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From days to hours: Can MALDI-TOF MS system replace both conventional and molecular typing methods with new cut off level for Vancomycin Resistant Enterococcus *faecium*

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ABSTRACT

Vancomycin-Resistant *E. faecium* (VRE) strains from clinical specimens were identified by conventional methods before. Following the phenotype-based identification, all strains were also identified using both BD Phoenix and VITEK MS bioMérieux System. Strains were typed with the Bruker MALDI-TOF MS system, pulsed field gel electrophoresis (PFGE) and 16S rRNA gene sequencing analysis and then the sensitivity compared for each. A cut off value of 850 assigned with Bruker MALDI-TOF MS system was found to give equal sensitivity to that of PFGE. Results obtained were compared with those of molecular typing. The main advantage of MALDI-TOF MS technology over the others was the much shorter analysis time which lasted only a few hours rather than days or a whole week. Also, the Bruker MALDI-TOF MS system was used for typing and compared with the gold standard method and this study is first to report the determined cut off level for typing of VRE strains.

1. Introduction

Enterococci, a cause of nosocomial infections and endocarditis have developed resistant strains to a number of antibiotics including penicillin and vancomycin (Moellering, 2005; Linden et al., 1996; Murray, 2000). In order to maintain control and to prevent the spread of hospital-originated epidemics of Vancomycin-Resistant enterococci (VRE), carriers of VRE members have to be identified as fast as possible.

When we compared MALDI-TOF MS systems with phenotype-based diagnostic methods for identification, phenotype-based diagnostic methods based on relatively expensive and lengthy procedures, often produced ambiguous results (Risch et al., 2010). Molecular typing methods therefore serve as essential epidemiological tools for the identification of pathogens at subspecies level. MLST (multilocus sequence typing), MLVA (Multilocus variable-number tandem repeat analysis), PFGE (Pulsed Field Gel Electrophoresis) and RAPD (Random amplified polymorphic DNA) often provide very useful information on the epidemiology of VRE infections (Chuang et al., 2010; Sabat et al., 2013; De Angelis et al., 2014). PFGE is considered the gold standard typing method since it often yields reproducible results and also because of its high discriminatory power (Liu et al., 2016; Swaminathan et al., 2006). This method on the other hand has limited sample size, and requires optimization, and much longer periods of experiment time (Werner et al., 2015). The typing technology used in this study was a cost-effective Bruker Maldi-TOF MS system which can identify bacterial strains at subspecies level much faster and with adequate accuracy (Hsu and Burnham, 2014; Tekippe and Burnham, 2014). The system produces results within a matter of minutes using intact microorganisms and relies on the differences in protein content between organisms. The differences were read as the mass to charge ratio of each protein (Dieckmann et al., 2010; Schröettner et al., 2014). In this study, we established a cut off level for identifying VRE members. Clinicians may solve hospital infectons' source with Bruker Maldi TOF MS instead of expensive and hard classification methods.

2. Materials and methods

2.1. Bacterial isolates and antimicrobial susceptibility testing

The VRE isolates used in this study were frozen strains of clinical

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Table 1
General features of Vancomycin-Resistant *Enterococcus faecium* isolates (M: Male; F: Female).

Strain No	Gender	Patient date of birth	Examples acquisition date	Polyclinic/service
ST1	M	14.02.2011	12.02.2014	Pediatry Service
ST2	F	15.09.2014	12.01.2015	Pediatry Service
ST3	M	01.04.1998	05.03.2010	Pediatry Service
ST4	F	30.09.2014	13.01.2015	Pediatry Service
ST 5	M	21.01.1960	06.09.2013	Anesthesia Intensive Care Unit
ST 6	M	07.09.1993	17.10.2013	Pediatry Service
ST 7	F	21.03.1932	05.06.2010	Emergency Poly.
ST 8	M	19.11.2009	31.01.2014	Anesthesia Intensive Care Unit
ST 9	F	12.09.2014	12.01.2015	Pediatry Service
ST 10	F	09.04.1999	30.03.2014	Pediatry Service
ST 11	F	24.06.1998	21.04.2011	Internal diseases service
ST 12	F	15.02.1995	07.02.2013	Anesthesia Intensive Care Unit
ST 13	M	07.03.2010	11.01.2013	Pediatry Service
ST 14	M	13.06.1963	19.11.2012	Hemodialyzer
ST 15	F	24.06.1998	21.04.2011	Internal diseases service
ST 16	F	05.02.2011	05.02.2011	New born ICU
ST 17	F	08.03.2013	15.12.2012	Pediatry Service
ST 18	M	05.11.1947	06.09.2013	Anesthesia Intensive Care Unit
ST 19	M	01.01.2009	18.10.2011	New born ICU
ST 20	M	01.07.1928	19.09.2013	Chest Diseases Service
ST 21	M	21.02.1949	04.02.2013	Nephrology Service
ST 22	M	01.07.1928	19.09.2013	Chest Diseases Service
ST 23	F	01.01.1965	23.09.2013	Rheumatology
ST 24	M	22.09.2006	14.01.2015	Pediatry Service
ST 25	F	09.04.1999	29.01.2013	Pediatry Service
ST 26	M	10.06.2011	23.07.2012	Pediatry Service
ST 27	F	23.04.2010	10.09.2013	Pediatry Service
ST 28	F	26.07.2009	13.01.2015	Pediatry Service
ST 29	M	05.03.1978	15.08.2011	Anesthesia Intensive Care Unit
ST 30	F	25.11.1942	18.09.2013	Nephrology Services
ST 31	F	09.02.2011	20.01.2011	New born ICU
ST 32	F	06.03.1950	27.10.2013	Internal diseases service
ST 33	F	15.04.2014	15.09.2014	New born ICU
ST 34	F	15.06.2009	20.07.2010	Pediatric Emergency
ST 35	M	25.07.2014	12.01.2015	Pediatry Service
ST 36	F	02.02.2012	16.09.2014	Pediatry Service
ST 37	M	27.01.2005	12.09.2013	Anesthesia Intensive Care Unit
ST 38	M	15.02.2002	15.09.2015	Anesthesia Intensive Care Unit
ST 39	M	01.07.1928	20.02.2015	Urology Sevice
ST 40	M	19.07.1961	07.02.2013	cardiology care unit
ST 41	F	08.03.2013	15.12.2012	Pediatry Service
ST 42	F	28.07.2013	30.20.2013	Anesthesia Intensive Care Unit
ST 43	M	02.03.2010	02.03.2010	New born ICU
ST 44	M	30.12.2014	30.12.2014	Anesthesia Intensive Care Unit
ST 45	F	07.10.1938	17.10.2010	Medical Oncology
ST 46	M	04.07.1944	13.01.2015	Urology Servıce
ST 47	M	31.03.2011	31.03.2011	New born ICU
ST 48	F	21.08.2014	06.10.2013	New born ICU
ST 49	F	05.06.2010	02.11.2010	Hemodialysis
ST 50	F	01.06.1978	13.12.2012	New born ICU
ST 51	M	09.09.2014	14.01.2013	Pediatry Service
ST 52	M	04.07.1944	13.01.2015	Urology Servıce
ST 53	F	07.03.1952	01.09.2010	Internal Medicine Services
ST 54	M	07.11.2010	18.01.2011	neurosurgery service
ST 55	F	07.03.1952	01.09.2010	Internal Medicine Services
ST 56	M	15.09.2014	15.09.2014	New born ICU
ST 57	M	01.03.2008	11.09.2012	Pediatric Hematology Services
ST 58	M	04.12.2013	12.12.2013	New born ICU
ST 59	F	02.11.2010	20.01.2011	Pediatry Service
ST 60	M	01.07.1933	04.08.2014	Anesthesia Intensive Care Unit
ST 61	F	15.04.2014	15.09.2014	New born ICU

specimens, isolated and identified by conventional methods at the Clinical Microbiology Laboratory Turkey. The isolates were first grown on 5% sheep-blood Columbia agar (Becton Dickinson, MD, USA). Colonies were picked and the cells were subjected to a number of morphological, physiological, and biochemical tests: motility, Gram staining, catalase activity, growth in 6.5% NaCl and in the presence of tellurite, esculin hydrolysis, pigment production, arginine hydrolysis, the profile of sugar (arabinose, insulin, mannitol, lactose, raffinose, sorbitol, sorbose, and sucrose) fermentation, and production of acid from methyl-alphaD-glucopyranoside (MGP) (Table 1). The strains that were considered to belong to the *Enterococcus* spp. were then analyzed using a BD Phoenix automated identification system (Becton Dickinson). Susceptibility testing for Vancomycin-Resistance was performed by agar dilution according to Clinical and Laboratory Standards Institute criteria (CLSI, 2009).

2.2. BD Phoenix automated identification system

The BD Phoenix™ automated identification and susceptibility testing system provides rapid, accurate and reliable detection of known and emerging antimicrobial resistance. The V3.34A/V3.54A software was used for this study and the ID was coated with bacterial colonies adjusted to the 0.5 McFarland standard using a CrystalSpec nephelometer (BD Diagnostics) according to the manufacturer's recommendations. The suspension was then discarded on the ID side of the Phoenix panel. After the instance name was saved to the system, it was uploaded to the device within the specified time. Control procedures were performed according to the manufacturer's recommendations.

2.3. VITEK MS bioMérieux system

Following the phenotype based identification, all strains were also identified using the VITEK MS system. In the system, a small amount of bacteria which was taken from isolated colonies from plates using a disposable 1 µL loop was inoculated as a thin layer into the separated areas on single-use target slides (bioMérieux, France). Then, 1 µL of α-cyano-(Cetinkaya et al., 2000)hydroxy cinnamic acid (CHCA) matrix solution (bioMérieux) was added to the inoculated bacteria and air dried. The inoculated isolates were first covered with 0.5 µL of formic acid, air dried, and then covered with 1 µL of matrix and air dried again. The prepared slides were processed in the VITEK MS device and the obtained spectra were automatically analyzed via the MYLA software (bioMérieux) (Karagoz et al., 2015).

2.4. Bruker MALDI-TOF mass spectrometry (MS) system

MALDI-TOF MS measurement was performed using an ethanol-formic acid extraction procedure. A loopful of bacterial material was suspended in 300 µL distilled water, and 900 µL ethanol were added. The cell suspension was centrifuged for 2 min at 17,000 × *g*, and the supernatant was discarded. The centrifugation was repeated, and the residual ethanol was discarded. The pellet was air dried and thoroughly resuspended in up to 50 µL formic acid-water (70:30, vol/vol) depending on the size, and finally, an equal volume of acetonitrile was

added. After centrifugation for 2 min at 17,000 × *g*, 1 µL of the supernatant was transferred to the MALDI target plate and allowed to dry at room temperature before being overlaid with 1 µL of matrix solution. The acquisition and analysis of mass spectra were performed in a Microflex LT mass spectrometer (Bruker Daltonik, Germany) using the MALDI Biotyper software package (version 3.0) with the reference database version 3.1.2.0 (3995 database entries). Scores of ≥2.0 were accepted for species assignment and scores of ≥1.7 but < 2.0 for identification to the genus level. Scores below 1.7 were considered unreliable.

2.5. Pulsed field gel electrophoresis

Bacterial cells were lysed in agarose plugs as described (Morrison et al., 1999). Chromosomal DNA in each plug was digested with 40 U *Sma*I (Fermentas, Life Technology, USA) and was run in a CHEF DR II system at 6 V cm^{-2} for 20 h with (Chao et al., 2014)30 s pulse times at 14 °C Gozalan et al. (2015).

The image of the DNA banding patterns was used for UPGMA (Unweighted pair group method with arithmetic mean) analysis, based on Dice's coefficient (Bionumerics software version 6.0, Applied Maths, Inc., Belgium). The analysis was performed at 1% tolerance and 1% optimization. According to the Dice similarity coefficient, > 95% similarities were accepted as the same PFGE types; between 95% and 80%, closely related; and < 80%, as clearly different patterns. Results were interpreted as indicated (Tenover et al., 1995).

2.6. 16S rRNA gene sequencing analysis

The bacterial isolates were further identified by16S rRNA gene sequencing (Beckman Coulter Inc., USA). The sequences from 16S rRNA gene PCR products that were generated using universal bacterial primers (8F, 5′-AGAGTTTGATCCTGGCTCAG-3′, and 787R, 5′-CGACTAC CAGGGTATCTAAT-3′) were used to determine the identities of the *E. faecium* isolates (Ryu et al., 2013). The procedure was modified from Ryu et al., 2013. Nucleotide comparisons were performed using the GenBank database and the BLAST algorithm. Similarity of > 99% between the query- and reference sequence proved that the isolates belonged to *E. faecium* and 1% identity is to *E. durans*. The aim of 16S rRNA gene sequencing analysis was confirmation of the results of BD Phoenix, VITEK MS (bioMérieux) and Bruker MALDI-TOF MS at the genus and species level.

3. Results

3.1. Genus and species identification

According to the 16S rRNA sequencing results, all of the 61 isolates were determined to be *E. faecium.* BD Phoenix, VITEK MS and Bruker MALDI-TOF MS correctly identified 100% (61/61) isolates at the genus level and 78.6% (48/61) at the species level. BD Phoenix system misidentified 21.3% (13/61) of the organisms at species level. VITEK MS system on the other hand identified 96.7% (59/61) of the isolates at species level according to the 16S rRNA sequencing results, and misidentified 3.2% (2/61) of the members as *E. gallinarum*. Notably, all of the 61 isolates were determined to be *E. faecium* by MALDI-TOF MS system (Table 2). So that, this system was found to have the strongest discriminating power at the genus and species level (Fig. 1).

3.2. Genotyping by PFGE

An analysis of genomic DNA by the PFGE was used to characterize clonal diversity and relationships among VRE members in the local hospital. After the VRE members were examined by the PFGE method using *Sma*I macrorestriction enzyme-digested DNA, profiles containing between (Griffin et al., 2012)20 bands were obtained. The clustering rate was (77.04%) (47/61) and 16 PFGE types and two PFGE groups (A and B) were recognized based on the enforcement to a similarity coefficient higher than 80%. This rate is also in accordance with the differences in band patterns when the Tenover et al. (1995) criteria are considered. Group B was the most prevalent in the VRE members, whereas only three isolates were recognized in Group A. These results emphasized that a VRE members circulates within the hospital, resulting in ongoing transmission among the clinics. However, a different VRE members in the urology clinic had been identified in 2015, and transmission to other clinics has not yet been determined. The PFGE dendrogram is presented in Fig. 2.

Table 2
Identification results level of species for four different systems.

Strain no	BD Phoenix identification	VITEKMS identification	MALDI-TOF MS (Bruker) identification	16S rRNA gene sequencing
13	*E. gallinarum*	*E. faecium*	*E. faecium*	*E. faecium*
20	*E. gallinarum*	*E. faecium*	*E. faecium*	*E. faecium*
26	*E. gallinarum*	*E. faecium*	*E. faecium*	*E.faecium*
32	*E. feacalis*	*E. feacalis*	*E. faecium*	*E. faecium*
41	*E. feacalis*	*E. faecium*	*E. faecium*	*E. faecium*
43	*E. casseliflavus*	*E. faecium*	*E. faecium*	*E. faecium*
50	*E. gallinarum*	*E. faecium*	*E. faecium*	*E. faecium*
54	*E. feacalis*	*E. faecium*	*E. faecium*	*E. faecium*
19	*E. gallinarum*	*E. faecium*	*E. faecium*	*E. faecium*
28	*E. gallinarum*	*E. faecium*	*E. faecium*	*E. faecium*
50	*E. feacalis*	*E. feacalis*	*E. faecium*	*E. faecium*
59	*E. feacalis*	*E. faecium*	*E. faecium*	*E. faecium*

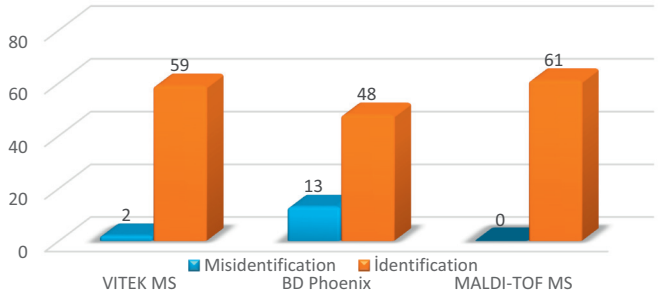


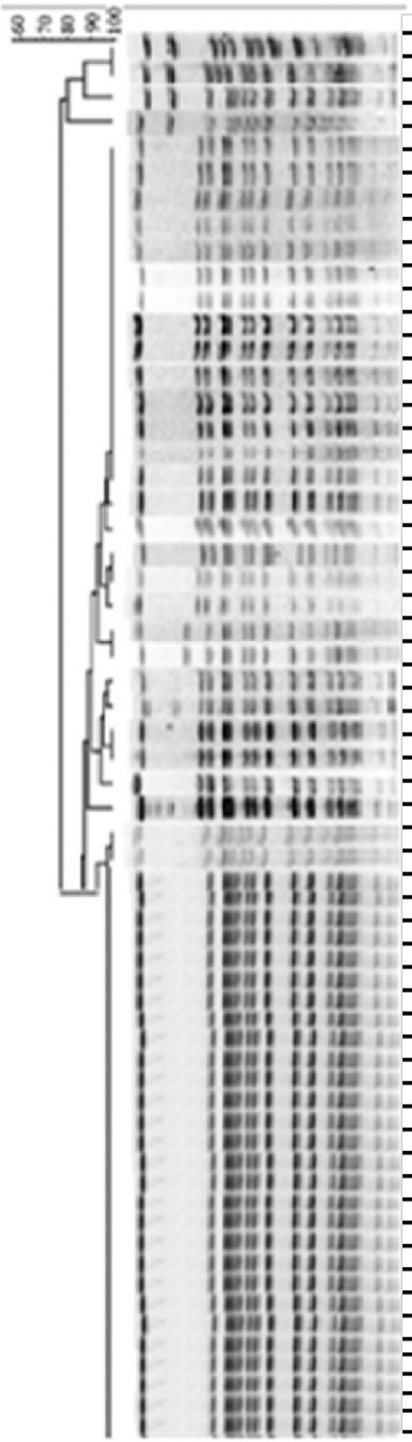
Fig. 1. Identification and misidentification levels of three automated system.

3.3. Dendrograms by MALDI-TOF MS

A dendrogram was obtained with the cut-off value of 850 by using the mass spectrometry results of the 61 VRE members (Fig. 3). As can be seen the isolates were divided into two main groups: the larger group was further divided into four sub-groups, and the smaller one had only three of the isolates.

3.4. Comparison of MALDI-TOF MS and PFGE Dendrograms

The dendogram obtained from MALDI-TOF MS cluster analysis and the PFGE dendogram were matched in Fig. 4. In here, when we designated cut off value of 850 for Vancomycin-Resistant *E. faecium*, a similar discriminatory ability was observed between MALDI-TOF MS and PFGE assignments Cluster separation in PFGE dendogram was performed according to the Tenover criteria (Tenover et al., 1995). When the similarity rate coincided with the criteria, values below 80% were accepted as isolates of different clones. The cut-off values are quite important in determining the clonal relationships by the MALDI-TOF MS Bruker system; these values must be compared with those of different methods in regard to microorganisms, and thus they have to be optimized.



Strain No	PFGE Type	PFGE Group	Gender	Sample received date	The relevant departments
ST 46	1	A	F	06.09.2013	Anesthesia Intensive Care Unit
ST 52	1	A	M	04.08.2014	Anesthesia Intensive Care Unit
ST 39	2	A	M	19.09.2013	Chest Diseases Service
ST 33	3	B	F	15.09.2014	New born ICU
ST 50	4	B	F	13.12.2012	New born ICU
ST 26	4	B	M	23.07.2012	Pediatry Service
ST 5	4	B	M	13.01.2015	Urology Service
ST 32	4	B	F	27.10.2013	Internal diseases service
ST3	4	B	M	05.03.2010	Pediatry Service
ST 41	4	B	F	15.12.2012	Pediatry Service
ST 37	4	B	M	12.09.2013	Anesthesia Intensive Care Unit
ST 49	4	B	F	02.11.2010	Hemodialysis
ST 8	4	B	M	31.01.2014	Anesthesia Intensive Care Unit
ST 20	4	B	M	20.12.2010	Urology Service
ST 22	4	B	M	19.09.2013	Chest Diseases Service
ST 45	4	B	F	17.10.2010	Medical Oncology
ST 43	4	B	M	02.03.2010	New born ICU
ST 35	4	B	M	12.01.2015	Pediatry Service
ST 47	5	B	M	31.03.2011	New born ICU
ST 11	6	B	F	21.04.2011	Internal diseases service
ST 18	7	B	M	06.09.2013	Anesthesia Intensive Care Unit
ST 42	7	B	F	30.20.2013	Anesthesia Intensive Care Unit
ST 9	8	B	F	12.01.2015	Pediatry Service
ST 51	9	B	M	14.01.2013	Pediatry Service
ST 38	9	B	M	15.09.2015	Anesthesia Intensive Care Unit
ST 25	10	B	F	29.01.2013	Pediatry Service
ST 59	11	B	F	20.01.2011	Pediatry Service
ST 24	12	B	M	14.01.2015	Pediatry Service
ST 12	12	B	F	07.02.2013	Anesthesia Intensive Care Unit
ST 14	13	B	M	19.11.2012	Hemodialyzer
ST2	14	B	F	12.01.2015	Pediatry Service
ST 31	15	B	F	20.01.2011	New born ICU
ST 10	15	B	F	30.03.2014	Pediatry Service
ST 19	16	B	M	18.10.2011	New born ICU
ST 44	16	B	M	30.12.2014	Anesthesia Intensive Care Unit
ST 56	16	B	M	15.09.2014	New born ICU
ST 30	16	B	F	18.09.2013	Nephrology Services
ST4	16	B	F	13.01.2015	Pediatry Service
ST 16	16	B	F	05.02.2011	New born ICU
ST1	16	B	M	12.02.2014	Pediatry Service
ST 6	16	B	M	17.10.2013	Pediatry Service
ST 23	16	B	F	23.09.2013	Rheumatology
ST 29	16	B	M	15.08.2011	Anesthesia Intensive Care Unit
ST 15	16	B	F	21.04.2011	Internal diseases service
ST 54	16	B	M	18.01.2011	neurosurgery service
ST 28	16	B	F	13.01.2015	Pediatry Service
ST 58	16	B	M	12.12.2013	New born ICU
ST 61	16	B	F	15.09.2014	New born ICU
ST 13	16	B	M	11.01.2013	Pediatry Service
ST 55	16	B	F	01.09.2010	Internal Medicine Services
ST 7	16	B	F	05.06.2010	Emergency Poly.
ST 60	16	B	M	13.01.2015	Urology Service
ST 57	16	B	M	11.09.2012	Pediatric Hematology Services
ST 21	16	B	M	04.02.2013	Nephrology Service
ST 17	16	B	F	15.12.2012	Pediatry Service
ST 36	16	B	F	16.09.2014	Pediatry Service
ST 48	16	B	F	05.10.2013	New born ICU
ST 53	16	B	F	01.09.2010	Internal Medicine Services
ST 27	16	B	F	10.09.2013	Pediatry Service
ST 40	16	B	M	18.07.1951	cardiology care unit
ST 34	16	B	F	15.06.2009	Pediatric Emergency

Fig. 2. Dendogram of the *Sma*I PFGE typing of 61 Vancomycin-Resistant *Enterococcus faecium* (M: Male; F: Female).

4. Discussion

Enterococi are very significant pathogens because they often present as opportunistic background flora in hospitals. Members of *E. faecium* are recognized as the most common nosocomial pathogens worldwide and this has paralleled the frequency of antibiotic resistant strains (Gozalan et al., 2015; Cattoir and Leclercq, 2013). > 20% of the enterococci isolated from intensive care units are resistant to vancomycin (Cattoir and Leclercq, 2013). The incidences of Vancomycin-Resistant *E. faecium* strains are 20% to 40% in the United States, and above 10% in some of the European countries (Cetinkaya et al., 2000). The highest prevalence of Vancomycin-Resistant Enterococci (VRE) in Europe (2007) is in Greece and Portugal (45%) (Werner et al., 2008). It is therefore important to detect hospitalized patients that have VRE members (Arslan et al., 2013).

As was mentioned previously, phenotypic characterisation methods are often lengthy experimental procedures and may produce ambiguous results especially in the identification of Enterococci. This necessitated the adoption of automated or semi-automated systems in diagnostic studies for detection. Such systems are often based on the analysis of macro molecules such as DNA, lipids or proteins (Cekin et al., 2013).

This study shows direct comparison between the two automated

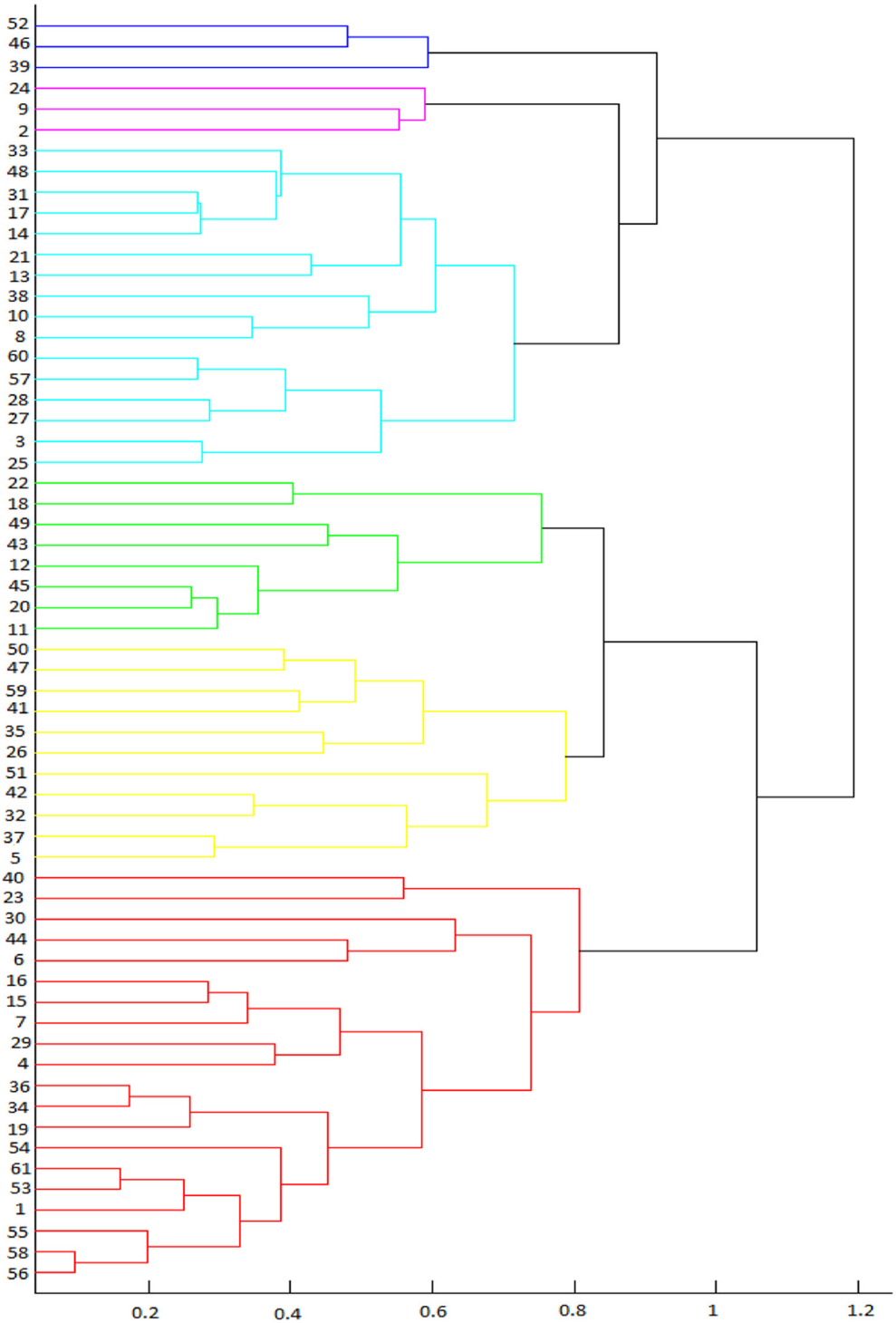


Fig. 3. MALDI-TOF MS dendogram for tested isolates with cut-off value of 850.

systems, VITEK-2 and BD Phoenix for the identification of *E. faecium* at the species level. A few similar studies have been reported for other bacteria, such as *Staphylococcus saprophyticus* (Lee et al., 2013; Kim et al., 2008; Chatzigeorgiou et al., 2011). It has been reported that MALDI-TOF identified 30 urinary isolates with 100% accuracy, followed by the VITEK 2 system with 93.3%. The efficiencies of two other systems, MicroScan GP33 and BBL CHROMagar Orientation Phoenix ID were 86.7% and 46.7%, respectively. In another study, the efficiencies of MALDI-TOF MS (Bruker) and BD Phoenix and VITEK-2 were compared for the identification of coagulase-negative Staphylococci and the former system was found to be more accurate (93.2%) (Dupont et al., 2010). Other studies have also produced similar results (Chao et al., 2014; Saffert et al., 2011). Schlebusch et al., compared whole genome sequencing (WGS) and MALDI-TOF MS genotyping methods for investigation of *E. faecium* outbreaks. WGS has the highest level of discrimination, but is not generally suitable for outbreak investigation because of cost and turnaround time. However, MALDI-TOF MS is a suitable and attractive method for typing because of its rapid times and low cost (Schlebusch et al., 2016). Griffin et al., used the MALDI- TOF MS method to identify Vancomycin-Resistant Enterococci and

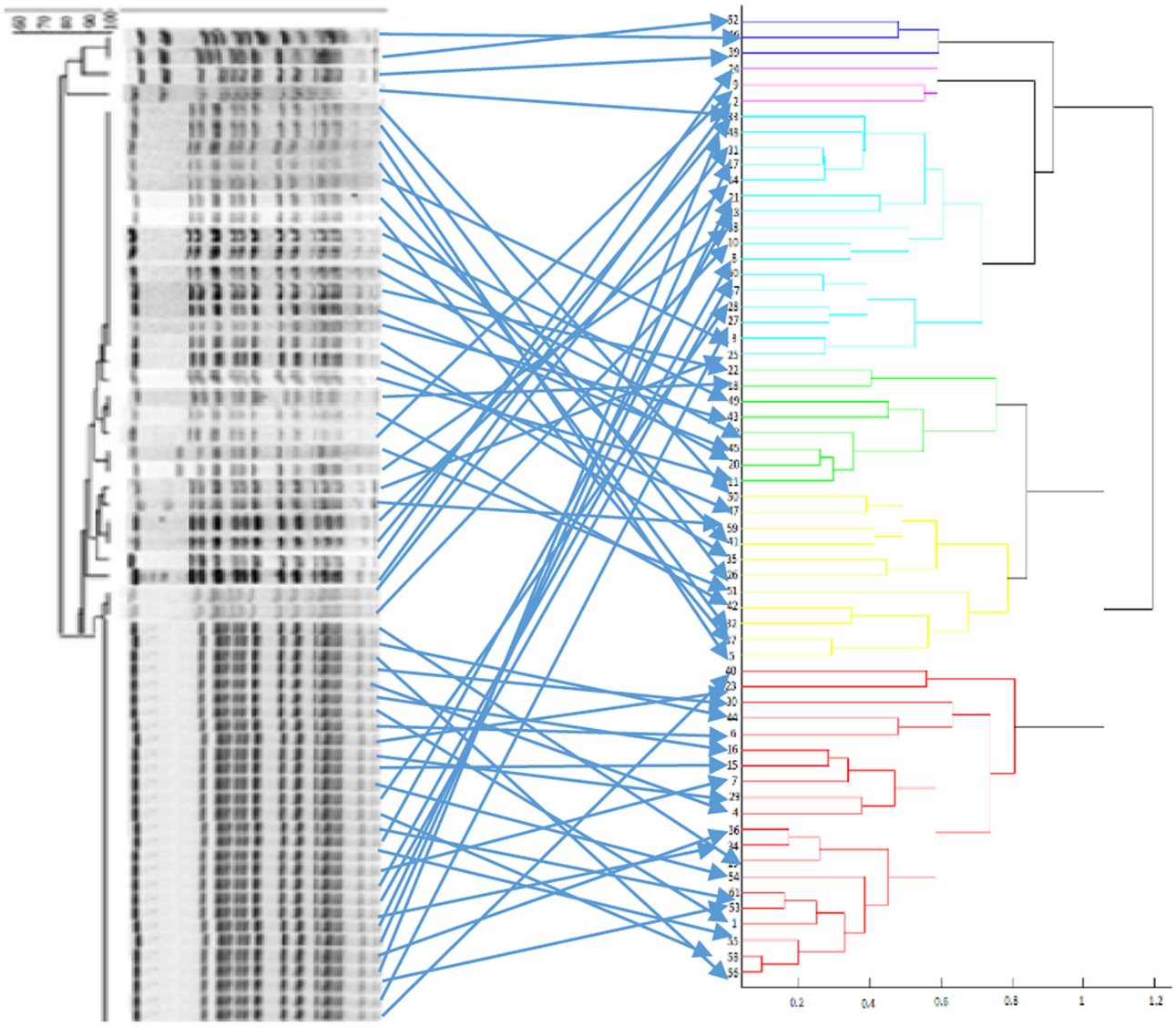


Fig. 4. Comparison between PFGE (left) and the MALDI-TOF MS dendogram using the protein extraction method (right).

investigate the epidemiology of an outbreak. Also they compared only 4 unrelated isolates with both this system and PFGE typing method. According to them, typing is possible with MALDI-TOF MS and it should be more cost effective than PFGE (Griffin et al., 2012). When we compare this study with ours, we compared all isolates with both methods and our aim is detect a cut of level for typing *E. faecium* without the need of PFGE method.

In order to determine the epidemiological characteristics of microorganisms; clonal relationships between the isolates also have to be demonstrated by molecular methods. Here, the PFGE method has been accepted to be the "gold standard" due to its high discriminatory power and the reproducibility of its results (Swaminathan et al., 2006).

In this study, the results indicate that MALDI-TOF MS could distinguish easily and reliably the *E. faecium* isolates. It also shows that MALDI-TOF MS technology is able to indicate the relatedness of *E. faecium* strains to a similar degree as the gold standard method.

Conflict of interest

The authors have declared that no competing interests exist.

Author contributions

Conceived and designed the experiments: SS, AK, GH. Performed the experiments: GH, AK, MP, Analyzed the data: SS, AK, GH. Wrote the manuscript: SS.

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