulence factors, vancomycin resistant, multilocus sequence typing, Iran

Introduction

Nosocomial infections (NIs) are a widespread problem in the current clinical setting and occurs in approximately 4% to 10% of hospitalized patients annually.[1,2] Previous studies indicate that the most common bacterial isolates from hospitalized patients are methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), multidrug-resistant gram-negative bacteria, and *Clostridium difficile*.[3] Enterococci, in particular, VRE, is one of the most important nosocomial pathogens,

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submit your manuscript | www.dovepress.com
DovePress
http://doi.org/10.2147/IDR.S217718

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that causes serious infections such as bloodstream infections (BSIs), catheter-associated urinary tract infections (CAUTIs), and intra-abdominal and intra-pelvic abscesses.[4,5] According to previous reports, prolonged hospitalization, immunodeficiency, and uncontrolled antibiotic administration as a high risk of conditions are responsible for acquiring enterococcal infections in patients.[6] Moreover, their ability to acquire specific genetic traits, such as virulence and antibiotic resistance determinants, plays an important role in the success and survival of enterococci in the hospital environment.[7] Virulence factors through the ability to adhere to a range of extracellular matrix proteins and followed by colonization and invasion into the host tissues lead to modulation of the host immunity and production of pathological changes directly through the production of enzymes and toxins or indirectly through induction of inflammation, contributing to pathogenesis and severity of enterococcal infections.[8]

Therefore, characterization of adhesions and invasion factors such as enterococcal surface protein (Esp), aggregation substance (AS) proteins (Asa1), collagen-binding protein (Ace), gelatinase (GelE), hyaluronidase (Hyl) and cytolysin (CylA) can be useful to improve our understanding and assessment of the pathogenicity of enterococcal infections.[9,10,11] Different molecular typing methods have been developed for the epidemiological investigations of enterococci.[12] Among the known enterococcal molecular typing methods, pulsed-field gel electrophoresis (PFGE) was approved as a standard and efficient typing method with a high degree of discrimination,[13] but PFGE remains difficult to standardize between laboratories due to its unsuitability for long-term epidemiology studies or for phylogenetic or population structure studies; this method is more susceptible to small genetic changes.[14,15] However, the most appropriate technique for global and long-term epidemiology studies is multilocus sequence typing (MLST).[15,16] In addition, MLST provides an unambiguous nomenclature for genotypes, and clones and data are easily stored in databases that can be exchanged between different laboratories via the Internet.[16,17,18] The emergence of antimicrobial resistant virulent enterococci is a serious problem for hospital infection control practitioners and clinicians treating infected patients.[19,20] Previous studies have demonstrated that *Enteroccus faecalis* is an important and virulent pathogen causing various infections.[21] Although there are several reports on the endemicity of vancomycin-resistant enterococci (VRE) in Iran, limited information is available in relation to the virulence determinants and molecular relatedness of vancomycin-resistant *E. faecalis* isolates in Isfahan (Center city of Iran). Therefore, in the present study, we investigated the molecular characterization of vancomycin-resistant *E. faecalis* isolates among Iranian hospitalized patients as a first study using MLST technique.

Materials And Methods

Study Design And Samples

This cross-sectional study was performed during the 7-month period from April 2017 to October 2017 at four teaching hospitals affiliated to Isfahan University of Medical Science, Isfahan, Iran. The study was approved by the Ethics Committee and was in accordance with the declaration of Helsinki [IR.MUI.REC.1396.3.066].

A total of 53 vancomycin-resistant *E. faecalis* isolates (VRE) were obtained from true infections including urinary tract infections (UTIs) (n = 35), respiratory tract infections (n = 5), bloodstream infections (n = 4), wound infections (n=4), abdominal infections (n = 3), eye infection (n = 1), and meningitis (n=1).

The samples were cultured on blood agar (Merck, Germany) and then were incubated at 37°C for 24 hrs. Enterococcal isolates were identified according to conventional microbiological tests such as Gram staining (FARA CO.) (Gram positive), catalase reaction (catalase negative), growth on brain heart Infusion agar (Conda, Madrid, Spain) with 6.5% NaCl, and bile-esculin test (positive) (Merck, Germany). Furthermore, *E. faecalis*was confirmed by amplification of the *ddlE* gene using species-specific primers (*ddl E. faecalis* F- 5′- ATCAAGTACAGTTAGTCT-3′ and R-5′-ACGATTCAAAGCTAACTG-3′).[22] The PCR protocol consisted of a pre-denaturation step at 95°C for 5 mins, followed by 30 cycles of 60 s at 95°C, 45 s at 48°C and 1 min at 72°C. A final extension step was performed at 72°C for 5 mins. However, high levels of vancomycin resistance and gentamicin resistance were detected by the E-test strips (Liofilchem, Italy) and gentamicin (120 μg) disk (Mast Group Ltd., UK.), respectively, on the Mueller–Hinton agar (Merck, Germany) in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines.[23]

DNA Extraction And Detection Of Virulence Genes

Genomic DNA was extracted from fresh colonies as described previously.[21] PCR was performed for detecting

seven of the genes encoding virulence factors including enterococcal surface protein (*esp*), gelatinase (*gelE*), aggregation substance (*asa1*), collagen-binding protein (*ace), Enterococcus faecalis* endocarditis antigen (*efa*), hyaluronidase (*hyl*), and cytolysin (*cylA*). Primers were synthesized as previously described and were as follows: *esp, hyl, gelE, asa1*,[24] *cylA*,[25] *ace*,[26] and *efa*.[27] The PCR products were separated by electrophoresis in 1% agarose gels with 1× TAE (Tris/Acetate/EDTA) buffer, stained with safe stain load dye (CinnaGen Co., Tehran, Iran) and visualized under ultraviolet illumination.

Multilocus Sequence Typing (MLST)

A total of 25 vancomycin-resistant *E. faecalis* isolates (VRE) with high levels of gentamicin resistance from internal (n=12) and intensive care units (ICU) (n=13) wards were subjected to MLST. Seven of *E. faecalis* housekeeping genes including *gdh* (glucose-6-phosphate dehydrogenase), *gyd* (glyceraldehyde-3-phosphate dehydrogenase), *pstS* (phosphate ATP-binding cassette transporter), *gki* (glucokinase, putative), *aroE* (shikimate 5-dehydrogenase), *xpt* (xanthine phosphoribosyl transferase), and *yqil* (acetyl-CoA acetyltransferase) were amplified by PCR method. Information of the *E. faecalis* MLST scheme, sequences of PCR primers, and the PCR conditions are available at MLST website (https://pubmlst.org/efaecalis/). STs were analyzed to determine clonal complex (CCs), single-locus variants (SLV), double-locus variants (DLV), and singletons using the eBURST algorithm (http://eburst.mlst.net/). Sequence types that were not grouped into a CC were defined as singletons. Also, the genetic relationship among strains was analyzed by constructing a dendrogram using eBURST program.

Statistical Analysis

The analysis was performed by using SPSS™ software, version 21.0 (IBM Corp., USA). The results are presented as descriptive statistics in terms of relative frequency.

Results

During 6 months of the study, a total of 53 VRE isolates were collected randomly with the possibility of true infections. Totally, of 53 confirmed VRE isolates, 56.6% and 43.4% isolates were obtained from female and male subjects, respectively.[28] The most frequent isolates 35 (66%) were obtained from UTIs and followed that from respiratory infections and bloodstream infections. Meanwhile, 32% (17/53) and 26.4% (14/53) isolates were obtained from hospitalized patients in ICUs and internal wards, respectively. According to MDR definition, all of 53 *E. faecalis* strains were MDR (100%) and more than 80% of the isolates were high-level gentamicin resistance (HLGR) (Table 1).[28]

Distribution Of Virulence Genes

According to the results of PCR assay, the *efa* was most prevalent virulence gene (100%), followed by *gelE* (92.4%), *asa1*(90.6%), *ace* (86.8%), *esp* (75.5%), *cylA* (39.6%), and *hyl* (18.9%). However, none of the isolates contained all the virulent genes. The distribution of genes encoding virulence factors among VRE isolates and different STs is shown in Table 1.

Clonal Lineages Identified By MLST

MLST analysis was performed for 25 selected VRE isolates from internal and ICU wards that were classified into eight different STs: ST6, ST422, ST28, ST448, ST531, ST328, ST421, and ST495. Using the BURST v3 algorithm, STs were grouped into two CCs including CCA (ST6, ST422, ST448, ST531) and CCF (ST28, ST421) and two singletons (ST328, ST495). Two STs were found to be highly prevalent and comprised more than half of the isolates; 40% (10 isolates) of the isolates were ST6 and 24% (six isolates) were ST422, followed by ST28, ST448, and ST531 (two isolates), whereas other three STs were represented by a single isolate (ST328, ST421, and ST495). 91.6% (11/12) of the selected strains from internal wards were ST6 (seven isolates) and ST422 (four isolates), while selected isolates of ICU ward showed a higher diversity.

Phylogenetic analysis of the concatenated sequences, including all seven MLST genes, from the 25 VRE isolates examined in this study is demonstrated in Figure 1. The phylogenetic tree showed that there is a close phylogenetic relationship between most of the strains in our study.

Overall, the distribution of virulence genes indicated a high frequency of those among different STs, so that the *asa1, efa, gelE, and ace* genes were present in all STs and the prevalence of *esp, cylA* and *hyl* genes was 84.0%, 44.0% and 28.0%, respectively.

Discussion

Despite the fact that many studies have investigated antibiotic resistance patterns and the virulence factors of *Enterococcus* spp. isolates, only few studies have systematically investigated vancomycin-resistant *E. faecalis*

Table 1 The Phenotypic And Genotypic Characteristics Of The 53 Vancomycin-resistant *E. faecalis* Isolates From Clinical Samples

Number Of Isolates	Gender	Infections	Ward	Virulence Genes	HLGR/Non-HLGR	ST
1	M	UTI	Infectious diseases	*efa, gelE, ace*	HLGR	
2	M	UTI	ICU	*asa1, efa, gelE, ace, esp,cylA,*	HLGR	421
3	M	BSI	ICU	*asa1, efa, gelE, ace, esp,cylA,*	HLGR	6
4	F	UTI	Emergency	*asa1, efa, gelE, esp*	HLGR	
5	F	UTI	Outpatient	*efa, gelE, ace, esp,*	HLGR	
6	F	WI	Surgery	*asa1, efa, gelE, ace, esp,cylA,*	HLGR	
7	M	BSI	Infectious diseases	*asa1, efa, gelE, ace, hyl*	HLGR	
8	M	UTI	Emergency	*asa1, efa, gelE, ace, esp,cylA,*	HLGR	
9	M	UTI	Internal	*asa1, efa, gelE, ace,,*	HLGR	6
10	F	UTI	Emergency	*asa1, efa, gelE, ace, esp*	Non-HLGR	
11	F	BSI	Respiratory	*asa1, efa, gelE, esp*	HLGR	
12	F	UTI	Internal	*asa1, efa, gelE, ace, esp*	HLGR	6
13	F	UTI	Internal	*asa1, efa, gelE, ace, esp,cylA,*	HLGR	422
14	M	UTI	Internal	*asa1, efa, gelE, ace, esp*	HLGR	422
15	F	UTI	ICU	*asa1, efa, gelE, ace, esp,cylA,*	Non-HLGR	
16	F	UTI	Emergency	*asa1, efa, ace*	HLGR	
17	F	UTI	Surgery	*asa1, efa, gelE, esp,cylA,hyl*	HLGR	
18	F	UTI	Rheumatology	*asa1, efa, gelE, ace, cylA*	Non-HLGR	
19	M	UTI	ICU	*asa1, efa, gelE, ace,esp, cylA*	HLGR	422
20	M	UTI	Surgery	*asa1, efa, gelE, ace, esp,hyl*	HLGR	
21	F	RTI	ICU	*asa1, efa, gelE, ace, cylA*	HLGR	28
22	F	WI	Rheumatology	*asa1, efa, ace, esp*	Non-HLGR	
23	F	UTI	Urology	*asa1, efa, ace,,cylA*	HLGR	
24	M	WI	Internal	*asa1, efa, gelE, ace*	Non-HLGR	
25	M	UTI	ICU	*asa1, efa, gelE,ace, cylA,*	HLGR	531
26	F	UTI	NICU	*asa1, efa, gelE, ace, esp,cylA,*	HLGR	448
27	M	WI	Infectious diseases	*efa, esp*	HLGR	
28	F	EI	NICU	*asa1, efa, gelE, ace*	Non-HLGR	
29	M	RTI	ICU	*asa1, efa, gelE, ace, esp,cylA,*	HLGR	422
30	F	UTI	Internal	*efa, gelE, esp*	Non-HLGR	
31	F	RTI	ICU	*asa1, efa, gelE, ace, esp*	HLGR	28
32	F	UTI	Respiratory	*asa1, efa, gelE, ace, esp,cylA,*	HLGR	
33	F	UTI	ICU	*asa1, efa, gelE, ace, esp*	HLGR	328

(*Continued*)

Table 1 (Continued).

Number Of Isolates	Gender	Infections	Ward	Virulence Genes	HLGR/Non-HLGR	ST
34	M	RTI	Surgery	*efa, gelE*	HLGR	
35	M	UTI	ICU	*asa1, efa, gelE, ace, esp,cylA,*	HLGR	6
36	M	UTI	Internal	*asa1, efa, gelE, ace, cylA, hyl*	HLGR	422
37	M	AI	Internal	*asa1, efa, gelE, ace, esp,hyl*	HLGR	6
38	M	AI	ICU	*asa1, efa, gelE, ace, esp,hyl*	HLGR	531
39	F	RTI	Internal	*asa1, efa, gelE, ace, esp,cylA,*	HLGR	422
40	F	UTI	ICU	*asa1, efa, gelE, esp,cylA*	Non-HLGR	
41	M	UTI	Emergency	*asa1, efa, gelE, ace, esp,cylA,*	HLGR	
42	F	UTI	Internal	*asa1, efa, gelE, ace, esp,hyl*	HLGR	6
43	F	UTI	ICU	*asa1, efa, gelE, ace, esp,cylA,*	HLGR	495
44	F	Me	CCU	*asa1, efa, gelE, ace, esp*	HLGR	
45	F	UTI	CCU	*asa1, efa, gelE, esp, cylA,*	HLGR	
46	F	AI	Internal	*asa1, efa, gelE, ace, esp, hyl*	HLGR	448
47	M	UTI	ICU	*asa1, efa, gelE, ace, esp,cylA,*	HLGR	6
48	M	UTI	Internal	*asa1, efa, gelE, ace, esp*	HLGR	6
49	F	BSI	ICU	*asa1, efa, gelE, ace, esp*	Non-HLGR	
50	F	UTI	Internal	*asa1, efa, gelE, ace, esp, hyl*	HLGR	6
51	M	UTI	Surgery	*asa1, efa, gelE, ace*	Non-HLGR	
52	F	UTI	Internal	*asa1, efa, gelE, ace, esp,hyl*	HLGR	6
53	M	UTI	Emergency	*asa1, efa, gelE, ace, esp,cylA*	HLGR	

Abbreviations: F, female; M, male; UTI, urinary tract infection; BSI, bloodstream infection; WI, wound infection; RTI, respiratory tract infection; EI, eye infection; AI, abdominal infection; Me, meningitis; ST, sequence type.

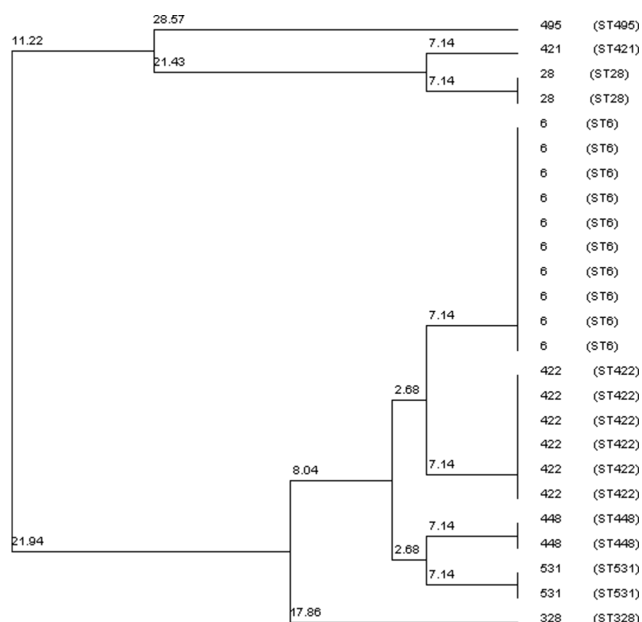

Figure 1 Cluster tree of sequence types (ST) of VRE strains. The tree constructed with the neighbor-joining method. In each branch is shown the corresponding bootstrap NJ values, taken over 1000 replicates,which assign confidence values for the groupings in the tree.

isolates originating from clinical specimens in our country. Therefore, we characterized the vancomycin-resistant *E. faecalis* (VRE) collected from different clinical specimens during a 7-month period in 2017 from various teaching hospitals in Isfahan city, Iran. Similar to other reports from Iran, resistance to multiple classes of antibiotics was common in *E. faecalis* strains as observed in the current study.[21,29,30] In this study, 81% of VRE strains were HLGR. Resistance to high concentrations of gentamicin among *E. faecalis* isolates has been showed in previous researches.[30,31] High-level resistance to gentamicin is caused by the aminoglycoside-modifying enzymes, reducing the effect of aminoglycosides except for streptomycin.[32] Antibiotic resistance is not sufficient for the pathogenesis of enterococcal infections and the presence of virulence factors plays an important role in the severity of infection.[10] The VRE isolates characterized in this study showed a high prevalence of virulence genes. Our findings indicated that all of the isolates harbored *efa* gene and similar frequencies of the *efaA* were reported in various studies.[21,26,33] It seems that the *efaA* gene is always present in *clinical E. faecalis strains*, whereas the strains isolated from other sources possessed the less frequency of EfaA determinant.[26] As reported in other studies, *gelE* was identified as the second most common virulence factor among the isolates in the current study (92.45%).[31,33] GelE seems to mediate virulence through effects such as degradation of host tissues and participates in the activation of autolysin, leading to the release of extracellular DNA and formation of a biofilm.[11] The aggregation substance gene (*asa1*) was present in 90.56% of all isolates. These results are in agreement with those obtained by Choi et al and Nasaj et al who showed a high incidence of *asa1* genes in clinical isolates of *E. faecalis*.[31,34] AS proteins encoded by *asa1* cause clumping of *E. faecalis* cells and mediate the high-frequency transfer of plasmid DNA between donor and recipient bacteria.[8] These high rates of *asa1* gene among all of our isolates can facilitate the exchange of resistance and virulence-associated genes via pheromone-responsive plasmids in a hospital setting. The results of our study indicated that the *ace* gene was present in 86.79% of isolates. Similarly, a high incidence of this gene in *E. faecalis* has been reported in previous studies.[21,35,36] *E. faecalis* Ace is a collagen and laminin adhesin, which seems to be effective in endocarditis and UTIs.[11] In the current study, the prevalence of the *esp* gene was also 75.47% in VRE strains. This finding in accordance with other studies indicate a possible role of the Esp as a colonization factor in UTI.[10,26,34] The frequency of *cylA* and *hyl* genes was 39.62% and 18.86%, respectively. Previous studies by Heidari et al in Tehran and Shiraz showed that the virulence genes *cylA* and *hyl* were present at varying levels in *E. faecalis* isolates.[21,33] These findings are in agreement with the results of Wanxiang et al, who showed that *cylA* and *hyl* genes were 19.4% and 19.6% in the Enterococcus isolates, respectively.[37] During this study, we also identified that *hyl*-positive strains were HLGR, and this may be due to the co-presence of the *hyl* and *aac(60)-Ie-aph(200)-Ia* genes in a common transmissible plasmid.

Molecular typing of 25 strains of VRE isolated in Isfahan using MLST provided the first data for the knowledge on the genetic population structure for this species in Iran, allowing us to compare it to those obtained worldwide, and to know the spread of some *E. faecalis* clones to Iran. In the current study, eight different STs were found among the 25 VRE isolates, and distributed into two CCs, including CCA (ST6, ST422, ST448, and ST531), CCF (ST28, ST421), and two singletons (ST328, ST495). ST422 and ST531 represented an SLV of ST6 and ST448, respectively. ST531 is a DLV of ST422 belonging to CCA and is also reported in Denmark.[38] Furthermore, ST531 represented SLV of ST6 belonging to CCA. ST28 is

an SLV of ST421 belonging to CCF. However, our study results showed that most of the strains examined had a common genetic origin. ST6, which is the ancestor of CCA, was the most frequently found ST compared to other STs (40%), and ST422 was the second most detected ST among the isolates (24%). Furthermore, it was found that CCA contained the greatest number of STs identified in this study (20/25; 80%). *E. faecalis* strains identified as ST6 showed similar characteristics to ST6 clones isolated in Portugal and Poland[39,40] and were characterized by high-level resistance to aminoglycosides, and vancomycin, but differed from the strains described in Cuba and Spain where they were vancomycin susceptible.[12,41] ST6 has been reported worldwide, and it is probably the major lineage in CCA, which is often associated with invasive infections and resistance to multiple drugs, owing to its adaptability to hospital environment as a result of the acquisition of pathogenicity islands and antimicrobial resistance genes by recombination and horizontal gene transfer.[14,42,43,44,45] Notably, 6 of the 25 isolates were found to be ST422 (Table 1). According to a review of studies worldwide, there are few reports of ST422 in *E. faecalis*, so that Hammerum et al report ST422 as HLGR, agreeing with our results.[38] Other STs reported in this study were also found previously from different parts of the world.[12,31,38,42,46] A high prevalence of virulence genes was also detected among STs described in this study, and there was no significant difference in the distribution of virulence factors between HLGR and non-HLGR isolates. There were some limitations related to the present study. First, a small number of VRE isolates were typed by MLST. Second, identification of the source of infection, taking preventive measures in the hospital setting, and molecular analysis of environmental specimens were required.

Conclusion

In summary, this research provided the first insight into the population structure of *E. faecalis* in Isfahan, Iran, and most of the strains examined in this study were related to European strains, and CCA was evidently circulating in Isfahan hospitals, being associated with MDR and virulence traits. In addition, our data indicate that *E. faecalis* strains isolated from clinical samples possess distinctive patterns of potential virulence factors with a high incidence of genes encoding virulence factors among isolates. It is recommended that some programs be performed to prevent the colonization of such virulent strains in the hospital environment, including better stewardship of antimicrobial agents and better awareness of the source for pathogen transmission in the hospital environment.

Ethics approval

The study protocol was approved by the Ethics Committee of Isfahan University of Medical Sciences (IR.MUI.REC.1396.3.066). However, we did not have human participants. The study used bacteria isolated from clinical samples in the clinical microbiology laboratory.

Acknowledgment

We are thankful to all members of the Department of Microbiology, School of Medicine, at Isfahan University of Medical Sciences. This was an original research paper from a Ph.D thesis.

Funding

This study was funded, in part, by a grant from the Isfahan University of Medical Sciences, [Grant no 396066].

Disclosure

The authors declare that they have no competing interests in this work.

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