RESEARCH ARTICLE

Mass Spectrometry Technology and qPCR for Detection of *Enterococcus faecalis* in Diabetic Foot Patients

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Abstract

Diabetic foot ulcer (DFU) is one of the most serious and costly complications of diabetic patients. *Enterococcus faecalis* (*E. faecalis*) represents one of the most virulent microorganisms in diabetic foot infections (DFIs). We therefore aimed to study the frequency and precise identification of *E. faecalis* in DFU. Six hundred thirty specimens collected from diabetic foot patients were used in the current investigation. Biochemical identification was carried out by the Vitek® 2 system. Proteomic analysis was implemented by MALDI-TOF MS and confirmed by SYBER Green real-time polymerase chain reaction (SYBER Green qPCR). According to the results, the overall frequency of *E. faecalis* in patients with DFU was 8.25% (52/630). Out of 52 *E. faecalis* strains, 40 isolates were isolated from males and 12 from females. The results of biochemical identification revealed that 92.30% (48/52) of *E. faecalis* isolates were properly recognized at the species level. Whereas 100% (52/52) of *E. faecalis* isolates were properly recognized by MALDI-TOF MS as 44.23% (23/52), 51.92% (27/52) and 3.84% (2/52) with a score value ranging from 2.300 to 3.000, 2.000-2.299 and 1.700-1.999 Da, respectively. Seven *E. faecalis* virulence genes, including *asa1, GelE, cylA, esp, hy1, VanA, and VanB*, were detected by SYBER Green RT-PCR. In conclusion, *E. faecalis* was the utmost predominant single organism isolated from the DFIs. MALDI-TOF mass spectrometry is considered a fast, trustworthy and economic detection method for various significant microorganisms. *E. faecalis* isolates were also found to carry several virulence genes. Our findings may serve as an urgent issue for supplementary investigations of contagions caused by *E. faecalis*.

Keywords: Diabetic foot infections; *E. faecalis*; Incidence; Identification; MALDI-TOF MS.

INTRODUCTION

Diabetes mellitus (DM) is a chronic deteriorating disease associated with many complications. Diabetic foot ulcer (DFU) is considered one of the major problems of DM with a yearly incidence of 10% among diabetic patients. It is calculated that 15% of diabetic patients are suffering from ulcers through their lifetime, and 10%–30% of these cases ultimately progress to amputation [1]. The infection rate is a significant causative issue in this case, according to this review of literature; about 60% of these amputations are preceded by the presence of infected ulcers. Saudi Arabia is one of the top ten Middle Eastern/Arab countries with the highest diabetes prevalence in adults, which will certainly lead to high rates of foot ulcers along with increasing morbidity and costs [2]. Most diabetic foot infections (DFIs) present with a polybacterial etiology caused by enterococcal strains, which are part of the multifaceted diabetic foot infection. Former researches stated that the genus *Enterococcus* is one of the utmost virulent microorganisms in DFIs [3]. Numerous microbes, can lead to DFIs, of which *Enterococci* are the common type of bacteria [4]. Former researches indicated that the genus *Enterococcus* genus is the most public organisms in DFIs, leading to increase the severity of infection and even death [5,6]. Recently, the genus *Enterococcus* is composed of thirty-eight species; *Enterococcus faecalis* (*E. faecalis*) represents the common significant species of this genus [7]. Their identification is established mostly on morphological and biochemical and molecular techniques [8,9]. This diversity of techniques permits a consistent detection of Gram-positive cocci. Nevertheless, phenotypic and genetic approaches have many limitations such as laborious, expensive and labor-intensive, and the molecular methods do not usually exist in numerous investigative test centers.

In recent times, numerous studies assessed the application of matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) for the detection of various microorganisms in the diagnostic laboratories [10,11,12,13,14,15,16]. Throughout this technology, microorganisms are recognized by means of either whole cells or cell extracts. The procedure is quick, delicate, and inexpensive. This skill has been willingly approved by specialists who have described the application of MALDI-TOF for a quantity of determinations such as microbiological identification, characterization, and discrimination [17,18]. In Germany, Werner et al. [19] indicated that MALDI TOF MS detected 64 *E. faecalis* and 37 *E. faecium* isolates, which were established by PCR. In Zagreb, Dobranic et al. [20] stated that MALDI-TOF MS identification presented 100% concordance with API 20 Strep in the identification of *E. faecalis*.

Therefore, the possibility for MALDI-TOF to rapidly describe a broad range of microbes, comprising fungi, bacteria, and viruses increase its possible use in various parts of biological identification. Consequently, this equipment can be applied for quick identification of numerous types of pathogens at comparatively low cost and also represents another powerful technique for traditional and molecular methods. The main impact of this technique consists of a significant improvement in limiting the time consumed from preparing the samples till identification.

From the previously mentioned date, the current study was delineated to examine several parameters: (i) Determine the colonization rate of *E. faecalis* in DFI in Saudi Arabia; (ii) Biochemical identification of *E. facials* using the VITEK 2 System; (iii) Proteomic identification of *E. faecalis* using MALDI-TOF MS confirmed by qPCR.

MATERIALS AND METHODS
Sample collection

The current investigation was conducted from June 2016 to January 2017 at King Saud Medical City, Riyadh region and Bukyriah General Hospital (BGH) Qassim region. The King Saud Medical City was opened in 1970 with a capacity of 1500 beds and provides hospital services to the province of Riyadh city. BGH opened in 1985 and accommodates 150 beds and covers hospital services to the province of Bukayriyah and surrounding areas. It is a patient-referral hospital that covers more than 20 health centers in addition to three small (30 beds) hospitals. A total of 630 samples were surveyed during June 2016 to January 2017. Swabs were collected from patients' DFI deep wound and abscess specimens. Pus from the lesions should be put in a sanitary sterilized bottle and/or an anaerobic transport bottle (Procedures Guidelines for the Microbiology Laboratory). Swabs were microscopically examined

and classified. Before sample collection, a sterile cotton swab was soaked with sterile saline solution. Bacterial description and proof of identity procedures were accomplished by standard protocols. From air dried and heat fixed smears, gram staining and cell morphology were implemented. Epidemiological data were recorded with respect to gender, age, and weight.

Isolation of *E. faecalis*

Fifty-two strains of *E. faecalis* were isolated from 630 diabetic foot patients. From the primary routine cultures, all specimens were cultured into blood agar and MacConkey agar plates (both from Sigma-Aldrich, USA). After incubation of all plates at 37°C for 24 to 48 h, *E. faecalis* colonies appeared as small, round, white colonies. The purified isolates were then subjected to presumptive identification tests, including Gram staining, catalase and bile esculin, growth at 45°C, and 6.5% of NaCl. Catalase-negative and bile esculin-positive, Gram-positive cocci arranged in pairs and/or chains forming alpha-hemolytic or non-hemolytic colonies on blood agar media were recognized as *Enterococci* and then they were spectated with the API Rapid Strep system.

Biochemical identification of *E. faecalis*

The identification- gram positive card (ID-GPC) of the VITEK® 2 system (Biomérieux, France) was used in this study for detection of *E. faecalis* isolates according to the manufacturer's instructions. Briefly, 2-3 fresh colonies were inoculated with 5 ml of salt solution and thoroughly mixed. The bacterial turbidity was tested in the range from 0.50 and 0.63 using DensiChekTM (BioMe2 rieux, France). A test tube filled with 5 ml of this suspension was loaded into the machine. The filled cassette was finally sited in the device, and the data from an unknown bacterium were compared to the particular catalogue to define a quantitative value for proximity to each of the database taxa. Both ATCC 19433 *E. faecalis* and ATCC 25923 *S. aureus* were utilized as quality control isolates.

Proteomic investigation of *E. faecalis* by MALDI-TOF MS

Preparation of bacterial samples

We then applied the MALDI biotyper device (Bruker Daltonik, Bremen, Germany) for fast and precise detection of *E. faecalis* isolates. A single colony from freshly streaked agar incubated

for 18-24h at 37°C, was utilized for each isolate and inoculated onto two spots of the target plate. Each colony was then enclosed with one microliter of matrix solution. The microbial bands were directly produced by applying compass software, and evaluation was conducted with a MALDI biotyper machine. We used *Escherichia coli* as a positive control (bacterial test standard) according to the recommendations of Bruker Corporation.

Data analysis and clustering

The score value of unidentified spectrum ranging from 0 to 3 was determined by comparing the corresponding unknown spectrum with the known spectrum in the Bruker library. The accuracy of strain recognition was detected by Bruker Daltonik, Bremen, Germany. The precise detection of species was performed using the Microflex LT device when the score value ranges from 2.3 to 3.0. Species and genus levels were also recognized in the range from 2.00 to 2.29 and 1.700 to 1.999, respectively. Furthermore, a score of 0.00 to 1.69 means that the proof of identity is not reproducible. The diverse spectra created with the Microflex LT compass software were measured in a m/z range from 2, 000 to 20, 000 Da. The official standards depended on fifty laser shots per spot. According to the Microflex LT library, which contains 5989 bacterial and fungal sub-species, a dendrogram was generated from the minimal spanning tree (MSP) data set. The MSP dendrogram was generated based on evaluation of the main spectra of the various species.

Molecular identification of *E. faecalis*

Genomic DNA isolation

Extraction of bacterial DNA was done by QuickGene-810 (Fujifilm, Tokyo, Japan) with a special DNA isolation kit (QuickGene DNA Tissue Kit S). All procedures were carried out based on the manufacturer recommendations. The purity and concentration of extracting DNA were detected by NanoDrop™ 2000 UV Spectrophotometer.

SYBER Green Real time PCR identification

The SYBER Green RT-PCR for *E. faecalis* was then carried out by 7500 Fast RT-PCR System (Applied Biosystems, USA). Seven different virulence genes of *E. faecalis*, including *asa1, GelE, cylA, esp, hy1, VanA and VanB. E. faecalis* were detected as described by the manufacturer's instructions of Thermo Fisher Scientific, USA. Lists of selected primers, qPCR conditions, and

amplified products are presented in Table 1. Briefly, a total of 20 µl reaction volume containing 10 µl of SYBER Green PCR master mix, 1 µl each of forward and reverse primer, 1 µl target DNA, and 7 µl of RNAse/DNase free water was used. All reactions were performed in duplicate. Preparation of a standard curve dilution series was done by adding 90 µl of template preparation buffer to five tubes and labeling them. The positive control template (*E. faecalis* [ATCC 19433]) was considered as tube 1, and 10 µl was transferred from this tube to tube 2 and mixed thoroughly. Ten microliters from tube 2 were then transferred into tube 3, continuing in the same manner up to the 9th tube. Finally, for the standard curve, 5 µl of the standard template was transferred to each well, making the final volume in each well 20 µl.

Amplifications were then completed in the RT-PCR system. Thermal cycling consisted of 37 °C for 15 min with AmpErase® uracil N-glycosylase (UNG) in order to prevent PCR carryover contamination and 95 °C for 2 min for enzyme activation followed by 40 cycles with every denaturation step at 95 °C for 10 s. Annealing and extension was performed at 60 °C for 60 s. Data were analyzed using sequence detection system (SDS) software. Amplification findings were expressed by plotting Delta Rn (DRn) versus cycle number for the interpretation of infection [21].

RESULTS

Incidence of *E. faecalis* in diabetic foot patients

The incidence of *E. faecalis* was investigated in 630 DFI samples. The frequency

Table 1. Primers utilized in the RT-PCR for *E. faecalis* detection

Target gene	Virulence factor	Primer	Sequence (59–39)	Product size (bp)
asa1	Aggregation substance	ACA11	GCACGCTATTACGAACTATGA	375
		ACA12	TAAGAAAGAACATCACCACGA	
GelE	Gelatinase	Gel11	TATGACAATGCTTTTTGGGAT	213
		Gel12	AGATGCACCCGAAATAATATA	
cylA	Cytolysin	CYT I	ACTCGGGGATTGATAGGC	688
		CYT IIb	GCTGCTAAAGCTGCGCTT	
esp	Enterococcal surface protein	ESP 14F	AGATTTCATCTTTGATTCTTGG	510
		ESP 12R	AATTGATTCTTTAGCATCTGG	
hyl	Hyaluronidase	HYL n1	ACAGAAGAGCTGCAGGAAATG	276
		HYL n2	GACTGACGTCCAAGTTTCCAA	
VanA	Vancomycin-resistant A	vanA 1	GGGAAAACGACAATTGC	175–191
		vanA2	GTACAATGCGGCCGTTA	907–891
VanB	Vancomycin-resistant B	Van B1	ATGGGAAGCCGATAGTC	173–189
		vanB 2	GATTTCGTTCCTCGACC	807–791

Table 2. Distribution of *E. faecalis* and its associated organisms among males & females

Isolates	Total isolates = 74		
	Males	Females	Total
Enterococcus faecalis	40	12	52
Acinetobacter baumanni	6	2	8
Staphylococcus aureus	4	0	4
Citrobacter freundii complex	2	0	2
Klebsiella pneumoniae	2	0	2
Staphylococcus epidermidis	2	0	2
Enterobacter aerogenes	0	2	2
Escherichia coli	2	0	2

of the organisms in the DFI is shown in fig. 1. According to the results, of the 630 DFI samples, 74 samples were positive as 52 *E. faecalis*, 8 *Acinetobacter baumanni* (*A. baumanni*), 4 *Staphylococcus aureus* (*S. aureus*), 2 *Citrobacter freundii* (*C. freundii*), 2 *Klebsiella pneumoniae* (*K. pneumoniae*), 2 *Staphylococcus epidermidis* (*S. epidermidis*), 2 Enterobacter aerogenes (*E. aerogenes*) and 2 *Escherichia coli* (*E. coli*).

Distribution of *E. faecalis* and its associated organisms among males & females

As presented in fig. 2, 40 *E. faecalis* isolates were recovered from males and 12

from females. Six isolates of *A. baumanni* were recovered form males and 2 from females. All *S. aureus*, *C. freundii*, *K. pneumoniae*, *S. epidermidis* and *E. coli* isolates were recovered from males

only, whereas *E. aerogenes* isolates were isolated from females only.

The effect of age on the prevalence of bacterial DFIs

To assess the effects of age on the prevalence of DFIs, patients enrolled in this study were categorized into different age groups varying from ≤1 year to ≥80 years. As illustrated in Table 3, patients in the age group ≤1- 10, 21–30 years, and 41–50 years showed the highest prevalence of bacterial DFIs at frequencies of 20.2%, 18.9%, and 17.5%, respectively, whereas patients in the age groups 11–20 years, 31–40 years, and 61–70 years exhibited the lowest risk of bacterial DFIs with a frequency of 5.4%.

Identification of *E. faecalis* and associated organisms

Biochemical identification

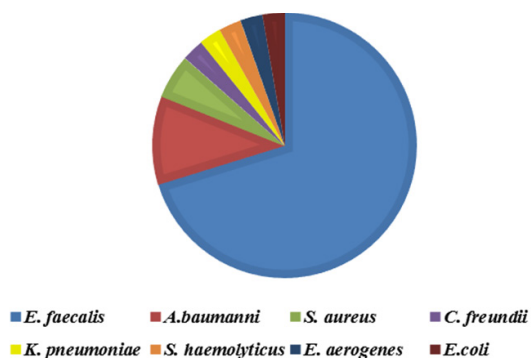A total of 52 *E. faecalis* and other 22 organisms recovered from DFI were assessed by

Fig. 1. Distribution of recovered organisms from 74 isolates among 630 DFIs swabs
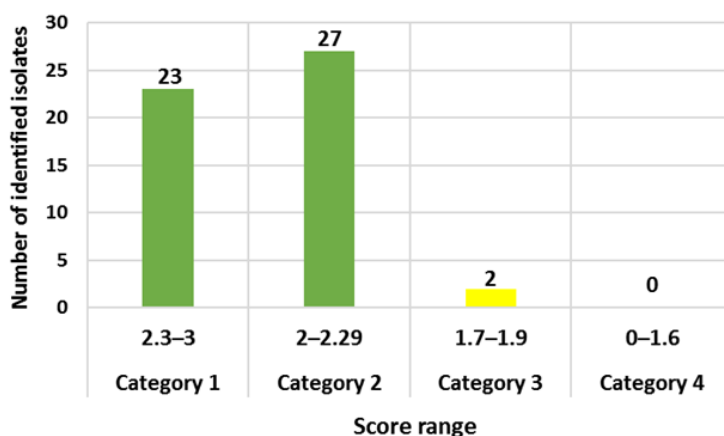

Fig. 2. Score values of 52 *E. faecalis* strains identified by Microflex LT

Table 3. Distribution of recovered DFIs bacteria in relation to age groups

Age Group	Total bacteria isolaes (n=74)		Total gram positive (n= 58)		Total gramNegative (n=16)	
	N	%	N	%	N	%
≤1 – 10 y	15	20.2	13	22.4	2	12.5
11 – 20 y	4	5.4	2	3.4	2	12.5
21 – 30 y	13	17.5	2	3.4	0	0
31 – 40 y	4	5.4	2	3.4	0	0
41 – 50 y	14	18.9	10	17.2	4	25
51 – 60 y	12	16.2	8	13.7	4	25
61 – 70 y	4	5.4	4	6.8	0	0
71 – 80≥ y	10	13.5	6	10.3	4	25

the Vitek™ 2 system for biochemical identification. As shown in Table 2, a total of 70 out of 74 isolates were correctly identified as 49/52 (94.23%) *E. faecalis,* 7/8 (87.2%) *A. baumanni,* 4/4 (100%) *S. aureus,* 2/2 (100%) *C. freundii complex,* 2/2 (100%) *K. pneumoniae,* 2/2 (100%) *S. haemolyticus,* 2/2 (100%) *E. aerogenes,* and 2/2 (100%) *E. coli.*

Proteomic identification of *E. faecalis*

According to our findings, 52 *E. faecalis* strains recovered from patients suffering from DFI were properly recognized by MALDI Biotyper (MBT). In the current study, all isolates were investigated using the MBT device, and the obtained spectra were matched with the storing spectra in the Bruker taxonomy of compass software. As seen in fig. 2, it was noticed that 52 *E. faecalis* strains were sufficiently well recognized at the species level as 44.23% (23/52), 51.92% (27/52) and 3.84% (2/52) with a score value ranging from 2.300 to 3.000 and 2.000-2.299 Da, respectively. In contrast, only two isolates (3.84%) were identified at the genus level with a log score ranging from 1.7 to 1.99. Ion peaks were observed in the line spectra of 2,000-16,000 Da. The strong peaks were concentrated in between 3,000 and 5,000 Da, which corresponded with different types of bacterial strains assessed by the library of compass software (fig. 3). A current gel spectral
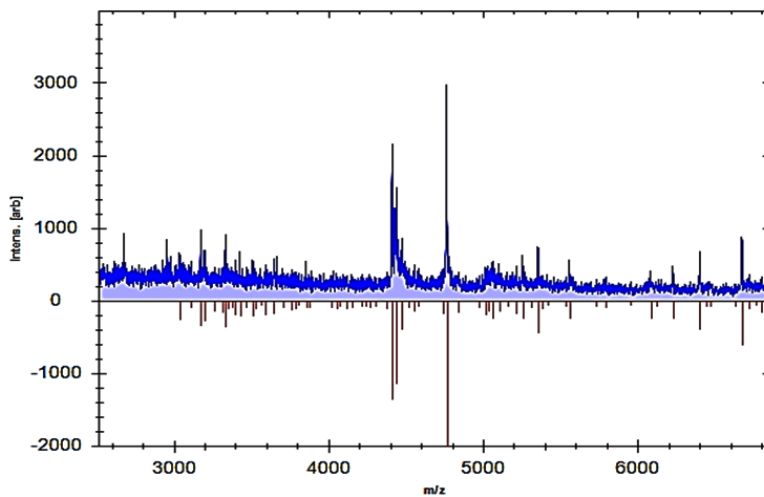

Fig. 3. Comparison of mass spectrum protein profiles of unknown samples (blue) with reference strain *E. faecalis* 20371 DSM (red)
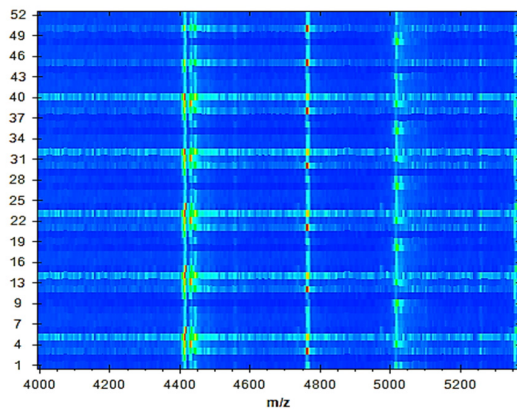

Fig. 4. A gel protein spectrum for 52 *E. faecalis* strains demonstrates that most peaks are condensed between 4, 400 and 5, 000 Da

image detected 52 *E. faecalis* strains. Numerous spectra were scattered within the range from 2, 000 to 15, 000 Da, and most peaks were condensed between 4, 400 and 5,000 Da (fig. 4). Furthermore, to clarify if the Microflex LT compass software could distinguish clonally-associated strains at the species level, spectra from 52 well-identified *E. faecalis* strains were investigated as shown on the gel. The various spectra were then developed to generate a novel cross-wise minimal spanning tree (MSP) dataset (fig. 5). According to the results, the dendrogram exhibited that analyzed *E. faecalis* strains were closely related to 11 *E. faecalis* reference strains in the Bruker taxonomy at a distance of 200.
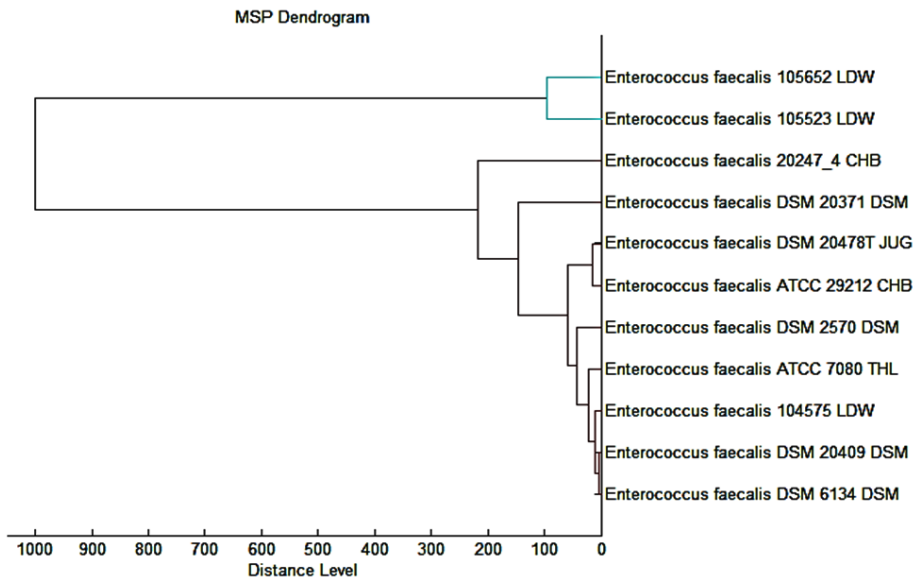
Fig. 5. The MSP dendrogram for 52 *E. faecalis* strains exhibited a strong relationship with 11 *E. faecalis* reference strains in the Bruker taxonomy
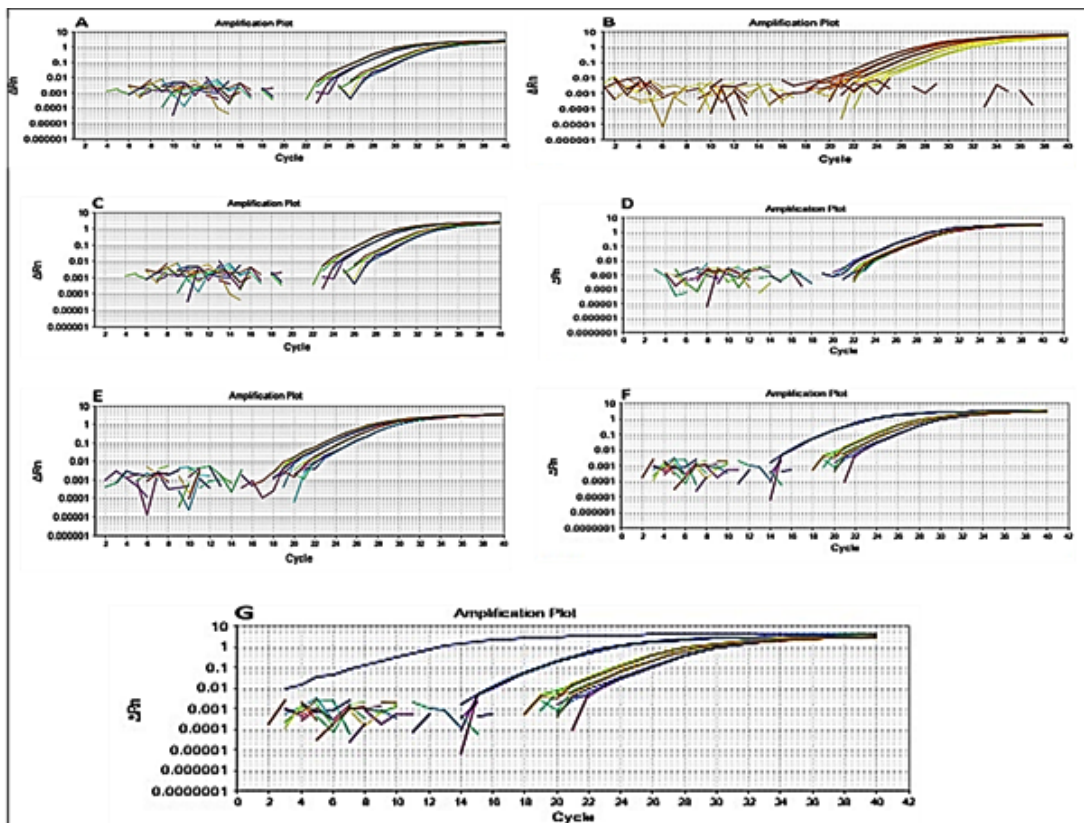

Fig. 6. Uniplex SYBR®Green Real time PCR amplification plot for detection of certain 6 virulence genes in *E. faecalis* strains. A: *asa1*; B: *GelE*; C: *cylA*; D: *esp*; E: *hy1*; F: *vanA*, and G: *vanB*

Molecular identification of *E. faecalis*

The qPCR method using the fast qPCR 7500 (Applied Biosystems) was used for detection of *E. faecalis* isolates. SYBR®Green qPCR was performed on DNAs extracted from seven reference strains as positive controls for the seven possible genes, including *asa1*, *GelE*, *cylA*, *esp*, *hy1*, *VanA*, *and VanB*. Specific primers designed using a reference for seven gene sequences representing *asa1, GelE, cylA, esp, hy1, VanA, and VanB* were collected from the GenBank database. As shown in fig. 6, all seven genes of *E. faecalis* were detected by qPCR.

DISCUSSION

Foot ulcer represents one of the major complications in diabetic patients and currently represent the most public diabetes-related cause of hospitalization. Patients suffering from diabetes have approximately a 25% chance of rising a foot ulcers in their life. DFIs lead to high morbidity rates and a lower extremity amputation may be resulted. Recently, DFIs represent the major cause of lower extremity amputations in many parts of the world [22].

In our study, 52 (8.25%) *E. faecalis* strains were recovered from 630 pus swabs from DFI. Similar results were obtained by [23,24], who stated that *E. faecalis are rarely encountered in DFIs.* Spichler et al. [22] established that the primary bacterial genera *in DFIs* were *Staphylococcus* (29.7%), *Peptoniphilus* (6.9%), *Rhodopseudomonas* (6.9%) and *Enterococcus* (6.4%). The incidence of *E. faecalis* in DFIs was also studied in Malaysia by Raja [25] who found that Gram-negative bacteria were the major isolated pathogens followed by *S. aureus* (44%), Group B *Streptococci* (25%), and *Enterococcus* spp. (9%). *Enterococci* are among the most common Gram-positive bacteria, and they are also characterized as significant nosocomial pathogens. Moreover, Edmonds [26], Mathangi and Prabhakaran [27], Roudbary et al. [28] and Sousa Lages et al. [29] reported that the frequency of *E. faecalis* in DFIs was 2%, 6.4%, 13.5% and 8.97%, respectively.

Recently, traditional techniques applied for microbial detection in the clinical biological laboratories have been based mainly on morphological and biochemical analyses, but these methods are still laborious, need multifaceted measures, and require huge quantities of biological agents, which is predominantly hard to accomplish for problematic microorganisms with unusual biochemical features [30]. Different molecular techniques, including *16S rRNA* gene sequencing, have been established as a compromise; nevertheless, they are not routinely applied as a result of their high price and high workload on technicians. Although, cultivation and other conventional methods have been established for identification of *E. faecalis*, numerous restrictions have been detected. Therefore, we used MBT as a rapid technique for detection of *E. faecalis* recovered from DFIs in Saudi Arabia. This technology is recently used as a potent technique for recognition of various pathogens [16,18,17,31].

In our study, recognition and clustering of *E. faecalis* strains were performed by proteomic fingerprinting and the occurrence of accurate identfication at the species level was 52/52 (100%) with the score levels of 2,000-3,000 Da. Similar findings were documented formerly by Werner et al. [19] who detected 64 *E. faecalis* and 37 *E. faecium* strains recovered from milk samples of mastitic cows by MBT. They established that the maximum identfication rate was 99.3%. Another study was conducted by Benagli et al. [32], who identified *E. faecalis* by MALDI-TOF MS with a confidence level of 100%. In addition, Griffin et al. [33] identified vancomycin resistant *E. faecalis* (VRE) stains by MBT with high identification rate of 96.7% and 98.1%, correspondingly.

In this context, the lower score values for two *E. faecalis* isolates at the species level (1.700 to 1.999) might be related to sample handling, and the amount of matrix in the sample were not accurate. Consequently, it is very significant to apply other standard methods such as PCR as an additional technique to support the suspected findings detected by MALDI-TOF MS [34]. The data of the present investigation were evaluated by MALDI-TOF MS, and it was confirmed that the furthest spectral peaks for the examined *E. faecalis* strains ranged from 3,000 to 10,000 Da. A similar range was obtained in numerous other studies on bacteriological recognition using MALDI-TOF MS [35,36,37]. Benagli et al. [32] indicated that MALDI-TOF MS is considered a trustworthy, economic, and rapid identification technique for clinically significant microorganisms, including *Enterococci*.

In addition, Pavlovic et al. [38] demonstrated that this technology has the ability to identify various pathogens through the generation of fingerprints of extremely plentiful proteins, followed by association with the reference spectra in the Bruker taxonomy. Other studies conducted by Pavlovic et al. [38] and Quintela et al. [39] stated that there are incomplete data on the application of this technique for identification of various strains of *Enterococci*. Moreover, Chabros et al. [40] showed that MBT may be a valuable instrument to discriminate between the species *E. faecium* and *E. gallinarum*. Although MALDI-TOF is considered an innovative method compared with the other biochemical methods, they found some complications with the identification of the *Enterococcus* species.

The cost and maintenance of this technique still represent the most important disadvantage of this method[13,38,41]. However, this technology is considered a potent, innovative method that has the possibility to replace classical identification methods for common routine isolates in the hopes of solving the problem of DFIs using an accurate and primary diagnostic technique.

In our investigation, we also applied an easy-to-use qPCR technique based on the amplification of fragments of *asa1, GelE, cylA, esp, hy1, VanA, and VanB* genes with SYBER green probe qPCR. This specific method permits identifying of the seven possible genes for *E. faecalis*. The seven fluorescence results are readable on a 7500 Fast Applied Biosystems. Based on our findings, the six *E.* faecalis genes were detected. The developed sensitivity of the qPCR technique could be explained by the fact that it possibly targets free-floating DNA and DNA from non-viable and cultivable viable cells [42]. In addition, our results were matched with the results obtained by Rocas et al. [43], who used a PCR method to detect *E. faecalis* in endodontic infections.

CONCLUSIONS

This study showed that several bacterial species were recovered from diabetic foot patients. *E. faecalis* was the most predominant single organism isolated from the lesions. These findings show the significance of *E. faecalis* in diabetic foot infections. *E. faecalis isolates* were found to carry several virulence genes, suggesting that additional researches on the dissemination and the possibility of virulent strains to carry these genes may be necessary. In addition, MALI Biotyper is a fast, trustworthy and a beneficial detection method for various microorganisms. Our outcomes may serve as a source for supplementary investigation studies of infections caused by this bacterium.

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