Comparative Immunology, Microbiology and Infectious Diseases 62 (2019) 13–18

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

Potential role of host defense antimicrobial peptide resistance in increased virulence of health care-associated MRSA strains of sequence type (ST) 5 versus livestock-associated and community-associated MRSA strains of ST72

Kyoung-Mi Kang[a,1], Jong-Hwan Park[b,1], So Hyun Kim[c,d], Soo-Jin Yang[a,*]

[a] School of Bioresources and Bioscience, Chung-Ang University, Anseong, Republic of Korea
[b] Laboratory Animal Medicine, College of Veterinary Medicine, Chonnam National University, Gwangju, Republic of Korea
[c] Asian Pacific Foundation for Infectious Disease (APFID), Seoul, Republic of Korea
[d] Division of Infectious Diseases, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Republic of Korea

ARTICLE INFO

Keywords:
CA-MRSA
HA-MRSA
Antimicrobial peptides
Virulence

ABSTRACT

The most significant community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) in Korea is sequence type (ST) 72 with staphylococcal cassette chromosome *mec* (SCC*mec*) type IV (ST72-MRSA-IV). Although the impact of CA-MRSA on the clinical outcomes versus healthcare-associated (HA)-MRSA remains unclear, it has recently been revealed that ST5 HA-MRSA-II is associated with higher mortality compared with ST72 CA-MRSA-IV, suggesting higher virulence in ST5 HA-MRSA-II strains.

In this investigation, human-/animal-originated ST72-MRSA-IV strains were examined for virulence phenotypes and compared with those of ST5-MRSA-II strains, the established HA-MRSA in Korea. Overall, ST5 HA-MRSA-II strains demonstrated higher levels of resistance to host defense-cationic antimicrobial peptides of human (LL-37), bovine (BMAP-28), and bacterial (polymyxin B) origins versus ST72-MRSA-IV strains via enhanced surface positive charge. Hemolysis profiles, gelatinase activity, and staphylococcal superantigen gene profiles were not different between ST72 CA-MRSA and ST5 HA-MRSA strains. However, ST5 HA-MRSA strains were able to downregulate initial cytokine response in murine macrophages.

1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) causes a wide range of clinical syndromes, ranging from skin infections to life-threatening bacteremia both in animals and humans [1,2]. In addition to healthcare-associated (HA) and community-associated (CA)-MRSA infections, livestock-associated (LA)-MRSA strains have emerged and spread widely in many species of animals, especially in European pigs [3]. MRSA clonal lineages can differ among geographical regions. Currently, the most significant CA-MRSA clone in Korea is sequence type (ST) 72 with staphylococcal cassette chromosome *mec* (SCC*mec*) type IV (ST72-MRSA-IV) [4]. However, the ST72-MRSA-IV strains have recently become a significant MRSA clone in Korean hospitals, where one of the most frequent HA-MRSA clones has been ST5-MRSA [5]. Although previous studies reported significant differences in clinical outcomes between CA-MRSA and HA-MRSA infection [6,7], the impact of ST72 CA-MRSA-IV on the development and progression of infection compared with ST5 HA-MRSA-II remains unclear.

Both bacterial and host factors may play crucial roles in the transmission of MRSA strains between different hosts, and one of the critical elements in host innate immune response against MRSA infection is the host defense cationic antimicrobial peptides (HD-CAPs) [8,9]. The persistence and progression of staphylococcal infections inevitably requires the organism to resist the bactericidal action of cytoplasmic membrane (CM)-targeting HD-CAPs. Although staphylococcal strategies deployed to overcome HD-CAP killing are not fully characterized, the relative surface positive charge of the staphylococcal cell envelope has been shown to be frequently associated with resistance to various HD-CAPs [10–12]. In the current study, potential genotype-specific susceptibility profiles to LL-37 (human cathelicidin found in epithelial cells and neutrophils) and BMAP-28 (bovine myeloid antimicrobial peptides) were examined using ST72-MRSA-IV and ST5-MRSA-II strains from human patients or bovine mastitis. Furthermore, net surface positive charges and antibiotic resistance profiles were analyzed to

* Corresponding author at: Department of Animal Science and Technology, Chung-Ang University, 4726 Seodong-daero, Daedeok-myeon, Anseong-si, Gyeonggi-do, 456-756, Republic of Korea.
E-mail address: soojin@cau.ac.kr (S.-J. Yang).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.cimid.2018.11.012
Received 9 August 2018; Received in revised form 22 November 2018; Accepted 27 November 2018

Table 1
Genotypes, virulence factors, and antibiotic resistance profiles in 26 MRSA strains.

Strain no.	MLST type	*spa* type	SCC*mec*	Virulence factors		Resistance profiles[a]	MIC (μg/ml)[b]		
				Hemolysis	Gelatinase activity		DAP	VA	OX
LA1	ST072	t148	IVa	α-β	+	–	0.38	1.5	8
LA2	ST072	t148	IVa	β	+	E-GM	0.5	2	32
LA3	ST072	t148	IVa	β	+	–	0.38	2	32
LA4	ST072	t148	IVa	β	+	–	0.5	1.5	4
LA5	ST072	t148	IVa	β	+	TE-E-GM-RA	0.38	1.5	16
LA6	ST072	t148	IVa	β	+	GM-RA	0.5	1.5	16
LA7	ST072	t148	IVa	β	+	–	0.38	1.5	16
HA1	ST005	t002	II	α	–	E	2	0.5	> 256
HA2	ST005	t601	II	α	–	E	1.5	0.5	> 256
HA3	ST005	t2460	II	α	–	TE-E-GM-RA	1.5	0.5	> 256
HA4	ST005	t2460	II	α	–	TE-E-GM	0.19	0.5	> 256
HA5	ST005	t002	II	α	–	TE-E-GM	0.19	0.38	> 256
HA6	ST005	t601	II	α	–	TE-E	0.125	0.5	> 256
HA7	ST005	t2460	II	α	–	TE-E-RA	0.38	0.5	> 256
HA8	ST005	t601	II	α	–	E	0.25	0.5	> 256
CA1	ST072	t324	IVa	α-β	+	E	1.5	1.5	128
CA2	ST072	t13921	IVa	α	+	–	0.5	0.1	32
CA3	ST072	t664	IVa	α	–	–	0.75	0.75	96
CA4	ST072	t2461	IVa	α	+	–	0.5	0.75	96
CA5	ST072	t324	IVa	α	–	E	0.5	0.75	64
CA6	ST072	t148	IVa	α	+	–	0.5	0.5	24
CA7	ST072	t324	IVa	β	+	–	0.5	0.5	64
CA8	ST072	t324	IVa	α-β	–	–	0.5	0.1	96
CA9	ST072	t324	IVa	α-β	+	E-RA	0.5	0.75	64
CA10	ST072	t664	IVa	β	+	–	0.5	0.75	32
CA11	ST072	t664	IVa	α	+	–	0.75	1.5	48

[a] TE, tetracycline; E, erythromycin; GM, gentamycin; RA, rifampicin.
[b] DAP, daptomycin; VAN, vancomycin; OX, oxacillin.

identify phenotypic correlates associated with in vitro HD-CAP resistance among the MRSA strains.

2. Materials and methods

2.1. MRSA strains and culture conditions

The 26 MRSA strains used in this study are listed in Table 1. Seven livestock-associated ST72-MRSA-IV strains were selected from 23 recently described MRSA strains isolated from cows with clinical or subclinical mastitis [13]. Eleven ST72-MRSA-IV strains from human patients were randomly selected from the MRSA collection in a previous investigation of invasive *S. aureus* infections [14]. These MRSA strains were isolated in a community setting or within 48 h of hospital admission. Eight ST5-MRSA-II strains employed in this study were clinical bloodstream isolates selected from the Asian Bacterial Bank of Asia Pacific Foundation for Infectious Disease (Seoul, Korea).

All MRSA strains were grown in Tryptic Soy broth (TSB; Difco Laboratories, Detroit, MI) or Mueller Hinton broth (MHB; Difco Laboratories) depending on the individual experiment. Liquid cultures were grown in Erlenmeyer flasks at 37 °C with shaking (200 rpm) in ≤ 10% of the flask volume.

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility was determined by standard disc diffusion methods according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2017) for the following 6 antibiotics: tetracycline, erythromycin, gentamicin, chloramphenicol, rifampin, and sulfamethoxazole-trimethoprim (BD, Franklin Lakes, NJ).

The MICs of the MRSA strains to daptomycin, vancomycin, and oxacillin were determined by standard E-test (AB Biodisc, Dalvagen, Sweden) according to the manufacturer's suggested protocol. Three independent experimental runs were performed to determine MICs to each of the antibiotics.

2.3. Analyses of staphylococcal virulence phenotypes

A gelatin hydrolysis test was performed as described previously [13]. To assess gelatinase activity, 4 ml of medium containing 12% gelatin (yeast extract 10 g/L, peptone 15 g/L, and gelatin from bovine skin 120 g/L; Sigma, St. Louis, MO) was inoculated from a single colony of MRSA and incubated for 7 ~ 14 days at 37 °C.

Hemolytic activity was determined on a 5% sheep blood agar plate (Komed, Seongnam, Korea) after 24–48 h incubation at 37 °C, as described previously [13].

2.4. DNA isolation and molecular characterization

Genomic DNA was isolated from the MRSA strains using the Genenmed Plasmid kit (Genenmed, Seoul, Korea) according to the manufacturer's protocol.

Multi Locus Sequence Typing (MLST) was performed according to the protocol described by Enright et al [15]. The alleles and sequence types were assigned according to the MLST database (https://pubmlst.org/saureus/). The *spa* repeat regions were amplified using a specific primer set [16], sequenced, and the *spa* type was determined according to the SpaServer database (http://spa.ridom.de/). Sequencing of all DNA samples was performed at Cosmo Genetech (Seoul, Korea).

To detect genes for Panton-Valentine leukocidin (PVL), toxic shock syndrome toxin-1 (TSST-1), and exfoliative toxins (ETs), PCR-based assays were performed using specific primer sets as previously described [17–19]. The presence of staphylococcal enterotoxin (SE) genes (*sea, seb, sec, sed*, and *see*) was determined by PCR-based methods described by Jennifer et al. [20].

2.5. Host Defense Cationic Antimicrobial Peptide (HD-CAP) susceptibility assays

Human cathelicidin (LL-37) [21], found in epithelial cells and neutrophils, was purchased from Peptide International (Louisville, KY). Bovine myeloid antimicrobial peptide-28 (BMAP-28) [22] was synthesized at GL Biochem (Shanghi, China) with purity > 95%. Polymyxin B (PMB) was purchased from Sigma Chemicals Co. (St. Louis, MO).

Since standard MIC assays in nutrient broth may underestimate bactericidal activities of antimicrobial peptides, in vitro susceptibility assays were performed with LL-37 and BMAP-28 as previously described using the 2-h microdilution method in Eagle's minimal essential medium [23,24]. The HD-CAP susceptibility assays were performed with LL-37 (4 μg/ml) and BMAP-28 (10 μg/ml) using an initial bacterial inoculum of 5×10^3 CFUs. These LL-37 and BMAP-28 concentrations were selected based on extensive preliminary experiments exhibiting their inability to completely eradicate the initial inocula of the MRSA strains over the 2-h assay period. Data are presented as the relative % of surviving CFU (± standard deviations) of peptide-treated versus peptide-untreated cells. A minimum of three independent experiments was carried out for each HD-CAP.

2.6. Net cell surface charge

The binding of fluorescein isothiocyanate (FITC)-labeled cationic poly-$_L$-lysine (PLL) to the *S. aureus* cell surface was assessed to measure the relative surface positive charge using a flow cytometric analysis as detailed previously [24,25]. Briefly, overnight-grown MRSA cells were washed twice with HEPES buffer (20 mM, pH 7.25) and then resuspended in the same buffer to an OD_{600} of 0.25. Then, FITC-PLL (10 μg/ml) was added to the cell suspension, and the samples were subjected to flow cytometric analyses after 15 min incubation at RT. A total of 15,000 events were counted, and 3 separate runs were performed using the FACS Aria II system (BD Bioscience, Franklin Lakes, NJ). Higher fluorescence units (FUs) correlate with a less relatively positive surface charge [25–27].

2.7. Infection of cultured murine macrophages and assessment of cytokine response

Bone marrow-derived macrophages (BMDMs) were isolated from the femur and tibia of C57BL/6 mice as previously described [28]. The BMDM cultures were maintained in Iscove's modified Dulbecco's medium (Gibco, Grand Island, NY) containing L929 cell culture supernatant (30% v/v). After incubation for 16–18 hours at 37 °C with 5% CO_2 in 48-well pates ($\sim 3 \times 10^5$ cells/well), BMDMs were infected with selected MRSA strains (4 CA-MRSA, 2 LA-MRSA, and 4 HA-MRSA strains) at a multiplicity of infection (MOI) of 1 or 5 for 1 h, and extracellular bacterial growth was inhibited by adding gentamycin (50 μg/ml). The culture supernatant was then collected at 24 h after the infection to determine inflammatory cytokine levels. The concentrations of IL-6 and TNF-α in the supernatant were determined using the ELISA kits (R&D Systems, Minneapolis, MN). Susceptibilities to gentamycin and MOI for MRSA infections were determined in prior pilot experiments in all *S. aureus* strains. The animal experiments were conducted according to the protocols approved by the Animal Research Committee (IACUC) of Chonnam National University, Gwangju, Korea (Approval No. CNU IACUC-YB-2017-56).

2.8. Statistical analysis

The data were analyzed by Mann-Whitney U test using IBM SPSS Statistics 23 software (IBM SPSS Statistics 23 for Windows, Chicago, IL, USA). Significance was determined at P values < 0.05.

3. Results

3.1. Antibiotic resistance profiles of MRSA strains

As noted in Table 1, disk diffusion tests revealed that 3/7 ST72-LA-MRSA isolates were resistant to more than 2 antibiotic agents, while 4/7 ST72-LA-MRSA strains were susceptible to all 6 antibiotic agents. Except for three MRSA strains (CA1, CA5, and CA9), the other 8 ST72-MRSA strains isolated from human patients were susceptible to all 6 antibiotics. In contrast to ST72-MRSA strains, all the ST5-MRSA-II strains were resistant to at least one of the 6 antibiotics. Of note, all the ST5-MRSA-II strains were resistant to erythromycin (100%), and 5/8 isolates were resistant to multiple antibiotics (> 2 antibiotic agents). All isolates were susceptible to VAN and DAP, except for 3 ST5-MRSA (HA1, HA2, and HA3) and 1 ST72-MRSA (CA1) strains with DAP MICs ≥ 1.5 μg/ml. All 8 ST5-MRSA strains were highly resistant to OX, with OX MICs ≥ 256 μg/ml.

3.2. Virulence factors and toxin genes

Phenotypic virulence factor analyses revealed that all MRSA isolates were hemolytic; 6/7 ST72-LA-MRSA isolates were beta-hemolytic, all the ST5-MRSA isolates were alpha-hemolytic, 6/11 ST72-MRSA isolates from human patients were alpha-hemolytic, and none of the isolates were gamma-hemolytic. Only 4 ST72-MRSA strains (LA1, CA1, CA8, and CA9) displayed an alpha-beta-hemolytic phenotype.

Although 100% of ST72-LA-MRSA isolates and 73% (8/11 isolates) of human ST-72-MRSA isolates were gelatinase positive, none of the ST5-MRSA isolates showed gelatinase activity.

None of the 26 MRSA strains harbored genes for PVL, TSST-1, ETs (*eta* and *etb*), or SEs (*sea, seb, sec, sed,* and *see*) (data not shown).

3.3. HD-CAP susceptibilities among MRSA strains

To assess whether there is difference in susceptibilities to distinct HD-CAPs among the three groups of MRSA strains, we examined the in vitro susceptibility profiles of MRSA strains against LL-37, BMAP-28, and PMB. LL-37 (representative of HD-CAPs of cathelicidin family encountered in human cutaneous and endovascular infections), BMAP-28 (a bovine HD-CAPs of the cathelicidin family), and polymyxin B (a cyclic bacterial-derived CAPs) were selected to represent HD-CAPs of different origins and encompass CM-targeting cationic molecules. Generally, ST5 HA-MRSA strains exhibited an overall higher survival profile when exposed to the three prototypical HD-CAPs (Fig. 1A–C). When compared as collective groups of LA-MRSA, CA-MRSA, and HA-MRSA strains, HA-MRSA strains displayed significantly higher resistance against all three HD-CAPs than those of the LA-MRSA and CA-MRSA strains (Table 2). Of note, although there was no statistical significance for susceptibilities to LL-37 and BMAP-28 between the LA-MRSA and CA-MRSA strain groups, CA-MRSA strains exhibited significantly higher survival when exposed to PMB ($P < 0.01$).

3.4. Net surface positive charge

FITC-PLL binding analyses of all 26 MRSA strains revealed that the ST5 HA-MRSA strains had a significantly increased positive surface charge compared to those of ST72 LA-MRSA and ST72 CA-MRSA strains (Fig. 2). In a group comparison (Table 2), enhanced surface positive charges in HA-MRSA strains were associated with significantly enhanced resistance against LL-37, BMAP-28, and PMB. Next, when the two groups of ST72 MRSA strains, LA-MRSA and CA-MRSA, were compared for net surface positive charge, there was no significant difference between the two groups of MRSA strains.

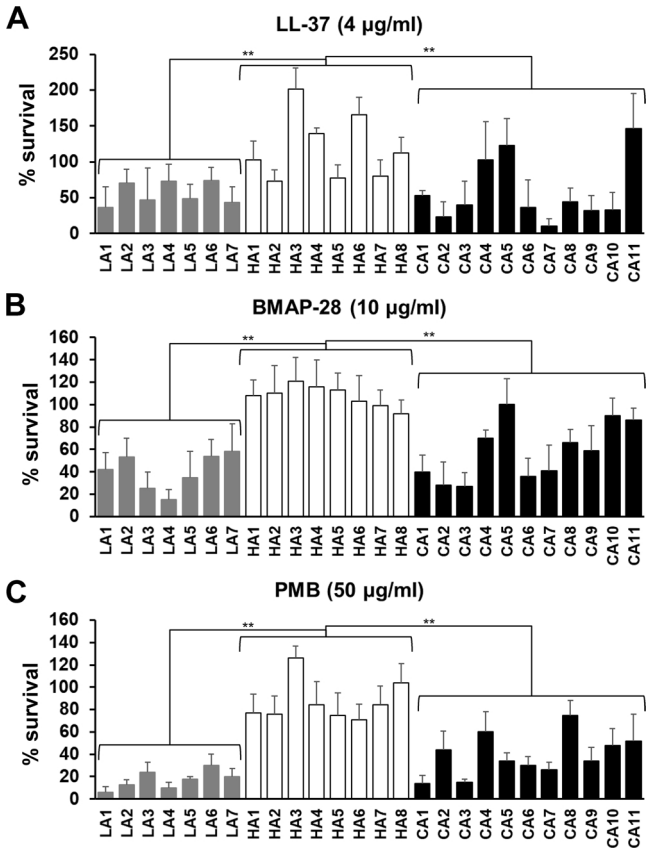

Fig. 1. in vitro susceptibility profiles of the MRSA strains to LL-37 (A), BMAP-28 (B), and PMB (C). in vitro staphylococcal survival assays were performed with LL-37 (4 μg/ml), BMAP-28 (10 μg/ml), and PMB (50 μg/ml) as described in the Materials and Methods section. These data represent the mean ± standard deviation of three independent runs. *$P < 0.05$; **$P < 0.01$

3.5. Innate immune response in murine macrophages

Macrophages play an essential role in the first line of host defense in innate immune system against MRSA infections. Thus, MRSA isolates from ST72 LA-MRSA, ST72 CA-MRSA, and ST5 HA-MRSA groups were assessed for induction of innate immune response by analyzing cytokine profiles in the murine macrophage infection model. All the MRSA strains elicited cytokine responses in BMDMs in a bacterial dose-dependent manner (data not shown). Importantly, ST72 CA-MRSA strains induced significantly higher levels of IL-6 and TNF-α cytokines than those of ST5 HA-MRSA strains (Fig. 3A and B). Interestingly, the two ST72 LA-MRSA strains (LA1 and LA2) induced even higher levels of IL-6 and TNF-α cytokines versus the ST72 CA-MRSA strains.

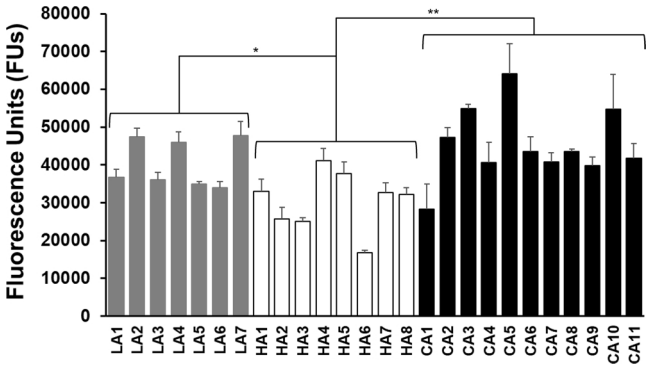

Fig. 2. Binding of positively charged FITC-labeled PLL to whole *S. aureus* cells. The graph shows relative fluorescence units (± SD) measured by flow cytometric analyses. In this assay, the level of fluorescence intensity inversely reflects the relative surface positive charge in *S. aureus* cells. The data are representative of three independent assays in duplicate samples. *$P < 0.05$; **$P < 0.01$.

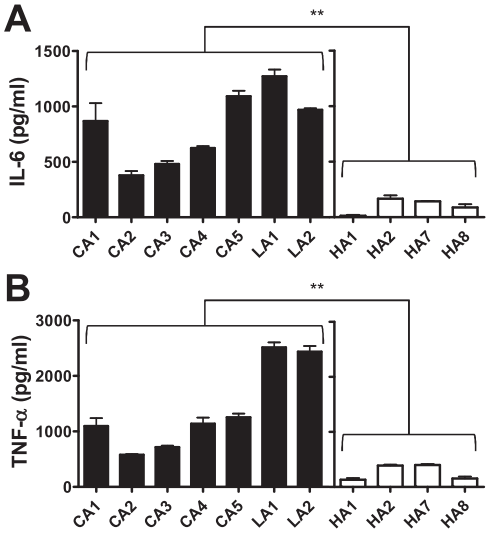

Fig. 3. Cytokine stimulation profiles in murine macrophages infected with MRSA strains. Mouse bone marrow-derived macrophages (BMDMs) were infected with each MRSA strain at an MOI of 5 for 24 h, and levels of IL-6 (A) and TNF-α (B) cytokines in the culture supernatants were determined. The data are the mean (± SD) of three independent experiments performed on separate days. *$P < 0.05$; **$P < 0.01$.

4. Discussion

Several recent studies have demonstrated that the most significant CA-MRSA clone in Korea is ST72-MRSA-IV [4,5,29,30] and this clonal lineage, usually lacking Panton-Valentine leucocidin (PVL), has become

Table 2
Group comparison of host defense antimicrobial peptide susceptibilities and positive surface charge among LA-MRSA, HA-MRSA, and CA-MRSA strains.

Parameter	MRSA Groups:			*P* value for:		
	LA-MRSA (n = 7)	HA-MRSA (n = 8)	CA-MRSA (n = 11)	LA vs HA	LA vs CA	HA vs CA
% survival after 2 h exposure to:	**31.4 ± 15.9**	**95.3 ± 34.5**	**29.8 ± 36.8**	**< 0.001**	**NS**	**< 0.001**
LL-37 (4 μg/ml)						
BMAP-28 (10 μg/ml)	**9.4 ± 6.1**	**73.6 ± 18.7**	**7.9 ± 8.8**	**< 0.001**	**NS**	**< 0.001**
PMB (50 μg/ml)	**17.3 ± 8.3**	**87.1 ± 18.7**	**39.3 ± 18.7**	**< 0.001**	**< 0.01**	**< 0.01**
Positive surface charge:	40412.8	30535.5	45414.1	**< 0.05**	**NS**	**< 0.01**
Relative fluorescence	**± 6283.7**	**± 1091.9**	**± 2821.7**			

[§]NS, not significant.

a major nosocomial clone [31,32]. Thus, ST72-MRSA-IV has been encroaching on nosocomial settings in Korea, replacing major HA-MRSA clones such as ST5-MRSA-II and ST239-MRSA-III [5,29,33]. In addition to CA-MRSA and HA-MRSA, animal host adapted MRSA (LA-MRSA) strains have been identified in various livestock animals and foods of animal origin [34–36]. Thus, there is an increased number of cases where humans are infected from animal originated MRSA strains [1,35]. Although the effect of CA-MRSA on the clinical outcomes versus HA-MRSA remains unclear, a recent prospective cohort study of the effect of ST72 CA-MRSA-IV on clinical outcomes revealed that ST5 HA-MRSA-II is associated with higher mortality compared with ST72 CA-MRSA-IV, suggesting higher virulence in ST5 HA-MRSA-II strains [7].

In the current investigation, we studied a total of 26 unique ST5-MRSA-II and human-/animal-originated ST72-MRSA-IV strains to evaluate potential relationships between the HD-CAP resistance phenotypes and enhanced virulence in ST5-MRSA-II.

First, it has been previously shown that the persistence and progression of *S. aureus* infections unambiguously requires the pathogen to resist the microbicidal action of cytoplasmic membrane (CM)-targeting HD-CAPs. In addition to damaging target bacterial CMs by a variety of mechanisms, HD-CAPs may also affect vital intracellular processes including biosynthesis of nucleic acids, proteins, and cell wall components [23,37]. As shown by the results in Fig. 1A, ST5 HA-MRSA-II isolates showed higher levels of resistance against the human cathelicidin, LL-37, than the two groups of ST72-MRSA-IV isolates, although CA4, CA5, and CA11 strains displayed somewhat increased resistance to LL-37 relative to the rest of the ST72-MRSA-IV isolates. Furthermore, ST5-HA-MRSA-II isolates exhibited highest levels of resistance to the bovine cathelicidin, BMAP-28, and the bacterial derived PMB (Fig. 1B and C), indicating that enhanced resistance to HD-CAPs in ST5-MRSA-II strains is likely to be associated with higher virulence than the ST72-MRSA-IV strains. Although there were no significant differences in resistance to LL-37 and BMAP-28 between the two groups of ST72-MRSA-IV strains, human patient-originated ST72 CA-MRSA strains displayed significantly reduced susceptibilities only to PMB versus the bovine-originated ST72 LA-MRSA strains ($P < 0.01$). These data also suggest that ST72-MRSA-IV strains developed different levels of HD-CAP resistance during infections in different host species.

Second, recently published studies have suggested that resistance to HD-CAP in *S. aureus* is often associated with distinct cell envelope adaptations such as increased positive surface charge, facilitating the charge repulsion mechanism for HD-CAP resistance [10,38]. In line with these prior observations, the overall ST5-MRSA-II strain group exhibited enhanced surface positive charge compared with those of the two ST72-MRSA-IV strain groups (Fig. 2 and Table 2). It is speculated that the increased positive surface charge in ST5-MRSA-II strains might be caused by either increased expression of *mprF* and/or *dltABCD* [26,27], upstream transcriptional regulators for *mprF* and *dltABCD* such as the *graRS* two-component regulatory system [11,39], or single nucleotide point mutations in the *mprF* open reading frame (ORF) [12]. In this regard, transcription profiles and sequence analyses of *graRS*, *mprF*, and *dltABCD* in these same MRSA strain groups are in progress.

Third, to model the effect of HD-CAP resistance in virulence of ST5-MRSA-II strains, we employed bone marrow-derived murine macrophage (BMDM) cultures [40] to test the innate immune responses to the three groups of MRSA strains. Macrophages are recognized as key components of innate immunity to *S. aureus* infection and recruitment of neutrophils to the site of infection. It has also been reported that mouse macrophages express the cathelicidin CRAMP to fight infections caused by bacterial pathogens [41]. As shown in Fig. 3A and B, ST72-MRSA-IV strains induced higher levels of the two pro-inflammatory cytokines, IL-6 and TNF-α, than ST5-MRSA-II strains in BMDMs. The observation that ST5-MRSA-II strains induce significantly less cytokines than ST72-MRSA-IV strains is likely to be correlated with the higher virulence of the ST5-MRSA-II strains [7] by downregulating inflammatory cytokine response in macrophages during the early infection stage.

Lastly, previous studies have suggested that reduced vancomycin susceptibility and presence of staphylococcal superantigen genes were associated with higher mortality in MRSA strains [7,42]. However, the ST5-MRSA-II strains did not show enhanced vancomycin MICs versus the two groups of ST72-MRSA-IV strains (Table 1), and none of the MRSA strains in our current studies possessed the three superantigen genes. In addition, hemolysis profiles and gelatinase activity were not associated with higher virulence in ST5-MRSA-II strains (Table 1).

It is important to recognize that there are several limitations in the current study. Our data were generated from limited number of LA-MRSA, CA-MRSA, and HA-MRSA strains. It will be necessary to assess HD-CAP resistance and surface positive charges along with clinical outcomes associated with virulence levels of ST72 CA-MRSA and ST5 HA-MRSA in larger strain collection. Moreover, we did not determine possible dysregulation of genetic factors associated with HD-CAP resistance phenotype, such as *graRS*, *dltABCD*, and *mprF* [10–12]. Furthermore, future studies will continue to focus on other physicochemical factors in staphylococcal cell membrane (CM) than the surface positive charge, including CM fluidity/rigidity, CM fatty acid composition, and CM phospholipid profiles [8,12,27]. Current studies are in progress to address these limitations.

In summary, our results suggest that (i) ST5 HA-MRSA-II strains tend to have higher levels of resistance to HD-CAPs compared with ST72 CA-MRSA-IV and ST72 LA-MRSA-IV strains; (ii) the higher HD-CAP resistance in ST5 HA-MRSA-II strains appears to be correlated with enhanced surface positive charge; and (iii) in addition to the HD-CAP resistance, ST5 HA-MRSA strains are able to avoid the host's innate immune defense by attenuating initial cytokine response in macrophages.

Acknowledgements

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education (NRF-2015R1D1A1A01057442 to S.J.Y.).

References

[1] C. Cuny, L.H. Wieler, W. Witte, Livestock-associated MRSA: the impact on humans, Antibiotics 4 (4) (2015) 521–543.
[2] H.A. Grema, Y.A. Geidam, G.B. Gadzama, J.A. Ameh, A. Suleiman, Methicillin resistant *Staphyloccus aureus* (MRSA): a review, Adv. Anim. Vet. Sci. 3 (2) (2015) 79–98.
[3] P. Butaye, M. Argudín, T. Smith, Livestock-associated MRSA and its current evolution, Curr. Clin. Microbiol. Rep. 3 (1) (2016) 19–31.
[4] E.S. Kim, J.S. Song, H.J. Lee, P.G. Choe, K.H. Park, J.H. Cho, W.B. Park, S.H. Kim, J.H. Bang, D.M. Kim, K.U. Park, S. Shin, M.S. Lee, H.J. Choi, N.J. Kim, E.C. Kim, M.D. Oh, H.B. Kim, K.W. Choe, A survey of community-associated methicillin-resistant *Staphylococcus aureus* in Korea, J. Antimicrob. Chemother. 60 (5) (2007) 1108–1114.
[5] J.-H. Song, P.-R. Hsueh, D.R. Chung, K.S. Ko, C.-I. Kang, K.R. Peck, J.-S. Yeom, S.-W. Kim, H.-H. Chang, Y.-S. Kim, Spread of methicillin-resistant *Staphylococcus aureus* between the community and the hospitals in Asian countries: an ANSORP study, J. Antimicrob. Chemother. 66 (5) (2011) 1061–1069.
[6] S.Y. Chen, C.H. Liao, J.L. Wang, W.C. Chiang, M.S. Lai, W.C. Chie, W.J. Chen, S.C. Chang, P.R. Hsueh, Methicillin-resistant *Staphylococcus aureus* (MRSA) staphylococcal cassette chromosome *mec* genotype effects outcomes of patients with healthcare-associated MRSA bacteremia independently of vancomycin minimum inhibitory concentration, Clin. Infect. Dis. 55 (10) (2012) 1329–1337.
[7] K.-H. Park, Y.P. Chong, S.-H. Kim, S.-O. Lee, S.-H. Choi, M.S. Lee, J.-Y. Jeong, J.H. Woo, Y.S. Kim, Community-associated MRSA strain ST72-SCC *mec* IV causing bloodstream infections: clinical outcomes and bacterial virulence factors, J. Antimicrob. Chemother. 70 (4) (2014) 1185–1192.
[8] M.R. Yeaman, A.S. Bayer, S.-P. Koo, W. Foss, P.M. Sullam, Platelet microbicidal proteins and neutrophil defensin disrupt the *Staphylococcus aureus* cytoplasmic membrane by distinct mechanisms of action, J. Clin. Invest. 101 (1) (1998) 178.
[9] R.E. Hancock, H.-G. Sahl, Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies, Nat. Biotechnol. 24 (12) (2006) 1551.
[10] C. Weidenmaier, A. Peschel, V.A. Kempf, N. Lucindo, M.R. Yeaman, A.S. Bayer, DltABCD-and MprF-mediated cell envelope modifications of *Staphylococcus aureus* confer resistance to platelet microbicidal proteins and contribute to virulence in a rabbit endocarditis model, Infect. Immun. 73 (12) (2005) 8033–8038.
[11] S.-J. Yang, A.S. Bayer, N.N. Mishra, M. Meehl, N. Ledala, M.R. Yeaman, Y.Q. Xiong,

A.L. Cheung, The *Staphylococcus aureus* two-component regulatory system, GraRS, senses and confers resistance to selected cationic antimicrobial peptides, Infect. Immun. 80 (1) (2012) 74–81.
[12] S.-J. Yang, N.N. Mishra, A. Rubio, A.S. Bayer, Causal role of single nucleotide polymorphisms within the mprF gene of *Staphylococcus aureus* in daptomycin resistance, Antimicrob. Agents Chemother. 57 (11) (2013) 5658–5664.
[13] J.W. Song, S.J. Yang, S. Shin, K.S. Seo, Y.H. Park, K.T. Park, Genotypic and phenotypic characterization of methicillin-resistant *Staphylococcus aureus* isolated from bovine mastitic milk in Korea, J. Food Prot. 79 (10) (2016) 1725–1732.
[14] E.S. Kim, I.-G. Bae, J.E. Cho, Y.J. Choi, I.-H. Kim, G.-S. Kang, H.-y. Sin, K.-H. Song, C. Park, D.-G. Lee, Clinical and molecular characterization of invasive hetero-resistant vancomycin-intermediate *Staphylococcus aureus* infections in Korean hospitals, J. Clin. Microbiol. 54 (3) (2016) 760–763.
[15] M.C. Enright, N.P. Day, C.E. Davies, S.J. Peacock, B.G. Spratt, Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of Staphylococcus aureus, J. Clin. Microbiol. 38 (3) (2000) 1008–1015.
[16] D. Harmsen, H. Claus, W. Witte, J. Rothgänger, H. Claus, D. Turnwald, U. Vogel, Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for spa repeat determination and database management, J. Clin. Microbiol. 41 (12) (2003) 5442–5448.
[17] K. Becker, R. Roth, G. Peters, Rapid and specific detection of toxigenic *Staphylococcus aureus*: use of two multiplex PCR enzyme immunoassays for amplification and hybridization of staphylococcal enterotoxin genes, exfoliative toxin genes, and toxic shock syndrome toxin 1 gene, J. Clin. Microbiol. 36 (9) (1998) 2548–2553.
[18] W. Johnson, S. Tyler, E. Ewan, F. Ashton, D. Pollard, K. Rozee, Detection of genes for enterotoxins, exfoliative toxins, and toxic shock syndrome toxin 1 in *Staphylococcus aureus* by the polymerase chain reaction, J. Clin. Microbiol. 29 (3) (1991) 426–430.
[19] G. Lina, Y. Piémont, F. Godail-Gamot, M. Bes, M.-O. Peter, V. Gauduchon, F. Vandenesch, J. Etienne, Involvement of panton-valentine leukocidin—producing *Staphylococcus aureus* in primary skin infections and pneumonia, Clin. Infect. Dis. 29 (5) (1999) 1128–1132.
[20] J. Hait, S. Tallent, D. Melka, C. Keys, R. Bennett, *Staphylococcus aureus* outbreak investigation of an Illinois bakery, J. Food Saf. 32 (4) (2012) 435–444.
[21] J. Turner, Y. Cho, N.-N. Dinh, A.J. Waring, R.I. Lehrer, Activities of LL-37, a cathelin-associated antimicrobial peptide of human neutrophils, Antimicrob. Agents Chemother. 42 (9) (1998) 2206–2214.
[22] S. Takagi, S. Hayashi, K. Takahashi, H. Isogai, L. Bai, H. Yoneyama, T. Ando, K. Ito, E. Isogai, Antimicrobial activity of a bovine myeloid antimicrobial peptide (BMAP-28) against methicillin-susceptible and methicillin-resistant Staphylococcus aureus, Anim. Sci. J. 83 (6) (2012) 482–486.
[23] Y.Q. Xiong, K. Mukhopadhyay, M.R. Yeaman, J. Adler-Moore, A.S. Bayer, Functional interrelationships between cell membrane and cell wall in antimicrobial peptide-mediated killing of Staphylococcus aureus, Antimicrob. Agents Chemother. 49 (8) (2005) 3114–3121.
[24] T. Jones, M.R. Yeaman, G. Sakoulas, S.-J. Yang, R.A. Proctor, H.-G. Sahl, J. Schrenzel, Y.Q. Xiong, A.S. Bayer, Failures in clinical treatment of *Staphylococcus aureus* infection with daptomycin are associated with alterations in surface charge, membrane phospholipid asymmetry, and drug binding, Antimicrob. Agents Chemother. 52 (1) (2008) 269–278.
[25] K. Mukhopadhyay, W. Whitmire, Y.Q. Xiong, J. Molden, T. Jones, A. Peschel, P. Staubitz, J. Adler-Moore, P.J. McNamara, R.A. Proctor, *In vitro* susceptibility of *Staphylococcus aureus* to thrombin-induced platelet microbicidal protein-1 (tPMP-1) is influenced by cell membrane phospholipid composition and asymmetry, Microbiology 153 (4) (2007) 1187–1197.
[26] A. Peschel, M. Otto, R.W. Jack, H. Kalbacher, G. Jung, F. Götz, Inactivation of the *dlt* operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides, J. Biol. Chem. 274 (13) (1999) 8405–8410.
[27] N.N. Mishra, S.-J. Yang, A. Sawa, A. Rubio, C.C. Nast, M.R. Yeaman, A.S. Bayer, Analysis of cell membrane characteristics of *in vitro*-selected daptomycin-resistant strains of methicillin-resistant *Staphylococcus aureus*, Antimicrob. Agents Chemother. 53 (6) (2009) 2312–2318.
[28] A. Celada, P.W. Gray, E. Rinderknecht, R.D. Schreiber, Evidence for a gamma-interferon receptor that regulates macrophage tumoricidal activity, J. Exp. Med. 160 (1) (1984) 55–74.
[29] S.H. Park, C. Park, J.-H. Yoo, S.-M. Choi, J.-H. Choi, H.-H. Shin, D.-G. Lee, S. Lee, J. Kim, S.E. Choi, Emergence of community-associated methicillin-resistant *Staphylococcus aureus strains* as a cause of healthcare-associated bloodstream infections in Korea, Infect. Control Hosp. Epidemiol. 30 (2) (2009) 146–155.
[30] J.R. Mediavilla, L. Chen, B. Mathema, B.N. Kreiswirth, Global epidemiology of community-associated methicillin resistant *Staphylococcus aureus* (CA-MRSA), Curr. Opin. Microbiol. 15 (5) (2012) 588–595.
[31] S.S. Lee, Y.J. Kim, D.R. Chung, K.-S. Jung, J.-S. Kim, Invasive infection caused by a community-associated methicillin-resistant *Staphylococcus aureus* strain not carrying panton-valentine leukocidin in South Korea, J. Clin. Microbiol. 48 (1) (2010) 311–313.
[32] E.-J. Joo, D. Chung, Y. Ha, S. Park, S.-J. Kang, S. Kim, C.-I. Kang, K. Peck, N. Lee, K. Ko, Community-associated Panton–Valentine leukocidin-negative meticillin-resistant *Staphylococcus aureus* clone (ST72-MRSA-IV) causing healthcare-associated pneumonia and surgical site infection in Korea, J. Hosp. Infect. 81 (3) (2012) 149–155.
[33] H.Y. Cha, D.C. Moon, C.H. Choi, J.Y. Oh, Y.S. Jeong, Y.C. Lee, S.Y. Seol, D.T. Cho, H.-H. Chang, S.-W. Kim, Prevalence of the ST239 clone of methicillin-resistant *Staphylococcus aureus* and differences in antimicrobial susceptibilities of ST239 and ST5 clones identified in a Korean hospital, J. Clin. Microbiol. 43 (8) (2005) 3610–3614.
[34] S.-K. Lim, H.-M. Nam, H.-J. Park, H.-S. Lee, M.-J. Choi, S.-C. Jung, J.-Y. Lee, Y.-C. Kim, S.-W. Song, S.-H. Wee, Prevalence and characterization of methicillin-resistant *Staphylococcus aureus* in raw meat in Korea, J. Microbiol. Biotechnol. 20 (4) (2010) 775–778.
[35] H. Graveland, B. Duim, E. Van Duijkeren, D. Heederik, J.A. Wagenaar, Livestock-associated methicillin-resistant *Staphylococcus aureus* in animals and humans, Int. J. Med. Microbiol. 301 (8) (2011) 630–634.
[36] S.-K. Lim, H.-M. Nam, G.-C. Jang, H.-S. Lee, S.-C. Jung, H.-S. Kwak, The first detection of methicillin-resistant *Staphylococcus aureus* ST398 in pigs in Korea, Vet. Microbiol. 155 (1) (2012) 88–92.
[37] K.A. Brogden, Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat. Rev. Microbiol. 3 (3) (2005) 238.
[38] P. Staubitz, H. Neumann, T. Schneider, I. Wiedemann, A. Peschel, MprF-mediated biosynthesis of lysylphosphatidylglycerol, an important determinant in staphylococcal defensin resistance, FEMS Microbiol. Lett. 231 (1) (2004) 67–71.
[39] M. Meehl, S. Herbert, F. Götz, A. Cheung, Interaction of the GraRS two-component system with the VraFG ABC transporter to support vancomycin-intermediate resistance in Staphylococcus aureus, Antimicrob. Agents Chemother. 51 (8) (2007) 2679–2689.
[40] J.-Y. Lee, M.-S. Lee, D.-J. Kim, S.-J. Yang, S.-J. Lee, E.-J. Noh, S.J. Shin, J.-H. Park, Nucleotide-binding oligomerization domain 2 contributes to limiting growth of mycobacterium abscessus in the lung of mice by regulating cytokines and nitric oxide production, Front. Immunol. 8 (2017) 1477.
[41] C.M. Rosenberger, R.L. Gallo, B.B. Finlay, Interplay between antibacterial effectors: a macrophage antimicrobial peptide impairs intracellular *Salmonella* replication, Proc. Natl. Acad. Sci. U. S. A. 101 (8) (2004) 2422–2427.
[42] S. Van Hal, T.P. Lodise, D.L. Paterson, The clinical significance of vancomycin minimum inhibitory concentration in *Staphylococcus aureus* infections: a systematic review and meta-analysis, Clin. Infect. Dis. 54 (6) (2012) 755–771.