의학석사 학위논문

황색포도알균 임상 분리 균주 타이핑을 위한 변형 다중 유전자좌 일렬반복수변이 지문분석법 및 반복염기서열 기반 PCR의 유용성, 표준방법인 간헐영역겔전기이동과의 비교

Utility of modified Multiple-locus Variable-number Tandem-repeat Fingerprinting and Repetitive-Sequence-Based PCR in Comparison with Pulsed-field Gel Electrophoresis for Typing Clinical Isolates of *Staphylococcus aureus*

2013년 2월

서울대학교 대학원

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지도교수 김 의 종

이 논문을 의학석사 학위논문으로 제출함

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서울대학교 대학원
의학과 검사의학 전공
정 소 이

정소이의 의학석사 학위논문을 인준함

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Utility of modified Multiple-locus Variable-number Tandem-repeat Fingerprinting and Repetitive-Sequence-Based PCR in Comparison with Pulsed-field Gel Electrophoresis for Typing Clinical Isolates of *Staphylococcus aureus*

Soie Chung

(Directed by Eui-Chong Kim)

A thesis submitted to the Department of Medicine in partial fulfillment of the requirements for the Degree of Master of Science in Laboratory medicine at Seoul National University College of Medicine

December 2012

Approved by Thesis Committee:

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학위논문 원문제공 서비스에 대한 동의서

본인의 학위논문에 대하여 서울대학교가 아래와 같이 학위논문 저작물을 제공하는 것에 동의합니다.

1. 동의사항

① 본인의 논문을 보존이나 인터넷 등을 통한 온라인 서비스 목적으로 복제할 경우 저작물의 내용을 변경하지 않는 범위 내에서의 복제를 허용합니다.

② 본인의 논문을 디지털화하여 인터넷 등 정보통신망을 통한 논문의 일부 또는 전부의 복제.배포 및 전송 시 무료로 제공하는 것에 동의합니다.

2. 개인(저작자)의 의무

본 논문의 저작권을 타인에게 양도하거나 또는 출판을 허락하는 등 동의 내용을 변경하고자 할 때는 소속대학(원)에 공개의 유보 또는 해지를 즉시 통보하겠습니다.

3. 서울대학교의 의무

①서울대학교는 본 논문을 외부에 제공할 경우 저작권 보호장치(DRM)를 사용하여야 합니다.

②서울대학교는 본 논문에 대한 공개의 유보나 해지 신청 시 즉시 처리해야 합니다.

논문제목 : 황색포도알균 임상 분리 균주 타이핑을 위한 변형 다중 유전자좌 일렬반복수변이 지문분석법 및 반복염기서열 기반 PCR의 유용성, 표준 방법인 간헐영역겔전기이동과의 비교

학위구분 : 석사

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Abstract

Introduction: Multiple-locus variable-number tandem-repeat fingerprinting (MLVF) is based on multiplex PCR, utilizing variable number tandem repeat. Similarly, repetitive sequence-based PCR (rep-PCR) uses primers that target non-coding repetitive sequences interspersed in bacterial genomes. Our goal was to compare the performance of MLVF and rep-PCR in distinguishing clinical *Staphylococcus aureus* isolates with that of pulsed-field gel electrophoresis (PFGE), which has traditionally been the gold standard.

Methods: Sixty-three clinically significant *S. aureus* isolates were tested using MLVF, rep-PCR, and PFGE. Multiplex PCR for MLVF was performed using PCR primers for *clfA*, *clfB*, *sdrCDE*, *sspA*, and *spa*. Rep-PCR was performed using DiversiLab *S. aureus* kit for DNA fingerprinting. PFGE was performed with genomic DNA fragments generated by *Sma*I endonuclease digestion. Banding patterns of MLVF or PFGE were analyzed using InfoQuestFP software. The data obtained by rep-PCR was analyzed by internet-based DiversiLab software

Results: The hands-on time of our modified MLVF method and rep-PCR was about 3 h, on average, for each of 18 or 12 isolates. PFGE (80% cutoff) or MLVF (75% cutoff) or rep-PCR (95% cutoff) separated all of the 63 isolates into 13, 12, 12 types, respectively. Three types generated by PFGE were identical to those generated by MLVF. However, no identical types were found between PFGE and rep-PCR. Only two of the types clustered similar isolates between PFGE and rep-

PCR. PFGE and MLVF yielded similar Simpson's diversity indices, indicating similar discriminatory power which is slightly higher than that of rep-PCR. The overall concordance between PFGE and MLVF was low, as represented by adjusted Rand indices (0.266−0.278). PFGE predicted MLVF type better than MLVF predicted PFGE type, as reflected by Wallace coefficients (PFGE cutoff 80% vs. MLVF cutoff 75%, 0.389 vs. 0.233). Between PFGE and rep-PCR, similar result of the adjusted Rand indices (0.234–0.250) and Wallace coefficients (PFGE cutoff 80% vs. rep-PCR cutoff 95%, 0.628 vs. 0.156) were observed. Analysis of the relationship between a pair of isolates showed 91.0% concordance between the PFGE (80% cutoff) and MLVF (75% cutoff).

Conclusions: Our simple, low-cost, modified MLVF protocol can effectively discriminate between *S. aureus* clinical isolates. MLVF can replace PFGE for the hospital infection control of *S. aureus*.

Key words: *Staphylococcus aureus*, pulsed-field gel electrophoresis, multiple-locus variable-number tandem-repeat fingerprinting, repetitive-sequence-based PCR

Student Number: 2011-21858

List of Tables

List of Figures

List of Abbreviations and Symbols

CDC	Centers for Disease Control and Prevention
KL	Kullback-Leibler
MLVF	Multiple-locus variable-number tandem-repeat fingerprinting
MRSA	Methicillin-resistant *Staphylococcus aureus*
NHSN	National Healthcare Safety Network
PFGE	Pulsed-field gel electrophoresis
Rep-PCR	Repetitive-sequence-based PCR
UPGMA	Unweighted pair-group method with arithmetic mean
VNTR	Variable number tandem repeat

Contents

Introduction

Although 20–30% of the general population is asymptomatically colonized with *Staphylococcus aureus*, colonization increases the risk of *S. aureus* infection when the host immune system is compromised [1]. *S. aureus* infections vary from superficial wound infections to invasive diseases, such as deep abscesses, osteomyelitis, and bacteremia [2]. Methicillin-resistant *S. aureus* (MRSA) is of especially great concern because MRSA infection extends the length of hospital stay and increases antibiotic use, costs, and mortality [3].

Traditionally, MRSA strain typing was accomplished by pulsed-field gel electrophoresis (PFGE) [4]. However, the operational time of PFGE is at least 72 h, the cost is relatively high, and the technique requires specialized training [5]. Sabat *et al.*[6] therefore designed a multiplex PCR scheme called multiple-locus variable-number tandem-repeat fingerprinting (MLVF), using 5 variable number tandem repeat (VNTR) loci. Studies have shown that MLVF can distinguish among MRSA strains and simultaneously provide results that parallel those of PFGE [5, 7, 8].

A recently invented commercially available semi-automated repetitive-sequenced-based PCR (rep-PCR) system, supplied with easy-access Web-based software, has shown to be an interesting option for MRSA typing [9].

We aimed to investigate the usefulness of MLVF and rep-PCR for typing

S. aureus isolates of clinical significance. We also tried to modify the MLVF method described previously, in order to maximize the technical advantage of MLVF, in terms of time, cost, and simplicity, compared with PFGE.

Materials and Methods

1. Bacterial isolates

Sixty-three hospital-acquired *S. aureus* isolates recovered in 2009 were selected according to Centers for Disease Control and Prevention (CDC) / National Healthcare Safety Network (NHSN) criteria [10]. Isolates that were regarded as contaminant or non-pathogenic were excluded. Among the 63 isolates, 15 were methicillin-susceptible, and 48 were methicillin-resistant. Most of the bacterial strains were isolated from blood culture and groups of 2 or 3 isolates that were considered epidemiologically related were chosen for analysis. The isolates were recovered from the following clinical sources: blood culture (N = 43), ascitic fluid (N = 2), cerebrospinal fluid (N = 3), sputum (N = 3), endotracheal aspiration (N = 3), pus (N = 2), T-cannula tip (N = 2), wounds (N = 2), throat (N = 1), tissue (N = 1), and drain (N = 1).

2. MLVF typing

Bacterial isolates were subcultured in 5% sheep blood agar overnight at 37°C. Total genomic DNA was extracted from *S. aureus* isolates using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Purified DNA was stored at -20°C.

The multiplex PCR assay containing *clfA*, *clfB*, *sdrCDE*, *spa*, and *sspA* primers was performed as previously described by Sabat *et al.* [6], with modifications. For convenience, the *AccuPower* HotStart PCR PreMix kit (Bioneer Co., Daejeon, Korea) was used in the PCR reaction. The kit contained a mixture of 1 unit of HotStart DNA polymerase, 1× PCR buffer, 250 μM of each dNTP, and 1.5 mM $MgCl_2$ in each reaction tube. A mixture of the following concentrations of each primer and 1 μL of template DNA (20–50 ng) was added to the reaction tube: 0.15 μM of clfA-F (forward) and clfA-R (reverse), 0.2 μM of clfB-F and clfB-R, 0.15 μM of sdrCDE-F and sdrCDE-R, 0.1 μM of spa-F and spa-R, and 0.3 μM of sspA-F and sspA-R. The thermal cycling was performed in a PTC-100 Thermal Cycler (MJ Research, Waltham, MA, USA) as follows: 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, with a final extension at 72°C for 7 min.

The amplified PCR products and 100-bp DNA ladder marker (Roche Applied Science, Indianapolis, IN, USA) were resolved by electrophoresis in a 2% agarose gel in 0.5× Tris-borate-EDTA (TBE) buffer at 50 V for 80 min using Mupid-2plus (Takara Bio Inc., Otsu, Japan). The gel image was saved as a TIFF file and analyzed using InfoQuestFP Software (Bio-Rad Laboratories Inc., Hercules, CA, USA) under the following tolerance settings: optimization, 0.5%, and position tolerance, 1.25% [5]. The band patterns were clustered into MLVF types using Dice's coefficient and the unweighted pair-group method with arithmetic mean (UPGMA). The effect of grouping similar MLVF patterns together into a smaller number of MLVF types was examined using cutoffs of >75% and >70% relatedness.

3. Repetitive-sequence-based PCR

Total genomic DNA was extracted from *S. aureus* isolates using the UltraClean microbial DNA isolation kit (Mo Bio Laboratories, Solana Beach, CA). Then, the DNA amount was subsequently measured by using a Nanodrop 2000 (Witec, Lucerne, Switzerland) to adjust the DNA concentration to 25–50 ng of genomic DNA/μL. A mixture of the following concentrations of each reagent was added to the reaction tube according to the manufacturer's instruction: 18μL of the kit-supplied rep-PCR mastermix (MM1), 2.5μL of GeneAmp 10 × PCR buffer (Applied Biosystems, Foster City, CA, USA), 2.0μL of kit-supplied Primer Mix LL, 0.5μL of AmpliTaq DNA polymerase(Applied Biosystems), and 2μL of genomic DNA(25–50 ng/μL). The thermal cycling was performed in a PTC-100 Thermal Cycler (MJ Research) as follows: 94°C for 2 min, followed by 30 cycles at 94°C for 30 s, 45°C for 30 s, 70°C for 90 s, with a final extension at 70°C for 3 min. Separation and detection of rep-PCR products were done by micro-fluidic chips of the Diversilab LabChip Devices (bioMérieux, Boxtel, The Netherlands) according to the manufacturer's instruction. On single microfluidic labchip, a total of 12 samples could be loaded in addition to a molecular size marker. Loading volume per sample was 1 μL. The labchip was loaded on Agilent 2100 Bioanalyzer (Agilent Technologies, Palo alto,

CA) provided by bioMérieux was used to separate the amplified fragments. The electrophoresis data were analyzed by the website-based DiversiLab software (version 3.4) with the Kullback-Leibler (KL) coefficients to determine the distance matrices. The UPGMA was used to create dentrograms, scatterplots, and electropherograms. The similarity index cut-off of 95%, 97%, and 98% was analyzed. Isolates were determined as different when one or more band difference(s) were notices with the overlay analysis [9].

4. PFGE

Genomic DNA of the isolates was prepared, digested by restriction enzyme *Sma*I (Roche Diagnostics GmbH, Mannheim, Germany), and separated in a GenePath system (Bio-Rad Laboratories Inc.) according to the CDC PulseNet protocol with modification [11]. *Sma*I PFGE patterns were saved in TIFF files. The file was exported to the InfoQuestFP software (Bio-Rad Laboratories Inc.) and analyzed using the Dice coefficient-UPGMA. A dendrogram was generated to examine the relatedness of PFGE patterns for all study isolates, and cutoff levels of 80% and 75% were applied to this dendrogram.

5. Calculation of concordance

Simpson's index of diversity was calculated to measure the discriminatory power of the typing systems. This index indicates the probability that 2 strains sampled randomly from a population will belong to 2 different types [12]. The formulas for Simpson's index (D) and the confidence interval (CI) are presented in the following equations:

$$\mathbf{D} = \mathbf{1} - \frac{\mathbf{1}}{\boldsymbol{N(N-1)}} \sum_{\boldsymbol{j=1}}^{\boldsymbol{S}} \boldsymbol{n_j(n_j-1)}$$

$$\boldsymbol{\sigma^2} = \frac{\mathbf{4}}{\boldsymbol{N}} \left[\sum \boldsymbol{\pi_j^3} - \left(\sum \boldsymbol{\pi_j^2} \right)^{\mathbf{2}} \right]$$

$$\mathbf{CI} = \left[\boldsymbol{D - 2\sqrt{\sigma^2}}, \boldsymbol{D + 2\sqrt{\sigma^2}} \right],$$

where N is the total number of strains in the sample population, S is the total number of types described, n_j is the number of strains belonging to the $_j$th type, and π_j is the frequency n_j/N.

To compare the 2 sets of results produced by PFGE and MLVF, we used 2 clustering comparison coefficients (adjusted Rand and Wallace) generated by a script from http://biomath.itqb.unl.pt/ClusterComp, as previously described [12].

Adjusted Rand are symmetric coefficients, i.e., they do not take into consideration which partition is considered the standard, while others, like

the ones proposed by Wallace do. It is also important to note in this context that none of the partitions tested are considered the "correct" partition in terms of microbial typing.

Given two partition schemes of the same data set, P and P', all these coefficients are calculated based on the fact that a pair of points (in microbial typing, a pair of points is a pair of isolates under study) from the data set will fall into one of the following conditions: a, the number of point pairs that are in the same cluster in P and P'; b, the number of point pairs that are in the same cluster in P but not in P'; c, the number of point pairs that are in the same cluster in P but not in P'; or d, the number of point pairs that are in different clusters in P and P'. n represents the total sample size, n_i the number of species belonging to the cluster i of partition A, and n_j the number of species belonging to the cluster j of partition B.

Wallace's coefficients provide an estimate of, given a typing method, how much new information is obtained from another typing method. A high value of Wallace's coefficient indicates that partitions defined by a given method could have been predicted from the results of another method, suggesting that the use of both methodologies is redundant.

P’ \ P	Pair in same cluster	Pair not in same cluster
Pair in same cluster	a	b
Pair not in same cluster	c	d

Rand index=$\frac{a+d}{a+b+c+d}$

Adjusted Rand index $= \frac{index - expected\ index}{maximum\ index - expected\ index}$

$$= \frac{a + d - n_c}{a + b + c + d - n_c}$$

where: $n_c = \frac{n(n^2+1)-(n+1)\sum n_i^2-(n+1)\sum n_j^2+2\sum\sum\frac{n_i^2 n_j^2}{n}}{2(n-1)}$

Wallace’s coefficients $W_{P'\to P}=\frac{a}{a+b}$ $W_{P\to P'}=\frac{a}{a+c}$

We also evaluated the concordance of each pair of isolates as previously described [13]. All possible pairs of isolates were cross-classified on the basis of matched or mismatched types. The resulting 2 × 2 table was evaluated using the chi-square statistic, and the percentage of concordant cells was calculated.

Results

1. Technical aspects of MLVF and rep-PCR

Using a commercial kit containing a premixture of PCR reagents, we were able to perform MLVF more easily by simply transferring the template DNA and primers into the reaction tubes. This MLVF protocol, modified from a previous study [6], is sufficiently simple for use in clinical laboratories, and 18 isolates can be typed in a single working day if pure cultures of bacterial isolates are available. The hands-on time for 18 samples was approximately 3 h on average (2 h for DNA extraction, 30 min for preparation of PCR reaction, and another 30 min for MLVF pattern reading), with most of the time spent on the extraction of DNA. Experienced personnel would be able to analyze the MLVF patterns by computer software within 30 min.

Similarly, using the rep-PCR method, the hands-on time for 12 samples was approximately 3 h on average (2 h for DNA extraction, 30 min for preparation of PCR reaction, and another 30 min for rep-PCR pattern reading).

2. Molecular typing by PFGE, MLVF, and rep-PCR

All 63 *S. aureus* clinical isolates could be typed by PFGE (Fig. 1), MLVF (Fig. 2), and rep-PCR (Fig.3). The strain type classification and characteristics of each isolate is described in Table 1. Because defining clonality in the PFGE using a similarity value of 80% as a cutoff is considered the gold standard [14], we used 80% and 75% cutoff values for PFGE and 75% and 70% for MLVF. We also tried an 80% cutoff for MLVF; however, we found that some isolates were not grouped into the same type predicted by PFGE (data not shown). For rep-PCR, strain relatedness was defined as a minimum of >95% similarity as described previously [15]. PFGE distinguished the isolates into 13 or 14 types and an additional 24 or 20 unique patterns at cutoff values of 80% and 75%, respectively (Fig. 1). Similarly, MLVF separated the 63 *S. aureus* isolates into 12 or 13 types and additional 16 or 12 unique patterns at cutoff values of 75% and 70%, respectively (Fig. 2). Rep-PCR distinguished the isolates into 12 types and an additional 9 unique patterns at cutoff values of 95%. PFGE types P10 and P13 were identical to MLVF types M1 and M5, respectively. A very good correlation was observed between PFGE types P8, P5, and P6 and MLVF types M3, M4, and M9, respectively. In these types, only 1 or 2 isolates composing each MLVF type were different from the isolates composing the PFGE type. Although no identical types were found between PFGE types and rep-PCR types, DL10 and DL13 clustered similar isolates those are composing P13 and P6. However, MLVF type M8, which was composed of 9

isolates at both 75% and 70% cutoff values, was divided into 5 different PFGE types. Rep-PCR type DL11 also divided into 5 different PFGE types (P1, P2, P3, P11, P12), indicating that MLVF or rep-PCR had a poorer discriminatory power than did PFGE.

Table 1. Characteristics and strain type classification by methods

No	Ward	Specimen	Sensitivity to methicillin	PFGE*	MLVF†	DL‡
1	Hemato-Oncology	Blood	MSSA			DL7
2	Hemato-Oncology	Blood	MSSA		M10	DL3
3	Hemato-Oncology	Blood	MSSA	P8	M3	
4	Hemato-Oncology	Blood	MSSA	P8		DL2
5	Hemato-Oncology	Blood	MSSA	P8	M3	DL2
6	Hemato-Oncology	Blood	MSSA			DL2
7	Gastroenterology	Blood	MSSA	p9		DL4
8	Gastroenterology	Blood	MRSA			
9	Gastroenterology	Blood	MRSA	P3	M8	DL10
10	Gastroenterology	Blood	MRSA		M2	DL9
11	Hemato-Oncology	Ascitic	MRSA	P3	M12	DL11
12	Hemato-Oncology	Ascitic	MRSA		M4	DL2
13	Emergency Medicine	Blood	MSSA	P9	M6	
14	Emergency Medicine	Blood	MSSA			DL4
15	Pediatric Neurosurgery	Wound	MRSA			DL6
16	Pediatric Neurosurgery	Cerebrospinal fluid	MRSA	P13	M5	DL9
17	Pediatric Neurosurgery	Cerebrospinal fluid	MRSA	P13	M5	DL9
18	Pediatric Neurosurgery	Wound	MRSA	P10	M1	DL6
19	Pediatrics	pus	MRSA	P10	M1	DL5
20	Pediatrics	Blood	MRSA	P13	M5	DL9
21	Pediatrics	Throat	MRSA	P10	M1	DL6
22	Pediatrics	Blood	MRSA	P10	M1	DL6
23	Pediatrics	Pus	MRSA	P10	M1	DL6
24	Pediatric Hemato-Oncology	Sputum	MRSA		M2	
25	Pediatric Hemato-Oncology	Blood	MRSA		M12	DL10
27	EICU	Blood	MRSA	P2		DL10
28	EICU	Blood	MRSA	P2	M8	DL10
29	EICU	Blood	MRSA	P2	M12	DL10
30	EICU	Blood	MRSA	P2	M12	DL10
31	MICU	Blood	MSSA	P6	M6	DL8
32	MICU	Blood	MRSA	P2		DL10
33	MICU	Blood	MSSA		M7	DL8
35	MICU	Blood	MRSA	P2		DL10
36	PICU	Blood	MRSA		M7	DL2

37	PICU	T-cannul tip	MRSA		M2	DL9
38	PICU	T-cannul tip	MRSA		M11	DL10
39	PICU	Tissue	MSSA			DL7
40	PICU	Endotracheal Aspiration	MRSA		M3	DL3
41	PICU	Drainage	MRSA	P2	M8	DL10
42	PICU	Blood	MRSA	P7	M2	DL9
44	PICU	Endotracheal Aspiration	MRSA		M10	DL2
48	SICU	Blood	MRSA	P12	M10	DL10
49	SICU	Blood	MRSA	P12	M8	DL10
50	SICU	Blood	MRSA	P11	M8	DL10
51	SICU	Blood	MRSA	P11	M8	DL10
52	SICU	Cerebrospinal fluid	MSSA	P1	M8	DL10
53	SICU	Blood	MRSA	P1	M8	DL10
54	SICU	Blood	MRSA	P3	M8	DL10
55	Hemato-Oncology	Blood	MRSA	P6	M9	
56	Hemato-Oncology	Blood	MRSA	P6	M9	
57	Hemato-Oncology	Blood	MSSA	P7		DL5
58	Hemato-Oncology	Blood	MSSA			
59	Hemato-Oncology	Blood	MRSA	P5	M4	DL5
60	Hemato-Oncology	Blood	MRSA	P4	M11	
61	Rehabilitation medicine	Blood	MRSA		M4	DL6
62	Rehabilitation medicine	Blood	MRSA	P4		DL11
63	Pediatric Neurosurgery	Blood	MRSA	P5	M4	DL1
64	Pediatric Neurosurgery	Blood	MRSA		M10	
65	Pediatric Neurosurgery	Endotracheal Aspiration	MRSA			DL11
66	Pediatric General Surgery	Blood	MRSA			
67	Pediatric General Surgery	Blood	MRSA	P7	M4	DL1
68	Pediatric General Surgery	Sputum	MRSA		M2	
69	Pediatric General Surgery	Sputum	MRSA		M6	DL1

Abbreviations: PFGE, pulsed-field gel electrophoresis; MLVF, multiple-locus variable-number tandem-repeat fingerprinting; DL, DiversiLab repetitive sequence-based PCR; EICU, emergency intensive care unit; MICU, medical intensive care unit; SICU, surgical intensive care unit.

The isolate that was not classified into any of the strain type was remained in blank by each typing

methods.

* Cutoff of 80%

† Cutoff of 75%

‡ Cutoff of 95%

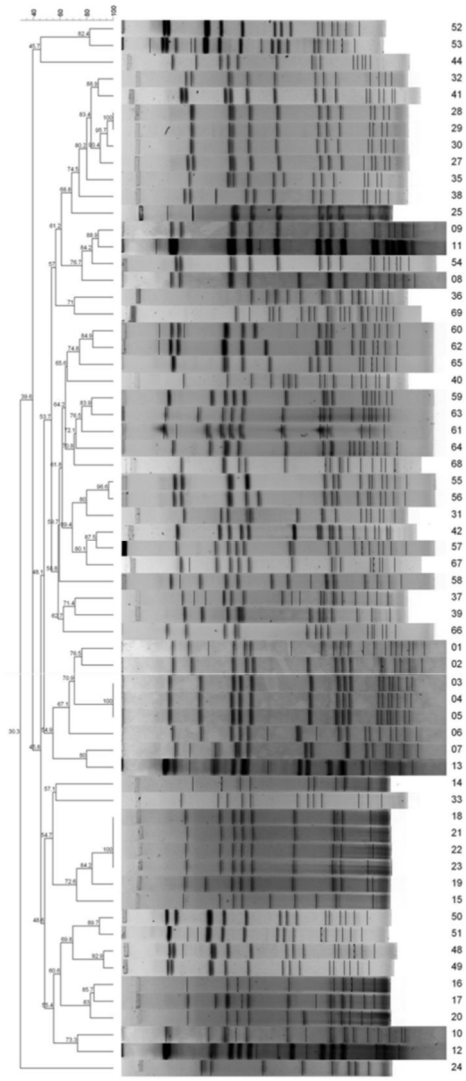


Fig 1 Pulsed-field gel electrophoresis (PFGE) types of the study isolates generated by the UPGMA algorithm, using 80% and 75% similarity cutoff values. The isolates that were not included in the above types were considered to be unique patterns. Isolate numbers presented in boldface were not included in any types generated by the 80% cutoff.

PFGE type (75% cutoff)	Isolate number
P1	52, 53
P2	27, 28, 29, 30, 32, 35, 41
P3	**8**, 9, 11, 54
P4	60, 62
P5	59, **61**, 63
P6	31, 55, 56
P7	42, 57, 67
P8	3, 4, 5
P9	**1, 2**
P10	18, 19, 21, 22, 23
P11	50, 51
P12	48, 49
P13	16, 17, 20
P14	7, 13

PFGE type (80% cutoff)	Isolate number
P1	52, 53
P2	27, 28, 29, 30, 32, 35, 41
P3	9, 11, 54
P4	60, 62
P5	59, 63
P6	31, 55, 56
P7	42, 57, 67
P8	3, 4, 5
P9	7, 13
P10	18, 19, 21, 22, 23
P11	50, 51
P12	48, 49
P13	16, 17, 20

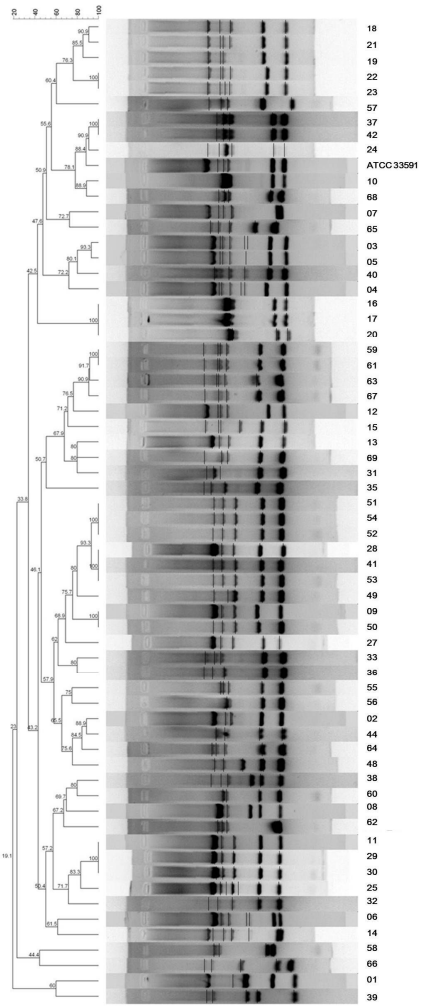
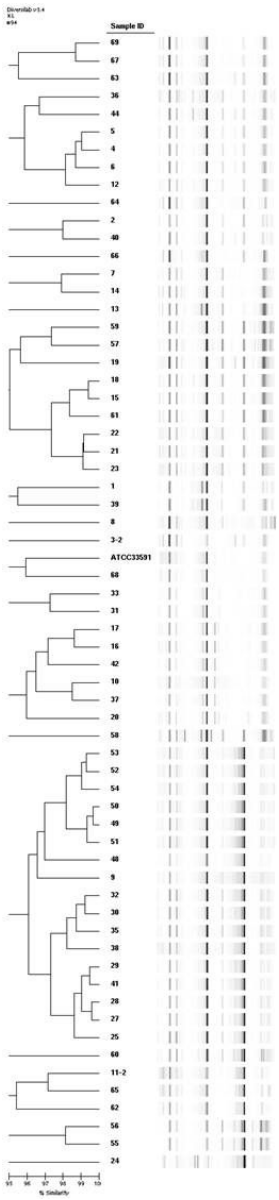


Fig. 2 Multiple-locus variable-number tandem-repeat fingerprinting (MLVF) types of the study isolates generated by the UPGMA algorithm using 75% and 70% similarity cutoff values. The isolates that were not included in the above types were considered to be unique patterns. Isolate numbers presented in boldface were not included in any types generated by the 75% cutoff.

MLVF type (75% cutoff)	Isolate number
M1	18, 19, 21, 22, 23
M2	10, 24, 37, 42, 68
M3	3, 5, 40
M4	12, 59, 61, 63, 67
M5	16, 17, 20
M6	13, 31, 69
M7	33, 36
M8	9, 28, 41, 49, 50, 51, 52, 53, 54
M9	55, 56
M10	2, 44, 48, 64
M11	38, 60
M12	11, 25, 29, 30

MLVF type (70% cutoff)	Isolate number
M1	18, 19, 21, 22, 23
M2	10, 24, 37, 42, 68
M3	3, **4**, 5, 40
M4	12, **15**, 59, 61, 63, 67
M5	16, 17, 20
M6	13, 31, 69
M7	33, 36
M8	9, 28, 41, 49, 50, 51, 52, 53, 54
M9	55, 56
M10	2, 44, 48, 64
M11	38, 60
M12	11, 25, 29, 30
M13	**7, 65**



DL type (KL 95% cutoff)	Isolate number
DL1	69,67,63
DL2	36,44,5,4,6,12
DL3	2,40
DL4	14,7
DL5	59,57,19
DL6	18,15,61,22,21,23
DL7	1,39
DL8	31,33
DL9	17,16,42,10,37,20
DL10	53,52,54,50,49,51,48,9,32,30,35,38,29,41,28,27,25
DL11	11,65,62
DL12	56,55

Fig.3 Repetitive sequence-based PCR (rep-PCR) types of the study isolates generated by the UPGMA algorithm using 95% similarity cutoff value. Similarity between overlaid peaks was calculated using the Kullback Leibler correlation. The isolates that were not included in the above types were considered to be unique patterns.

3. Comparison of typing methods

To compare the discriminatory power of PFGE, MLVF, and rep-PCR, we determined the mean number of isolates per type and Simpson's diversity index for all the isolates (Table 2). MLVF (75% cutoff) and PFGE (80% cutoff) yielded similar D-values of 0.954 (95% CI 0.933–0.974) and 0.972 (95% CI 0.957–0.988), respectively. However, the D-value of rep-PCR (95% cutoff) was slightly lower than that of MLVF or PFGE [0.897(0.848–0.946)].

To compare the congruence between type assignments using PFGE and MLVF, we calculated adjusted Rand indices (Table 3) and Wallace coefficients (Table 4) for each cutoff value of PFGE and MLVF. The overall congruence MLVF and rep-PCR compared to PFGE was presented by adjusted Rand indices, ranging from 0.234 to 0.278. As expected from the Wallace coefficients, PFGE predicted MLVF or rep-PCR type better than MLVF or rep-PCR predicted PFGE type (Wallace coefficients, PFGE cutoff 80% vs. MLVF cutoff 75%, 0.389 vs. 0.233; PFGE cutoff 80% vs. MLVF cutoff 70%, 0.426 vs. 0.232; PFGE cutoff 75% vs. MLVF cutoff 75%, 0.359 vs. 0.256; PFGE cutoff 75% vs. MLVF cutoff 70%, 0.391 vs. 0.253; PFGE cutoff 80% vs. DL cutoff 95%, 0.625; PFGE cutoff 75% vs. DL cutoff 95%, 0.512; MLVF cutoff 75% vs. DL cutoff 95%, 0.542; PFGE cutoff 70% vs. DL cutoff 95%, 0.505).

Although the congruence between PFGE and MLVF in typing these clinical isolates was poor, notable agreement was found when the relationship

between a pair of isolates was analyzed (Table 5). Cross-classification of the isolates, based on matched or mismatched schemes by PFGE (80% cutoff) and MLVF (75% cutoff), showed that the 2 typing systems were 91.0% concordant. Using different cutoffs, the PFGE and MLVF showed 88.3% to 90.1% concordance (data not shown). Any given pair of isolates distinguished by one method tended to be distinguished by the other. Since the maximum samples for printed similarity matrix, the cross-classification of the isolates by rep-PCR and PFGE could not be obtained.

Table 2. Simpson's diversity indices of the genotyping methods for all isolates typed

Methods	No. of types	No. of isolates per type (mean ± SD)	D (95% CI)
PFGE (80% cutoff)	13	3.0 ± 1.47	0.972 (0.957−0.988)
PFGE (75% cutoff)	14	3.1 ± 1.44	0.967 (0.951−0.983)
MLVF (75% cutoff)	12	3.9 ± 1.98	0.954 (0.933−0.974)
MLVF (70% cutoff)	13	3.9 ± 2.02	0.949 (0.929−0.970)
DL (95% cutoff)	12	4.5 ± 4.27	0.897 (0.848−0.946)

Abbreviations: D, Simpson's index; CI, confidence interval; PFGE, pulsed-field gel electrophoresis; MLVF, multiple-locus variable-number tandem-repeat fingerprinting; DL, DiversiLab repetitive sequence-based PCR.

Table 3. Adjusted Rand index for PFGE, MLVF, and repetitive-sequence-based PCR using different cutoffs

Typing method	Adjusted Rand index				
	PFGE cutoff 80%	PFGE cutoff 75%	MLVF cutoff 75%	MLVF cutoff 70%	DL cutoff 95%
PFGE cutoff 80%	1				
PFGE cutoff 75%	0.860	1			
MLVF cutoff 75%	0.266	0.271	1		
MLVF cutoff 70%	0.275	0.278	0.950	1	
DL cutoff 95%	0.250	0.234	0.321	0.321	1

Abbreviations: PFGE, pulsed-field gel electrophoresis; MLVF, multiple-locus variable-number tandem-repeat fingerprinting; DL, DiversiLab repetitive sequence-based PCR.\

$$\text{Adjusted Rand index} = \frac{index - expected\ index}{maximum\ index - expected\ index} = \frac{a+d-n_c}{a+b+c+d-n_c}$$

where: $n_c = \frac{n(n^2+1)-(n+1)\sum n_i^2-(n+1)\sum n_j^2+2\sum\sum\frac{n_i^2 n_j^2}{n}}{2(n-1)}$

Given two partition schemes of the same data set, P and P', all these coefficients are calculated based on the fact that a pair of points (in microbial typing, a pair of points is a pair of isolates under study) from the data set will fall into one of the following conditions: a, the number of point pairs that are in the same cluster in P and P'; b, the number of point pairs that are in the same cluster in P but not in P'; c, the number of point pairs that are in the same cluster in P but not in P'; or d, the number of point pairs that are in different clusters in P and P'. n represents the total sample size, n_i the number of species belonging to the cluster *i* of partition A, and n_j the number of species belonging to the cluster *j* of partition B.

Table 4. Wallace coefficients for PFGE and MLVF using different cutoffs

Typing method	Wallace coefficient (95% CI)				
	PFGE cutoff 80%	PFGE cutoff 75%	MLVF cutoff 75%	MLVF cutoff 70%	DL cutoff 95%
PFGE cutoff 80%		0.944 (0.886–1.000)	0.389 (0.275–0.503)	0.426 (0.308–0.544)	0.628 (0.496–0.761)
PFGE cutoff 75%	0.797 (0.706–0.888)		0.359 (0.254–0.465)	0.391 (0.276–0.506)	0.512 (0.388–0.637)
MLVF cutoff 75%	0.233 (0.145–0.321)	0.256 (0.161–0.350)		1.000 (1.000–1.000)	0.542 (0.435–0.648)
MLVF cutoff 70%	0.232 (0.146–0.319)	0.253 (0.158–0.347)	0.909 (0.836–0.982)		0.505 (0.401–0.278)
DL cutoff 95%	0.156 (0.033–0.278)	0.151 (0.028–0.274)	0.228 (0.066–0.278)	0.235 (0.072–0.278)	

Abbreviations: CI, confidence interval; PFGE, pulsed-field gel electrophoresis; MLVF, multiple-locus variable-number tandem-repeat fingerprinting, DL, DiversiLab repetitive sequence-based PCR.

Wallace's coefficients $W_{P'\rightarrow P}=\frac{a}{a+b}$ $W_{P\rightarrow P'}=\frac{a}{a+c}$

Given two partition schemes of the same data set, P and P', all these coefficients are calculated based on the fact that a pair of points (in microbial typing, a pair of points is a pair of isolates under study) from the data set will fall into one of the following conditions: a, the number of point pairs that are in the same cluster in P and P'; b, the number of point pairs that are in the same cluster in P but not in P'; c, the number of point pairs that are in the same cluster in P but not in P'; or d, the number of point pairs that are in different clusters in P and P'.

Table 5. Cross-classification of all possible pairs of isolates based on matched or mismatched PFGE and MLVF types

PFGE (80% cutoff)	MLVF (75% cutoff)		Sum
	Match	Mismatch	
Match	31	30	61
Mismatch	145	1747	1892
Sum	176	1777	1953

$P < 0.001$

Concordance $= \frac{31+1747}{1953} \times 100 = 91.0$ (%)

Abbreviations: PFGE, pulsed-field gel electrophoresis; MLVF, multiple-locus variable-number tandem-repeat fingerprinting.

Discussion

Several studies based on VNTR schemes have been published for *S. aureus* strain typing [5-8, 16-18]. Most of the studies, however, were performed using selected MRSA isolates of certain limited PFGE types, which might introduce a selection bias. In this study, we chose clinically significant *S. aureus* isolates to evaluate the utility of MLVF and rep-PCR, with respect to infection control in a hospital setting. Because PFGE is labor intensive, time consuming, and expensive, routine use of this procedure in a clinical laboratory is rather demanding. Therefore, we tried to determine whether PFGE could be replaced with the more "simplified" MLVF and rep-PCR. We used a commercial premix PCR kit, instead of preparing a mixture of PCR reagents before amplification. After PCR amplification, we performed electrophoresis for only 80 min with a 12-cm length system, which can easily be utilized in clinical laboratories. Our modified MLVF method could save much time, compared to PFGE, which requires approximately 3 days (2 days for DNA agarose disc preparation, followed by 1 day of pulsed-field gel electrophoresis), as well as the previous MLVF procedure that utilized a master mix, which was prepared by adding each

ingredient to each reaction tube, plus electrophoresis using an extended gel, which required 3 h 30 min for running [6]. Even the cost of our modified MLVF was much lower than that of PFGE ($10 vs. $15 per test). Although the high cost ($74 per test) of the rep-PCR pointed as a drawback, the total process accomplished using reagent or kit supplied by the manufacturer provided handiness to the technician.

As shown by Simpson's diversity index, in our study, MLVF showed discriminatory power similar to that of PFGE. The rep-PCR, however, demonstrated low discriminatory power compared to PFGE and MLVF. This finding is consistent with the findings of a previous study that used 8 different VNTR loci, including *spa* and *sspa* for MLVF [16] and other studies regarding rep-PCR showing Simpson's diversity index of 0.905 for PFGE and 0.860 for rep-PCR [19], which argues against the use of DL typing as a standalone typing technique and the authors recommend that rep-PCR typing should be used to screen isolates, followed by testing strains that share the same rep-PCR type with a more discriminative method [20, 21].

The overall congruence between types defined by PFGE, MLVF, and rep-PCR was fairly low, as reflected by adjusted Rand index and Wallace coefficients. The adjusted Rand index allows a quantitative

evaluation of the global congruence between the 2 partitions [12]. On the other hand, Wallace coefficients, given a particular typing method, provide an estimate of how much new information is obtained from another method. The adjusted Rand index between PFGE and MLVF calculated in our study was similar to that calculated in 2 previous studies [18, 22]. However, the Wallace coefficient of PFGE predicting MLVF type in our study was lower than that calculated in the 2 previous studies. The discrepancy in the results may be explained by the difference in the *S. aureus* isolates used. Since the electrophoretic pattern of MLVF is based on staphylococcal interspersed repeats, the difference in the bacterial isolates might have affected the clustering profile. Those previous studies enrolled EMRSA-15 (ST22) and EMRSA-16 (ST36/ST30) or ST398 MRSA. In our study, we used various clinical isolates with diverse PFGE or MLVF patterns that could be classified into 37 types by PFGE (80% cutoff) and into 28 types, including unique types by MLVF (75% cutoff). Moreover, 15 of 65 isolates were methicillin-susceptible *S. aureus* (MSSA), which is known to be more polyclonal than MRSA [23].

Another explanation for the relatively low Wallace coefficients of PFGE predicting MLVF types in our study could be that we used only *Sma*I for DNA digestion in the PFGE. In the study by Rasschaert *et al.*, *Bst*ZI,

*Sac*II, *Apa*I, and combinations of these enzymes were used [22]. The combination of all 3 restriction enzymes yielded a Wallace coefficient of 1.00 in predicting MLVF types in that study.

However, when the relationship between 2 specific isolates was analyzed, PFGE (cutoff 80%) and MLVF (cutoff 75%) showed 91.0% concordance, calculated by each cell (Table 5). In other words, every pair of isolates can be distinguished by either MLVF or PFGE. Although the InfoQuestFP software provided excellent dendrograms by applying multilinkage where branches degenerate, every dendrogram would confront loss of information while converting the data set of similarity values into a simplified hierarchical structure [24]. Furthermore, similar to PFGE analysis, MLVF also required careful visual inspection to confirm the results owing to small band shifts.

Even though the Wallace coefficients for MLVF predicting PFGE type (gold standard cutoff of 80%) were similar at both MLVF cutoffs of 75% and 70% (0.233 vs. 0.232), we suggest that a 75% cutoff be used for MLVF, since it showed higher discriminatory power.

The adjusted Rand index between PFGE (cutoff 80%) and rep-PCR was fairly low but slightly higher than that observed in previous study (0.250 vs. 0.083) [25]. The Wallace coefficient of PFGE predicting rep-PCR

type (0.628) showed similar result from previous study (0.540) [25]. However, the Wallace coefficient of rep-PCR predicting PFGE type showed slightly higher value compared to that of previous study indicating that the rep-PCR performed in this study demonstrated better correlation with PFGE strain typing compared to the previous study [25]. The adjusted Rand index and Wallace coefficient obtained between rep-PCR and PFGE or MLVF showed that MLVF has discriminatory power compared to rep-PCR and more effectively predict PFGE types.

We could confirm that the previous studies describing rep-PCR as a technically rapid and easy platform with short hands on time and the whole process is performed by kit-supplied reagent that one technician could complete the typing within 8 h [20, 21, 25]. The DNA extraction step however, was laborious and took most of the hands on time that the manufacturer might consider replacing it to utilizing automated equipment.

In conclusion, our modified protocol of MLVF demonstrated good performance in typing of clinically significant *S. aureus* isolates selected on the basis of the CDC/NHSN criteria. Furthermore, we were able to shorten the running time by using a PCR premixture kit and performing electrophoresis using a 12-cm length system. Because MLVF has large advantage over PFGE in terms of technical aspects, MLVF could be used in

place of PFGE, especially when urgent investigation of a hospital *S. aureus* outbreak is required. The commercially available rep-PCR method, although rapid and easy to perform, has limited discriminatory power and therefore should be complemented by more discriminative method in isolates that share identical patterns.

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국문 초록

다중 유전자좌 일렬반복수변이 지문분석법은 다중 유전자좌를 이용한 다중핵산증폭검사에 기반한다. 이와 비슷하게 반복염기서열 기반 PCR은 세균의 유전체 사이사이에 반복적으로 나타나는 염기서열을 표적으로 하여 검사하는 방법이다. 이번 연구의 목적은 다중 유전자좌 일렬반복수변이 지문분석법과 반복염기서열 기반 PCR을 임상에서 분리된 황색포도알균의 균주 타이핑에서 전통적으로 표준방법으로 쓰여왔던 간헐영역겔전기이동과 비교하고자 하였다.

서울대학교병원 임상 검체에서 분리된 황색포도알균을 대상으로 다중 유전자좌 일렬반복수변이 지문분석법, 반복염기서열 기반 PCR, 간헐영역겔전기이동을 이용하여 각각 분석하였다. 다중 유전자좌 일렬반복수변이 지문분석법은 *clfA*, *clfB*, *sdrCDE*, *sspA*, *spa* 의 5가지 시발체를 이용하였다. 반복염기서열 기반 PCR 디버시랩의 황색포도알균 키트를 사용하여 분석하였다. 간헐영역겔전기이동에는 *Sma*I의 제한효소를 이용하였다. 다중 유전자좌 일렬반복수변이 지문분석법 과 간헐영역겔전기이동 검사에서 얻어진 밴드 패턴은 인포퀘스트 소프트웨어를 이용하여 분석하였고, 반복염기서열 기반 PCR에서 얻어진 전기영동 그래프는 회사에서 제공하는 인터넷 기반 디버시랩 소프트웨어를 이용하여 분석하였다.

다중 유전자좌 일렬반복수변이 지문분석법 및 반복염기서열 기반 PCR을 이용하였을 때 검사자가 들인 소요시간은 3시간 정도였다. 간헐영역겔전기이동, 다중 유전자좌 일렬반복수변이 지문분석법, 반복염기서열 기반 PCR 검사시 각각 63개의 임상균주를 13, 12, 12

개의 타입으로 분류할 수 있었다. 그 중에서 3개의 타입은 간헐영역겔전기이동 및 다중 유전자좌 일렬반복수변이 지문분석법 에서 동일하게 분류되었다. 하지만 반복염기서열 기반 PCR 검사를 간헐영역겔전기이동과 비교하였을 때 일치하는 균주 군은 발견할 수 없었다. 오직 2개의 균주 군만이 두 검사 방법에서 비슷한 임상균주를 포함함을 알 수 있었다. 간헐영역겔전기이동과 다중 유전자좌 일렬반복수변이 지문분석법은 비슷한 정도의 심슨의 다양성 인덱스를 나타냄으로써 비슷한 정도의 균주 변별력을 가짐을 시사하였다. 전반적인 균주 타이핑의 일치율은 간헐영역겔전기이동과 다중 유전자좌 일렬반복수변이 지문분석법에서 0.266-0.278 정도로 낮았고, 간헐영역겔전기이동이 다중 유전자좌 일렬반복수변이 지문분석법의 균주 타이핑을 예측할 수 있는지 여부는 윌러스 계수로 판단할 때 0.389로 낮았다. 간헐영역겔전기이동과 반복염기서열 기반 PCR을 비교하였을 때 두 검사간의 전반적인 일치율은 0.234-0.250으로 낮았고, 간헐영역겔전기이동이 반복염기서열 기반 PCR을 예측하는 정도는 0.628로 그 반대의 경우(0.156)보다 월등히 높았다.

결론적으로 간단하면서 비용이 싼 다중 유전자좌 일렬 반복수변이 검사는 반복염기서열 기반 PCR 및 간헐영역겔전기이동과 비교하였을 때 비교적 빠르게 효과적으로 황색포도알균의 균주 타이핑에 도움이 될 것이며 향후 병원 감염 관리에서 역할이 기대된다.

주요어 : 황색포도알균, 다중 유전자좌 일렬 반복수변이 지문분석법, 반복염기서열 기반 PCR, 간헐영역겔전기이동

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