이학 박사학위 논문

Comparison of Multilocus sequence typing change patterns of Vancomycin resistant *Enterococcus faecium* in the past nine years

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I submit this thesis as the Doctoral thesis in Medical Sciences.

2, 2018

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The Doctoral thesis of JOON KIM in Medical Sciences is hereby approved.

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Ajou University Graduate School
December, 22th, 2017

- Abstract-

Comparison of Multilocus sequence typing change patterns of Vancomycin resistant *Enterococcus faecium* in the past nine years

Background : To understand the spread of Vancomycin Resistant Enterococcus (VRE) is an important component of hospital infection control measures. Multilocus sequence typing (MLST) is useful in determining the long–term evolutionary process and minimizes differences in experimental results across individuals and laboratories. It is also useful in determining evolutionary origins and backgrounds of bacterial species. This study carries out MLST analysis on *vanA* Vancomycin resistant *Enterococcus faecium* isolated from patient specimen in a single university hospital in the past nine years in order to observe changes in genetic evolution over time.

Method : During the years from 2007 to 2015, 45 clinical isolates of *vanA*-containing *E. faecium* were collected from Ajou university hospital in Korea. Species were identified through the VitekII system (BioMerieux, Hazelwood, MO) and antibiotic susceptibility testing was performed by disk diffusion and E-test according to Clinical and Laboratory Standards Institute (CLSI) guidelines. To determine genetic relatedness, Matrix Assisted Laser Desorption Ionization-Time Of Flight mass spectrometry (MALDI-TOF MS) was employed. The multilocus sequence types (MLST) were determined to characterize the clonal diversity of the *vanA* Vancomycin resistant *Enterococcus faecium* isolates.

Result : All isolates were highly resistant to ampicillin, teicoplanin, ciprofloxacin, and vancomycin whereas they were most susceptible to linezolid and quinupristin-

dalfopristin. 45 clinical isolates were genetically unrelated according to MALDI-TOF MS analysis. MLST showed that the clinical isolates had 7 sequence types (ST), ST17(n=19) being the most common, followed by ST78(n=13), ST192(n=6), ST64(n=4), ST262(n=1), ST414(n=1) and ST981(n=1).

Conclusion : The MLST analysis showed that the sequence types of most isolates belonged to clonal complex 17(CC17). This is consistent with outbreaks in hospitals. The study found that the most common type of separation was changed from ST78 to ST17. We had single observations for ST262, ST414 and ST981 but they appear to be random occurrences. MLST can be useful for speculating the genetic evolution of *vanA* containing *E. faecium* isolates.

Keywords : MLST, ST, MALDI-TOF MS, *E,faecium*

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Ⅰ. Introduction

Since vancomycin-resistant enterococci (VRE) were first reported in Europe in 1986, the frequency of isolation has increased worldwide to date, and nowadays it is indigenous to almost all medical institutions. In Korea, the incidence of *Enterococcus durans*, which is highly resistant to vancomycin, has been reported to have increased up to the present time after being reported by Park et al. [1] in 1992. Although enterococci are relatively weakly pathogenic, infections caused by VRE are often found in long-term inpatients with impaired immunity, and the treatment is not effective and the mortality rate for VRE bacteremia is approximately 37% [2]. In particular, VanA type VRE is highly resistant to vancomycin and is difficult to treat, leading to high mortality from bacteremia [3,4].

The VRE resistant types known to date are VanA, VanB, VanC, VanD, VanE, VanG, VanL, VanM and VanN, and of them, VanA and VanB types account for most of the global epidemic [5-8]. In Korea, the incidence of VRE is similar to that of the United States, which is spreading to hospital infections. Therefore, it has begun to occur mainly in large hospitals and to occur recently in small hospitals, showing aspects of indigenization [9]. VRE, which is the subject of hospital infection control, is VanA type and VanB type, which are proven resistant, and *vanA* or *vanB* gene is transferred among VanC type, so that the same VRE is included in the subject [10]. Molecular genetic epidemiological analysis is usually directed to VanA type *Enterococcus faecium* (*E. faecium*). Traditionally, pulsed field gel electrophoresis (PFGE) has been used to analyze VRE propagation patterns. This reflects only short-term genetic correlations and the standardization of test methods has not been established so that comparisons between laboratories are difficult. Also long-term

genetic correlations and gene evolution cannot be detected. Therefore, molecular genetic epidemiological methods other than PFGE were introduced.

Multilocus sequence typing (MLST) analyzes the sequence of 6 to 7 housekeeping genes (450-500 bp) containing information on the production of proteins involved in essential cell functions and is available on the MLST website (http: // www. mlst.net) for analysis of genetic diversity and type determination (ST). The different sequences of the house keeping genes are distinguished by alleles, which provide an allelic profile and serve as identification markers for strain typing. In the case of *E. faecium*, seven genes including *adk* (adenylate kinase), *atpA* (ATP synthase, alpha subunit), *ddl* (D-alanine:D-alanine ligase), *gyd* (glyceraldehyde-3-phosphate dehydroge nase), *gdh* (glucose-6-phosphate dehydrogenase), *purK* (phosphoribosylaminoimidazol e carboxylase ATPase subunit), *pstS* (phosphate ATP-binding cassette transporter), etc. are used. Bacterial identification by MLST has sufficient resolution to distinguish even very closely related strains. The MLST method reflects the long-term dynamics of genes and is a method for estimating the epigenetic origins and evolutionary backgrounds of the same strains without error between experimenter and laboratory [11].

Matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) was developed for the analysis of polymeric materials in the 1980s. This method is known to be suitable for biopolymers and synthetic polymers which are massive and unstable in heat, by vaporization/ionization without decomposition of the sample [12,13,14]. The principle of analysis is to detect the ion of the analysis target itself and to predict the mass of the analysis target. It uses the post-source-decay method to spontaneously proceed with peptide degradation and ionization in the flight tube, and peptides or proteins can be

identified by providing direct information about the sequence of an unknown peptide or protein (Fig. 1).

MALDI-TOF MS can be divided into a laser desorption ionization stage, which irradiates the sample with a strong pulsed laser to disintegrate the sample-matrix to release ions, and a Time of Flight (TOF) stage to measure ion flight time. The TOF method can be classified into linear TOF using the principle that light ions arrive at the detector before heavy ions and reflectron TOF using the principle that high-speed ions fly longer distances in the reflectron [15]. By using MALDI-TOF MS analysis, the strains can be identified by comparing the protein information of the bacteria to be known with the information of each strain already constructed. It has been proven that the efficiency of the classification and identification of the microorganisms in the hospital is high, and it has been used to identify *S. aureus, E. coli, Klebsiella,* and *Salmonella* other than *Enterococci,* and to verify the classification model [16]. Thus, on the target of *vanA* vancomycin-resistant *E. faecium* that was isolated for 9 years in a university in Korea, this study attempted to compare the results of analyzing MALDI-TOF M/S and MLST and investigate the characteristics of house-keeping gene variation. Therefore, this study tried to find out the prevalence of strains belonging to the CC17 in hospital and to use it as a useful data for management to reduce it.

1. The target slide is prepared and introduced to a high-vacuum environment.
2. A precise laser burst ionizes the sample.
3. A "cloud" of proteins is released and accelerated by an electric charge.
4. After passing through the ring electrode, the proteins' Time of Flight is recorded using a formula from the time recorded.
5. Proteins are detected with a sensor to create a spectrum that represents the protein makeup of each sample.

Fig. 1 Schematic diagram showing the work-flow in a MALDI-TOF MS.
(http://www.biomerieux-diagnostics.com/vitek-ms).

Ⅱ. MATERIALS AND METHODS

A. Research Materials

1. Target strains

From a *vanA* vancomycin-resistant *E. faecium* isolated from a urine culture study commissioned to the Department of Diagnostic Medicine, Ajou University hospital for 9 years between 2007 and 2015, 5 strains were collected every year from 2007 to 2015, and a total of 45 strains (strain code name vanA 01 ~ vanA 45) were tested. To determine the long-term dynamics of the gene of interest, the target strains were selected based on similar conditions, if possible. The isolated strains were collected from urine specimens that were relatively easy to obtain, and the 45 strains of *vanA* vancomycin-resistant *E. faecium* isolated at 2-3 month intervals a year from 2007 to 2015 were collected.

Each strain was tested for vancomycin resistance using brain heart infusion (BHI) agar containing 6 μg/mL vancomycin. All strains identified as VRE were identified using the Vitek Ⅱ system (BioMerieux, Hazelwood, MO) and biochemical techniques. *E. faecium* BM4147 was used as a positive control strain and vancomycin susceptible *E. faecalis* (ATCC 29212) and vancomycin susceptible *E. faecium* 2 strains were used as negative control strains.

B. Research Methods

1. Antimicrobial susceptibility test

The antimicrobial susceptibility test for the target strain was performed by disk diffusion method for vancomycin, ampicillin, tetracycline, teicoplanin, ciprofloxacin, linezolid, and quinupristin-dalfopristin according to the method described by Clinical and Laboratory Standards Institute (CLSI) M100-S26. The target strain was suspended in a Mueller hinton broth with a MacFarland nephelometer to a turbidity of 0.5, inoculated on a Mueller-Hinton medium, incubated with each antimicrobial disc, incubated for 16-18 hours, and then the inhibition band was measured. The minimum inhibitory concentration (MIC) for vancomycin and teicoplanin was measured using an E-test strip (BioMérieux, Marcy-l'Etoile, France). Table 1 shows the tolerance standards used for each antimicrobial agent.

Table 1. Criteria of Antisusceptibility test (CLSI 2016 M100-26)

Antimicrobial agent	Disk content	Zone diameter Interpretive Criteria (nearest whole mm)			MIC Interpretive Criteria (μg/mL)		
		S	I	R	S	I	R
Ampicillin	10 μg	≥17	-	≤16			
Tetracyclin	30 μg	≥19	15-18	≤14			
Teicoplanin	30 μg	≥14	11-13	≤10	≤8	16	≥32
Vancomycin	30 μg	≥17	15-16	≤14	≤4	8-16	≥32
Ciprofloxacin	5 μg	≥21	16-20	≤15			
Linezolid	30 μg	≥23	21-22	≤20			
Quinupristin-dalfopristin	15 μg	≥19	16-18	≤15			

2. Real-time PCR

Real-time PCR was performed according to the manufacturer's method using the Anyplex™ VanR Real-time Detection kit (Seegene, Sankt Ingbert, Germany) to confirm the vancomycin-resistant genotype of the target strain. The method was as follows: Brain heart infusion broth containing Vancomycin 6 μg/mL was inoculated with target strains and cultured at 35°C for more than 1 day. The DNA extraction solution in the kit was vortexed and added 200 μL into a 1.5 mL microcentrifuge tube. 50 μL of cultured broth was added to a 1.5 mL microcentrifuge tube. Tightly closed microcentrifuge tube using cap lock were vigorously vortexed and boiled for 10 minutes in heat block (100℃). After incubation at room temperature for 5 minutes, the tube centrifuged at 13,000 rpm for 5 minutes. 100 μL of supernatant transferred to a new tube, and used 3 μL for PCR. For PCR, added 17 μL of PCR Mastermix [Seegene, Sankt Ingbert, Germany: 4 μL 5X Van OM, 3μL 8- MOP solution, 10 μL 2X detection mix] and 3 μL of sample DNA in a PCR tube and mixed softly and briefly centrifuged. PCR was performed at 95°C for 2 min, 1 cycle at 95°C for 30 sec, and 45 cycles at 60°C for 30 sec using the CFX96™ Real-time PCR System (Bio-Rad Laboratories, Inc, Hercules, CA, USA.). The target analyte and Fluorophore used are shown in Table 2.

Table 2. Fluorophore used for Real-time PCR.

Analyte	Fluorophore	Minimum Single Threshold Value
vanA	FAM	500
vanB	Quasar 670	200
vanC	CAL Red 610	300
Internal Control	CAL Gold 540	50

3. MALDI-TOF M/S

MALDI-TOF M/S was used to analyze genetic relatedness of target strains. A single colony was selected on a blood agar culture medium (KOMED, Seoul, Korea) cultured at 37°C for 24 hours and thinly coated on a target slide. Then, 1 μL of α-cyano-4-hydroxycinnamic acid (CHCA) matrix (BioMérieux, La Balme-les-Grottes, France) and prepared a sample slide that was dried at room temperature. The prepared sample slides were loaded into a VITEK MS (BioMérieux, Marcy l'Etoile, France) system and spectra were obtained at a range of 2,000-20,000 Da mass spectrometry using the Research Use Only Mode (RUO) software Lanchpad V2.84.

Each strain was repeated for analysis for three times. The spectra obtained from the instrument were identified using the Spectral ARchive And Microbial Identification System (SARAMIS) V 4.13 database and the VITEK MS RUO system was calibrated using the *E.coli* ATCC 8739 standard strain before and after the test. To analyze the correlation of 45 *Enterococcus faecium* strains analyzed by MLST, all spectra obtained from the equipment were exported to the ASCII format peak list through the processing process, and the peak list was to be taken to the SARAMIS database for further analysis (Fig. 2). The software that compared the mass spectra generates a score value based on the similarities between the test strain and stored data sets. This score value provides information about the propriety of the identification. A score value above 2.0 is considered to be a reliable species level identification. Values between 1.7 and 2.0 represent reliable genus level identification[15,25]. Each spectra was compared with each other in SARAMIS to perform cluster analysis and the results were expressed as dendrograms.

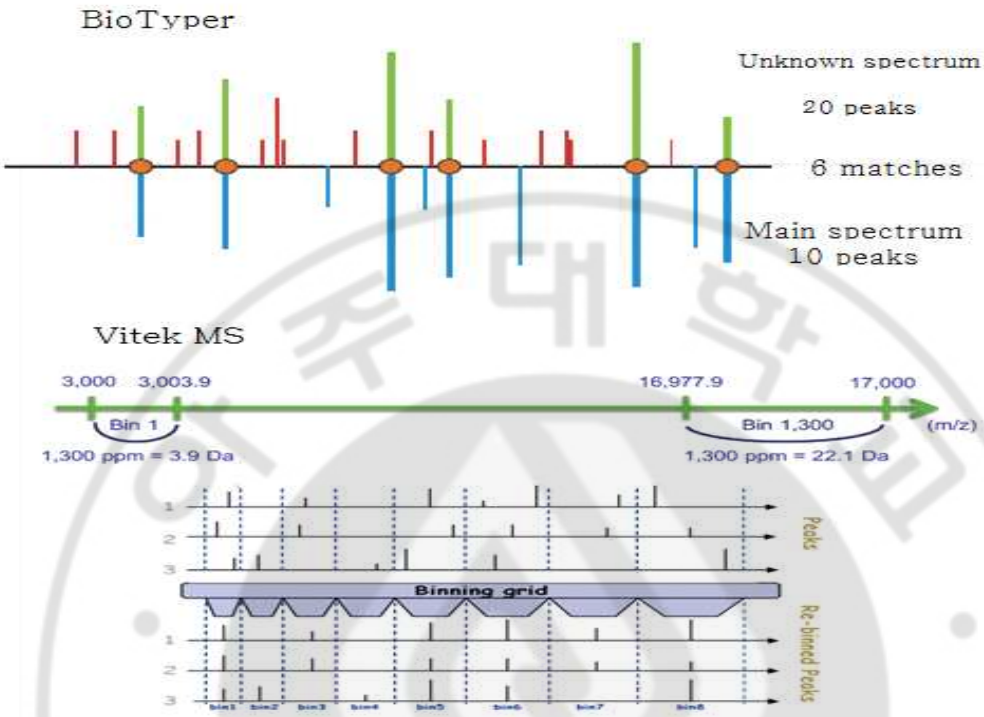


Fig. 2 Algorithms and result sheets of two commercially available MALDI-TOF analysis platforms[17].

4. Multilocus sequence typing

(A) Genomic DNA extract

45 strains were streaked on blood agar plates and incubated at 37 ℃ for 18-24 hours. 5-15 colonies were collected into 200 μL sterilized purified water, centrifuged at 12,000 rpm for 5 min. The precipitate was used to extract genome DNA with Quick Gene DNA whole blood kit (KURABO Industries Ltd, japan) according to operating instruction.

(B) Gene amplification

PCR was performed according to the method described in website (http://efaecium.mlst.net.) The total amount of the PCR reaction was adjusted to 50 μL, and the reaction was carried out using 1.25 U of TaKaRa Ex Taq^{TM}, 2 × buffer (25 mM TAPS, 50 mM KCl, 2 mM $MgCl_2$, 1 mM 2-mercaptoethanol), 0.4 mM dNTPs mixture, 10 pM primer 1 μL and 10 ng of purely isolated chromosomal DNA.

All PCR amplifications were carried out in a GeneAmp PCR System 9600 (Perkin-Elmer Corp., Norwalk, CT.) under the following conditions: Initial denaturation at 95 ℃ for 15 min, 35 cycles of 30s at 94 ℃, 30s at 52 ℃, 30s at 72 ℃, followed by 5 min 72 ℃. PCR products were directly purified from the reaction mixture with the QIAquick PCR purification kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's recommendations. After purification, electrophoresis was carried out at 120 V for 20 minutes in 1.2% agarose gel. Table 3 shows the primers used for each housekeeping gene.

Table 3. Oligonucleotide primers used in this study

Primer	Sequecce(5'→ 3')	Product size (bp)	Target gene
adk1n adk2n	GAACCTCATTTTAATGGGG TGATGTTGATAGCCAGACG	437	*adk*
atpA1 atpA2	CGGTTCATACGGAATGGCACA AAGTTCACGATAAGCCACGG	556	*atpA*
ddl1 ddl2	GAGACATTGAATATGCCTTATG AAAAAGAAATCGCACCG	465	*ddl*
gdh1 gdh2	GGCGCACTAAAAGATATGGT CCAAGATTGGGCAACTTCGTCCCA	530	*gdh*
gyd1 gyd2	CAAACTGCTTAGCTCCAATGGC CATTTCGTTGTCATACCAAGC	395	*gyd*
purK1n purK2n	CAGATTGGCACATTGAAAG TTCATTCACATATAGCCCG	492	*purK*
pstS1 pstS2	TTGAGCCAAGTCGAAGCTGGAG CGTGATCACGTTCTACTTCC	583	*pstS*

Abbreviation: *adk,* adenylate kinase; *atpA,* ATP synthase alpha subunit; *ddl,* D-alanine:D-alanine ligase; *gyd,* glyceraldehyde-3-phosphate dehydrogenase; *gdh,* glucose-6-phosphate dehydrogenase; *purK,* phosphoribosylaminoimidazole carboxylase ATPase subunit; *pstS,* phosphate ATP-binding cassette transporter; 1, forward; 2, reverse.

(C) Housekeeping gene sequencing

Sequencing reactions were performed in the DNA Engine Tetrad 2 Peltier Thermal Cycler (BIO-RAD) using the ABI BigDye(R) Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using primer. The fluorescent-labeled fragments were purified by the method that Applied Biosystems recommends as it removes the unincorporated terminators and dNTPs. The samples were injected to electrophoresis in an ABI 3730xl DNA Analyzer (Applied Biosystems).

(D) Database analysis for allele profile and ST identification

To confirm the sequence type (ST), PCR products of 7 house-keeping genes were submitted to Macrosen (Seoul, Korea) for sequencing. Each of the analyzed sequences was submitted to the Multiallign website (http://multalin.toulouse.in.ra.fr/multalin) and PubMLST (https://pubmlst.org/databases/) to confirm the respective allele number and the final ST was confirmed for *E. faecium* (Fig. 3). Each strain was analyzed using the eBURST program (https://eburst.mlst.net/v3/mlst_datasets/) on the PubMLST website with the nucleotide sequence of each gene and corresponding ST.

Online BURST algorithm, simple and speedy for epidemiological surveillance disregarding much of the evolutionary information contained in the nucleotide sequence, was used for analysis[18]. 45 isolates for this study in PubMLST database were clustered into separate clonal complexes by BURST (n-3, or more mathes).

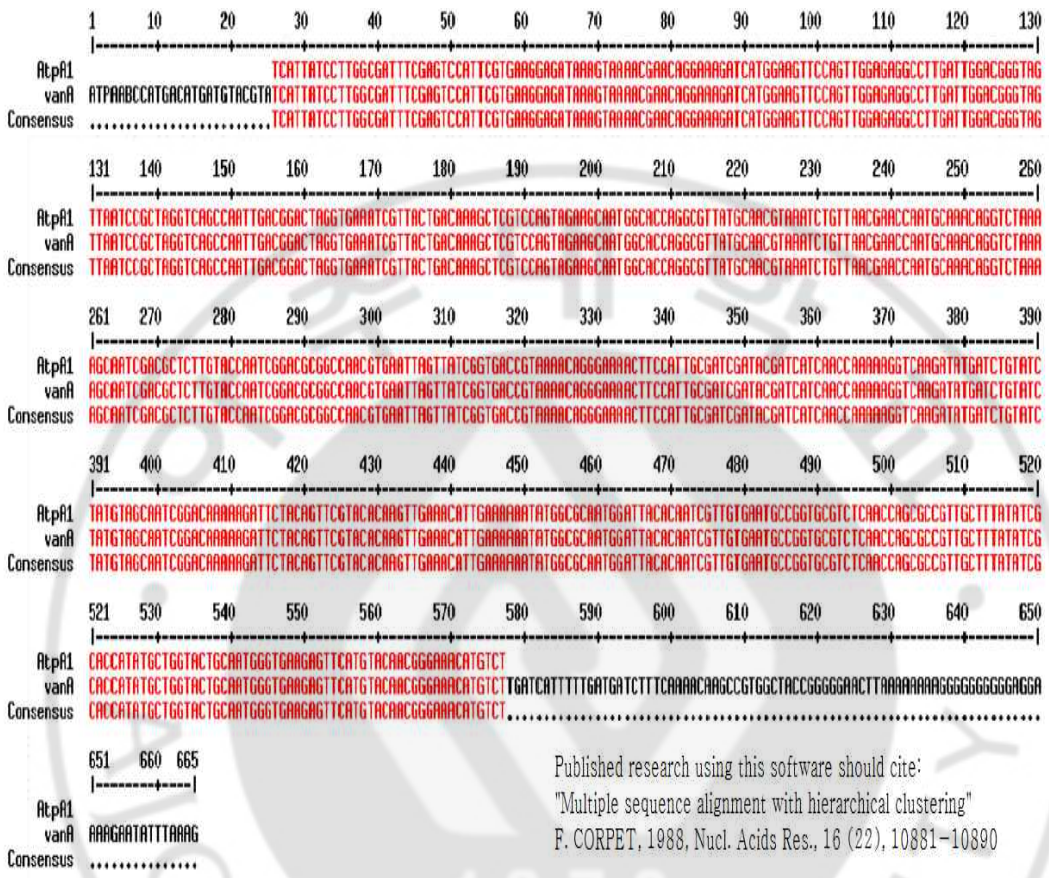


Fig. 3 Representative image of Multiallign website for compare allelic profiles with 45 strains in this study.

Ⅲ. RESULTS

1. Antimicrobial susceptibility test and resistant genotype

The antimicrobial susceptibility tests performed on 45 strains according to the CLSI standard showed that the vancomycin MIC values were all highly resistant to ≥256 μg/mL. The MIC values of teicoplanin were moderately resistant to vanA 03 and 35 strains at 16 μg/mL, vanA 04, 10, 17 and 39 strains at 8 μg/mL and vanA 18 strain at 4 μg/mL, respectively. vanA 06, 11, 12, 31, and 41 strains showed resistance at 32 μg/mL, and the remaining 33 strains were resistant at 64 μg/mL. Ampicillin 10 μg/mL and ciprofloxacin 5 μg/mL showed resistance to all strains, all strains were susceptible to linezolid 30 μg/mL and quinupristin-dalfopristin 15 μg /mL, and tetracyclin 30 μg/mL was susceptible to 34 strains, resistant to 10 strains, and moderately resistant to 1 strain (Table 4).

To confirm the resistance genotypes, real-time PCR with three fluorophores for *vanA, vanB* and *vanC* genes was performed it was observed that all 45 strains had *vanA* resistant genes, and that *vanB* and *vanC* genes were negative.

Table 4. Antibiotic susceptibility profiles of 45 strains

strain	MIC (μg/mL)		Resistance to antibiotics					Year
	VA	TP	AM	TE	CIP	LZD	SYN	
vanA01	>256	64	R	S	R	S	S	2007
vanA02	>256	64	R	S	R	S	S	2007
vanA03	>256	16	R	S	R	S	S	2007
vanA04	>256	8	R	R	R	S	S	2007
vanA05	>256	64	R	S	R	S	S	2007
vanA06	>256	32	R	S	R	S	S	2008
vanA07	>256	64	R	S	R	S	S	2008
vanA08	>256	64	R	S	R	S	S	2008
vanA09	>256	64	R	S	R	S	S	2008
vanA10	>256	8	R	R	R	S	S	2008
vanA11	>256	32	R	S	R	S	S	2009
vanA12	>256	32	R	I	R	S	S	2009
vanA13	>256	64	R	R	R	S	S	2009
vanA14	>256	64	R	S	R	S	S	2009
vanA15	>256	64	R	S	R	S	S	2009
vanA16	>256	64	R	S	R	S	S	2010
vanA17	>256	8	R	S	R	S	S	2010
vanA18	>256	4	R	S	R	S	S	2010
vanA19	>256	64	R	R	R	S	S	2010
vanA20	>256	64	R	S	R	S	S	2010
vanA21	>256	64	R	S	R	S	S	2011
vanA22	>256	64	R	S	R	S	S	2011
vanA23	>256	64	R	S	R	S	S	2011
vanA24	>256	64	R	S	R	S	S	2011
vanA25	>256	64	R	S	R	S	S	2011
vanA26	>256	64	R	R	R	S	S	2012
vanA27	>256	64	R	R	R	S	S	2012
vanA28	>256	64	R	R	R	S	S	2012
vanA29	>256	64	R	S	R	S	S	2012
vanA30	>256	64	R	S	R	S	S	2012
vanA31	>256	32	R	R	R	S	S	2013
vanA32	>256	64	R	S	R	S	S	2013
vanA33	>256	64	R	S	R	S	S	2013
vanA34	>256	64	R	S	R	S	S	2013
vanA35	>256	16	R	S	R	S	S	2013
vanA36	>256	64	R	S	R	S	S	2014
vanA37	>256	64	R	S	R	S	S	2014
vanA38	>256	64	R	S	R	S	S	2014
vanA39	>256	8	R	R	R	S	S	2014
vanA40	>256	64	R	S	R	S	S	2014
vanA41	>256	32	R	S	R	S	S	2015
vanA42	>256	64	R	S	R	S	S	2015

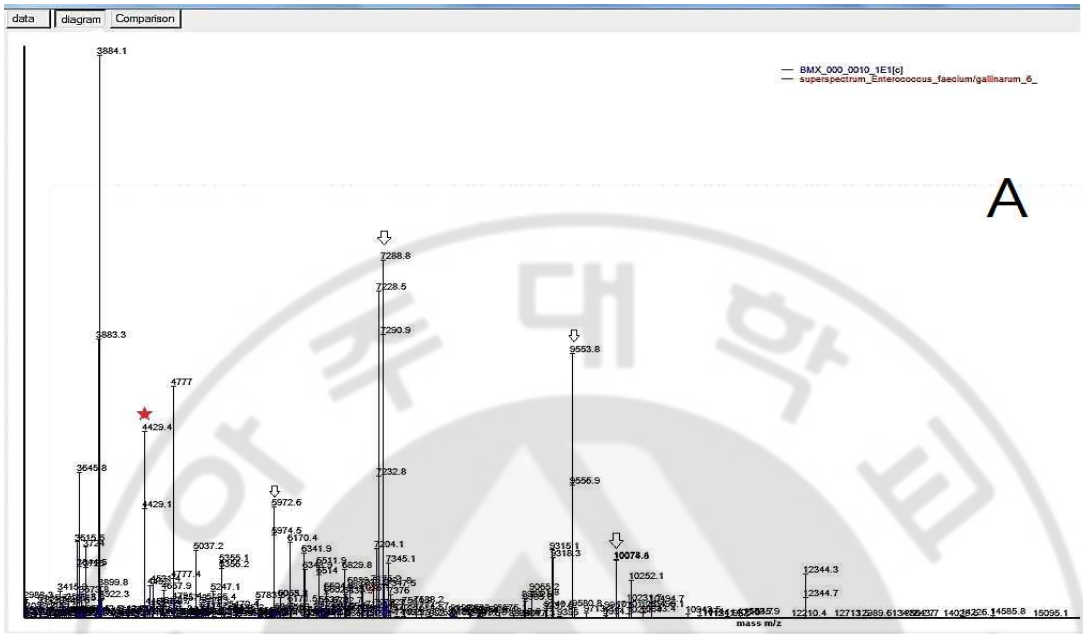
vanA43	>256	64	R	R	R	S	S	2015
vanA44	>256	64	R	S	R	S	S	2015
vanA45	>256	64	R	S	R	S	S	2015

Abbreviation: MIC, minimum inhibitiory concentration; VA, vancomycin; TP, teicoplanin; AM, ampicillin; TE, tetracyclin; CIP, ciprofloxacin; LZD, linezolid; SYN, quinupristin-dafopristin; ST, sequence type.

2. MALDI-TOF M/S

The *E. faecium* 45 strains isolated from the urine specimens were identified as *E. faecium* in the VITEK MS RUO system, and were consistent with biochemical differentiation tests other than VITEK 2 Gram-positive (GP) Identification card (BioMerieux, Hazelwood, MO). All of the obtained spectra showed good resolution, with a variety of peaks and specific spectral profiles for each strains. Total mass lists for each of the 45 strains were generated with the SARAMIS system by calculating the arithmetic means for the m/z values of the three replicate spectra acquired by MALDI-TOF M/S. The peak measured for each strain showed a peak mass spectra of 227 on average from a minimum of 179 peaks (vanA 02) to a maximum of 257 peaks (vanA 31). A total of 18 common peaks were identified, of which m/z 4429 Da±1 peaks found in both 45 strains and control strains, was reported by Quintela et al.[19] (m/z 4426 Da±1). It was a genus specific biomarker that confirmed its presence in all *Enterococcus spp.*

The other 17 major peak masses were m/z 3884 Da±1, 4776 Da±1, 5354 Da±2, 5972 Da±2, 5989 Da±3, 6038 Da±2, 6052 Da±2, 6169 Da±1, 6182 Da±2, 6341 Da±2, 6889 Da±2, 7288 Da±1, 8120 Da±3, 8162 Da±2, 9062 Da±2, 9553 Da±1, 10074 Da±2, etc. (Fig. 4). The results of the genotyping analysis based on the spectra of 45 strains obtained using MALDI-TOF MS showed that the strains showed genetic diversity and were not associated between strains (Fig. 5).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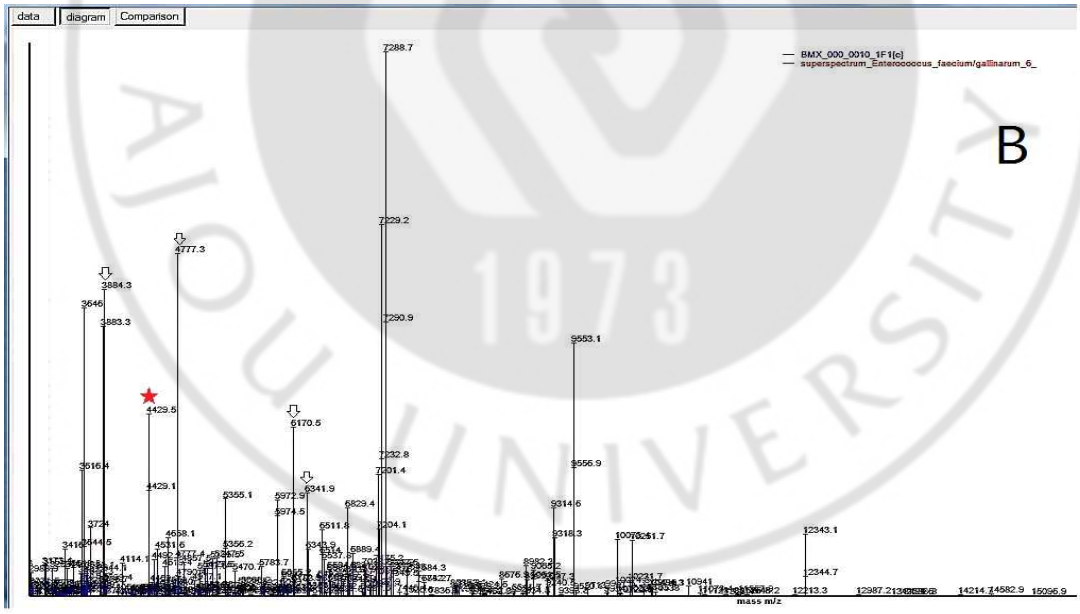
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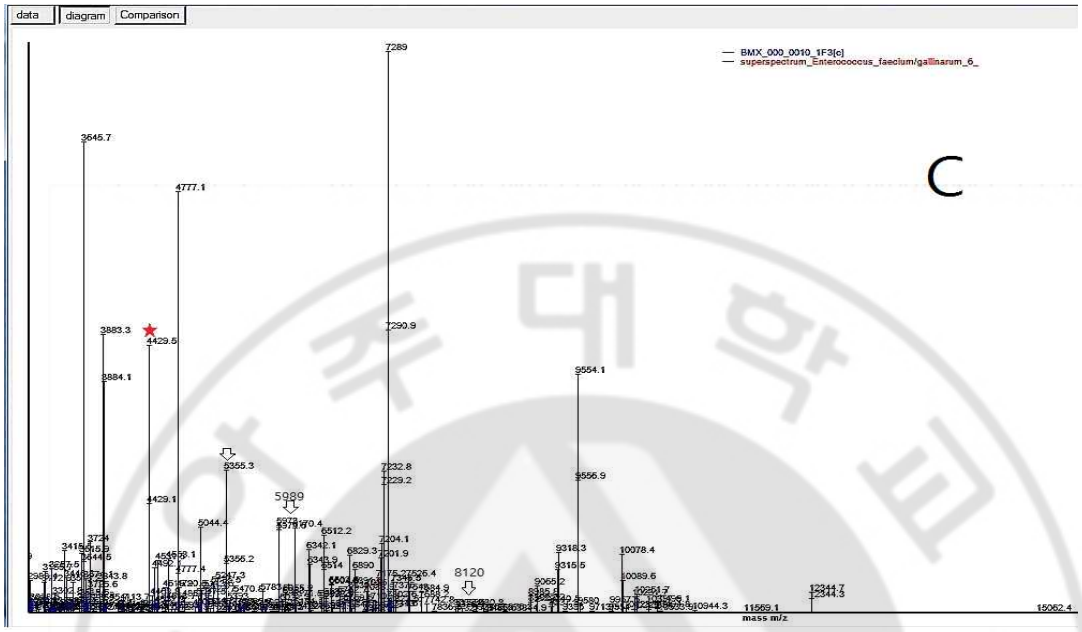
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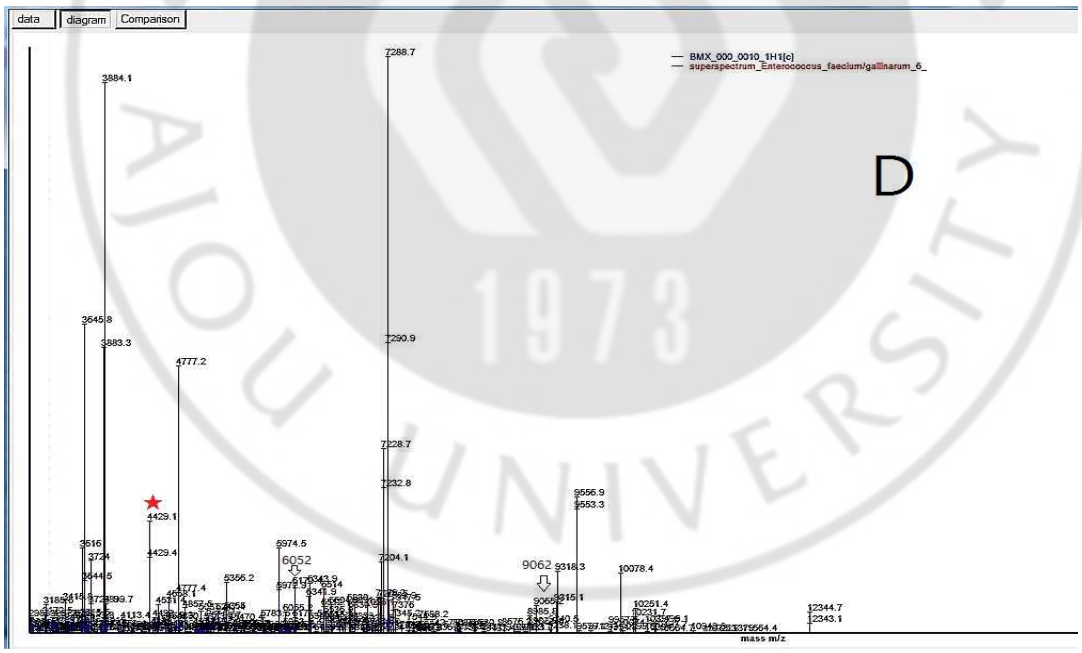
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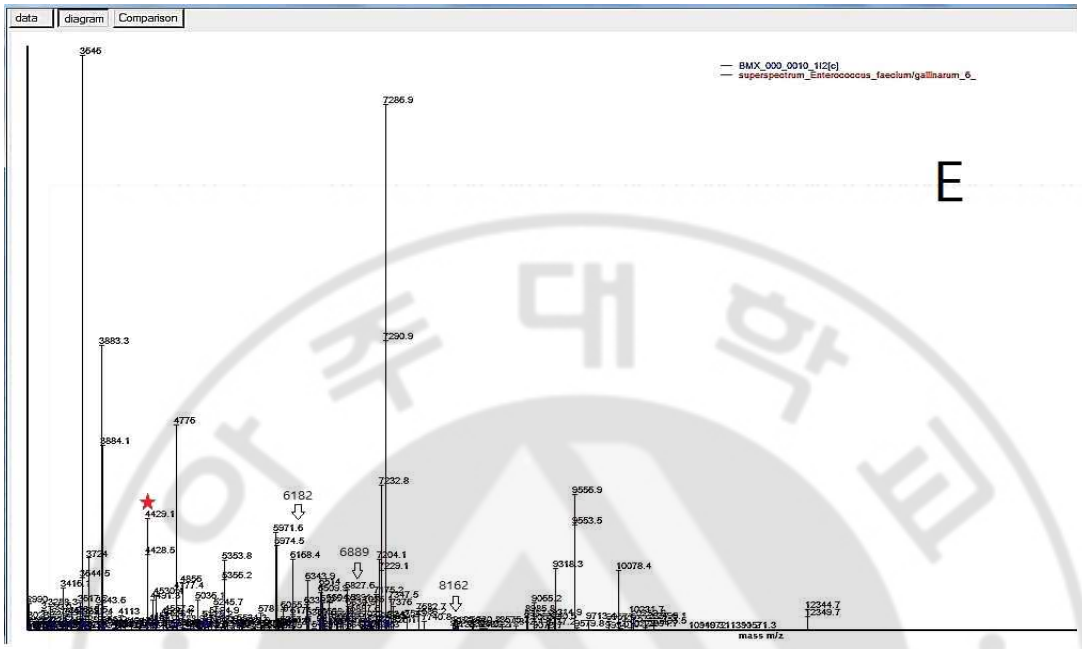
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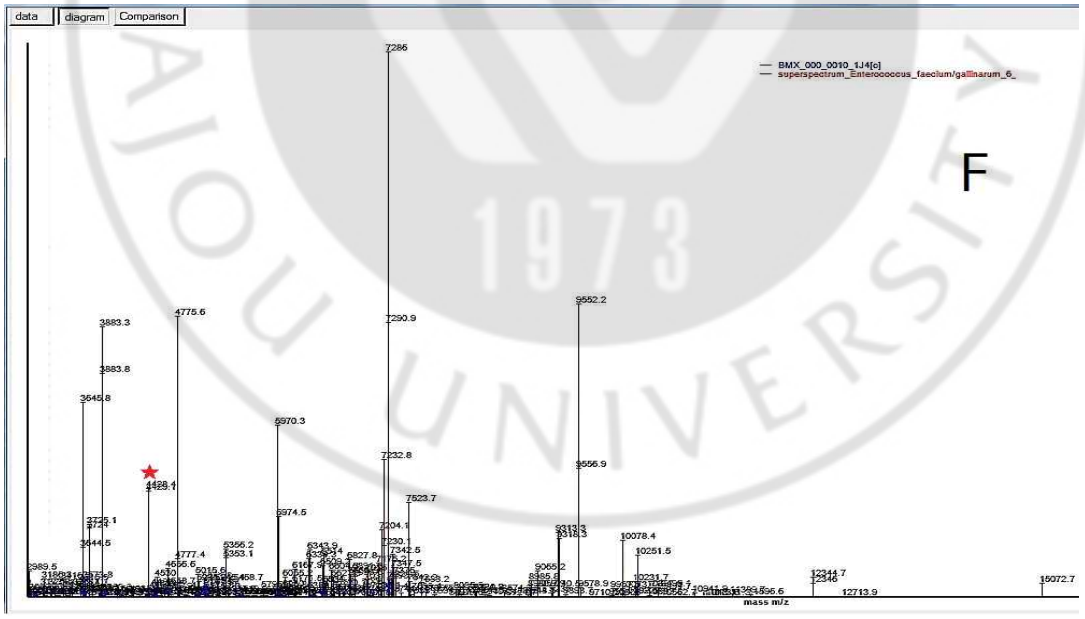
E
mass m/z


data
diagram
Comparison
BMX_000_0010_1J4[c]
superspectrum_Enterococcus_faecium/gallinarum_6_
F
mass m/z

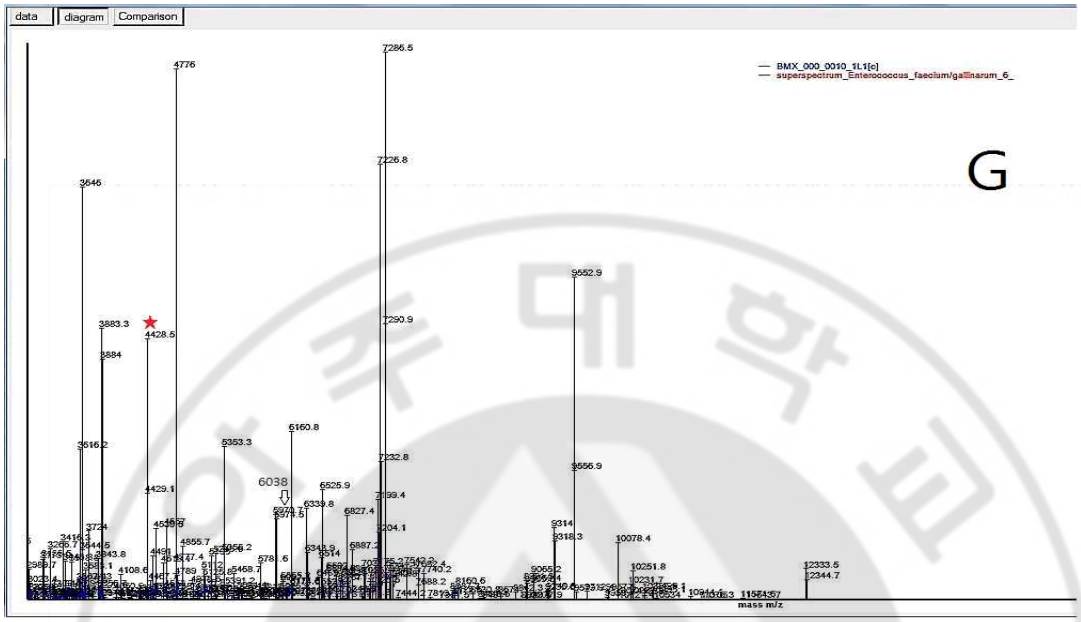


Fig. 4 Representative MALDI-TOF mass spectra of *vanA E. faecium* strains representing the seven STs established in this study, showing the high overall similarity of mass spectral patterns. The intensity is shown on the y-axis, and the mass to charge ratio (m/z) is shown on x-axis. Characteristic peaks are highlighted with symbols: ★ represents genus specific peaks present in all the studied *E. faecium* strains, ⇩ represents peaks in 17 common for this study. (A) ST17. (B) ST78. (C) ST192. (D) ST64. (E) ST262. (F) ST414. (G) ST981.

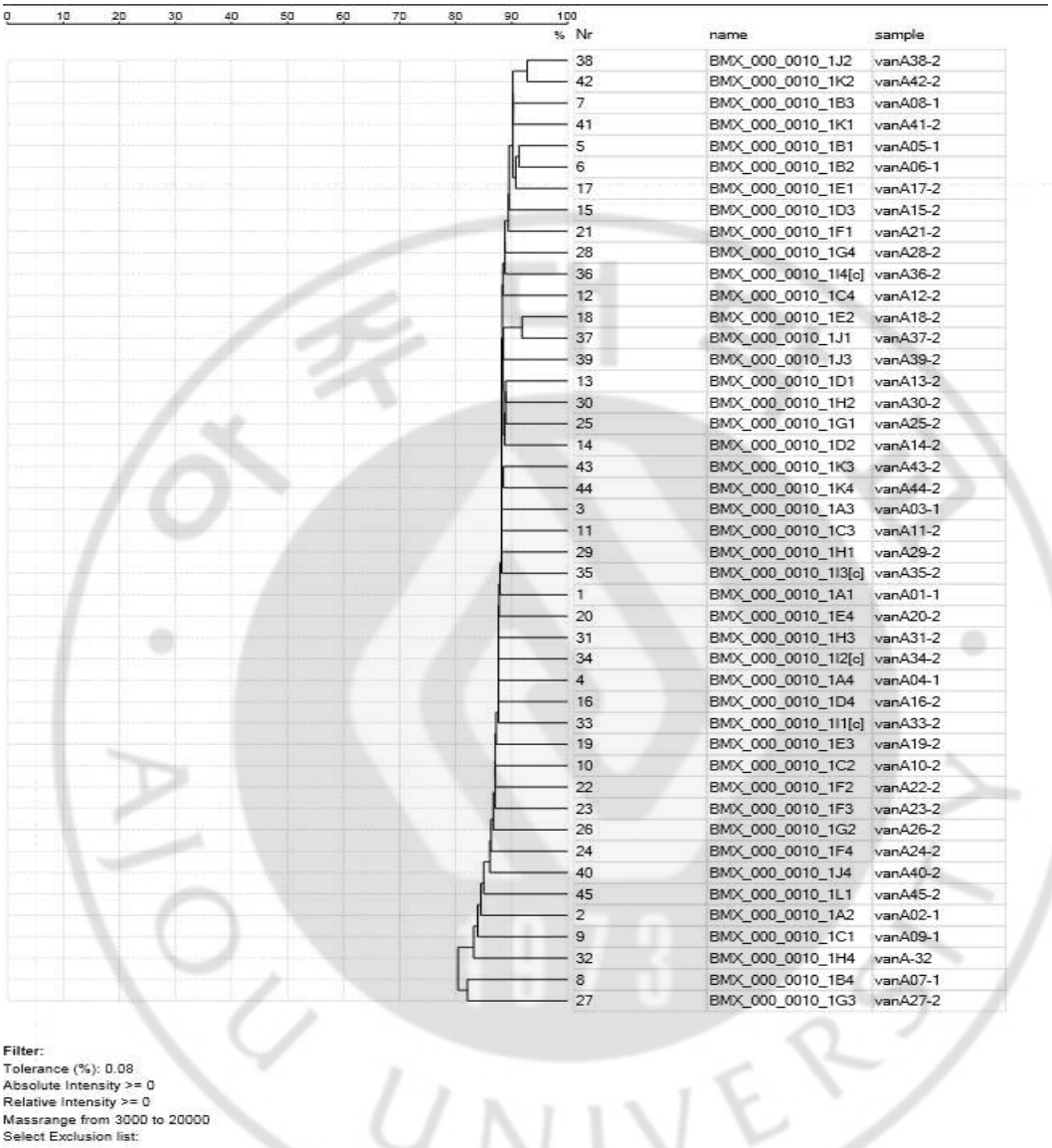


Fig. 5 Dendrogram of 45 *vanA E. faecium* obtained after MALDI-TOF MS analysis.

3. Multilocus sequence typing analysis

The MLST of *vanA E. faecium* isolated showed seven STs (Table 5). The *atpA* and *pstS* genes have three allelic numbers of 1, 7, 15 and 1, 7, and 20, respectively. The two allelic numbers of *ddl* and *gyd* are 1 and 5 and *adk* gene have 1 and 3. The remaining *gdh* and *purK* genes are 1 allelic number. To confirm the diversity of the clones, the seven STs were classified into n-3, or more mathes by the eBURST program (Fig. 6), all belonging to Clonal Complex 17 (CC17). ST17 (19 strains), ST78 (13 strains) and ST192 (6 strains) were common types in seven STs. The most common ST17 was distributed evenly over 9 years, and ST78 and ST192 were also uniformly distributed over a period of 9 years. In addition, ST64 (4 strains), ST262 (1 strain), ST414 (1 strain) and ST981 (1 strain) were isolated, and ST64 (4 strains) was recently isolated. This study compared seven STs with the major ST types isolated from domestic prevalence strains by the eBURST programs (Fig. 7).

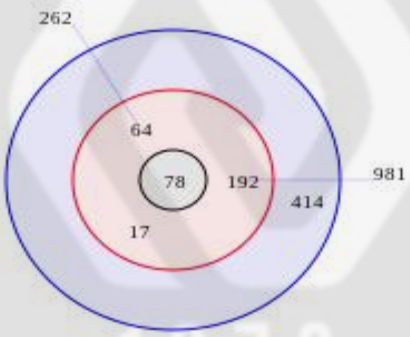
Table 5. Distribution of sequence types of 45 strains

ST(allelic profile) (*atpA-ddl-gdh-gyd-pstS-purK-adk*)	No. of isolates									
	2007	2008	2009	2010	2011	2012	2013	2014	2015	Total (n=45)
17 (1-1-1-1-1-1-1)	3	1	3	3	1	2	2	3	1	19
78 (15-1-1-1-1-1-1)	1	4	2	1	2	0	2	0	1	13
192 (15-1-1-1-7-1-1)	1	0	0	1	1	2	0	0	1	6
64 (7-1-1-1-1-1-1)	0	0	0	0	0	0	1	2	1	4
262 (7-1-1-5-7-1-1)	0	0	0	0	0	1	0	0	0	1
414 (15-5-1-1-20-1-1)	0	0	0	0	1	0	0	0	0	1
981 (15-5-1-1-7-1-3)	0	0	0	0	0	0	0	0	1	1

Abbreviation: ST, sequence type.

Group definition: 4 or more matches
Groups with central ST will be displayed as an image.

group: 1				
ST	Frequency	SLV	DLV	SAT
17	1	2	1	3
64	1	2	2	2
78*	1	3	1	2
192	1	1	5	
262	1	0	2	4
414	1	0	3	3
981	1	0	2	4



SVG file (right click to save)

Fig. 6 eBURST diagram of 45 *vanA E. faecium* STs of this study.
(n-3, more matches condition).

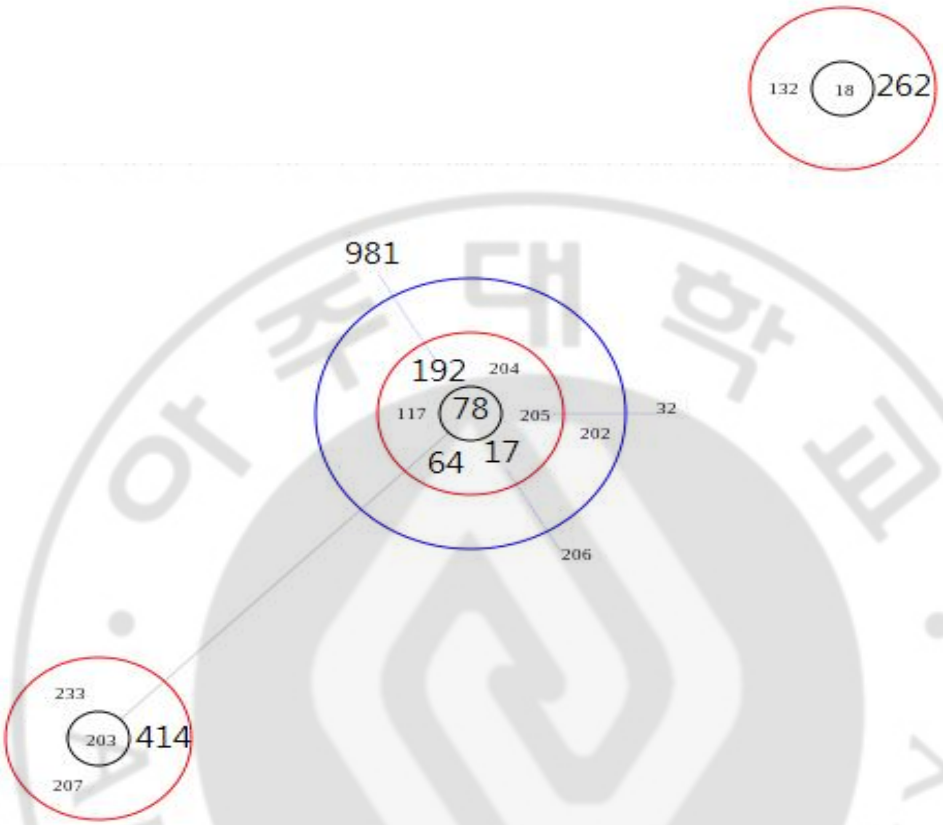


Fig. 7 Clustering analysis of 7 STs against korea major prevalence STs.

Each STs highlighted with large number. Domestic prevalence STs highlighted with small number.

IV. DISCUSSION

Of *vanA E. faecium* target strains, the MIC for vancomycin was ≥256 μg/mL in all 45 strains, which indicates high resistance. On the other hand, the MIC values of teicoplanin were lower than those of vanA 04,10,17,39 strains at 8 μg/mL, and vanA 18 strain at 4 μg/mL, respectively, and the susceptible VanB phenotype-*vanA* genotype. In general, vancomycin genotype and phenotype are consistent, but VanB phenotype-*vanA* genotype VRE, which is resistant to vancomycin and susceptible to teicoplanin, is frequently reported. The VanB phenotype-*vanA* genotype VRE occurring in Korea is the main cause of the *vanX, vanY* and *vanZ* gene loss associated with the insertion of *IS1216V* [20-22], unlike foreign countries where most of the *vanS* domain is mutated in *Tn1546*. On the other hand, vanA 03 and 35 strains were moderately resistant to 16 μg/mL, and all other strains were resistant to teicoplanin, VanA phenotype-*vanA* genotype. All strains showed resistance to ampicillin 10 μg/mL and ciprofloxacin 5 μg/mL. All strains were susceptible to linezolid 30 μg/mL and quinupristin-dalfopristin 15 μg/mL. Although enterococci resistant to linezolides have been found and reported in the early 2000s [23], they have not been found in this study.

MALDI-TOF MS is capable of analyzing samples with not dissolve or little volatility, and unlike conventional mass spectrometry, it is not necessary to use extreme conditions requiring modification of materials such as pyrolysis, and since the column is not used, it has the advantage that it is relatively easy to manipulate [14]. In addition, microbiological identification using MALDI-TOF MS allows for low proficiency, low analysis cost, and rapid identification compared to other commercial identification systems (VITEK 2 system, 16s rRNA gene analysis) [24].

The analysis of genotypes of 45 strains using MALDI-TOF MS showed an average various 227 peaks mass from at least 179 peaks (vanA 02) to a maximum of 257 peaks (van A31). A total of 18 common peaks were identified, and a peak of m/z 4429 Da±1, found in both 45 strains and control strains, was a genus specific biomarker that could be detected in all *Enterococcus spp*. The other 17 major peaks were m/z 3884 Da±1, m/z 4776 Da±1, m/z 5354 Da±2, m/z 5972 Da±2, m/z 5989 Da±3, m/z 6038 Da±2, m/z 6052 Da±2, m/z 6169 Da ±1, m/z 6182 Da±2, m/z 6341 Da±2, m/z 6889 Da±2, m/z 7288 Da±1, m/z 8120 Da±3, m/z 8162 Da±2, m/z 9062 Da±2, m/z 9553 Da±1, m/z 10074 Da±2, etc. Two peaks of m/z 6052 Da±2 and m/z 6889 Da±2 were species specific biomarkers identified only in *E. faecium* [25]. However, 45 strains showed genetic diversity and showed no relationship between strains[26-28]. This suggests that, as it is typical in infection, the vancomycin-resistant genes in the enterococcal host were transferred horizontally.

MLST analysis is a method to determine the nucleotide sequence of seven house-keeping genes from bacterial chromosomal DNA, input it into the MLST database, and then determine the Sequence Type (ST) by combining allelic profiles. The variation of the gene sequence of the house-keeping gene was reflected, thus indicating a long-term evolution [29]. MLST can compensate for the disadvantages of PFGE, which reflects short-term gene mutations by reflecting long-term gene variation. It reflects the long-term evolution of the gene, so that it is possible to deduce what kind of clone is occurring in various countries, and thus it is possible to gauge the trend of the occurrence of bacterial infection for many years. These characteristics are particularly important in studies of the VRE epidemic. The first application of MLST analysis to VRE is Homan et al. [11]. MLST analysis has been

widely used to determine the long-term variation of VRE gene expression. A number of MLST analysis studies of VRE have been undertaken during about 10 years after Homan's report [30-34]. Clonal complex 17 (CC17) is an *E. faecium* resistant to ampicillin, which causes, epidemics in six continents around the world. *E. faecium*, belonging to CC17, is highly resistant to ampicillin and fluoroquinolone and has a novel putative pathogenicity island, so it is presumed to have acquired vancomycin resistance after being indwelled in hospitals for a long time [36,37]. The discovery of these global epidemic strains was made possible by MLST analysis. The primary founder of CC17 was ST22, but the distribution was epidemic around ST17, a secondary founder rather than ST22. Representative types belonging to CC17 are ST78, ST17, ST64, etc., and types with single locus variation (SLV) and types with double locus variation (DLV) occupy most of the global epidemic. In particular, enterococci belonging to CC17 are known to have a high vancomycin-resistant transposon acquisition rate [30]. For this reason, worldwide, CC17 vancomycin-resistant enterococci (VRE) account for the vast majority of hospital infection epidemics [31-33]. In the MLST results of this study, 19 strains (42%) were the most common as ST17 type, 13 strains(29%) were ST78 type, 6 strains(13%) were ST192 type, 4 strains(8%) were ST64 type, and ST262 type(2%), ST414 type(2%) and ST981 type(2%) were one strain each (Table 5). The most common ST17 type was distributed evenly over 9 years, and the ST192 type and ST78 type were found to be uniformly distributed over a period of 9 years. Other ST64 type (4 strains), ST262 type (1 strain), ST414 (1 strain) and ST981 (1 strain) were confirmed, and ST64 (4 strains) has recently been able to identify a large number of isolated cases, and it is necessary to continuously observe whether it is due to a temporary epidemic. All seven sequence types in this study belonged to CC17. Of

these, ST17, ST64, and ST78 are the main types, ST192 is the SLV of ST78, and the point mutation of the *pstS* gene is caused [35], and ST262 and ST414 are DLV of ST192. ST981 is DLV of ST192 and ST414. As the strains of this study, even after a period of 9 years, show that most of the CC17 lineages were found, it is suggested that CC17 survived predominantly in the hospital environment.

The MLST type for the VRE domestically isolated is ST17, ST18, ST32, ST78, ST64, ST117, ST132, ST192, ST202, ST203, ST204, ST205, ST206, ST207, ST233, ST262, ST414, etc., and all of the types found in this study were of the previously reported ST type [34,35]. However, in a study the authors conducted on VRE isolated from the same hospital between 2002 and 2004, ST78 (43%), ST203 (26%), ST205 (5%), ST17 (3%) and ST18 and ST32 type were one strain, respectively. In this study, which isolated VRE from 2007-2015, ST17 (43%), ST78 (29%), ST192 (14%) and ST64 (9%) and other ST262, ST414 and ST981 were 1 strain, respectively, and it was found that the change occurred with time [38]. The most commonly isolated type was changed from ST78 to ST17, and ST203, which accounted for 26%, disappeared [39], indicating that ST192 occupied the site. In addition, ST type showing sporadic occurrence also showed changes. From the above results, MLST analysis showed that the predominant type of ampicillin-resistant VRE in the hospital changed with the duration of the MLST type. In addition, the ST17 type isolated in this study is a type that is often isolated in most countries, including Europe and the United States, and this type occupies most of the global epidemics[40]. ST78 and ST64 have been reported mostly in European countries. ST262 and ST414 isolated with one strain were reported in Taiwan, China and Australia, and this type could be estimated sporadically by continent [41]. In this study, MLST analysis can be used to estimate the long-term strain variation of VRE

in the hospital. In the future, it is necessary to use MLST to continuously analyze the variation patterns of strains in hospitals and to compare epidemic strains. Since the VRE was first discovered in 1986, it is now difficult to eradicate it in hospitals. In the future, by analyzing using MLST and searching early, it will be possible to identify the strains belonging to CC17 in advance and that efforts should be made to reduce this.

V. CONCLUSIONS

Recently, the MALDI-TOF M/S method has been widely used for genetic analysis of experimental strains by replacing PFGE. The MLST method reflects the long-term dynamics of genes and is a method for estimating the epigenetic origins and evolutionary backgrounds of the same strains without error between experimenter and laboratory. For *vanA* vancomycin-resistant *E. faecium* isolated in a domestic university hospital for 9 years, this study was conducted to carry out MLST analysis to investigate gene mutation by period. The antimicrobial susceptibility test of *E. faecium* 45 strains with the *vanA* gene collected at Ajou University Hospital from 2007 to 2015 showed high resistance to ampicillin, teicoplanin, ciprofloxacin and vancomycin antibiotics, whereas linezolid and quinupristin-dalfopristin were all susceptible.

In the results of genetic typing of 45 strains using MALDI-TOF MS, 18 common peak masses including genus and species specific peak mass were confirmed, but genetic diversity indicated that there was no association between strains as similar peak patterns were observed. The results of MLST analysis showed 7 sequence types (ST). ST17 type was the most common with 19 strains, ST78 type 13 strains, ST192 type 6 strains, ST64 type 4 strains, and ST262 type, ST414 type and ST981 type were one strain respectively. In the MLST analysis, all strains belonged to clonal complex 17 (CC17). This is a commonly observed result in large hospital epidemics. It can be seen that the most commonly isolated type was changed from ST78 to ST17. ST262 type, ST414 type and ST981 type, which were isolated by one strain, were found to be sporadic occurrence. Therefore, MLST analysis is an effective method to estimate the evolution pattern of *vanA* VRE gene.

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국문요약

배경 : 반코마이신 내성 장알균의 유행을 이해하는 것은 의료 관련 감염관리에 있어서 중요한 요인이다. MLST법은 유전자의 장기적 역학 변동을 반영하고 실험자와 실험실간의 오차 없이 동일 균종들의 역학적 기원 및 진화적 배경들을 추정할 수 있는 방법이다. MALDI-TOF MS 분석법은 최근 들어 PFGE법을 대체하여 실험균주의 유전적 형별분석에 많이 사용되어지고 있다. 이에 본 연구는 국내 일개 대학병원에서 9년간 분리된 *vanA* 반코마이신 내성 *E. faecium*을 대상으로 MLST 분석을 시행하여 기간에 따른 유전자 변이를 알아보고자 하였다.

대상 및 방법 : 2007년부터 2015년까지 아주대학교병원에서 수집된 *vanA* 유전자를 지닌 *E. faecium* 45주를 대상으로 하였다. 수집된 균주는 VitekⅡ system (BioMerieux, Hazelwood, MO)및 생화학적 감별 시험으로 동정하였고, CLSI 기준에 따라 디스크확산법과 E-test 방법으로 항균제 감수성 검사를 실시하였다. MALDI-TOF 질량 분석법을 이용하여 각 균주의 유전적 연관성을 분석하였다. 그리고 MLST 분석을 이용하여 *vanA* 반코마이신 내성 *E. faecium* 클론의 다양성에 대하여 분석을 실시하였다.

결 과: 모든 균주는 ampicillin, teicoplanin, ciprofloxacin 과 vancomycin 항균제에 대해 고도의 내성을 갖고 있었으며 반면에 linezolid 와 quinupristin-dalfopristin에는 모두가 감수성 이였다. MALDI-TOF MS를 이용한 45균주의 유전적 형별분석 결과에서는 유전적 다양성을 보여 균주 간 연관성이 없는 것으로 나타났다. MLST 분석결과는 7개의 sequence type (ST)을 보였다. ST17형이 19주로 가장 많았고 ST78형 13주, ST192형 6주, ST64형 4주, ST262형과 ST414형 그리고 ST981형이 각각 1주였다.

결 론: MLST 분석에서 모든 균주는 clonal complex 17(CC17)에 속하였다. 이는 대형

병원 유행에서 흔히 관찰되는 결과이다. 본 연구에서는 가장 흔하게 분리되는 유형이 ST78에서 ST17로 바뀌었음을 알 수 있었다. 각각 1주씩 분리된 ST262와 ST414형 그리고 ST981형은 산발적 발생임을 알 수 있었다. 이에 MLST 분석은 *vanA* VRE 유전자의 진화 양상을 추정 할 수 있는 효과적인 방법이라 사료된다.