pISSN 1229-1153 / eISSN 2465-9223 *J. Food Hyg. Saf.*
Vol. 35, No. 4, pp. 391~397 (2020)
https://doi.org/10.13103/JFHS.2020.35.4.391

Journal of Food Hygiene and Safety
Available online at http://www.foodhygiene.or.kr

Biofilm Formation, Antimicrobial Peptide Resistance, and Hydrogen Peroxide Resistance in Livestock-Associated *Staphylococcus aureus* Isolates

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(Received May 14, 2020/Revised June 9, 2020/Accepted July 9, 2020)

ABSTRACT - Human infections with livestock-associated methicillin-resistant/-susceptible *Staphylococcus aureus* (LA-MRSA/LA-MSSA) have recently been increasing significantly. These LA-MRSA and LA-MSSA strains can be transmitted to individuals who have frequent contact with livestock animals and foods of animal origin. In this study, major virulence potentials of *S. aureus* such as biofilm formation, antimicrobial peptide resistance, and *in vitro* hydrogen peroxide (H_2O_2) resistance were assessed using 20 MRSA and MSSA strains isolated from raw milk, beef cattle, and workers in the livestock industry. Static biofilm formation assays revealed that there is no difference in levels of biofilm production between MRSA versus MSSA or bovine- versus human-associated strains. *In vitro* BMAP (bovine myeloid antimicrobial peptide)-28 susceptibility assays also revealed no difference in the resistance to the antimicrobial peptide between MRSA versus MSSA or bovine- versus human-associated *S. aureus* strains. However, LA-MRSA strains displayed increased resistance to H_2O_2, which may play an important role in survival and dissemination of the pathogen in livestock. These results provide an important basis for understanding pathogenic potentials of LA-MRSA and LA-MSSA strains in human and animal hosts.

Key words : *Staphylococcus aureus*, Biofilm, Antimicrobial peptide resistance, Hydrogen peroxide resistance

Staphylococcus aureus is a serious nosocomial pathogen, and finding an effective treatment has been becoming more challenging due to the development of antimicrobial resistance such as methicillin-resistant *S. aureus* (MRSA)[1]. Although less frequent than hospital-associated MRSA (HA-MRSA), community-associated MRSA (CA-MRSA) strains have also become increasingly resistant to multiple antimicrobial agents[2,3].

In addition to human host, infections with MRSA or methicillin-susceptible *S. aureus* (MSSA) have been reported in companion animals and domesticated livestock[4-6]. Recently, these livestock-associated MRSA (LA-MRSA) and MSSA (LA-MSSA) strains have emerged in food animals raised in concentrated animal feeding environments[4,5]. LA-MRSA and LA-MSSA strains can be transmitted to individuals who have frequent contact with livestock animals such as farmers, slaughterhouse workers, and retail market workers[4,5,7,8]. Since the colonization of food animals and dissemination of LA-MRSA and LA-MSSA into community through foods of animal origin are an important public health concern, at least several investigations were carried out to monitor the prevalence of *S. aureus* in chicken[9], beef[10], pork[11], and raw milk samples[12]. Our laboratory also investigated the prevalence of MRSA and MSSA in the pork and beef production system in Korea including swine/cattle farms, slaughterhouses, and retail markets[4,5,13].

Although some of the recent studies have suggested that LA-MRSA and LA-MSSA strains tend to lose genetic elements involved in human infection such as the human immune evasion cluster genes (*scn*, *chp*, *sak*, and *sep*) and Panton-Valentine leucocidin (PVL) genes (*lukS* and *lukF*)[14], skin and bloodstream infections in humans by the LA-*S. aureus* have been increasing in the USA and European countries[15-17]. Unlike the epidemiological studies with LA-MRSA and LA-MSSA strains, very few studies have examined the relative virulence of food animal-associated *S. aureus* strains compared to the *S. aureus* strains isolated from persons in frequent contact with livestock and foods of animal origin[7,18].

In this study, we examined the pathogenic potential of LA-MRSA and LA-MSSA strains isolated from beef cattle, raw

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milk, farm workers, slaughterhouse workers, and retail market workers. For the virulence assessment of the *S. aureus* strains, static biofilm formation assays, susceptibility assays to bovine myeloid antimicrobial peptide (BMAP-28), and *in vitro* hydrogen peroxide (H_2O_2) survival analyses were performed.

Materials and Methods

Staphylococcal isolates and culture condition

The 20 *S. aureus* strains (11 MRSA and 9 MSSA strains) used in this study were isolated from beef cattle, raw milk, or workers in the beef production chain in Korea (Table 1). The 7 ST72 MRSA strains were from bovine mastitic milk samples collected in Gyeonggi province in Korea[12]. The 3 ST5 MRSA strains and all of the MSSA strains were from the beef production system in Korea[13].

All staphylococcal isolates were cultured in brain heart infusion broth (BHI) (Difco Laboratories, Detroit, MI, USA) or tryptic soy broth (TSB) (Difco) depending on each experiment. Broth cultures were grown in Erlenmeyer flasks at 37°C with shaking at 225 rpm in a media volume that was less than 15% of the flask volume for maximum aeration.

Table 1. MRSA and MSSA strains used in this study

Strain[1)]	Origin	ST-type[2)]	SCC*mec*[3)]	Ref.
MRSA				
BGFA-222E	Beef cattle	ST5	II	(13)
BGFA-262E	Beef cattle	ST5	II	(13)
BGFA-292E	Beef cattle	ST5	II	(13)
BKFH-321E	Farm worker	ST72	IV_a	(13)
LA1	Raw milk	ST72	IV_a	(12)
LA2	Raw milk	ST72	IV_a	(12)
LA3	Raw milk	ST72	IV_a	(12)
LA4	Raw milk	ST72	IV_a	(12)
LA5	Raw milk	ST72	IV_a	(12)
LA6	Raw milk	ST72	IV_a	(12)
LA7	Raw milk	ST72	IV_a	(12)
MSSA				
BJFA-222	Beef cattle	N.T	-	(13)
BSFA-4104	Beef cattle	ST1	-	(13)
BKFA-211	Beef cattle	ST2416	-	(13)
BKFH-451	Farm worker	N.T	-	(13)
BSSH-171	Slaughterhouse worker	ST2199	-	(13)
BJMH-426	Retail market worker	ST72		(13)
BJMH-111	Retail market worker	ST7	-	(13)
BSMH-611	Retail market worker	ST1	-	(13)
BSMH-616	Retail market worker	ST188	-	(13)

[1)]MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-susceptible *S. aureus*.
[2)]ST, sequence type; N.T, non-typeable;.
[3)]SCC*mec*, staphylococcal cassette chromosome *mec*.

Biofilm formation under static conditions

In vitro static biofilm formation assays were carried out on all the staphylococcal strains as described before[19]. Briefly, *S. aureus* cells from fresh overnight cultures were adjusted to OD_{600nm} of 0.1, then diluted 1:100 with fresh BHI supplemented with glucose (30 mM). Next, 200 μL of the diluted staphylococcal culture was aliquoted to 96-well round bottom plates (SPL Life Sciences, Pocheon, Korea). After 48h incubation at 37°C, the 96-well plates were washed three times with phosphate-buffered saline (PBS, pH 7.4), and then air dried at room temperature. The dried 96-well plates were then stained with safranin for 5 min, 30% acetic acid (Sigma, St. Louis, MO, USA) was added and absorbance was measured at OD_{492nm}. A minimum of three independent experiments were carried out for each *S. aureus* strain.

In vitro antimicrobial peptide susceptibility assays

The antimicrobial peptide, BMAP-28 was synthesized at GL Biochem (GL Biochem, Shanghi, China) with a purity > 96%[20]. BMAP-28 belongs to the cathelicidin-derived antimicrobial peptides and has strong bactericidal activity[20,21].

Since standard minimum inhibition concentration (MIC) assays in Mueller-Hinton broth (MHB) may underestimate bactericidal activities of BMAP-28, *in vitro* susceptibility assays were performed as previously described using the 2-h microdilution method in RPMI-1640 medium supplemented with 5% Luria-Bertani (LB) broth (Difco)[22]. The *in vitro* antimicrobial peptide susceptibility assays were performed with 0.2 μg/mL of BMAP-28 using an initial staphylococcal inoculum of ~5×10^3 CFUs. This BMAP-28 concentration was selected based on extensive preliminary experiments using multiple *S. aureus* strains. The data were expressed as the relative % of surviving CFUs (± standard deviation) of BMAP-28 exposed versus unexposed cells. At least three independent experiments were performed on separate days.

Susceptibilities to hydrogen peroxide (H_2O_2)

In vitro susceptibilities to hydrogen peroxide were performed in PBS as described previously[23]. Briefly, ~2×10^9 CFUs of *S. aureus* cells were incubated in the presence of 1.5% of H_2O_2 at 37°C for 2h, and then 1,000 U/mL of catalase (Sigma) was added to stop the activity of residual H_2O_2. To enumerate surviving CFUs, 10-fold dilutions were plated on tryptic soy agar (TSA) and incubated at 37°C for 24h. The data are expressed as the mean % survival of (±

SDs) H_2O_2-treated versus H_2O_2-untreated controls. At least three independent assays were carried out on separate days.

Statistics

The data were analyzed for statistical significance using Mann-Whitney *U* test (GraphPad Software Inc., San Diego, CA, USA). Significance was determined at a *P*-value of < 0.05.

Results and Discussion

Recent emergence of MRSA and MSSA in food-producing animals has been raising considerable concern because this livestock-associated *S. aureus* would disseminate to humans through the food production system[4,5]. In fact, there has been an increase in the number of cases where humans are colonized or infected with LA-MRSA and LA-MSSA worldwide[16,24,25]. Although livestock-associated *S. aureus* strains have been known to lack genetic components necessary for human infection[14], several recent studies indicated that LA-MRSA and LA-MSSA strains isolated from swine or poultry farms have increased virulence or lethality in mice infection models compared to clinical MSSA strains isolated from human hosts[26,27]. More recently, Randad *et al*. suggested that clonal complex (CC) 398 LA-MRSA strains have enhanced pathogenicity and *in vivo* bacterial burden compared to CA-MRSA strains in murine skin and soft tissue infection model[28]. However, the virulence mechanisms and host immune responses associated with the increased pathogenicity in LA-MRSA and LA-MSSA still remain unknown.

Biofilm formation on host tissues, organs, and indwelling medical devices represents a significant host immune evasion mechanism for development of chronic infections[29]. *S. aureus* cells within biofilms are usually more tolerant to various antimicrobial agents and bactericidal action of host's immune systems[30-32]. In addition, biofilms can trigger resistance to environmental stresses including disinfection, cleaning, and sanitization which results in persistent contamination of processing equipment in the food industry[33]. As shown in Fig. 1A, biofilm formation assays under static condition revealed that there is no difference in the biofilm formation between MRSA and MSSA strain groups. In line with previous studies[13,34,35], this result suggests that *S. aureus* strains can develop biofilms independent of resistance to methicillin. However, when the *S. aureus* strains were grouped as bovine- and human-associated *S. aureus* strains, human-associated *S. aureus* strains displayed significantly increased levels of biofilm formation compared to the bovine-associated *S. aureus* strains ($P<0.01$) (Fig. 1B). Although the mechanisms underlying the increased biofilm formation in *S. aureus* strains from human host are unclear, these results, in combination with the previously published data[13], indicate that *S. aureus* strains need to modify various virulence determinants such as biofilm production to readily adapt to different niches in diverse hosts.

Antimicrobial peptides such as cathelicidins protect human and animal skin and mucosal epithelia against bacterial infections[36]. A bovine originated cathelicidin, BMAP-28, also induces cell membrane depolarization and death in *S. aureus*[20,21]. However, *S. aureus* has developed the means to resist host antimicrobial peptides such as defensins and cathelicidins[37-39]. Thus, the ability to overcome the killing effect of antimicrobial peptides is an important virulence factor for MRSA and MSSA strains. In contrast to the previously published data[13], no significant difference was found in resistance to BMAP-28 (0.2 μg/mL)

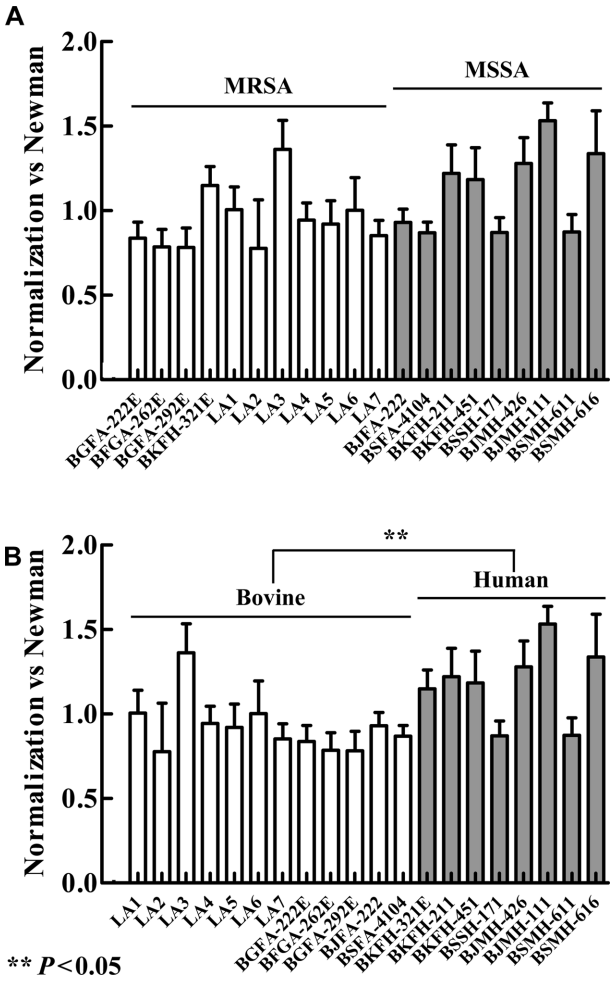

Fig. 1. Comparison of biofilm formation between MRSA and MSSA (A) or bovine- and human-originated *S. aureus* strains (B). Relative biofilm formation of each strain was quantified relative to the *Staphylococcus aureus* Newman strain. $^{**}P<0.05$.

between MRSA and MSSA strains (Fig. 2A, P=0.317), indicating that cathelicidin family peptides of different origin (i.e. PMAP-36, LL-37, and BMAP-28 from porcine, human, and bovine origins, respectively) have distinctive mechanisms for their bactericidal activities[36]. When the *S. aureus* strains were compared as bovine- and human-originated strain groups, no significant difference was observed, either (Fig. 2B, P=0.669). Collectively, in combination with the previous results[13], these data suggest that antimicrobial peptides of distinct structure exert differential actions against *S. aureus* strains that have diverse survival strategies under antimicrobial peptide exposure.

In humans and livestock animals, neutrophils are the major phagocytes and provide vital defense against staphylococcal infections[40]. Phagocytosis of *S. aureus* may lead to the formation of potent antibacterial reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and hydroxyl radicals[41]. Thus, the ability to overcome the oxidative stress by ROS produced by host neutrophil is an important virulence factor for *S. aureus*[41]. Hydrogen peroxide killing assays revealed that MRSA strains has significantly higher level of resistance to 1.5% of H_2O_2 compared to MSSA strains (P<0.05, Fig. 3A). In particular, MRSA strains isolated from raw milk samples exhibited highest level of H_2O_2 resistance among the *S. aureus* strains.

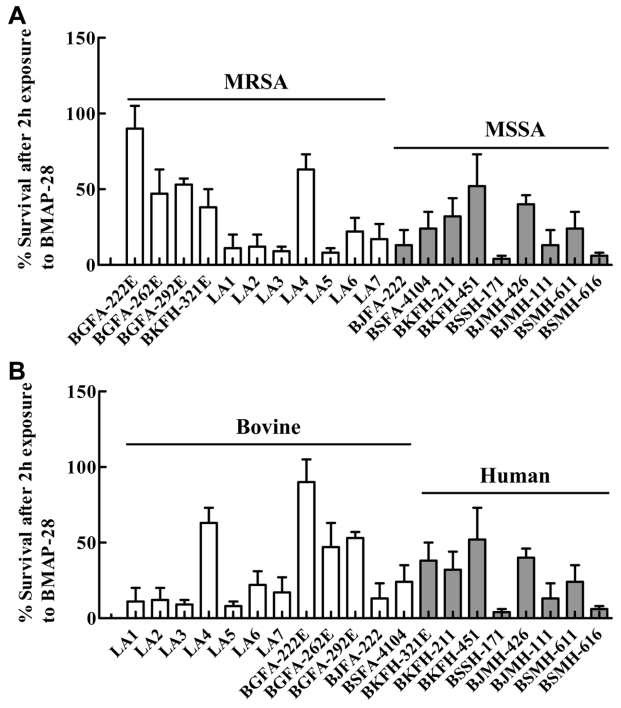

Fig. 2. Comparison of *in vitro* susceptibilities of *S. aureus* strains against BMAP-28 (0.2 μg/mL) between MRSA and MSSA (A) or bovine- and human-originated *S. aureus* strains (B). The data are presented as means (± SDs) of at least 3 runs in triplicate samples.

Contamination of raw milk by *S. aureus* can be resulted from direct excretion from the udders of cows with staphylococcal mastitis or from the handling and processing of raw milk[13,42]. Since raw milk can serve as a vehicle for the transmission of MRSA and MSSA, additional precautions are necessary to stop the contamination of milk production chain. Previous studies reported that *S. aureus* exerts several defensive mechanisms to combat ROS by using catalase, superoxide dismutase, and staphyloxanthin[23,41]. Therefore, future studies should examine these defensive molecules in MRSA and MSSA strains in association with the H_2O_2 resistance phenotype.

In conclusion, our data suggest that surveillance and monitoring of MRSA and MSSA in livestock, food of animal origin, and individuals of livestock industry. Although the LA-MRSA or LA-MSSA strains did not show enhanced biofilm formation compared to the *S. aureus* strains from humans, LA-MRSA strains exhibited increased resistance to hydrogen peroxide. The increased resistance to hydrogen peroxide may play an important role in the survival and colonization of MRSA in human and animal hosts, and thus dissemination of the pathogens. In addition, the hydrogen peroxide resistance in MRSA can affect the

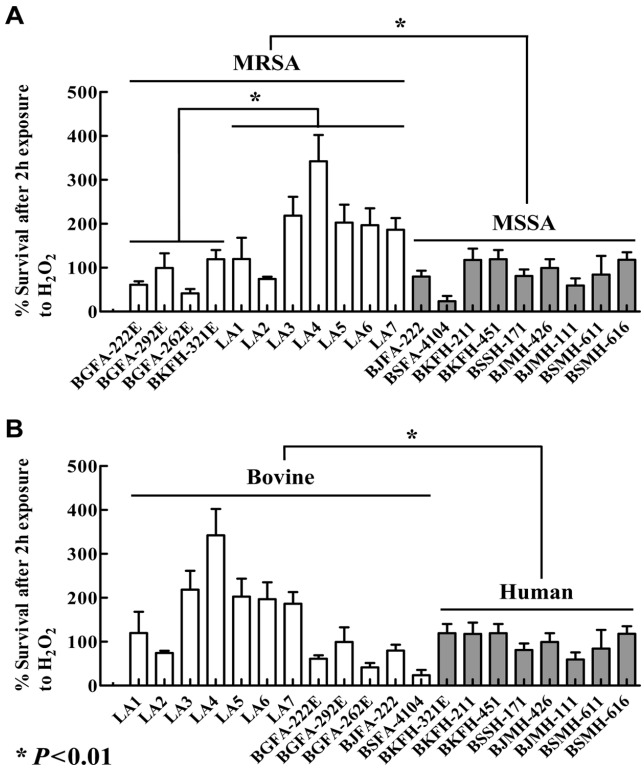

Fig. 3. Comparison of hydrogen peroxide (H_2O_2) resistance between MRSA and MSSA (A) or bovine- and human-originated *S. aureus* strains (B). The data are expressed as the mean % survival of (± SDs) H_2O_2-treated versus H_2O_2-untreated controls. $^*P$<0.01.

organism's ability to persist in the environment by enhancing survival under hydrogen peroxide disinfectant. Lastly, future studies are necessary to elucidate molecular mechanisms associated with the enhanced biofilm formation in *S. aureus* strains from human hosts and the hydrogen peroxide resistance in LA-MRSA strains.

Acknowledgement

This work was supported by a grant from the Basic Science Research Program, National Research Foundation of Korea (NRF-2019R1F1A1058397 to SJY).

국문요약

최근 가축에서 유래된 메티실린에 내성이 있는 황색포도상구균과 감수성을 보이는 황색포도상구균(LA-MRSA/LA-MSSA)에 의한 사람의 감염증이 증가하는 추세이다. 이러한 LA-MRSA 및 LA-MSSA균주는 가축을 비롯한 축산업에 종사하는 사람들에게 전파가 이루어질 수 있다. 본 연구에서는 원유, 육우, 축산 종사자에서 분리된 20개의 MRSA 및 MSSA 균주를 이용하여 생물막 형성, 항균 펩타이드에 대한 저항성 및 과산화수소 저항성과 같은 황색포도상구균의 주요 병원성 인자를 평가하였다. 생물막 형성 실험에서는 MRSA와 MSSA간의 차이는 없었으며, 동물 유래 분리주와 사람 유래 분리주들 간의 비교에서도 차이가 없음이 확인되었다. BMAP-28에 대한 감수성 시험 결과 MRSA-MSSA 또는 동물 분리-사람 분리 간의 차이가 없음을 확인하였다. 생물막 형성과 BMAP-28 감수성과는 달리, 원유에서 분리된 MRSA 균주들의 H_2O_2에 대한 내성 증가가 확인 되었다. 본 연구를 통하여 가축 및 축산업 종사자에서 분리된 LA-MRSA와 LA-MSSA 균주의 주요 병원성 인자를 확인하였으며, 숙주 및 환경에서의 생존과 전파 가능성을 이해하는데 기초 자료로 활용 될 수 있을 것이다.

Conflict of interests

The authors declare no potential conflict of interest.

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