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Multilocus sequence type-dependent activity of human and animal cathelicidins against community-, hospital-, and livestock-associated methicillin-resistant *Staphylococcus aureus* isolates

Running title: Clonotype-specific activity of cathelicidins against MRSA isolates

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Abstract

Sequence type (ST) 5 methicillin-resistant *Staphylococcus aureus* (MRSA) with staphylococcal cassette chromosome *mec* (SCC*mec*) type II (ST5-MRSA-II) and ST72-MRSA-IV represent the most significant genotypes for healthcare- (HA) and community-associated (CA) MRSA in Korea, respectively. In addition to the human-type MRSA strains, the prevalence of livestock-associated (LA) MRSA clonal lineages, such as ST541 and ST398 LA-MRSA-V in pigs and ST692 LA-MRSA-V and ST188 LA-MRSA-IV in chickens, has recently been found. In this study, clonotype-specific resistance profiles to cathelicidins derived from humans (LL-37), pigs (PMAP-36), and chickens (CATH-2) were examined using six different ST groups of MRSA strains: ST5 HA-MRSA-II, ST72 CA-MRSA-IV, ST398 LA-MRSA-V, ST541 LA-MRSA-V, ST188 LA-MRSA-IV, and ST692 LA-MRSA-V. Phenotypic characteristics often involved in cathelicidin resistance, such as net surface positive charge, carotenoid production, and hydrogen peroxide susceptibility were also determined in the MRSA strains. Human- and animal-type MRSA strains exhibited clonotype-specific resistance profiles to LL-37, PMAP-36, or CATH-2, indicating the potential role of cathelicidin resistance in the adaptation and colonization of human and animal hosts. The ST5 HA-MRSA isolates showed enhanced resistance to all three cathelicidins and hydrogen peroxide than ST72 CA-MRSA isolates by implementing increased surface positive charge and carotenoid production. In contrast, LA-MRSA strains employed mechanisms independent of surface charge regulation and carotenoid production for cathelicidin resistance. These results suggest that human- and livestock-derived MRSA strains use different strategies to counteract the bactericidal action of cathelicidins during the colonization of their respective host species.

Keywords: Methicillin-resistant *Staphylocuccus aureus*; CA-MRSA; HA-MRSA; LA-MRSA; Cathelicidin; Host adaptation

INTRODUCTION

Staphylococcus aureus is a commensal organism and opprtunistic pathogen frequently carried asymptomatically on the skin and mucous membranes of human and animal. Mechicillin-resistant *S. aureus* (MRSA) acquired resistance to methicillin and nearly all β-lactam antibiotics such as cephalosporins as a result of *mecA* or *mecC* gene acquisition [1]. Mostly, MRSA strains harbor the *mecA* within a large mobile genetic element known as staphylococcal casette chromosome *mec* (SCC*mec*) [2]. Besides the resistance to β-lactam antibiotics, MRSA usually exhibits multidrug resistance (MDR) phenotypes, and serious infections with such MRSA strains result in significantly enhanced morbidity and mortality [3, 4].

MRSA has been known to be widespread and common, but specific genetic lineages can vary depending on geographical regions and host species [5, 6]. MRSA strains adapted to humans have usually been divided into halthcare-associated (HA) and community-associated (CA) MRSA. Although the distinctions between the two groups of MRSA strains are becoming obscure, HA-MRSA and CA-MRSA have mainly been recognized in hopitalized patients and non-hospitalized population, respectively. In Korea, sequence type 5 MRSA with SCC*mec* type II (ST5-MRSA-II) and ST72-MRSA-IV represent the most significant clonal lineages for (HA- and CA-MRSA, respectively [7-9]. In addition to the human-adapted strains, colonization or infection with livestock-associated (LA) MRSA has been reported in several domesticated livestock animals [1, 6, 10]. These carrier animals not only serve as reservoirs for opportunistic infections in themselves but also can transmit LA-MRSA to other animal species or humans [10]. In this sense, the recent emergence of ST398 LA-MRSA-V and ST541 LA-MRSA-V in pigs and pig farm environments has posed a serious public health concern worldwide [11-14]. In fact, it has been reported that human workers were colonized with ST398 MRSA shortly after direct or indirect contact with pigs or carcasses in pig farms or slaughterhouses [12]. Although there is limited information on MRSA in poultry, previous studies identified two important clonal lineages of LA-MRSA strains, ST692 LA-MRSA-V and ST188 LA-MRSA-IV, in chickens, chicken carcasses, and slaughterhouse workers in Korea [15, 16]. Although various types of MRSA clonal lineages, including both human- and livestock-adapted MRSA strains, have been found in humans and animals, factors affecting the transmission of human-type MRSA lineages to animals or LA-MRSA to humans are poorly understood.

One of the crucial factors in host innate immune reactions against MRSA infection or colonization is the host defense cationic antimicrobial peptide (HD-CAP) [17]. The successful transmission and adaptation of MRSA in a

new host species inevitably require the ability to overcome the bactericidal action of HD-CAPs from new hosts [18-20]. Cathelicidins are small, positively charged antimicrobial peptides that constitute a unique family of HD-CAPs. Cathelicidins have been identified in neutrophils, natural killer cells, and the epithelial cells in the skin, respiratory, and gastrointestinal tracts of humans and various animals [21]. Thus, a better understanding of the potential differences in bactericidal activities of human- and animal-originated cathelicidins against human- or animal-adapted MRSA strains will facilitate new insight into host species-specific prevalence of MRSA clonotypes.

In this investigation, potential clonotype-specific resistance profiles to cathelicidins in humans (LL-37), pigs (PMAP-36), and chickens (CATH-2) were determined using six different MRSA strains derived from humans (ST5 HA-MRSA-II and ST72 CA-MRSA-IV), pigs (ST398 LA-MRSA-V and ST541 LA-MRSA-V), and chickens (ST692 LA-MRSA-V and ST188 LA-MRSA-IV). In addition, the net surface positive charges, carotenoid production, and hydrogen peroxide (H_2O_2) resistance profiles of the MRSA strains were analyzed to identify phenotypic determinants linked to cathelicidin resistance.

MATERIALS AND METHODS

MRSA strains and identification

A total of 45 MRSA isolates of human and animal origin were used in this investigation (Table 1). All human isolates were selected from two previous studies from our laboratory: eight ST5 HA-MRSA and eleven ST72 CA-MRSA isolates [22, 23]. Nine ST398 LA-MRSA and six ST541 LA-MRSA isolates were selected from a previous investigation of LA-MRSA in healthy pigs [11]. Six ST692 LA-MRSA and six ST188 LA-MRSA isolates from healthy broiler chickens were selected and obtained from the national surveillance of antimicrobial resistance in livestock performed during 2018-2019 by the Korean Centers for Disease Control and Prevention. This study was conducted under protocols approved by the Institutional Animal Care and the Use Committee of Chunag-Ang University, Anseong, Korea (Approval No. 2018-00112).

For isolation of MRSA, swab samples were inoculated into 4 mL of tryptic soy broth (Difco Laboratories, Detroit, MI, USA) containing 10% NaCl and incubated 18-20 h at 37 °C with shaking at 200 rpm. Next, 20 μl of the enriched cultures were streaked onto chromID MRSA SMART agar (bioMérieux, France), then grown for 18-20 h at 37 °C. Suspected MRSA colonies from each sample were selected and subcultured on tryptic soy agar (Difco Laboratories) for further identification. To confirm if they were indeed *S. aureus*, all 45 isolates were subjected to 16S ribosomal RNA sequencing [24] and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF; Microflex, Daltonics Bruker, Bremen, Germany). Briefly, presumptive *S. aureus* colonies were applied onto the matrix and the samples were then ionized in an autmated mode with a laser beam in the MALDI-Biotyper Realtime Classification system. The peptide mass fingerprints of bacterial samples were used to identify *S. aureus* (score values of ≥2.0) based on the spectral database (MALDI Biotyper 3.1). All identified MRSA isolates were then grown in Mueller-Hinton broth (Difco Laboratories, Detroit, MI, USA) or tryptic soy broth (TSB; Difco Laboratories), depending on each experiment.

Determination of antimicrobial susceptibility

The antimicrobial susceptibility of MRSA isolates was examined using the standard disc diffusion assay according to the 2018 Clinical and Laboratory Standards Institute (CLSI) guidelines [25]. The 13 antimicrobial drugs used in the disc diffusion assays were ampicillin (AMP, 10 μg), cefoxitin (FOX, 30 μg), penicillin (PEN, 10

μg), gentamicin (GEN, 10 μg), clindamycin (CLI, 2 μg), chloramphenicol (CHL, 30 μg), erythromycin (ERY, 15 μg), mupirocin (MUP, 200 μg), sulfamethoxazole-trimethoprim (SXT, 23.75/1.25 μg), rifampicin (RIF, 5 μg), quinupristin-dalfopristin (SYN, 15 μg), tetracycline (TET, 30 μg), and ciprofloxacin (CIP, 5 μg). Mupirocin discs were obtained from Oxoid (Hampshire, UK), and the remaining antimicrobial discs were purchased from BD BBL™ (Becton Dickinson, Franklin Lakes, NJ). A standard E-test® (bioMérieux, France) method was used to determined the minimum inhibitory concentrations (MICs) of oxacillin (OXA) according to the manufacturer's protocol. The MIC values of MRSA isolates were determined according to the CLSI M100 and VET08 documents [26, 27]. Two reference strains, *S. aureus* MW2 and *S. aureus* ATCC® 29213, were included in the antimicrobial susceptibility assays.

Molecular characterization of MRSA isolates

All confirmed MRSA strains were subjected to multilocus sequence typing (MLST) as described before [28]. MLST has widely been accepted for a moleuclar epidemiological method of sequence-based typing in *S. aureus*, which analyzes seven relatively conserved houskeeping genes that encode essential proteins. The seven target loci (*aroE*, *arcC*, *glpF*, *tpi*, *gmk*, *pta*, and *yqiL*) were amplified via PCR and sequenced. The STs were then determined as suggested in the MLST database (http://pubmlst.org/saureus/) [28]. The allelic profiles of each STs of MRSA strains were: ST5 (1-4-1-4-12-1-10), ST72 (1-4-1-8-4-4-3), ST398 (3-35-19-2-20-26-39), ST541 (3-35-19-60-20-26-39), ST692 (12-89-1-1-4-5-90), and ST188 (3-1-1-8-1-1-1). The types of staphylococcal cassette chromosome *mec* (SCC*mec*) were determined through a series of multiplex PCR analyses, as previously described [2]. SCC*mec* types were assigned based on combinations of *ccr* (*ccrA1-3*, *ccrB1-4*, and *ccrC*) and *mec* gene complexes [2, 24]. For *spa* typing, the *spa* repeat regions were PCR-amplified and sequenced to examine the tandem repeats, and *spa* types were determined in each MRSA isolate based on the SpaServer database (http://spa.ridom.de/) [29]. The *agr* types (I-IV) of MRSA isolates were assinged through a PCR-based method, as described previously [29].

***In vitro* susceptibility assays to cationic antimicrobial peptides**

Three different host defense cationic antimicrobial peptides (HD-CAPs) of human (LL-37), porcine (PMAP-36), and poultry (CATH-2) origin were used to assess the genotype- and host-specific susceptibility patterns of MRSA isolates to LL-37 (human cathelicidin) [30], PMAP-36 (porcine myeloid antimicrobial peptide) [31], and

CATH-2 (chicken cathelicidin), respectively [32]. Human cathelicidin LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) was obtained from Peptide International (Louisville, KY, USA). PMAP-36 (GRFRRLRKKTRKRLKKIGKVLKWIPPIVGSIPLGCG) and CATH-2 (RFGRFLRKIRRFR PKVTITIQGSARF) were synthesized at GL Biochem, Shanghai, China with a purity of > 90%. Based on the manufacturer' analytical data such as reverse-phase HPLC or MALDI-TOF MS, lyophilized peptides were resuspended in phosphate-buffered saline, aliquoted, and stored at -20°C.

In vitro susceptibility assays to LL-37, PAMP-36, and CATH-2 were performed as previously described using RPMI medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% Luria-Bertani (LB) broth [33]. Briefly, overnight cultures of *S. aureus* cells grown in TBS were collected, washed two times with RPMI media, and then adjusted to ~1×10^4 CFU/mL in the RPMI containing 10% LB broth. Next, a final *S. aureus* inoculum of ~5×10^3 CFUs was incubated at 37°C in the presence of LL-37 (10 μg/mL), PMAP-36 (1.0 μg/mL), or CATH-2 (0.5 μg/mL). After 3 h incubation with peptides, aliquots (0.1 mL) of samples were processed for quantification of surviving bacterial cells (CFU/mL) by plating 10-fold serial dilutions of each culture on TSA plates. The peptide concentrations were adopted from extensive preliminary assays showing their inability to completely eliminate the viability of the initial bacterial inocula over the 3-h experiment period. Data are shown as the mean % of surviving MRSA CFUs ± standard deviations (SDs) of cathelicidin-exposed versus unexposed bacterial cells. Three independent experiments were performed in triplicates for each MRSA isolate.

Measurement of net surface positive charge

To assess the relative staphylococcal cell surface positive charge that can affect susceptibility to HD-CAPs, cytochrome *c* binding assays were carried out on the 45 MRSA strains as described before [34, 35]. Briefly, MRSA isolates were grown overnight (16-18 h) in TSB, washed twice with 20 mM morpholinepropanesulfonic acid (MOPS, pH 7.0) buffer, and resuspended in MOPS at an OD_{600nm} of 1.0. Staphylococcal cells were then incubated with 50 μg/mL of cytochrome *c* (Sigma-Aldrich) for 15 min at room temperature. Next, the reaction mixture was centrifuged (×13,000 rpm) for 1 min to pellet the bacterial cells, and the quantity of free cytochrome *c* that remained in the supernatant was determined by measuring the OD_{530nm}. A higher concentration of unbound cytochrome *c* in the supernatant corresponds to a more positively charged staphylococcal cell surface [36]. Three independent experiments were performed for each MRSA isolate.

Determination of carotenoid production

MRSA isolates were cultured in TSB to the stationary growth phase (24 h) at 37 °C with shaking at 200 rpm to quantify carotenoid production. After incubation, staphylococcal cells were pelleted, washed three times with PBS, and then diluted in PBS to ~1.0×10^9 CFU/mL. The carotenoid contents of the MRSA isolates were collected using the methanol extraction method as previously described [37], and then quantified spectrophotometrically by measuring the OD_{462nm}. At least three independent assays for carotenoid quantification were performed for all MRSA isolates.

***In vitro* hydrogen peroxide susceptibility assays**

Susceptibility to hydrogen peroxide was determined as previously described by Liu *et al.* [38]. Briefly, ~2.0×10^9 CFUs of MRSA isolates were incubated with H_2O_2 (1.5% final concentration) at 37 °C for 2 h, and catalase (1,000 U/mL, Sigma-Aldrich) was added to remove residual H_2O_2. Ten-fold dilutions were then prepared and placed on TSA plates for the enumeration of surviving staphylococcal cells. Data are expressed as the mean % of surviving CFU ± SDs of H_2O_2-treated versus untreated cells.

Statistical analysis

The quantitative data obtained in this study were analyzed for statistical significance using the Kruska-Wallis analysis of variance (ANOVA) test with the Tukey post hoc correction for multiple comparisons. Statistical significance of experimental data was set at p values < 0.05.

RESULTS

Genotypic profiles of MRSA isolates

A total of 45 MRSA isolates derived from humans (n = 19), pigs (n = 14), and chickens (n = 12) were used in this study (Table 1). All human and pig isolates were selected from previous studies [11, 22, 23]: eight isolates of ST5 HA-MRSA-II, eleven isolates of ST72 CA-MRSA-IV, eight isolates of ST398 LA-MRSA-V, and six isolates of ST541 LA-MRSA-V. As shown in Table 1, MLST analyses revealed that 12 chicken-associated MRSA isolates were ST692 LA-MRSA-V and six ST188 LA-MRSA-IV. Except for eight ST5 HA-MRSA-II isolates, which belonged to *agr* type II, all other 37 MRSA isolates were *agr* type I.

As presented in Table 1, three (t002, t2460, and t601) and five (t324, t13921, t664, t2461, and t148) different types of *spa* were identified in ST5 HA-MRSA-II and ST72 CA-MRSA-IV isolates, respectively. Unlike the various *spa* types observed in CA-MRSA and HA-MRSA isolates, all animal isolates tended to have ST-type-specific *spa* sequences. Except for one ST398 LA-MRSA (t571), the other ST398 LA-MRSA isolates belonged to t18102 or t18103. All six ST541 LA-MRSA isolates belonged to *spa* type t034. The chicken-derived ST692 and ST188 LA-MRSA isolate groups belonged to t2247 and t189, respectively (Table 1).

Antimicrobial resistance profiles of MRSA isolates

In general, the antimicrobial resistance patterns of MRSA strains differed depending on their ST and *spa* type. All LA-MRSA isolates showed multidrug resistance phenotypes to three or more subclasses of antimicrobial agents (Table 1). In particular, three isolates of ST398 LA-MRSA-V with *spa* type t18103 displayed the highest level of multidrug resistance (> 8 subclasses of antibiotic agents). In contrast to human-associated MRSA isolates (ST5 and ST72 MRSA isolates), all MRSA isolates of livestock origin (ST398, ST541, ST692, and ST188 MRSA isolates) were resistant to tetracycline. Interestingly, three ST types (ST398, ST692, and ST188) of LA-MRSA strains were resistant to ciprofloxacin, whereas all six ST541 LA-MRSA strains were susceptible to ciprofloxacin. In agreement with previous reports [39], ST5 HA-MRSA strains displayed higher levels of multidrug resistance than ST72 CA-MRSA isolates.

When grouped into six different STs (ST5, ST72, ST398, ST541, ST692, and ST188) of strain groups, the ST5 HA-MRSA strains had the highest oxacillin MIC values (512 μg/mL), followed by ST692 LA-MRSA (32 - 256

239 μg/mL), ST188 LA-MRSA (128 μg/mL), ST72 CA-MRSA (32 - 64 μg/mL), ST541 LA-MRSA (16 - 32 μg/mL),
240 and ST398 LA-MRSA (4 - 24 μg/mL) (Table 1).

241

242 ***In vitro* susceptibilities to LL-37, PMAP-36, and CATH-2**

243 To assess the potential genotype-specific differences in susceptibility to HD-CAPs of human and animal origins
244 among the six ST groups of MRSA isolates, *in vitro* 2-h survival assays were carried out for all 45 MRSA isolates
245 against LL-37 (10 μg/mL), PMAP-36 (1.0 μg/mL), or CATH-2 (0.5 μg/mL). Interestingly, the ST5 HA-MRSA,
246 ST692 LA-MRSA, and ST188 LA-MRSA isolates exhibited overall higher survival profiles against LL-37 than
247 the ST72 CA-MRSA, ST398 LA-MRSA, and ST541 LA-MRSA isolates (Fig. 1A). The two STs of chicken origin,
248 ST692 and ST188 MRSA isolates, displayed similar survival levels against LL-37. However, human- and pig-
249 associated MRSA isolates exhibited significantly different levels of resistance to LL-37 between the two host-
250 specific ST types (ST5 MRSA > ST72 MRSA, $p < 0.01$; ST398 > ST541 LA-MRSA isolates, $p < 0.05$).

251 Susceptibility assays to PMAP-36 revealed that pig-associated LA-MRSA isolates were significantly more
252 resistant to porcine cathelicidin than the human-associated or chicken-associated MRSA isolates ($p < 0.01$) (Fig.
253 1B). Although there were no significant differences in susceptibility to PMAP-36 between the two STs of LA-
254 MRSA isolates (ST398 versus ST541 or ST692 versus ST188), ST5 HA-MRSA isolates exhibited significantly
255 higher survival than ST72 CA-MRSA isolates ($p < 0.01$).

256 **As** shown in Fig. 1C, similar to the host-specific resistance to porcine cathelicidin (PMAP-36) observed in
257 ST398 LA-MRSA strains isolated from pigs, ST692 LA-MRSA strains derived from chickens showed the highest
258 level of resistance to the chicken cathelicidin (CATH-2) among the six ST groups of MRSA isolates. In contrast,
259 ST188 LA-MRSA displayed significantly lower levels of resistance to CATH-2 than the ST692 LA-MRSA strains
260 ($p < 0.01$). The two groups of swine-associated MRSA strains, ST398 LA-MRSA and ST541 LA-MRSA, showed
261 the lowest levels of resistance to the CATH-2 peptide compared to the human- and chicken-associated MRSA
262 strains (Fig. 1C).

263

264 **Surface positive charge of MRSA isolates**

265 Cytochrome *c* binding assays revealed that ST5 HA-MRSA and ST188 LA-MRSA had the highest net surface
266 positive charge levels among the six ST groups of MRSA strains (Fig. 2). Unlike the human- and chicken-

associated MRSA isolates, which exhibited significant differences between ST5 HA-MRSA and ST72 CA-MRSA ($p < 0.05$) or ST692 vs. ST188 LA-MRSA ($p < 0.01$), swine-associated LA-MRSA isolates did not show significant differences in cell surface charge between the ST398 and ST541 MRSA groups.

Measurement of carotenoid production

Quantification of carotenoid production in all 45 MRSA strains revealed that ST5 HA-MRSA and ST188 LA-MRSA strains exhibited the highest carotenoid content among the six different ST groups (Fig. 3). When the strain groups were compared within their host species, ST5 HA-MRSA, ST398 LA-MRSA, and ST188 LA-MRSA showed significantly higher levels of carotenoid production than the ST72 CA-MRSA, ST541 LA-MRSA, and ST692 LA-MRSA strains ($p < 0.05$).

Susceptibility to hydrogen peroxide

As shown in Fig. 4, *in vitro* H_2O_2 susceptibility assays over a 2-h period revealed that the ST5 HA-MRSA and ST398 LA-MRSA strain groups exhibited significantly higher survival in the presence of 1.5% H_2O_2 than that of ST72 HA-MRSA and ST541 LA-MRSA strains ($p < 0.05$). In contrast, there was no significant difference in the mean survival against H_2O_2 between the two chicken-derived MRSA strains, ST188 and ST692. Of note, ST5 HA-MRSA strains showed the highest level of resistance to H_2O_2 among the six different ST groups.

DISCUSSION

MRSA is an important human and animal pathogen that can cause frequent and often life-threatening infections in both community and hospital settings [4, 40]. However, the mechanisms involved in the transmission of human- or animal-adapted MRSA strains to new host species remain poorly understood. One of the critical components in host defense against MRSA infections involves innate immune responses, including HD-CAPs such as cathelicidins [41-43]. Since MRSA infections in human and animal hosts are caused mainly by colonizing strains of staphylococci [3, 44], the pathogen needs to deploy survival and adaptation mechanisms against the cathelicidins of human and animal hosts.

It has been shown that certain clonal lineages of MRSA strains are found in limited numbers of animal species or geographical regions [1, 6, 10]. Some recent studies have reported that ST72-MRSA-IV and ST5-MRSA-II represent major community and hospital MRSA clones in Korea, respectively [7-9]. In addition to the human-adapted MRSA clones, several major LA-MRSA clones have been recognized in livestock animals and meat production chains in Korea. The nationwide spread and prevalence of ST398-MRSA-V and ST541-MRSA-V in pigs and pork production systems in Korea have been demonstrated in several previous studies [11, 13, 14, 16]. Furthermore, ST692 and ST188 LA-MRSA strains have also been isolated from chicken and chicken meat production chains in Korea [15, 16, 21]. The two groups of chicken-derived LA-MRSA clones used in this study were ST692-MRSA-V with *spa* type t2247 and ST188-MRSA-IV with *spa* type t189 (Table 1).

Both host and bacterial factors may play critical roles in the adaptation and transmission of MRSA within or between human and animal hosts [19, 40, 45]. In this regard, one of the crucial innate immune responses of human and animal hosts against newly colonizing MRSA strains is HD-CAPs, especially cathelicidins. A previous report from our laboratory revealed that ST5 HA-MRSA-II tended to show higher resistance to LL-37 and bovine myeloid antimicrobial peptide-28 (BMAP-28), suggesting a potential role of cathelicidin resistance in enhancing the virulence and mortality of ST5 HA-MRSA-II [22]. In line with these observations, ST5 HA-MRSA-II displayed significantly higher levels of resistance against cathelicidins of human (LL-37) and pig (PMAP-36) origins than the ST72 CA-MRSA-IV strains (Figs 1A and B). The two chicken-derived LA-MRSA clones, ST692 and ST188 LA-MRSA, displayed higher levels of resistance to LL-37 andthan the ST72, ST398, and ST541 MRSA strains ($p < 0.01$), while ST398 and ST541 exhibited the highest levels of resistance to the porcine cathelicidin PMAP-36 ($p < 0.01$). Besides being prevalent in poultry farms, ST188 MRSA strains have been recognized as

major CA-MRSA in Australis and China [46, 47]. ST692 MRSA has also been identified in slaughterhouse workers in Korea [16], indicating that the higher levels of resistance to human cathelicidin (LL-37) in ST188 and ST692 MRSA may help to colonize human host. The observation that ST692 MRSA isolates are significantly more resistant to CATH-2 than ST188 MRSA isolates is likely to be correlated with the higher prevalence of the ST692 in chicken farms in Korea [15]. ST398 MRSA has been isolated from pigs and pig farm environment in many countries including Korea [5, 7, 10, 11, 13]. Of note, the clonal lineage of ST541 MRSA has been detected in healthy pigs in Korea [11]. As shown in Fig. 1B, ST398 and ST541 MRSA isolates were more resistant to the cathelicidin of pig origin (PAMP-36), correlating with previous reports of predominant colonization of pigs with CC398 (ST398 and ST541) strains. However, significantly enhanced LL-37 resistance in ST398 might be associated with higher prevalence of ST398 versus ST541 MRSA strains [11]. Overall, these data indicated that MRSA strains of human or animal origin tended to show enhanced levels of resistance to the cathelicidins of the same host species from which they were derived.

Several recently published reports have demonstrated that the HD-CAP resistance phenotype in staphylococci is associated with physicochemical alterations in the cell membrane, such as increased net cell surface positive charge, altered cell membrane fluidity, and fatty acid composition [48-51]. Consistent with previously published data, the ST5 HA-MRSA-II strains exhibited an enhanced net surface positive charge than the ST72 CA-MRSA-IV strains [22], indicating that the increased net surface positive charge in the ST5 HA-MRSA-II strains contributed to the enhanced resistance to cathelicidins (Fig. 2). However, although the pig-derived LA-MRSA strains exhibited the highest level of resistance to PMAP-36 (Fig. 1B), these two ST groups (ST398 and ST541) had lower levels of net surface positive charge than the human (ST5)- and chicken-derived (ST692 and ST188) MRSA strains (Fig. 2). The ST188 MRSA strains also showed significantly elevated surface positive charges compared to the ST692 MRSA strains ($p < 0.01$), correlating with the LL-37 resistance phenotype (Fig. 1A), but not with CATH-2 resistance (Fig. 1C). The enhanced surface positive charge in *S. aureus* has been associated with altered expression/sequence variation of *dltABCD*, *mprF*, and *graRS* genes [34, 35, 48]. In addition, degree of *O*-acetylation in peptidoglycan cell wall has been proposed to be involved in antimicrobial peptide resistance via surface positive charge regulation in *S. aureus* [48]. These additional factors affecting surface positive charge in *S.aureus* need to be addressed in future studies. Moreover, aside from the surface positive charge, cell envelope perturbations such as celll membrane fluidity, altered cell membrane permeabilization by cathelicidins, and staphylococcal memebrane fatty acid composition need to be evaluated. Based on the results, it is likely that there are multiple mechanisms involved in the host- and clonotype-specific cathelicidin resistance profiles of MRSA

strains in addition to the net cell surface positive charge, especially for LA-MRSA strains of chicken and pig origins.

Carotenoid pigmentation in *S. aureus* has been shown to affect cell membrane fluidity and counteract oxidative host defense mechanisms. It has been demonstrated that increased carotenoid biosynthesis could contribute to HD-CAP resistance in *S. aureus* by decreasing cell membrane fluidity [51]. In agreement with previous reports [22], ST5 HA-MRSA strains with enhanced resistance to the three cathelicidins (Figs. 1A, B, and C) exhibited significantly increased levels of carotenoid production compared to the ST72 CA-MRSA strains (Fig. 3). In addition, correlating with the LL-37 resistance profiles (Fig. 1A), the ST5 HA-MRSA and ST188 LA-MRSA strains produced significantly higher carotenoid contents than the other four groups of MRSA strains ($p < 0.05$). Enhanced carotenoid production in *S. aureus* has previously been linked to enhanced resistance to human antimicrobial peptides and cell wall targeting antimicrobial agents [18]. However, although the pig-associated LA-MRSA strains (ST398 and ST541) exhibited the highest levels of resistance to PMAP-36 (Fig. 1B), these strain groups did not produce higher levels of carotenoids than the ST5, ST72, and ST188 strain groups (Fig. 3), indicating that changes in cell membrane fluidity via carotenoid production have a very limited impact on the PMAP-36 resistance phenotype in ST398 and ST541 strains. Furthermore, the significant difference in carotenoid production observed between ST692 and ST188 LA-MRSA strain groups did not correlate with a higher level of CATH-2 resistance in the ST692 strain group versus the ST188 strain group (Fig. 1C). Collectively, these results suggest that human-type MRSA and LA-MRSA strains deploy different strategies to counteract the bactericidal activity of HD-CAPs encountered during host adaptation and colonization.

Given the impact of carotenoid production on the antioxidant activity of MRSA against the host innate immune response [38, 52, 53], *in vitro* susceptibility to H_2O_2 was determined for the six clonotypes of MRSA strains. Consistent with the carotenoid pigmentation profiles (Fig. 3), the ST5 HA-MRSA and ST398 LA-MRSA strain groups exhibited significantly higher levels of resistance to H_2O_2 than the ST72 CA-MRSA and ST541 LA-MRSA strains, respectively (Fig. 4). Although the results failed to reach statistical significance in resistance to H_2O_2, higher levels of carotenoid production in ST188 MRSA strains were also correlated with the moderate increase in H_2O_2 resistance compared to the ST692 strains. In a previous study it has been suggested that the carriage of superantigen genes in ST5 HA-MRSA may play a role in the higher virulence versus ST72 CA-MRSA [54]. Based on our result it is likely that enhanced resistance to HD-CAPs and H_2O_2 via increased surface positive charge and carotenoid production also contribute to the higher virulence of ST5 HA-MRSA versus ST72 CA-MRSA strains. Recently, ST188 *S. aureus* has been identified as a major clonal lineage causing infections in multiple host species

in China [47]. Whole genome sequence analyses revealed that ST188 *S. aureus* strains can be transmitted more easily among different host species [47]. The higer level of LL-37 resistance (Fig. 1A), enhanced surface positive charge (Fig. 2) and higher carotenoid production in ST188 MRSA strains (Fig. 3) versus ST692 MRSA strains may help to colonize diverse host species. On the contrary, the highest level of CATH-2 resistance observed in ST692 MRSA strains may be linked to the prevalence of this lineage in chickens [15].

In conclusion, the results of the present investigation suggest that: (i) there are differences in the biological activities of human- and animal-derived cathelicidins against MRSA strains of human (ST72 CA-MRSA and ST5 HA-MRSA), pig (ST398 and ST541 LA-MRSA), and chicken (ST692 and ST188 LA-MRSA) origins; (ii) ST5 HA-MRSA strains have higher resistance to various types of cathelicidins than ST72 CA-MRSA strains, as correlated with an increased net surface positive charge, enhanced production of carotenoid pigments, and enhanced resistance to H_2O_2; (iii) in contrast to human-type MRSA strains, pig- and chicken-associated MRSA strains had cathelicidin resistance mechanisms other than surface positive charge regulation and carotenoid production; and (iv) clonotype-specific cathelicidin resistance profiles may contribute to increased virulence or adaptation within new host species. To the best of our knowledge, this study is the first to evaluate potential role of HD-CAP resistance in human and animal host adaptation of MRSA strains.

Figure Legends

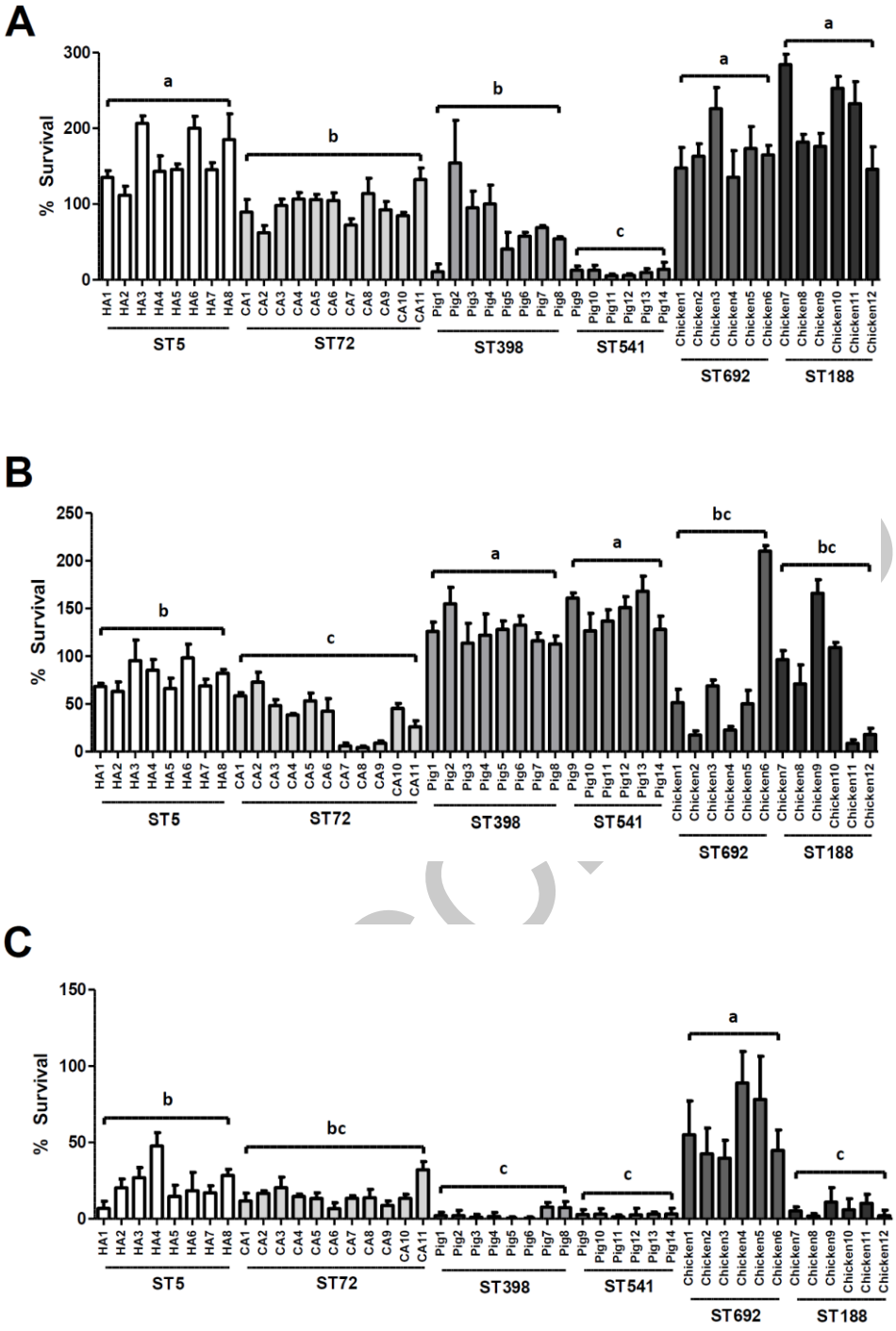
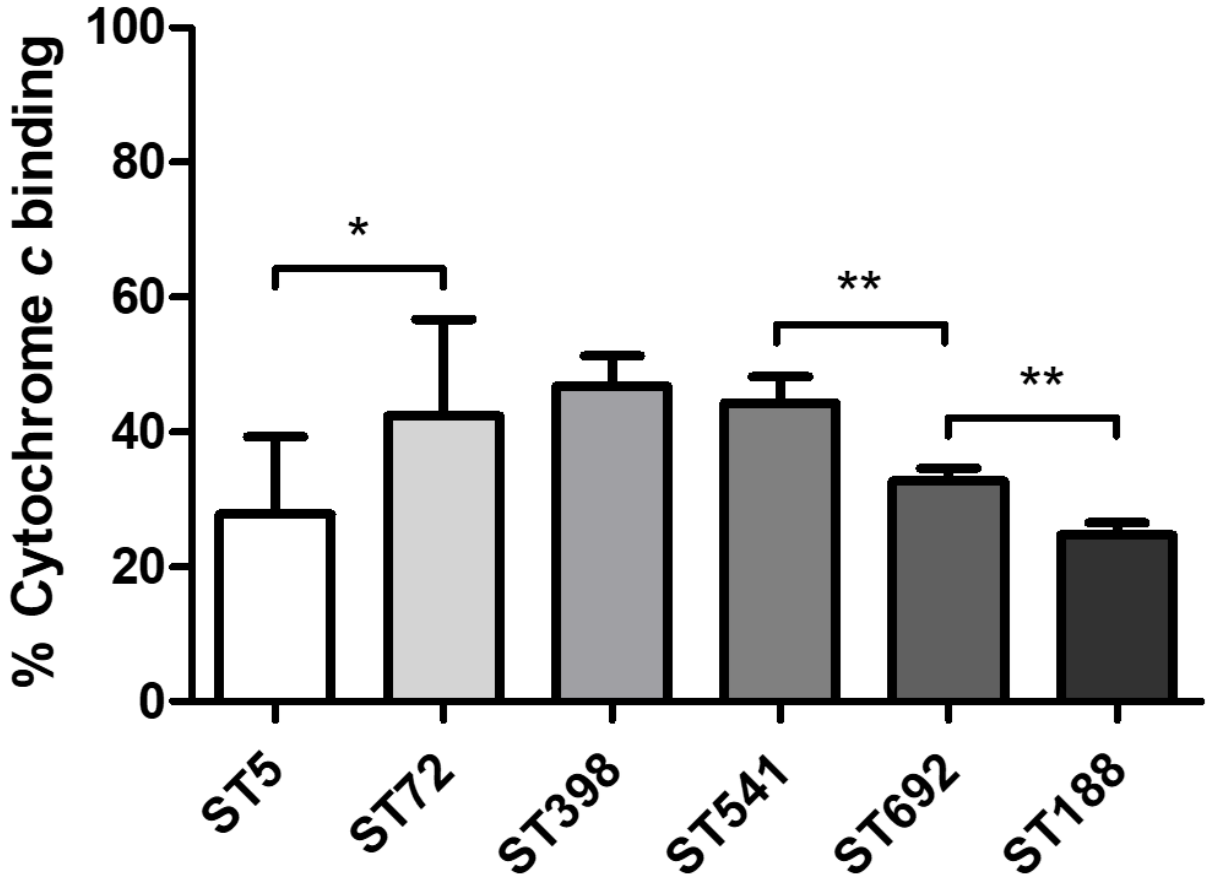


Figure 1. Susceptibility profiles of the different ST groups of MRSA strains to LL-37 (A), PMAP-36 (B), and CATH-2 (C). MRSA strains were exposed to LL-37 (10 μg/mL), PMAP-36 (1.0 μg/mL), and CATH-2 (0.5 μg/mL) and viability of the bacterial cells were determined. Data represent the means ± standard deviation of three independent experiments. Different letters indicate significant differences between the groups.



402

403 **Figure 2.** Cytochrome *c* binding assays to measure comparative surface positive charges of MRSA strains. These

404 data represent means ± standard deviation of three independent experiments. *$p < 0.05$; **$p < 0.01$

405

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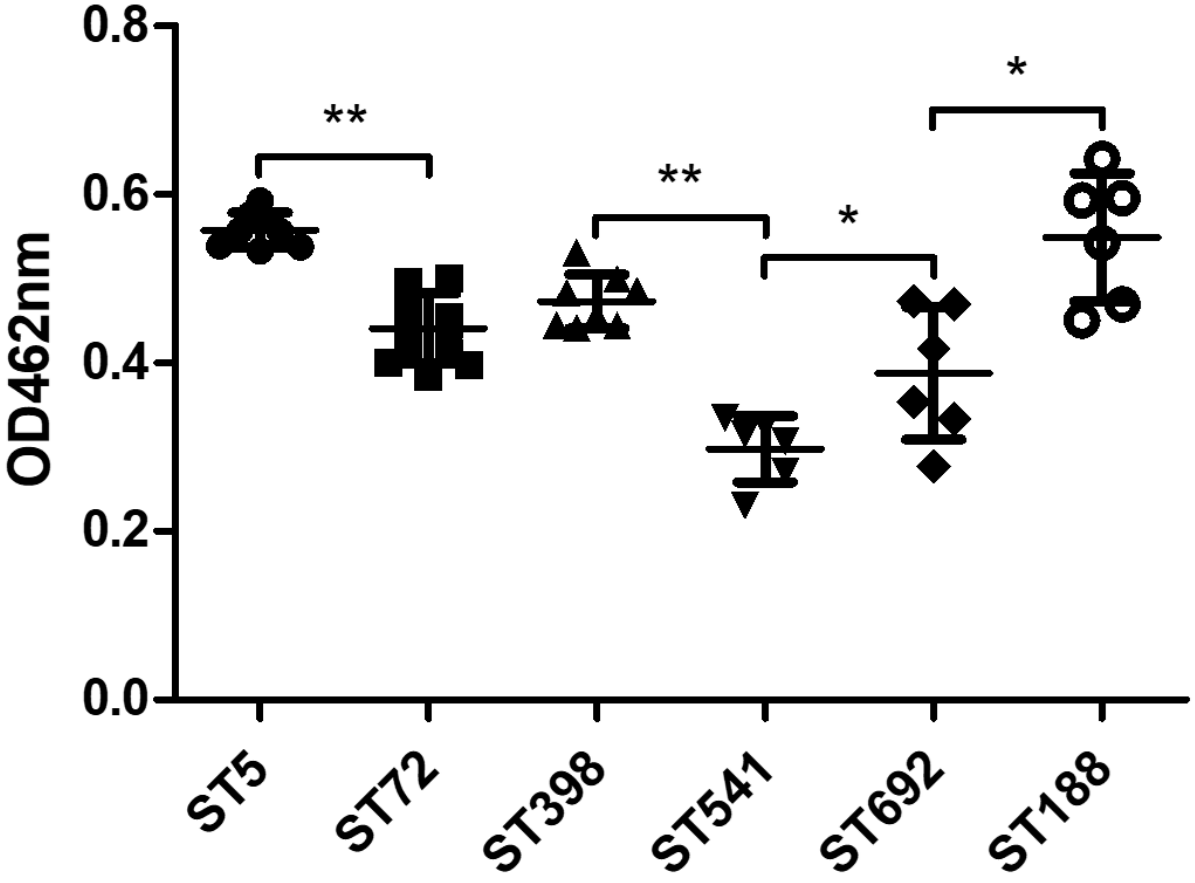


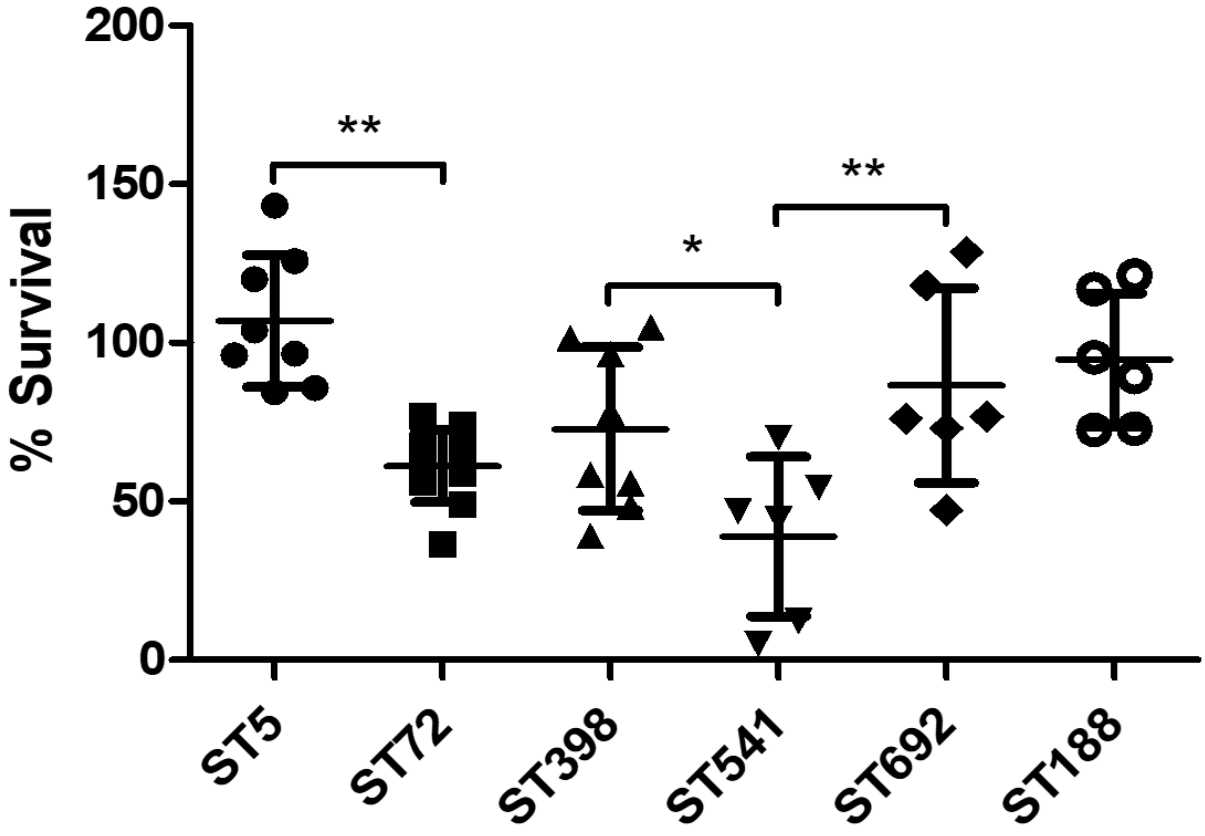
407

408 **Figure 3.** Measurement of staphyloxanthin production in different ST groups of MRSA strains. Bars represent the

409 means ± standard deviation of three independent experiments. *$p < 0.05$; **$p < 0.01$

410

411



412

413 **Figure 4.** Survival of different ST groups of MRSA strains against hydrogen peroxide. MRSA cells (~2.0×10^9
414 CFUs) were incubated with H_2O_2 (1.5% final concentration) at 37 ℃ for 2 h and surviving bacterial cells were
415 enumerated on TSA plates. Bars represent the mean % of surviving CFU ± SDs of H_2O_2-treated versus untreated
416 cells. *$p < 0.05$; **$p < 0.01$

417

418

Table 1. Genotype and antimicrobial resistance profiles of MRSA strains used in this study.

MLST	Strain ID	*spa*	SCC*mec*	*agr*	Antimicrobial Resistance Profiles	OX MIC (μg/mL)
ST5	HA1	t002	II	II	AMP-CIP-CLI-ERY-FOX-PEN	512
(n = 8)	HA2	t601	II	II	AMP-CIP-CLI-ERY-FOX-PEN	512
	HA3	t2460	II	II	AMP-CIP-CLI-ERY-FOX-FUS-GEN-PEN-RIF	512
	HA4	t2460	II	II	AMP-CIP-CLI-ERY-FOX-FUS-GEN-PEN	512
	HA5	t002	II	II	AMP-CIP-CLI-ERY-FOX-FUS-GEN-PEN	512
	HA6	t601	II	II	AMP-CIP-CLI-ERY-FOX-PEN	512
	HA7	t2460	II	II	AMP-CIP-CLI-ERY-FOX-FUS-PEN-RIF	512
	HA8	t601	II	II	AMP-CIP-CLI-ERY-FOX-PEN	512
ST72	CA1	t324	IV	I	AMP-ERY-FOX-PEN	32
(n = 11)	CA2	t13921	IV	I	AMP-FOX-PEN	64
	CA3	t664	IV	I	AMP-FOX-PEN	64
	CA4	t2461	IV	I	AMP-FOX-PEN	32
	CA5	t324	IV	I	AMP-ERY-FOX-PEN	64
	CA6	t148	IV	I	AMP-FOX-PEN	32
	CA7	t324	IV	I	AMP-FOX-PEN	32
	CA8	t324	IV	I	AMP-FOX-PEN	32
	CA9	t324	IV	I	AMP-ERY-FOX-PEN-RIF	64
	CA10	t664	IV	I	AMP-FOX-PEN	32
	CA11	t664	IV	I	AMP-FOX-PEN	32
ST398	Pig1	t571	V	I	AMP-CHL-CIP-FOX-FUS-PEN-TET	24
(n = 8)	Pig2	t18103	V	I	AMP-CHL-CIP-CLI-ERY-FOX-GEN-PEN-SYN-TET	4
	Pig3	t18103	V	I	AMP-CHL-CIP-CLI-ERY-FOX-GEN-PEN-SYN-TET	4
	Pig4	t18103	V	I	AMP-CHL-CIP-CLI-ERY-FOX-GEN-PEN-SYN-TET	8
	Pig5	t18102	V	I	AMP-CHL-CIP-FOX-PEN-TET	24
	Pig6	t18102	V	I	AMP-CHL-CIP-FOX-PEN-TET	16
	Pig7	t18102	V	I	AMP-CHL-CIP-FOX-PEN-TET	16
	Pig8	t18102	V	I	AMP-CHL-CIP-FOX-PEN-TET	16
ST541	Pig9	t034	V	I	AMP-CLI-ERY-FOX-PEN-TET	32
(n = 6)	Pig10	t034	V	I	AMP-CLI-ERY-FOX-PEN-TET	12
	Pig11	t034	V	I	AMP-CLI-ERY-FOX-PEN-TET	16
	Pig12	t034	V	I	AMP-CLI-ERY-FOX-PEN-TET	16
	Pig13	t034	V	I	AMP-CLI-ERY-FOX-PEN-TET	16
	Pig14	t034	V	I	AMP-CLI-ERY-FOX-PEN-TET	16

ST692	Chicken1	t2247	V	I	AMP-CIP-CLI-ERY-FOX-PEN-TET	128
(n = 6)	Chicken2	t2247	V	I	AMP-CIP-CLI-ERY-FOX-FUS-PEN-TET	256
	Chicken3	t2247	V	I	AMP-CIP-CLI-ERY-FOX-PEN-TET	256
	Chicken4	t2247	V	I	AMP-CIP-CLI-ERY-FOX-PEN-TET	128
	Chicken5	t2247	V	I	AMP-CIP-CLI-ERY-FOX-PEN-TET	64
	Chicken6	t2247	V	I	AMP-CIP-CLI-ERY-FOX-PEN-TET	32
ST188	Chicken7	t189	IV	I	AMP-CIP-CLI-ERY-FOX-GEN-PEN-SXT	128
(n = 6)	Chicken8	t189	IV	I	AMP-CIP-CLI-ERY-FOX-GEN-PEN-SXT	128
	Chicken9	t189	IV	I	AMP-CIP-CLI-ERY-FOX-GEN-PEN-SXT	128
	Chicken10	t189	IV	I	AMP-CIP-CLI-ERY-FOX-GEN-PEN-SXT	128
	Chicken11	t189	IV	I	AMP-CIP-CLI-ERY-FOX-GEN-PEN-SXT	128
	Chicken12	t189	IV	I	AMP-CIP-CLI-ERY-FOX-GEN-PEN-SXT	128

ST, sequence type; SCC*mec*, staphylococcal cassette chromosome *mec*; AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; CLI, clindamycin; ERY, erythromycin; FOX, cefoxitin; GEN, gentamicin; MUP, mupirocin; RIF, rifampicin; SXT, sulfamethoxazole-trimethoprim; SYN, quinupristin-dalfopristin; TET, tetracycline; OX, oxacillin.

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