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Bacteriological and Immunological Characteristics of Community-associated Methicillin-Resistant *Staphylococcus aureus* in Korea

울 산 대 학 교 대 학 원

의 과 학 과

조 영 건

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이 논문을 이학석사 학위 논문으로 제출함

2023년　8월

울 산 대 학 교　대 학 원

의 과 학 과

조 영 건

조영건의 이학석사 학위 논문을 인준함

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Abstract

Staphylococcus aureus is a major bacterial pathogen causing skin and soft tissue infections, pneumonia, and bacteremia in community and hospital infections. Since the 1960s, the frequency of methicillin-resistant *Staphylococcus aureus* (MRSA) has increased rapidly worldwide. In Korea, the frequency of hospital-associated methicillin-resistant *Staphylococcus aureus* (HA-MRSA) increased in the 1980s. Since the 2000s, community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) has increased not only in Korea but also worldwide. In this study, bacteriological and immunological studies were conducted to compare the main sequence type strain of CA-MRSA, which is currently prevalent in Korea, with the main sequence type strain of HA-MRSA, which was prevalent in the past. This laboratory has been collecting isolated and identified MRSA from clinical specimens of MRSA patients at Asan Medical Center in Seoul for 15 years. Strain types were obtained by confirming the molecular genotypes of all MRSA, and bacteriological studies including quantitative real time-polymerase chain reaction (qRT-PCR) and western blot were performed using subjected strains. As a result of the bacteriological study, four of the genes encoding surface proteins of *S. aureus* showed significant differences at mRNA level between the two types of strains. Following this, it was confirmed that there was a difference at the protein level as well. Several immunological studies, including intracellular cytokine staining (ICS) and enzyme-linked Immunosorbent assay (ELISA), additionally performed using patients' blood. This resulted in a significant difference in the concentration of IL-17A in serum or plasma. In this study, it was confirmed that there was a

difference in the expression level of surface proteins between the two types bacteriologically. Immunological studies did not reveal any remarkable differences between the two types. It is expected that further studies will reveal the cause of the differences in the infection route and clinical pathogenesis through the surface proteins of the two types of strains.

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Introduction

Staphylococcus aureus is a major bacterial pathogen that causes various infections such as bacteremia, endocarditis, osteoarticular infections, skin and skin structure infections. In the past, the mortality rate of *S. aureus* infection reached 90% when antibiotics were not used. However, the rate dropped dramatically with the use of penicillin. However, it was not long before most of *S. aureus* appeared resistant strains to penicillin. To treat these resistant strains, methicillin, a semi-synthetic beta-lactam antibiotic that is not degraded by beta-lactamase secreted by *S. aureus*, was developed in 1960. However, methicillin-resistant *S. aureus* (MRSA), which does not respond to this antibiotic, has emerged. MRSA first appeared in England in 1961, and since the late 1970s, its frequency has increased rapidly around the world, including England and Australia, and has been spread in hospitals in the 1980s. In Korea, the frequency of MRSA began to increase rapidly from the 1980s. Most of them were isolated from general hospitals or university hospitals, and among them, MRSA was known to be the main cause of hospital infections in Korea, with the rate exceeding 60-70%. Until the 2000s, patients who appeared to have at least one healthcare-associated risk factor were predominantly present. In other words, even community-acquired infections were often associated with hospital infections, and these cases were classified as hospital-associated MRSA (HA-MRSA). HA-MRSA appeared mainly in hospitals until the 2000s, and sequence type 5 (ST5) was the major strain type in any country including Korea. Since the 2000s, cases where infection occurred in the community without a healthcare exposure history were

classified as community-associated MRSA (CA-MRSA), and its influence has expanded worldwide. CA-MRSA stains have staphylococcal cassette chromosome *mec* type IV or V worldwide and belong to clonal complex 8, but multi locus sequence type (MLST) and *spa* types are different for each country and region. For example, ST8 in the US and ST72 in Korea. CA-MRSA in Korea has established sequence type 72 (ST72) as the major strain type and is still one of the dominant types. This CA-MRSA enters the hospital and competes with ST5-MRSA and methicillin-sensitive (susceptible) *S. aureus* (MSSA) (Fig. 1).

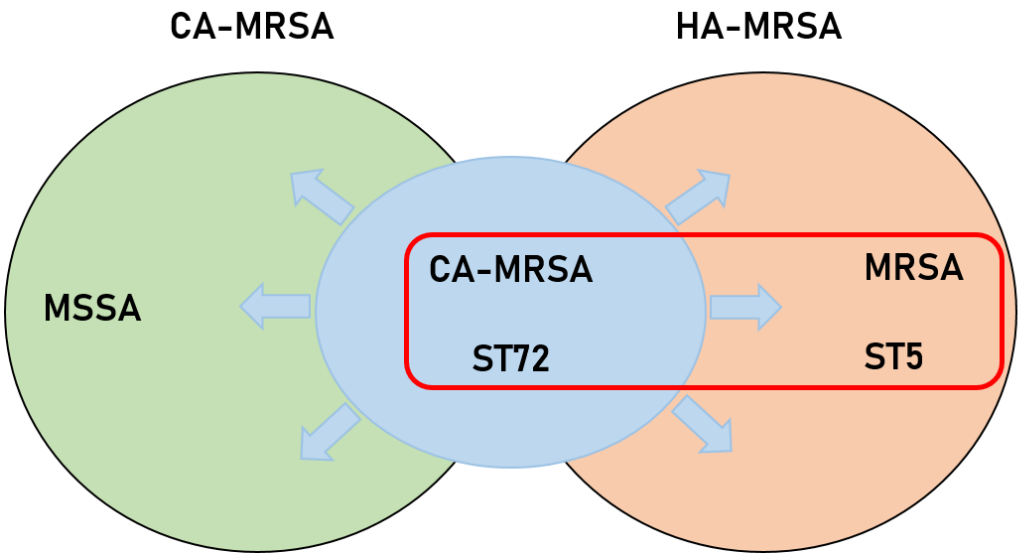


Figure 1. Prevalence patterns of CA-MRSA in Korea within communities and hospitals.

ST8 has characteristics that cause skin and skin structure infections such as skin abscess and pneumonia in the community, and occurred mainly in athletes, prisoners, and homosexuals who have frequent skin contact. Panton-Valentine Leukocidin (PVL) and PSM are known as the main virulence factors of ST8, and ST8 is known to be more toxic than ST5, the existing HA-MRSA. Unlike ST8, ST72, a major CA-MRSA strain in Korea, does not have PVL toxin and is characterized by causing bone and joint infection, but there is no known major virulence factor. In this study, I tried to find the factors associated with pathogenesis of the ST72-MRSA by comparing with the ST5-MRSA. I compared the molecular bacteriologic characteristics of the ST72-MRSA, the main CA-MRSA clone in Korea with that of ST5-MRSA, which had been main HA-MRSA clone. We also compared the immunologic characteristics of patients with ST72-MRSA bacteremia with that of patients with ST5-MRSA bacteremia. In *S. aureus*, surface proteins and secreted proteins play chief role in pathogenesis of *S. aureus* infection (Fig. 2).

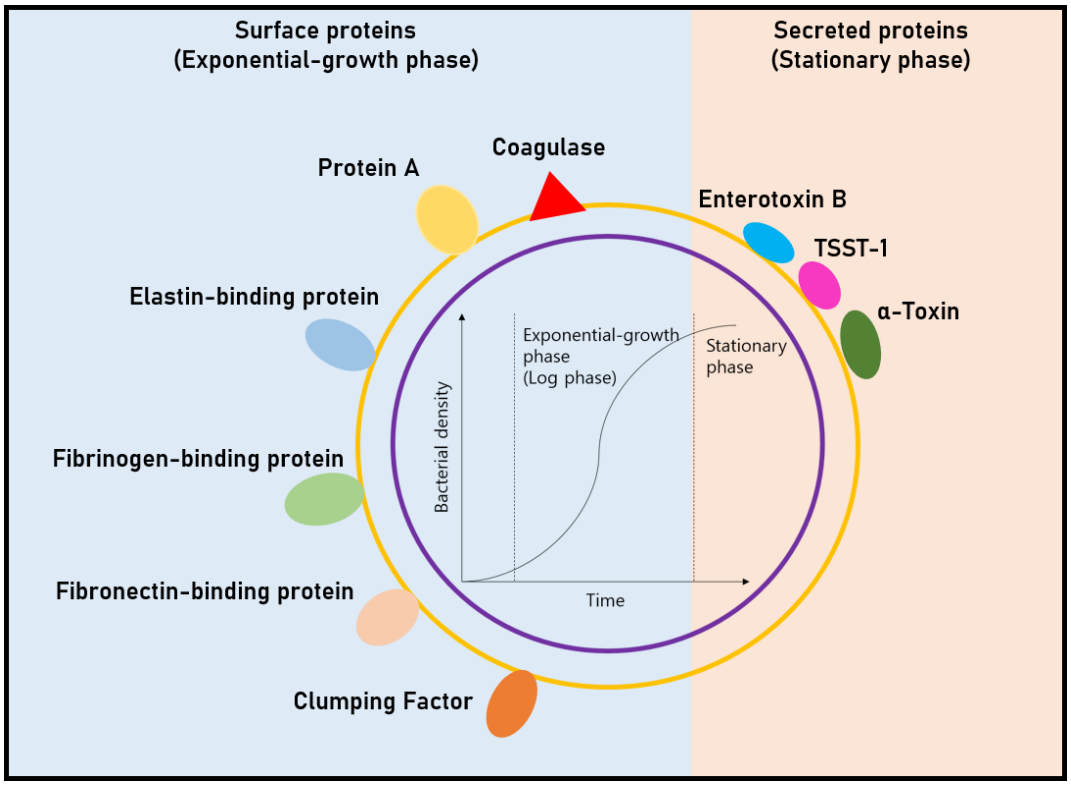


Figure 2. Virulence factors of *S. aureus* expressed during the growth cycle

One of the defining characteristics of *S. aureus* is its ability to form clumps in the presence of soluble fibrinogen, which likely has a protective benefit and facilitates adhesion to host cells or tissues [1]. Surface proteins play an important role in this role and are mainly expressed during the exponential growth phase of MRSA Bacteremia. Important bacterial virulence factors are surface factors that cause bacteria to adhere and colonize at the surfaces. These specific adhesive agents can bind to a variety of host proteins, particularly those in the extracellular matrix (ECM), and also to the host cells. This binding is mediated by a family of proteins called microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) [3, 24]. In the stationary

phase when the number of bacteria is sufficiently increased and the infection is established, the secreted protein plays an important role as a virulence factor. In other words, surface proteins play a role in attaching MRSA to cells or tissues in the early stage of infection, and after attached, the secreted proteins play a role in expressing toxicity to cells or tissues. *S. aureus* expresses various surface proteins including staphylococcus protein A (spa), clumping factor A (clfA), clumping factor B (clfB), fibronectin-binding protein A (fnbA), fibrinogen-binding protein (fib), elastin-binding protein S (ebpS), and coagulase (coa). As a bacteriological study, the difference in expression of surface proteins at the early stage of infection was compared at the mRNA and protein levels. In addition, there have few human immunologic studies especially on the T cell immune response studies using the blood of Patients with S. aureus bacteremia. I tried to characterize the cytokine profiles and T cell immune response of ST72-MRSA bacteremia by comparing with that of ST5-MRSA bacteremia. Using the advantage of the laboratory where patient's blood was well collected, we also tried to see if there was a difference in the host immune response to infection of each type of strain. In this study, I tried to find the factors associated with pathogenesis of the ST72-MRSA by studying the expression of surface proteins as a molecular bacteriologic study and the the cytokine profiles and T cell immune responses as an immunologic study.

Materials and Methods

1. Collection and Isolates of *Staphylococcus aureus*

This study samples were collected 1101 MRSA strains obtained with bacteremia patients from Asan Medical Center, Seoul, and Republic of Korea from 2008 to 2022. The strains collected using blood agar plate (BAP) at Department of Laboratory Medicine. The strains were streaked at fresh BAP and incubated overnight at 37℃. This overnight incubated strains were stored in 20% glycerol-Tryptic Soy Broth (TSB) at -80℃ for a long time store.

2. Genomic DNA (gDNA) extraction

Genomic DNA (gDNA) extraction was using WizPrep™ gDNA mini kit (Cell/Tissue) (wizbiosolution). gDNA of *S. aureus* was obtained from the single colony isolated from overnight sub-cultured *S. aureus* in BAP. Single colony of the cultured strain was collected with a plastic loop into E-tube, suspended in GT1 Buffer or lysozyme. GT2 buffer and proteinase K were added with vortex and incubated the mixture for 10 min in water bath at 56℃. 100% ethanol was added the mixture and transferred to spin column. The column was centrifuged for 1 min at 13,000 rpm. Then, the column was washed with W1 and W2 buffer for 1 min at 13,000 rpm. After washing steps, gDNA was obtained with 50 ul of elution buffer. The extracted gDNA was used for next molecular genotyping of MRSA.

3. δ-hemolysin activity test

To determine δ-hemolysin activity of strains, we used δ-hemolysin activity by cross-streaking vertically RN4220 strain and test strains on a BAP. The plate was incubated for 16 hours at 37℃ The next day, δ-hemolysin activity was presented by an enhanced area of hemolysis at the intersection streaks on BAP.

4. MRSA genotyping

4-1) Detection of *mecA* gene

MecA positive were determined with PCR product size by polymerase chain reaction (PCR). Primers information were listed in Table 1 from MRSA protocols [30]. The PCR condition was an initial denaturation at 2 min, followed by 35 cycles of 95℃ for 15 sec, 55℃ for 15 sec and 72℃ 1 min and final extension at 72℃ for 7 min. PCR products (2.5 ~ 3.5 ul) were separated by 1 % agarose gel in 0.5 X TAE buffer at 100 V and visualized with 20000X RedSafe. *MecA* gene positive was determined by the presence of a product band with a size of 286 bp in the gel image.

4-2) SCC*mec* genotyping.

SCC*mec* genotyping of strains were determined with PCR product size by two-steps multiplex PCR. Primers information were listed in Table 1 from MRSA protocols [30]. The first multiplex PCR (MPCR1) condition was an initial denaturation at 2 min, followed by 35 cycles of 95℃ for 15 sec, 55℃ for 15 sec and 72℃ 1 min and final extension at 72℃ for

7 min. The second multiplex PCR (MPCR2) conditions were an initial denaturation at 2 min, followed by 35 cycles of 95℃ for 15 sec, 55℃ for 15 sec and 72℃ 1 min 30 sec and final extension at 72℃ for 7 min. PCR products (2.5 ~ 3.5 ul) were separated by 1 ~ 1.5% agarose gel in 0.5 X TAE buffer at 100 V and visualized with 20000X RedSafe. SCC*mec* type determined according to size of product band in gel image.

Table 1. Primers used in the multiplex PCR for *mecA* detection and SCC*mec* genotyping

	Target genes	Primer name	Sequence (5'-3')	Size (bp)	Type
M-PCR1	mecA	*mA1*	TGCTATCCACCCTCAAACAGG	286	MRSA
		mA2	AACGTTGTAACCACCCCAAGA		
	ccrB	*Bc*	ATTGCCTTGATAATAGCCITCT		reverse
	ccrA1	*α1*	AACCTATATCATCAATCAGTACGT	695	I
	ccrA2	*α2*	TAAAGGCATCAATGCACAAACACT	937	II, IV
	ccrA3	*α3*	AGCTCAAAAGCAAGCAATAGAAT	1,791	III
	ccrC	*γR*	CCTTTATAGACTGGATTATTCAAAAT	518	III, V, VII
		γF	CGTCTATTACAAGATGTTAAGGATAAT		
	ccrAB4	*α4.2*	CTATCAATGCACCAGAACTT	1,287	VI, VIII
		β4.2	TTGCGACTCTCTTGGCGTTT		
M-PCR2		*mA7*	ATATACCAAACCCGACAACTACA		reverse
	Class A (mecA-mecI)	*m16*	CATAACTTCCCATTCTGCAGATG	1,965 1,797	II, VIII III
	Class B (mecA-IS1272)	*IS8*	GGTTTCACTCGGATGTCTGT	1,594 3,154	I, IV, VI IVA
	Class C2 (mecA-IS431)	*IS2*	TGAGGTTATTCAGATATTTCGATGT	804	V, IX

4-3) Determination of accessory gene regulator (agr) types

Polymerase chain reaction (PCR) method was performed for determination of *agr* genotype of MRSA. Primers were listed in Table 2. PCR condition was 1 cycle of initial denaturation for 1 min at 95℃, followed by 35 cycles of 95℃ for 15 sec, 55℃ for 15 sec and 72℃ 1 min and final extension at 72℃ for 7 min. PCR products (2.5~3.5 ul) were separated by 1% agarose gel in 0.5 X TAE buffer at 100 V and visualized with 20000 X RedSafe. *Agr* type was determined according to size of product band in gel image.

Table 2. Primers used for agr genotyping of Staphylococcus aureus.

Gene	Primers (5'-3')	Product size
F primer	PanF - ATG CAC ATG GTG CAC ATG C	-
agr I	R - GTC ACA AGT ACT ATA AGC TGC GAT	441 bp
agr II	R - TAT TAC TAA TTG AAA AGT GGC CAT AGC	575 bp
agr III	R - GTA ATG TAA TAG CTT GTA TAA TAA TAC CCA G	323 bp
agr IV	R - CGA TAA TGC CGT AAT ACC CG	659 bp

4-4) Multi locus sequence typing (MLST)

The multi locus sequence type of strains were determined by PCR method using the seven housekeeping genes of *S. aureus* : carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glpF*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), Triosephosphate isomersase (*tpi*), and acetyl coenzyme A acetyltransferase (*yqiL*). Primers of the seven genes

were listed in table 3 from online *S. aureus* MLST database (https://pubmlst.org/organisms/staphylococcus-aureus/primers).

PCR condition was 1 cycle of initial denaturation for 1 min at 95℃, followed by 35 cycles of 95℃ for 15 sec, 55℃ for 15 sec and 72℃ 1 min and final extension at 72℃ for 7 min. The completed products ordered sequencing at Cosmogenetech (http://www.cosmogenetech.com/main.jsp) and confirmed each sequence type of seven housekeeping genes by sequence query at online *S. aureus* MLST database.

Table 3. Primers used for multi locus sequence typing (MLST)

Gene	Primer	Sequence (5' - 3')	Product size
carbamate kinase (*arcC*)	*arcC*	F – TTGATTCACCAGCGCGTATTGTC R – AGGTATCTGCTTCAATCAGCG	456 bp
shikimate dehydrogenase (*aroE*)	*aroE*	F – ATCGGAAATCCTATTTCACATTC R - GGTGTTGTATTAATAACGATATC	456 bp
glycerol kinase (*glpF*)	*glpF*	F – CTAGGAACTGCAATCTTAATCC R – TGGTAAAATCGCATGTCCAATTC	465 bp
guanylate kinase (*gmk*)	*gmk*	F – ATCGTTTTATCGGGACCATC R – TCATTAACTACAACGTAATCGTA	417 bp
phosphate acetyltransferase (*pta*)	*pta*	F – GTTAAAATCGTATTACCTGAAGG R – GACCCTTTTGTTGAAAAGCTTAA	474 bp
triosephosphate isomerase (*tpi*)	*tpi*	F – TCGTTCATTCTGAACGTCGTGAA R – TTTGCACCTTCTAACAATTGTAC	402 bp
acetyl coenzyme A acetyltransferase (*yqiL*)	*yqiL*	F – CAGCATACAGGACACCTATTGGC R – GTTGAGGAATCGATACTGGAAC	516 bp

4-5) *spa* typing

Polymerase chain reaction (PCR) method was performed for *spa* typing of

subjected strains using *spa* primer (1117F/1514R) listed in table 4. PCR condition was 1 cycle of initial denaturation for 1 min at 95℃, followed by 35 cycles of 95℃ for 15 sec, 55℃ for 15 sec and 72℃ 1 min and final extension at 72℃ for 7 min. The completed products ordered sequencing at Cosmogenetech (http://www.cosmogenetech.com/main.jsp).

The sequenced samples were determined by Bionumerics version 7.6 (Applied Maths) for final *spa* typing. If samples were not performed PCR using 1117F/1514R primer, 794F/1738R primer was used at PCR with same condition.

Table 4. Primers used for amplification of the *spa* genes from staphylococcus aureus

Gene	Primer	sequence (5' - 3')
spa	1117F	GACGATCCTTCGGTGAGCAAAG
	1514R	CAGCAGTAGTGCCGTTTGCTT
spa	794F	CCTATTGTCAGAAGCTAAAAAGTT
	1738R	TTATAGTTCGCGACGACGTCC

5. Expression level mRNA of surface proteins

5-1) Total RNA extraction from MRSA

RNA extraction of all subjected strains used TRIzol reagent. Subjected MRSA isolates were subcultured into blood agar plate (BAP) and cultured overnight at 37℃. The strains were subcultured overnight in tryptic soy broth (TSB) at 37℃ in a 180 RPM shaking incubator. Overnight cultured bacteria was incubated into fresh TSB with 1:100 dilution at 37℃ shaking (180 RPM) for reaching OD_{600} 1.0 ~ 1.1 (mid-exponential phase). After reaching exponential-growth phase, strains were harvested by centrifugation at 13,000 rpm for 1 min. The harvested cell pellets were resuspended with 1 X TE buffer, lysostaphin (1mg/ml), and lysozyme (10ug/ml) for 30 ~ 60 minutes at 37℃. Total RNA was extracted according to the TRIzol Reagent manufacturer's aqueous phase separation and RNA isolation procedures. After followed steps, concentration and purity of total RNA was measured with Nano-Drop spectrophotometer.

5-2) RNA cleanup

20 ug of total RNA was used in DNase treatment step. The extracted RNA was incubated with 10X DNase I Buffer, Recombinant DNase I (RNase-free) (10 U), and DEPC-water for 30 min at 37℃. After DNA clear step, DNase-treated RNA was purified using RNeasy Mini Kit. Finally, the RNA sample was performed polymerase chain reaction (PCR) of DNA gyrase subunit B (gyrB) *staphylococcus aureus* housekeeping gene. PCR

products were separated by 1% agarose gel in 0.5 X TAE buffer at 135 V and visualized with 20000 X RedSafe. If DNA band was not presented in gel, the sample moved on to the next step.

5-3) cDNA synthesis

The cleanup completed RNA quantified using Nano-drop spectrophotometer. Then, 1 ug of RNA was used for cDNA synthesis. The synthesis was used Transcriptor First Strand cDNA synthesis Kit (Roche) by reverse transcriptase using PCR program: denaturation for 10 min at 65℃, incubation for 10 min at 25℃, incubation for 30 min at 55℃, inactivation enzyme for 5 min at 85℃ and holding at 4℃. cDNA was stored at -20℃.

5-4) Quantitative real-time polymerase chain reaction (qRT-PCR) of *staphylococcus aureus* surface proteins genes

cDNA template was used for qRT-PCR to estimate the relative expression of *spa*, *fnbA*, *ebpS*, *clfA*, *clfB*, *fib*, and *coa* genes. Primers of seven genes were listed in Table 5. The expressions of these surface protein genes were normalized by using a housekeeping gene, DNA gyrase B [2]. To perform qRT-PCR, SYBR Green I Kit (LightCycler® 480, Loche). SYBR Green Master Mix, PCR-grade water, cDNA diluted 1:50 of PCR grade water, and primer of the desired gene at a concentration of 5 pM were mixed into LightCycler® 480 Multiwell Plate 96, white (Loche). The equipment of qRT-PCR used LightCycler® 96 (Loche). The qRT-PCR conditions included pre-incubation at 95℃ for 10 min, 45 amplification

cycles of 95℃ for 10 sec, 60℃ for 10 sec and 72℃ for 10 sec and melting cycle of 95℃ for 10 sec, 65℃ for 1 min and 97℃ for 1 sec.

Table 5. Surface proteins gene primers of *S. aureus* used in quantitative real-time polymerase chain reaction (qRT-PCR).

Gene	primer	sequenece (5' – 3')
Target Gene		
staphylococcal protein A	*spa*	F - TATGCCTAACTTAAACGCTG R - TTGGAGCTTGAGAGTCATTA
fibronectin-binding protein A	*fnbA*	F - ACAAGTTGAAGTGGCACAGCC R - CCGCTACATCTGCTGATCTTGTC
elastin-binding protein S	*ebpS*	F – GGTGCAGCTGGTGCAATGGGTGT R - GCTGCGCCTCCAGCCAAACCT
clumping factor A	*clfA*	F – ATGTGACAGTTGGTATTGACTCTGG R - TAGGCACTGAAAAACCATAATTCAGT
clumping factor B	*clfB*	F - ATAGGCAATCATCAAGCA R - TGTATCATTAGCCGTTGTAT
fibrinogen-binding protein	*fib*	F - CGTCAACAGCAGATGCGAGCG R - TGCATCAGTTTTCGCTGCTGGTTT
coagulase	*coa*	F – GTAGATTGGGCAATTACATTTTGGAGG R – CGCATCAGCTTTGTTATCCCATGT
Reference gene		
gyrase subunit B	*gyrB*	F - CAACTATGAAACATTACAGCAGCGT R – TGTGGCATATCCTGAGTTATATTGAAT

6. Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western blot of staphylococcus protein A (spa)

In order to obtain cell wall-associated proteins of the exponential-

growth phase, cells were harvested after shaking incubation at 37°C to OD600 1.0 ~ 1.1. The cell pellet was treated with lysostaphin (1mg/ml), incubated at 37°C for 30 minutes, and further sonication was performed. The cell supernatant obtained through sonication was obtained and protein quantification was performed through BCA assay. The same amount of protein was mixed with 5 X SDS loading buffer and 1 X PBS and used for SDS-PAGE in a final 20 ul. Separating gel was made with 12% (DW, 1.5 M Tris-HCl (pH 8.8), 30% acrylamide, 10% SDS, 10% APS, TEMED), and stacking gel was made with 5%. Electrophoresis was performed on stacking gel at 80 V for 10 minutes and separating gel at 100 V for 1 hour 30 min with 1 X running buffer (1 X Tris-Glycine-SDS buffer). The transfer step used PVDF membrane with transfer buffer (1 X Tris-Glycine buffer with 20% of methanol) at 100 V or 250 mA for 1 hour 30 min. The membrane was washed with 1 X TBS with Tween-20 buffer and blocked with 5 % Skim milk for 2 hours at room temperature. As a primary antibody, monoclonal mouse anti-protein A (Sigma-Aldrich) diluted 1:5000 ~ 1:10000 in 1% skim milk or 1% BSA with 1 X TBS with Tween-20 buffer and incubated overnight shaking at 4℃. The overnight incubated membrane was washed three times and incubated shaking in 3 % skim milk with goat anti-mouse IgG (H+L) secondary antibody conjugate HRP (Invitrogen) of 1:10000 for 2 hours at room temperature. After washing 3 times, detection was performed. Clarity Western ECL Substrate (BIO-RAD) was used for detection. Images were obtained with ChemiDoc™ MP.

7. T cell immune response

7-1) Peripheral Blood Mononuclear Cell (PBMC) isolation of *staphylococcus aureus* bacteremia patients

Venous blood of *Staphylococcus aureus* bacteremia patient was collected in heparinized vacutainer tubes (BD Vacutainer®). First, serum or plasma was collected by centrifuging for 10 min at 2500 rpm for other experiment. The remained bloods were diluted with 1 X Dulbecco's Phosphate Buffered Saline (DPBS, WELGENE) of 1:2 or 1:3 (blood : DPBS). PBMCs were isolated by lymphocyte separation medium (LSM, CORNING) gradient separation for 20 min at 2200 rpm with brake 0. Then, the buffy coat layer was transferred to a new tube. The transferred PBMCs were washed with 1 X DPBS for 10 min at 1800 RPM, 4℃. If Red blood cells were remained with PBMCs, cells was resuspended and incubated with RBC lysis buffer (Red Blood Cell Lysing Buffer Hybri-Max™, SIGMA) for 5~15 min at room temperature. The RBC lysed PBMCs were washed with 1 X DPBS for 10 min at 1800 RPM, 4℃. The pellet was resuspended in 1 ml of 1 X DPBS, then counted the numbers of PBMCs with AOPI (ViaStain™ AOPI Staining Solution, Nexcelom). The counted sample was washed with 1 X DPBS for 10 min at 1800 RPM, 4℃. All steps completed PBMCs were stored in cell freezing media containing 90% Fetal Bovine Serum (FBS, Gibco) and 10% Dimethyl Sulfoxide (DMSO, Hybri-Max™, SIGMA), gradually frozen in a freezing container and stored in liquid nitrogen until analyzed for other experiments.

7-2) Heat-killed *S. aureus* preparation

All *S. aureus* were heat-killed for the human blood analysis. For heat killing, strains were prepared in fresh Tryptic Soy Broth (TSB) at OD_{600} of 1.0. Cell pellet was washed with 1 X phosphate-buffered saline (PBS) for 2 times. CFUs were counted by plating serial dilutions with saline onto Mueller Hinton Agar (MHA) plate. *S. aureus* suspensions were heat-inactivated at 90℃ for 45 minutes. The prepared heat-killed *S. aureus* was treated for PBMC analysis at the same ratio.

7-3) Intracellular cytokine staining (ICS) and flow cytometry analysis of PBMCs.

In this study, freezing PBMCs of SAB patients in LN2 tank were thawed and washed before counting of living cells with AOPI. In healthy donor's case, heat-killed *S. aureus* stimulation performed immediately PBMC isolation within 3 hours after blood draw. Cells were resuspended to final cells of 5 x 10^5 ~ 1 x 10^6 per well of 96 well plate (SPL cell culture plate) and stimulated 5 x 10^7 ~ 1 x 10^8 CFU/ml with heat-killed *S. aureus* of multiplicity of infection (MOI) 1:100 for 7 days. After incubation for 7 days, cell culture supernatant was collected to new tube for cytokine measurement. The remained cells were performed ICS for flow cytometry analysis. The cell viability was confirmed by Near-IR fluorescent reactive dye (Invitrogen), and then CD3 (BD horizon, clone UCHT1), CD4 (eBioscience, clone RPA-T4), and CD8 (eBioscience, clone RPA-T8) staining was performed. Cells were fixed and permeabilized

using the BD Cytofix/Cytoperm Fixation and Permeabilization Kit, followed by ICS with fluorochrome-conjugated antibodies against IFN-γ (eBioscience, clone4S.B3). Flow cytometric data was acquired with a BD FACSCanto II and analyzed using FlowJo software.

7-4) Measurement of interferon (IFN)-γ concentration in cell culture supernatant

The collected cell culture supernatant after 7 days was measured IFN-γ concentration by uncoated ELISA method (Human IFN-γ Uncoated ELISA kit, ThermoFisher). After completing all the procedures in the kit, the results were obtained at 450-570nm using a microplate reader (SpectraMAX190, Molecular Devices, San Jose, CA, USA).

8. Measurement of cytokines concentration in serum or plasma of SAB patients

The serum or plasma of SAB patients was used to measure cytokine concentraion. Concentration of IFN-γ, interleukin (IL)-10, IL-6, IL-17A and tumor necrosis factor (TNF)-α in serum or plasma were measured by using a Procartaplex™ high sensitivity multiplex immunoassay (ThermoFisher). If samples were not reached at standard limit, the samples were considered as 0.

9. Statistical analysis

This study compared ST72 and ST5 in strains and blood. Mann-Whitney test was used to compare the expression levels of genes and cytokine study results between the two groups. p value < 0.05 was considered statistically significant difference. In the bacteriological study results, the mean and standard deviation were shown as a bar graph. In the immunological study results, the mean and standard deviation were shown as a dot plot graph. All statistical analyses were performed using Prism 9.4.1 (GraphPad Software).

Results

1. Subjected strains and bloods from *S. aureus* bacteremia (SAB) patients.

The laboratory at the Department of Infectious Diseases, Asan Medical Center, Seoul, collected 1077 MRSA cases from August 2008 to September 2022. First, all strains checked whether *mecA* is positive and confirmed molecular characteristics including MLST, δ-hemolysin activity, *agr* genotype, SCCmec type, and *spa* type to determine if strains were suitable for research use. As previously known, CA-MRSA in Korea was dominated ST72-agr function-*agr*Ⅰ-SCC*mec*Ⅳ(A) with mainly three spa types, t324, t664, and t148 (Table 6). HA-MRSA, which has been prevalent in the past, was dominated ST5-agr dysfunction-*agr*Ⅱ-SCC*mec*Ⅱ(B) with mainly three *spa* types, t2460, t9353, and t002 (Table 7). 30 strains for each type were selected and surface proteins expression levels were compared. The proteins that could be identified were confirmed by western blotting. Bloods were collected from 606 patients with SAB at Seoul Asan Medical Center from January 2014 to December 2022. Only blood collected for the first time at the hospital before appropriate antibiotics were prescribed was used in this study. Of the 231 patients in these cases, 97 patients had MRSA. Among 97 MRSA patients, only patients with ST72 (n=35) and ST5 (n=21) were selected (Table 8). To examine adaptive immune response of *S. aureus*, the PBMC of blood collected from subjected patients was stimulated for 7 days to

analysis the Frequency of $CD4^+$ T cells secreting IFN-γ by flow cytometry, and IFN-γ concentration in cell culture supernatant was measured by ELISA. In addition, concentrations of 5 cytokines in serum or plasma of subjected patients were compared by multiplex ELISA. The difference in the Frequency of $CD4^+$ T cells secreting IFN-γ by flow cytometry was also compared after stimulating each type of strain in the blood of healthy persons under the same conditions.

Table 6. Clinical strains information of MRSA sequence type 72 (ST72) used in this study.

Isolates No.	Genetic characteristics						
	Clonal complex	ST	mecA	δ-hemolysin activity	Agr type	SCCmec type	SpA type
CA1	CC8	72	+	function	I	IVA	t324
CA2	CC8	72	+	function	I	IVA	t324
CA3	CC8	72	+	function	I	IVA	t324
CA4	CC8	72	+	function	I	IVA	t324
CA5	CC8	72	+	function	I	IVA	t324
CA6	CC8	72	+	function	I	IVA	t324
CA7	CC8	72	+	function	I	IVA	t324
CA8	CC8	72	+	function	I	IVA	t324
CA9	CC8	72	+	function	I	IVA	t324
CA10	CC8	72	+	function	I	IVA	t324
CA11	CC8	72	+	function	I	IVA	t324
CA12	CC8	72	+	function	I	IVA	t324
CA13	CC8	72	+	function	I	IVA	t324
CA14	CC8	72	+	function	I	IVA	t324
CA15	CC8	72	+	function	I	IVA	t324
CA16	CC8	72	+	function	I	IVA	t324
CA17	CC8	72	+	function	I	IVA	t324
CA18	CC8	72	+	function	I	IVA	t324
CA19	CC8	72	+	function	I	IVA	t324
CA20	CC8	72	+	function	I	IVA	t324

CA21	CC8	72	+	function	I	IVA	t324
CA22	CC8	72	+	function	I	IVA	t324
CA23	CC8	72	+	function	I	IVA	t324
CA24	CC8	72	+	function	I	IVA	t664
CA25	CC8	72	+	function	I	IVA	t664
CA26	CC8	72	+	function	I	IVA	t664
CA27	CC8	72	+	function	I	IVA	t664
CA28	CC8	72	+	function	I	IVA	t664
CA29	CC8	72	+	function	I	IVA	t148
CA30	CC8	72	+	function	I	IVA	t148

Table 7. Clinical strains information of MRSA sequence type 5 (ST5) used in this study.

Isolates No.	**MLST**		**Genetic characteristics**				
	Clonal complex	ST	mecA	δ-hemolysin activity	Agr type	SCCmec type	SpA type
HA1	CC5	5	+	dysfunction	II	II	t2460
HA2	CC5	5	+	dysfunction	II	II	t2460
HA3	CC5	5	+	dysfunction	II	II	t2460
HA4	CC5	5	+	dysfunction	II	II	t2460
HA5	CC5	5	+	dysfunction	II	II	t2460
HA6	CC5	5	+	dysfunction	II	II	t2460
HA7	CC5	5	+	dysfunction	II	II	t2460
HA8	CC5	5	+	dysfunction	II	II	t2460
HA9	CC5	5	+	dysfunction	II	II	t2460
HA10	CC5	5	+	dysfunction	II	II	t2460
HA11	CC5	5	+	dysfunction	II	II	t2460
HA12	CC5	5	+	dysfunction	II	II	t2460
HA13	CC5	5	+	dysfunction	II	II	t2460
HA14	CC5	5	+	dysfunction	II	II	t2460
HA15	CC5	5	+	dysfunction	II	II	t2460
HA16	CC5	5	+	dysfunction	II	II	t2460
HA17	CC5	5	+	dysfunction	II	II	t2460
HA18	CC5	5	+	dysfunction	II	II	t2460
HA19	CC5	5	+	dysfunction	II	II	t2460
HA20	CC5	5	+	dysfunction	II	II	t2460

HA21	CC5	5	+	dysfunction	II	II	t2460
HA22	CC5	5	+	dysfunction	II	II	t9353
HA23	CC5	5	+	dysfunction	II	II	t9353
HA24	CC5	5	+	dysfunction	II	II	t9353
HA25	CC5	5	+	dysfunction	II	II	t9353
HA26	CC5	5	+	dysfunction	II	II	t9353
HA27	CC5	5	+	dysfunction	II	II	t9353
HA28	CC5	5	+	dysfunction	II	II	t002
HA29	CC5	5	+	dysfunction	II	II	t002
HA30	CC5	5	+	dysfunction	II	II	t002

Table 8. Information of blood in ST72 and ST5 patients used in this study.

Characteristic	ST72 patients (n = 35) No. (%)	ST5 patients (n = 21) No. (%)
SCC*mec* type		
II	1 (2.8)	21 (100)
IV	31 (88.6)	-
NID	3 (8.6)	-
***agr* genotype**		
I	35 (100)	1 (5.3)
II	-	18 (84.2)
NID	-	2 (10.5)
δ-hemolysin activity		
Function	31 (88.6)	2 (9.5)
Dysfunction	4 (11.4)	19 (90.5)
Spa type		
t2460	-	7 (33.3)
t9353	-	3 (14.3)
t002	-	3 (14.3)
t324	16 (45.7)	-
t664	5 (14.3)	-
t148	7 (20)	-
other	7 (20)	8 (38.1)

2. qRT-PCR of seven surface proteins genes

The expression levels of seven surface protein-related genes (*spa, fnbA, ebpS, clfA, clfB, fib, coa*) among CA-MRSA major sequence type in Korea were compared. *spa, fnbA, fib*, and *coa* genes showed lower levels in ST72 than in ST5 (Fig 3). Among them, the *spa* and *fnbA* genes showed very significantly low levels in the ST72 strain, and the *fib* gene also seemed to be statistically different. On the other hand, in the case of the *ebpS, clfA*, and *clfB* genes, the ST72 showed higher levels than the ST5 strain (Fig 3). However, only the *ebpS* gene showed a significant difference. Finally, 4 surface proteins showed significant differences.

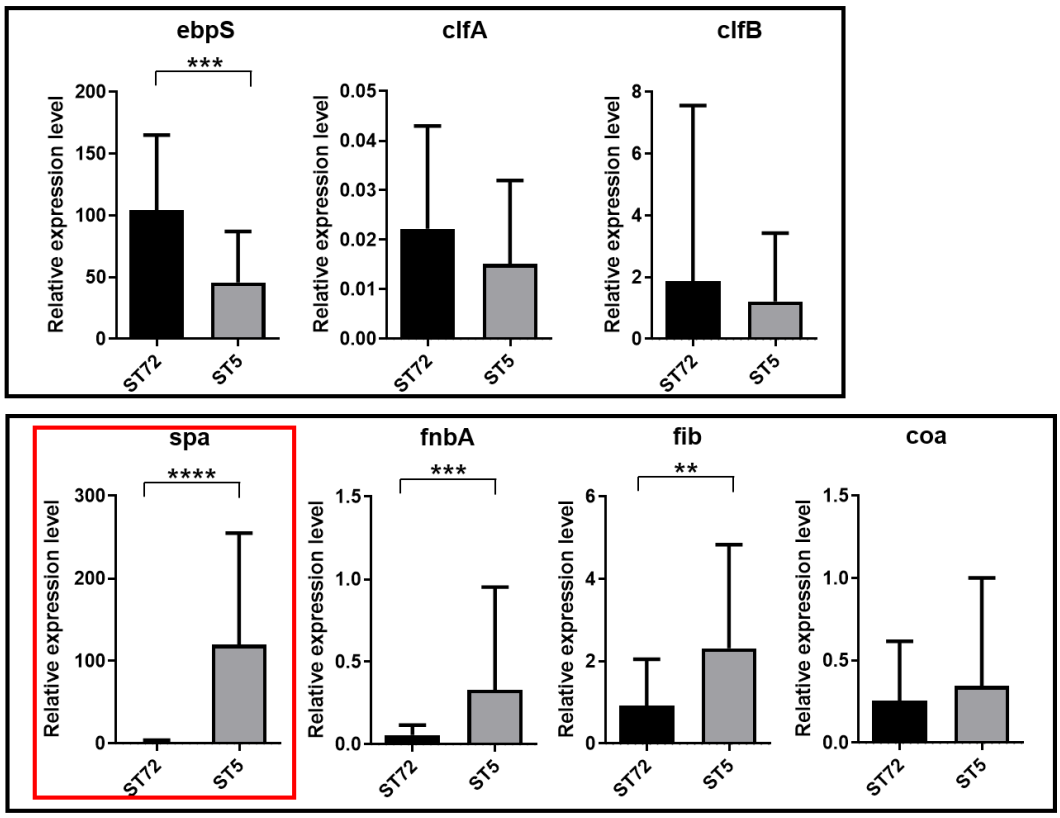


Figure 3. Comparison expression level of surface proteins genes in strains between ST72 (n=30) and ST5 (n=30) at mRNA level. qRT-PCR analysis to quantify the expression of *spa*, *fnbA, fib, coa, ebpS, clfA,* and *clfB* genes of ST72 and ST5 strains used in this study. ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001

3. Western blot for staphylococcus protein A (spa)

In order to confirm the correlation between the expression level at the protein and the mRNA, the expression level of the spa protein among the seven surface proteins was analyzed by western blot using an anti-protein A antibody. In ST72, most of them were faintly expressed, and in ST5, most of the bacteria were noticeably more expressed than in ST72. As in the qRT-PCR result of *spa*, it was confirmed that the expression of ST72 was much lower.

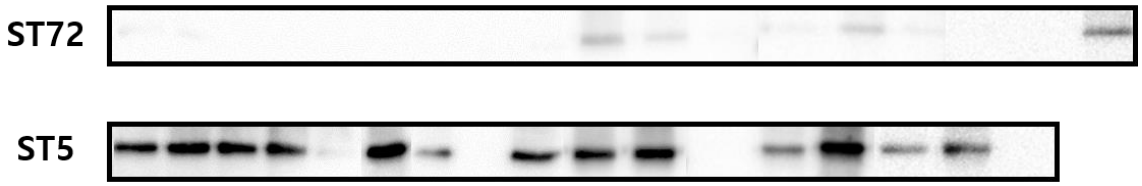
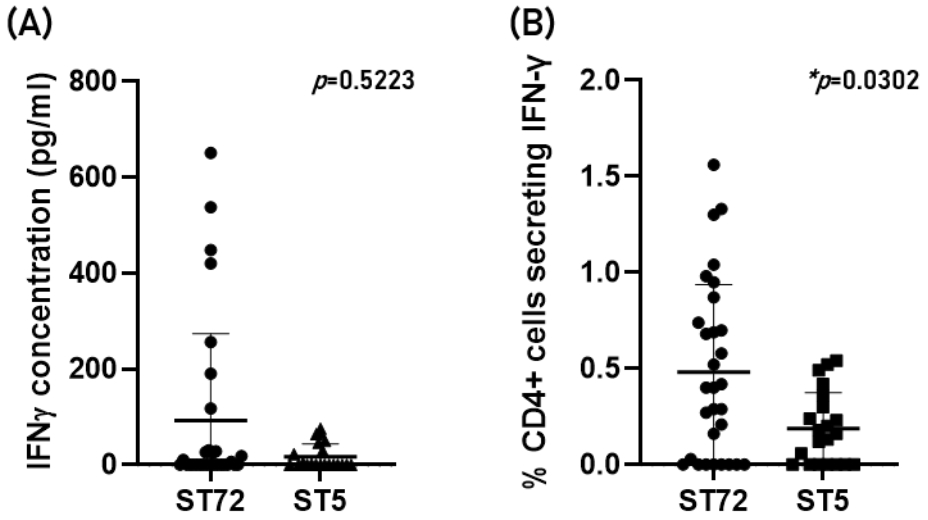


Figure 4. Comparison expression level of spa protein in strains between ST72 (n=18) and ST5 (n=17) at protein level.

4. In vitro activation of PBMC for IFN-γ detection by ELISA and flow cytometry

IFN-γ in cell culture supernatant was not detect at 16 samples of ST72 and 12 samples of ST5. In the case of ST5, the remaining detected samples were detected below 100 pg/ml. In the case of ST72, almost of them were detected below 30 pg/ml, but in some cases, up to 650 pg/ml was detected beyond 100 pg/ml (Fig 5A). Percentage of $CD4^+$ T cell secreting IFN-γ in PBMC was detected higher in the ST72 group than in the ST5 group (Fig 5B). Figure 4C showed the frequency of IFN-γ secreting cells in $CD4^+$ T cells analysed using flow cytometry at representative patient of both groups.



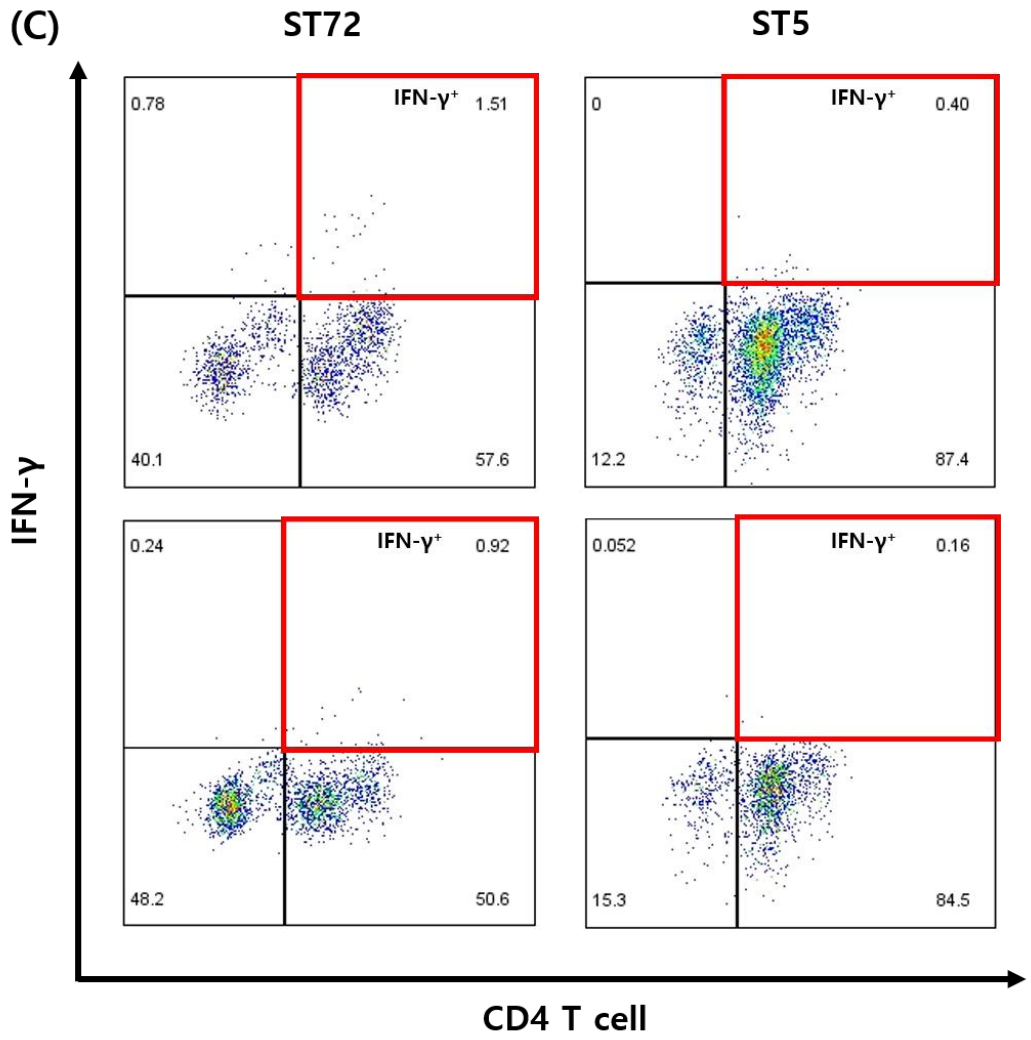


Figure 5. IFN-γ concentration in cell culture supernatant and Frequency of CD4+ T cells secreting IFN-γ in PBMC from MRSA bacteremia patients After stimulation of PBMCs using heat-killed S. aureus for 7 days, (A) the IFN-γ concentration in the cell culture media measured and (B) performed intracellular cytokine staining to analyze IFN-γ secreting cells in $CD4^+$ T cells in 51 patients of ST72(n=30) and ST5(n=21). Frequency of IFN-γ secreting cells in $CD4^+$ T cells

was analysed by gating on IFN-γ cells of the $CD4^+$ populations using flow cytometry (C). Results presented as individual patient values with mean and standard deviation indicated by bar. *p<0.05

5. Cytokine measurement by human high sensitivity multiplex immunoassay

There was no significant difference in cytokines in day 0 blood serum or plasma between the two groups. Only IL-17A (**p=0.0027) showed statistically significant between ST72 and ST5 group (Fig. 6B).

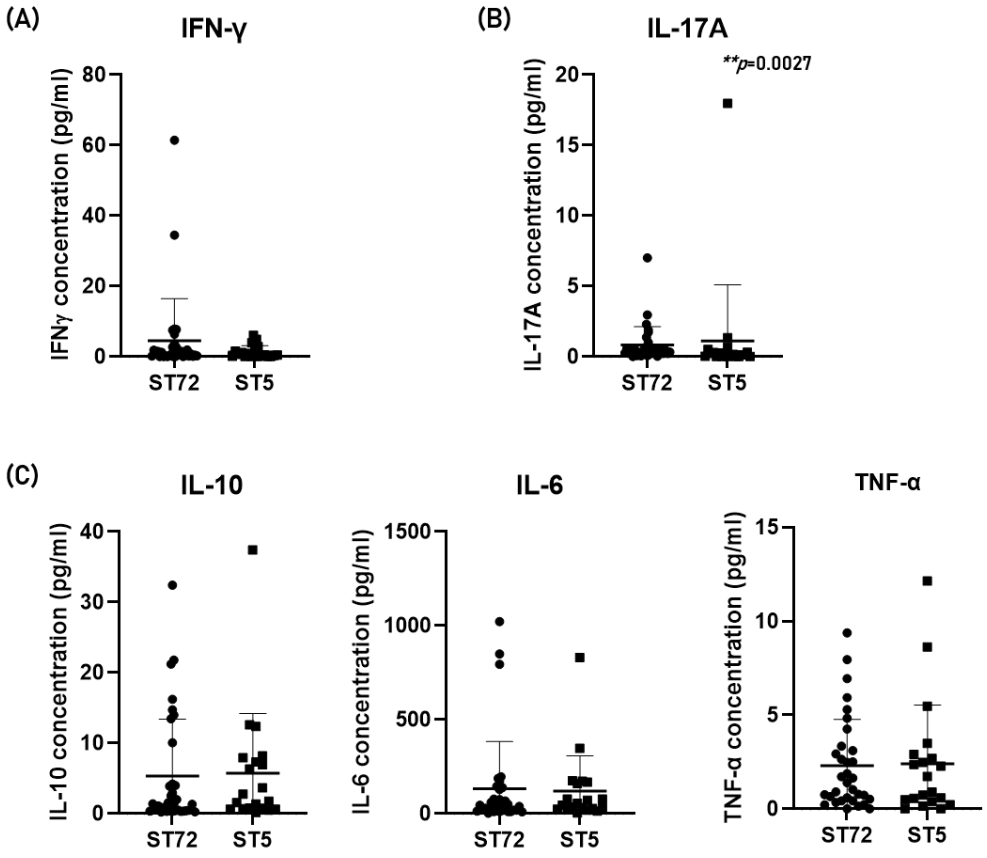


Figure 6. Cytokine concentration in serum or plasma of SAB patients Serum or plasma from SAB patients was collected on day 0 post-initial bacteremia and measured concentration of five cytokines by multiplex enzyme-linked immunosorbent assay (ELISA). Results presented as individual patient values with mean and standard deviation indicated by bar, ST72 (n=33) and ST5 (n=20). $**p<0.01$

6. Comparison of frequency of IFN-γ secreting cells in $CD4^+$ T cells into PBMC of healthy donors

Immediately after isolating PBMC from healthy people, heat-killed *S. aureus* for each type stimulated same condition for 7 days, and the Frequency of IFN-γ secreting cells in $CD4^+$ T cells by flow cytometry was analyzed. However, there was no remarkable difference or tendency after stimulation for each type (Fig. 7).

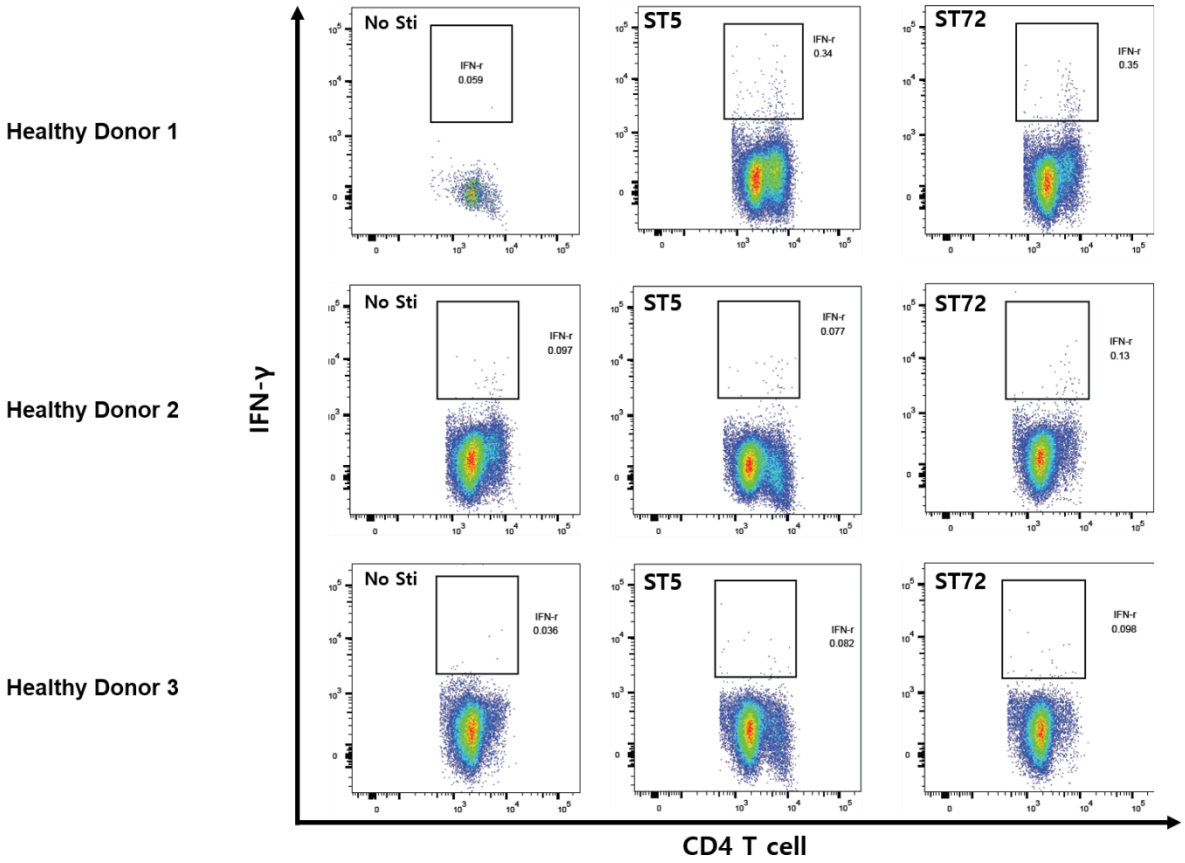


Figure 7. Frequency of IFN-γ secreting cells in CD4+ T cells analyzed using flow cytometry in PBMC of three healthy donors. All PBMCs were co-cultured with heat-killed *S. aureus* of each type strain for 7 days.

Discussion

Until the 1990s, ST5 was prevalent worldwide as HA-MRSA, but CA-MRSA, which appeared after the 2000s, ST8 was prevalent in the US, Australia, and Europe, while ST72 was representatively dominant in Korea. The types that are dominant in each country are different and is known that the pathogenesis caused clinically for each type is also different [31, 32]. Until now, ST72 and ST5 are the most dominant CA-MRSA in Korea. CA-MRSA ST72 is known to cause infection mainly in bones and joints compared to ST5. In this study, the goal was to compare the CA-MRSA ST72 strain, which is most prevalent in Korea, with the HA-MRSA ST5 strain, which was prevalent in the past, to find bacteriological differences. In addition, several immunological studies using the blood of *staphylococcus aureus* bacteremia (SAB) patients, for which there are not many previous studies, were also conducted. First, all characteristics of MRSA were confirmed to determine whether the strain was suitable for research use. For each group, 30 strains were selected according to the most major genotype. As already known, δ-hemolysin function-*agr* type Ⅰ-SCC*mec*Ⅳ(A) was the most major in ST72, and δ-hemolysin dysfunction-*agr* type Ⅱ-SCC*mec*Ⅱ(B) was the most major in ST5 [26, 27, 28]. Surface proteins, which are mainly expressed on exponential growth phages during *S. aureus* infection, play an adhesion role on host cells or tissues during the early stages of infection. There are various types of surface proteins of *S. arueus*. Among them, we compared the gene expression levels of seven surface proteins (*spa, ebpS, fnbA, clfA, clfB, fib, coa*) that mainly act on adhesion. The above 7 surface proteins have various binding mechanisms and show various functions

depending on the ligand (Table 9).

Table 9. Properties of surface proteins of *S. aureus* [6, 36, 37, 38]

Surface protein of *S. aureus*	Ligand and binding mechanism	Function
Clumping factor A (ClfA) [36]	Fibrinogen γ chain C-terminus (DLL)	Adhesion to immobilized fibrinogen, immune evasion by binding soluble fibrinogen
	Complement factor I Mechanism unknown	Immune evasion, degradation of C3b
Clumping factor B (ClfB) [36]	Fibrinogen α-chain repeat 5, keratin 10 and loricrin (DLL)	Adhesion to desquamated cells from nares and from skin of AD patients; nasal colonization and colonization of AD skin
Fibronectin binding protein A (fnbA) [36]	FnBPA A domain binds fibrinogen γ-chain C-terminus and elastin (FnBPA A domain, DLL)	Adhesion to ECM
Protein A (spa) [36]	IgG Fc IgM Fab V_H3 subclass TNFR-1 Von Willebrand factor Unknown ligand (region Xr)	Inhibits opsonphagocytosis B cell superantigen Inflammation Endovascular infection; endocarditis Inflammation
Elastin binding protein S (ebpS) [6]	N-terminal 30-kDa region of elastin	Bacterial colonization to facilitate pathogenesis
Fibrinogen binding protein (fib) [38]	α-chain of fibrinogen and its derivative fibrin	Enhances a non-functional interaction between fibrinogen and platelets Repression of fibrinogen-dependent platelet aggregation
Coagulase (coa) [37]	Prothrombin	Activate prothrombin and cleave fibrinogen to fibrin cables

The genes that showed the most significant difference among the 7 surface proteins were *spa* and *ebpS*. At group of ST72 strains, *spa* showed an expression level close to 0 compared to ST5 strains. Staphylococcus protein A (spa) is associated with Inflammation, induces endovascular infection; endocarditis, acts as a B cell superantigen, and inhibits opsonphagocytosis [3]. Spa is also a virulence factor in murine models of *S. aureus* kidney abscess formation, skin infection, pneumonia, sepsis, and septic arthritis [7].
Among these several functions, the most well-known major function of spa is to induce immune evasion by inhibiting opsonphagocytosis.

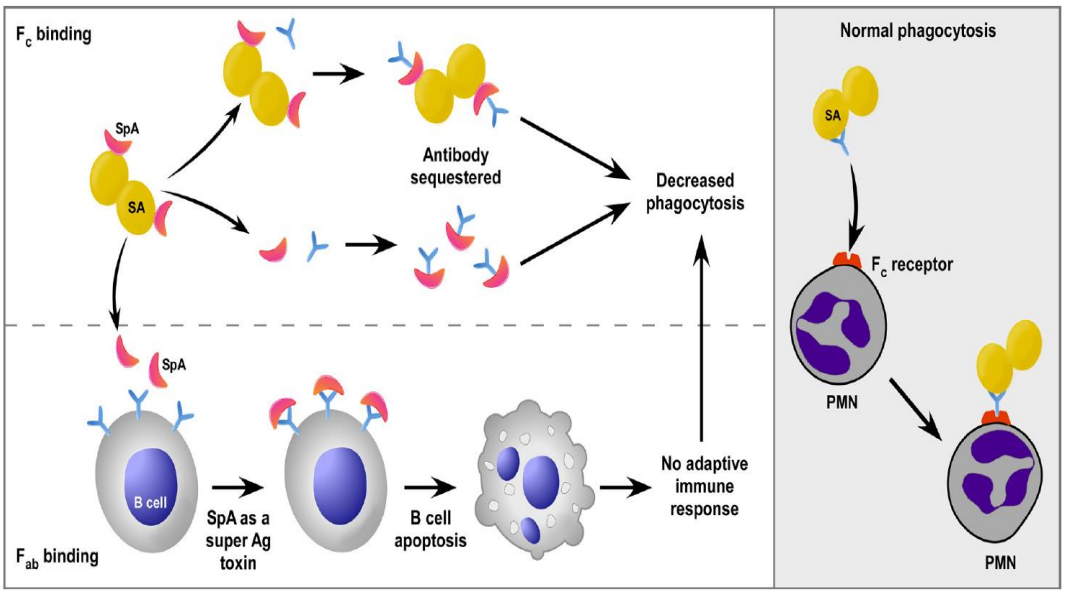


Figure 7. Mechanism of staphylococcus protein A (spa)-mediated immune evasion. PMN, polymorphonuclear leukocyte [33].

Although spa appears on the surface of *S. aureus*, it is secreted freely and blocks normal phagocytosis by binding to the antibody's Fc region. In addition, spa induces B-cell death and inhibits the occurrence of an adaptive immune response by binding to the Fab regions of the B-cell receptor, which prevents the production of specific antibodies to *S. aureus* [33]. It is possible that spa could have influenced the results of the immunological study in this study by promoting immune suppression. Recently, the novel application of spa as a diagnostic biosensor has been reported. Finally, as using spa as a drug for treatment of autoimmune diseases has recently been started, the further evaluation of spa effects on immune system and its animal and clinical studies should be highly recommended [23]. In addition to *spa*, *fnbA* and *fib* showed significant results in the same trend. FnbA have been demonstrated to be involved in not only adhesion to cells but also internalization by cells [34]. FnbA is also a mediator of signaling cell and actin cytoskeletal rearrangement. The adhesion gene *fnbA* leads to cardiovascular disease and cardiovascular system infection through platelet activation and also causes adhesions to artificial implants in the body [3]. Fibrinogen is one of the main proteins deposited on implanted biomaterials. The ability of S. aureus to adhere to fibrinogen is an important factor in promoting wound infection, foreign body infection, and endocarditis [35]. Finally, three genes, *spa, fnbA,* and *fib*, showed significantly lower results in ST72. These proteins are well known to be involved in endocarditis. In combination with the expression levels of these proteins at the gene level, it can be seen that ST5 has a higher incidence of endocarditis. However, previous clinical studies have shown that fnbA is not

involved in endocarditis. In this study, *ebpS* expression level in ST72 strains was higher than ST5 strains. In the case of ebpS, there was not much information because it was a protein that had not been studied much yet, and it was difficult to think of a hypothesis hastily. Among the seven surface proteins, the expression levels of all genes except for *ebpS* were low, ranging from 0 to 10, but only *ebpS* showed a particularly high level in ST72 strains with statistically significant difference. Due to these bacteriological differences, it is thought that more detailed studies on ebpS proteins are needed. The elastin-binding protein (ebpS) is an adhesion firmly associated with the cytoplasmic membrane that has been found to specifically interact with the host extracellular matrix component elastin [6, 25]. As described above, ST72, the most major sequence type of CA-MRSA in Korea, flowed into hospitals and entered a competitive trend with ST5, which was prevalent. Recently, it has become the main species of CA-MRSA in Korea and is clinically known to cause osteoarthritis in which infection occurs in bones or joints. Therefore, based on the results of the expression level of surface proteins, it is thought that *ebpS*, which is the only high expression level in ST72, may be involved in osteoarthritis. It is believed that the relationship between ebpS protein and osteoarthritis can be confirmed through cell study using osteoclast or osteoblast and a mouse osteoarthritis model study. In addition, in the case of bone joint infection, antibiotic permeability is poor and recurrence often occurs due to immune evasion mechanisms of *S. aureus* such as capsular polysaccharides or intracellular infiltration [9]. Therefore, it seems necessary to study this immune evasion mechanism together. However, the limitation and

consideration of this study is that multidisciplinary studies are needed because there are various other virulence factors or clinical characteristics involved in *S. aureus* infection.

In the production of *S. aureus* vaccines, there have been several model vaccines that have shown preventive effects against *S. aureus* infections in mouse models, but human clinical trials have not yet shown their effectiveness. The reason may be that there are too many immune response factors involved in *S. aureus* infection [29]. Previous studies have attempted to establish the contribution of T cell immunity during *S. aureus* infection, along with efforts to develop a vaccine against *S. aureus* infection [19]. The $CD4^+$ T helper (Th) cell immunity might be important against *S. aureus* infection in human [20]. There are several subsets of $CD4^+$ T helper (Th) cells, in particular Th1 cells produce IFN-γ, which promotes bacterial elimination inside macrophage phagosomes and plays an important role in immunological and inflammatory processes during *S. aureus* infection [19, 20, 21, 22]. Animal models have also reported that IFN-γ, a representative cytokine produced in helper T lymphocytes, plays an important role in preventing infection with *S. aureus* [12, 13]. In addition to IFN-γ, interleukin (IL)-17 is a cytokine produced by Th17, and studies have reported that the concentration of IL-17 is higher in patients with persistent bacteremia and metastatic infection [14]. There are also reports that the concentration of pro-inflammatory cytokines TNF-alpha and IL-6 and anti-inflammatory cytokine IL-10 are associated with persistent bacteremia, complicated infection, and mortality [11, 15, 16, 17]. Identification

of the immune response including cytokines in *S. aureus* infection is an important factor in understanding the mechanism of infection and complications and predicting severity and prognosis. Based on these previous studies, this study conducted several immunological studies to determine whether there were immunological differences between ST72-MRSA and ST5-MRSA patients. Among the cytokines described above, the study was conducted with a focus on detecting IFN-γ. Using the strength of collecting blood from SAB patients in this laboratory, several immunological studies using blood were attempted. The first blood collected from ST72-MRSA, ST5 patients, that is, the blood on day 0 was used. It was additionally confirmed that this blood was the blood before antibiotics were administered in the hospital in order to block the variables of the study results as much as possible. When the concentrations of five cytokines were measured in serum or plasma, almost cytokines showed no difference, but IL-17A was the only one with slightly significant statistics (**p=0.0027) (Fig 5B). However, it would not be possible to say that there is a difference between the two types of immune responses only by this result. And there is a plot showing a high value alone in the ST5 group of IL-17A results. ST5 appears to have a higher mean value, but if the plot with a high value alone is removed or left at 0, the result is reversed as the mean value of ST72 is higher (Fig 5B). Significance is also higher than **p=0.0027. And one notable point is that the result of the patient who corresponds to the plot with a high value alone is located in the highest plot of the ST5 group in IL-10, IL-6, and TNF-α results (Fig 5C). In the ST72 group, it was not possible to find a correlation in which a particular

cytokine was particularly high, and other cytokines were also high. However, this patient with ST5 uniquely showed the highest result in all cytokine results except for IFN-gamma. In the process of developing this study in the future, it seems necessary to consider all the clinical characteristics of this patient. In this regard, it seems that the limitations of immunological research, which must also consider the clinical characteristics of each patient, are revealed.
In a previous study, IFN-γ in the serial blood serum or plasma of SAB patients was not detected in most of the samples. However, in this study, more than 75% of samples were detected in both groups, although at a generally low level. Since the sample used in the previous study had a long storage period and was serially collected, it must have been blood after antibiotics were prescribed. It is possible that these conditions adversely affected cytokine detection. This is the reason why cell culture supernatant cultured with *S. aureus* for 7 days was used in the study. Therefore, when looking at this study results after culturing with heat-killed *S. aureus* for 7 days, IFN-γ was detected in cell culture supernatant and $CD4^+$ T cells (Fig 4). In addition, when looking at the IFN-γ concentration measured in cell culture supernatant (Fig 4A) and the frequency of cells secreting IFN-γ in $CD4^+$ T cells (Fig 4B) after culturing with heat-killed *S. aureus* for 7 days, the two results are similar graphically. The frequency of cells secreting IFN-γ in CD4+ T cells was also found to be statistically significant (*p=0.0302). Figure 4B is an experiment in which cytokine is confined in the cell and fixed and then detected, and Figure 4A is an experiment in which cytokine secreted as a supernatant is measured during the culture period. Therefore, there is a possibility that this result was

proportional to the result of Figure 4A due to the frequency of cells secreting IFN-gamma in $CD4^+$ T cells. The same experiment was performed by stimulating the two types of strains in the blood of healthy donors, but there was no remarkable difference either. Accordingly, we could analyze IFN-γ only after stimulation using heat-killed *S. aureus*. It is conceivable that memory Th1 cells which previously exposed to *S. aureus* induced IFN-γ response. The conclusion of immunological studies is that there is a difference in samples number of each type because it is blood collected with difficulty in hospitals, and there may be a problem with PBMC storage quality. However, in this study, when PBMC re-dissolved and measured viability and live cell count, only PBMC suitable for use in the study were used. Because some significance was shown in the frequency of cells secreting IFN-γ in $CD4^+$ T cells, it seems that the problem of PBMC storage quality can be excluded to some extent. However, since the cell experiment was conducted for 7 to 8 days, the cell can be easily damaged during the experiment, and the possibility that the number of live cells has decreased cannot be ignored. It is thought that the results without significance appeared due to various experimental difficulties and limitations of the preceding research, and it is necessary to seek additional PBMC research directions that can supplement them. These limitations made it difficult to explain the meaning of the results, so more in-depth research is needed.

By studying the representative strains of CA-MRSA in Korea, it will be possible to provide the basis for target discovery for prevention and treatment of

community spread in Korea and development of vaccines. It is expected that research on the pathogenesis of Korea's strains will further enable the discovery of targets for vaccines that are universal to foreign strains or the development of therapeutic agents. As for bacteriological studies, *S. aureus* has many and diverse proteins that act upon infection in addition to surface proteins, so it seems that more diverse fields of research are needed. Immunological research is a study on patient blood collected from hospitals, so there are too many things to consider because there are differences in the number of samples that we cannot control and the basic clinical characteristics of patients are different from person to person. For this reason, it is not easy to generalize, and it seems to be the biggest limitation of immunological research that this point affects the interpretation of the results.

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국문 요약

황색포도알균(*Staphylococcus aureus; S. aureus*)은 지역사회와 병원 감염의 중요한 원인균으로 피부 및 연조직 감염, 폐렴, 균혈증 등을 일으키는 주요 박테리아 병원체이다. 1960년대부터 전 세계적으로 메티실린 내성 황색포도알균(methicillin-resistant *Staphylococcus aureus*; MRSA)의 빈도가 급격히 증가하였다. 우리나라에서는 1980년에 들어서면서 병원 내 획득 메티실린 내성 황색포도알균 (hospital-associated methicillin-resistant *Staphylococcus aureus*; HA-MRSA) 의 빈도가 증가하게 되었다. 2000년대 이후로는 병원감염과 연관된 요소 없이 지역사회에서 감염이 발생한 경우의 지역사회 획득 메티실린 내성 황색포도알균 (Community-acquired methicillin-resistant *Staphylococcus aureus*; CA-MRSA)이 우리나라 뿐만 아니라 전 세계적으로 증가하게 되었다. 본 연구는 우리나라에서 현재 유행하고 있는 CA-MRSA의 주요 시퀀스 타입 ST72를 과거에 유행했던 HA-MRSA의 주요 시퀀스 타입 ST5에 비교해보고자 미생물학적, 면역학적 연구를 진행하였다.

서울아산병원 임상검체로부터 15년 동안(2008~2023) 분리 동정된 MRSA의 분자학적 유전자형을 확인하여 strain type을 확보하였다. 황색포도알균 감염에 중요한 역할을 하는 7 가지의 표면 단백질 유전자를 선택하여 발현 수준을 quantitative real-time polymerase chain reaction로 비교하였으며, western blot을 통하여 단백질 수준에서의 발현 차이도 확인하였다. 또한 황색포도알균 균혈증 환자 혈액의 peripheral blood mononuclear cell (PBMC), serum or plasma를 사용 하여 intracellular cytokine staining (ICS), enzyme-linked immunosorbent assay (ELISA) 기법으로 사이토카인 분석을 진행하였다.

실험에 사용된 7 가지의 표면 단백질을 암호화하는 유전자들 중 4 가지의 유전자(*ebpS, SpA, fnbA,* and *fib*)가 전 세계적으로 만연했던 ST5 균주들에 비해

국내의 주종인 ST72 균주들에서 유의미하게 상향 및 하향 발현하는 것으로 나타났다. 그 중 *ebpS* 단백질 유전자는 ST72에서 유일하게 상향 발현 수준을 보인 유전자이다. 면역학 연구 결과로는 serum or plasma 내의 IL-17A의 농도가 통계학적으로 차이가 있는 것으로 나타났다.

본 연구에서는 국내 CA-MRSA의 주종인 ST72가 ST5에 비교하여 표면 단백질의 발현 수준 차이가 있음을 확인하였다. 추후 미생물학적 및 면역학적 연구를 통해 두 타입 균주들의 차등 발현된 표면 단백질을 통한 감염 경로 및 임상적 병인 양상 차이의 원인을 밝히는 것이 필요하다.