Korean J. Food Sci. An. Vol. 35, No. 4, pp. 502~506 (2015)
© 2015 Korean Society for Food Science of Animal Recources
DOI http://dx.doi.org/10.5851/kosfa.2015.35.4.502
ISSN 1225-8563 eISSN 2234-246X

ARTICLE

Virulence Factors of *Staphylococcus aureus* Isolated from Korean Pork *bulgogi*: Enterotoxin Production and Antimicrobial Resistance

Byeong Su Jung[1], Yong Ju Lee[1], Na-Kyoung Lee[1], Hyoun Wook Kim[2], Mi-Hwa Oh[2], and Hyun-Dong Paik[1,3,*]

[1]*Department of Food Science and Biotechnology of Animal Resources, Konkuk University, Seoul 143-701, Korea*
[2]*Animal Products and Development division, National Institute of Animal Science, RDA, Suwon 441-706, Korea*
[3]*Bio/Molecular Informatics Center, Konkuk University, Seoul 143-701, Korea*

Abstract

The aim of this study was to investigate the antimicrobial resistance profiles of and the enterotoxin gene distribution in 4 strains of *Staphylococcus aureus* (S10-2, S10-3, S12-2, and S13-2) isolated from 90 *bulgogi* samples. The *S. aureus* enterotoxin H gene (*seh*) was found in all the strains, while the *S. aureus* enterotoxin A gene (*sea*) was found only in 3 of the 4 strains. The S10-2 strain expressed a combination of enterotoxin genes - *seg*, *seh*, *sei*, *sej*, *selm*, and *seln*. The strains S10-2 and S13-2 were resistant to ampicillin and penicillin G, and all the isolated strains were resistant to tetracycline. The S10-2 strain was the only *mec*A-positive strain; it was also resistant to β-lactam antibiotics. Thus, genes encoding enterotoxin as well as those conferring antibiotic resistance were identified in the *S. aureus* strains isolated from pork *bulgogi*. These results represents the potential occurrence of MRSA in pork *bulgogi*, and the need for a monitoring system for pork *bulgogi* in order to prevent an outbreak of staphylococcal food poisoning.

Keywords: foodborne pathogen, *Staphylococcus aureus*, enterotoxin, antibiotic susceptibility

Received June 15, 2015; Revised July 20, 2015; Accepted July 20, 2015

Introduction

Staphylococcus aureus is one of the most common causes of food borne diseases and is found in a variety of foods (Andreja, 2012). *S. aureus* is a public health concern because of its ability to produce enterotoxins and to survive in harsh conditions. Staphylococcal enterotoxins (SEs) are a leading cause of staphylococcal food poisoning in humans; they may also be involved in other types of infections. The symptoms of SE poisoning are increased saliva secretion, vomiting, abdominal cramping, and diarrhea, with blood in some cases. Staphylococcal enterotoxins A (SEA) through to staphylococcal enterotoxin E (SEE) are the most commonly isolated enterotoxins in food poisoning outbreaks (Argudín *et al*., 2010). However, a number of other enterotoxin types designated SEG-SEJ, SE*l*M, SE*l*N, and SE*l*O has been defined (Argudín *et al*., 2010; Yarwood *et al*., 2002).

The extensive therapeutic use of antibiotics in humans and animals has contributed to the increase in antibiotic resistance in pathogens. Especially problematic among these resistant pathogens are methicillin-resistant *S. aureus* (MRSA), which have become a serious concern due to the high rates of community and nosocomial-acquired infections. In addition, the phenomenon of horizontal gene transfer has resulted in the spread of methicillin resistance, increasing the pressure on a limited supply of alternative antibiotics. Antibiotic resistance in MRSA is determined by the *mec*A and *fem*A genes (Keun *et al*., 2011). The *mec*A gene encodes a penicillin binding protein (PBP-2a), which has a low binding affinity for methicillin and other β-lactam antibiotics.

Pork *bulgogi* is barbecued or pan-fired pork that has been marinated with a mixture of soy sauce, sugar, minced garlic, green onion, black pepper, and sesame oil. Its microbial safety was reported their bacterial condition (Ahn *et al*., 2012; Hong *et al*., 2011; Jo *et al*., 2004). Due to the high salt content of soy sauce, *bulgogi* does not provide an environment suitable for microbial growth (Jo *et al*, 2004). As a result, just 5 strains were isolated from 90 pork *bulgogi* samples (Ahn *et al*., 2012). The low num-

*Corresponding author: Hyun-Dong Paik, Department of Food Science and Biotechnology of Animal Resources, Konkuk University, Seoul 143-701, Korea. Tel: +82-2-2049-6011, Fax: +82-2-455-3082, E-mail: hdpaik@konkuk.ac.kr

bers of *S. aureus* isolates may be due to soy sauce, which has been shown to exert antimicrobial effects against *S. aureus*, *Shigella flexneri*, *Vibrio cholera*, *Salmonella* Enteritidis, and *Escherichia coli* (Kataoka, 2005). The aim of this study was to investigate the antimicrobial resistance profiles of and the enterotoxin gene distribution in *S. aureus* strains isolated from pork *bulgogi*.

Materials and Methods

Bacterial strains

The four *S. aureus* strains S10-2, S10-3, S12-2, and S13-2 were isolated from raw pork *bulgogi* using Baird-Parker agar plates enriched with egg yolk (EY) tellurite (Ahn *et al*., 2012). Five different *S. aureus* strains KCCM 11593, ATCC 25923, KCCM 40510, KCCM 40511, and KCCM 40512 were used as standards. Frozen stocks were maintained at -80°C in tryptic soy broth (TSB, Difco Laboratories, USA) containing 20% glycerol. The *S. aureus* strains were incubated at 37°C for 24 h in TSB, and used for further study.

S. aureus DNA extraction

Following overnight growth, the *S. aureus* strains were harvested by centrifugation at 8,900 × *g* for 15 min. The pelleted cells were used for genomic DNA extraction using the AccuPrep® genomic DNA extraction kit (Bioneer Co., Korea), together with lysozyme treatment (Sigma Chemical Co., USA), according to the manufacturer's instructions.

PCR detection of Staphylococcal enterotoxin genes

PCR was used to test for the presence of 23S rRNA, *nuc*, and staphylococcal enterotoxin (SE) genes defined (Argudín *et al*., 2010; Yarwood *et al*., 2002). The PCR reaction was performed in a 20 μL reaction volume containing 10 pmol of each primers (Table 1), 25 μL of 2×

Table 1. Used oligonucleotide primers and amplification conditions

Genes	Primer	Oligonucleotide sequences	Product size (bp)	Annealing temp. (°C)
23S rRNA	STAUR2	ACGGAGTTACAAAGGACGAC	1,250	64
	STAUR4	AGCTCAGCCTTAACGAGTAC		
nuc	*nuc*-1	GCGATTGATGGTGATACGGTT	279	55
	nuc-2	AGCCAAGCCTTGACGAACTAAAGC		
sea	SEA-1	AAAGTCCCGATCAATTTATGGCTA	219	55
	SEA-2	GTAATTAACCGAAGGTTCTGTAGA		
seb	SEB-1	TCGCATCAAACTGACAAACG	476	55
	SEB-2	GCAGGTACTCTATAAGTGCC		
sec	SEC-1	GACATAAAAGCTAGGAATTT	257	55
	SEC-2	AAATCGGATTAACATTATCC		
sed	SED-1	CTAGTTTGGTAATATCTCCT	317	55
	SED-2	TAATGCTATATCTTATAGGG		
see	SEE-1	TAGATAAAGTTAAAACAAGC	169	55
	SEE-2	TAACTTACCGTGGACCCTTC		
seg	SEG-1	AATTATGTGAATGCTCAACCCGATC	642	55
	SEG-2	AAACTTATATGGAACAAAAGGTACTAGTTC		
seh	SEH-1	CAATCACATCATATGCGAAAGCAG	376	55
	SEH-2	CATCTACCCAAACATTAGCACC		
sei	SEI-1	CTCAAGGTGATATTGGTGTAGG	577	55
	SEI-2	AAAAAACTTACAGGCAGTCCATCTC		
sej	SEJ-1	CATCAGAACTGTTGTTCCGCTAG	192	55
	SEJ-2	CTGAATTTTACCATCAAAGGTAC		
selm	SEM-1	TCTTAGGAACTATTATGGTAGC	471	55
	SEM-2	CCTGCATTAAATCCAGAA		
seln	SEN-1	GGAGTTACGATACATGATGG	292	55
	SEN-2	ACTCTGCTCCCACTGAAC		
selo	SEO-1	TGATGATTATATAAATAATCGATTTACG	249	55
	SEO-2	ATATGTACAGGCAGTATCC		
*mec*A	mecA-1	TCACCTTGTCCGTAACCTGA	678	57
	mecA-2	TCGTGTCACAATCGTTGACG		
*fem*A	femA-1	CGAGGTCATTGCAGCTTGCT	231	57
	femA-2	CCAGCATTACCTGTAATCTCGCCA		

Go Taq® Green Master Mix (Promega, USA), 2 μL of template DNA. The final volume was adjusted to 50 μL using RNase free sterile water. All amplification steps were carried out in a thermocycler (Bioer, Switzerland) with an initial denaturation step at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 2 min, annealing at 55-64°C for 30 s, and a 30 s extension at 72°C, follow by a final extension at 72°C for 10 min. The amplified PCR products were resolved by electrophoresis in a 1% agarose gel.

Antibiotic susceptibility

Antibiotic susceptibility of the *S. aureus* strains were tested using the disc agar diffusion method on Mueller Hinton agar (Dection Dickinson, France), following the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2007). The antibiotic discs used were as tetracycline (30 μg), streptomycin (10 μg), gentamicin (30 μg), vancomycin (30 μg), amoxicillin (20 μg) and clavulanic acid (10 μg), ampicillin (10 μg), penicillin (10 IU), oxacillin (10 μg), cefazolin (30 μg), and cephalothin (30 μg). The strains were classified as susceptible, intermediate, or resistant according to the supplier's instructions.

Detection of *mec*A and *fem*A genes

The *mec*A and *fem*A genes primers used as a biomarker for resistance. The FastPCR software program (http://primerdigital.com/fastpcr.hml) was used to analyze primers (Table 1) for self-dimerization, cross-dimerization, and optimum annealing temperature. PCR conditions was followed manufacture's instruction, and annealing temperatures are shown in Table 1.

Results and Discussion

Detection of enterotoxin genes of the *S. aureus* strains isolated from pork *bulgogi*

The *S. aureus* strains were isolated from pork *bulgogi* using a selective medium in our previous study and were identified as *S. aureus* by Gram staining, catalase testing, latex agglutination, and the API Staph System (Ahn *et al*., 2012). In this study, the 4 isolates were confirmed as *S. aureus* strains by PCR amplification of the 23S rRNA and *nuc* genes (Table 2).

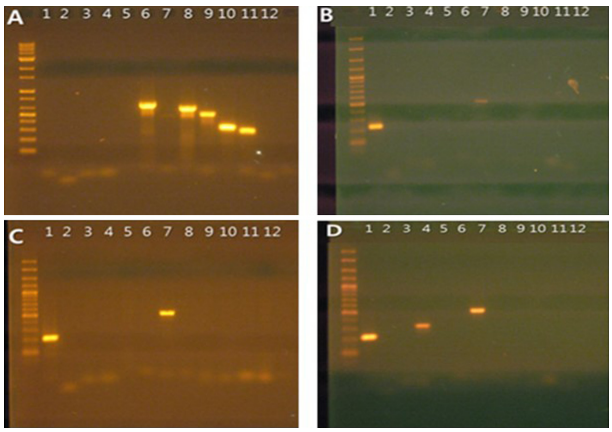

Fig. 1. Agarose gel electrophoresis analysis of PCR products of enterotoxin genes. (A) 10-2, (B) 10-3, (C) 12-2, (D) 13-2. Lane 1, *sea*; lane 2, *seb*; lane 3, *sec*; lane 4, *sed*; lane 5, *see*; lane 6, *seg*; lane 7, *seh*; lane 8, *sei*; lane 9, *sej*; lane 10, *selm*; lane 11, *seln*; lane 12, *selo.*

Staphylococcal enterotoxins (SEs) belong to a large family of staphylococcal pyrogenic exotoxins, which have been implicated in food poisoning, causing toxic shock-like syndromes, and several allergic and autoimmune diseases (Ortega *et al*., 2010). Fig. 1 shows the results for the PCR detection of the enterotoxin genes *sea* to *selo*. The strain S10-2 possessed 6 enterotoxin genes: *seg*, *seh*, *sei*, *sej*, *selm*, and *seln*. Strains S10-3 and S12-2 possessed the *sea* and *seh* enterotoxin genes. The strain S13-2 was shown to possess 3 genes encoding SEA, SED, and SEH. The *seh* gene, which encodes SEH, was detected in all the strains; however, there was no amplification of the enterotoxin genes *seb*, *sec*, *see*, and *selo*. Results of enterotoxin genes had a different as the strains. The enterotoxins SEA, SEB, and SED are the most common enterotoxins associated with human food-poisoning outbreaks (Argudín *et al*., 2010; Zschck *et al*., 2005). In addition, SEG, SEH, and SEI have also been shown to be emetic after oral administration in a primate model (Argudín *et al*., 2010).

Antibiotic susceptibility

S. aureus infections are primarily controlled through

Table 2. Characteristics of isolated *S. aureus* strains from pork *bulgogi*

Isolate	23S rRNA gene	nuc gene	SE genes	*femA* gene	*mecA* gene
S10-2	+	+	*seg, seh, sei, sej, selm, seln*	+	+
S10-3	+	+	*sea, seh*	+	-
S12-2	+	+	*sea, seh*	+	-
S13-2	+	+	*sea, sed, seh*	+	-

Table 3. Antibiotic resistance of control strains and isolated *S. aureus* from pork *bulgogi*

Antibiotic groups	Antibiotic	Strains								
		KCCM 11593	ATCC 25923	KCCM 40510	KCCM 40511	KCCM 40512	S10-2	S10-3	S12-2	S13-2
Tetracyclines	Tetracycline (30 μg)	S[a]	S	R[b]	S	S	R	R	R	R
Aminoglycosides	Streptomycin (10 μg)	S	S	R	S	S	S	S	S	S
	Gentamicin (30 μg)	S	S	S	R	S	S	S	S	S
Glycopeptide	Vancomycin (30 μg)	S	S	S	S	S	S	S	S	S
Amino penicillin	Amoxicillin (20 μg)+ Clavulanic acid (10 μg)	S	S	R	S	S	S	S	S	S
β-Lactam	Ampicillin (10 μg)	S	S	R	R	R	R	S	S	R
	Penicillin (10 IU)	S	S	R	R	R	R	S	S	R
	Oxacillin (10 μg)	S	S	R	R	R	R	S	S	S
Cephalosporins	Cefazoline (30 μg)	S	S	R	S	S	S	S	S	S
	Cephalothin (30 μg)	S	S	R	S	S	S	S	S	S

[a]Sensitive; [b]resistant.

antibiotics. It is therefore concerning how rapidly this organism develops resistance to a large number of antibiotics. Here, we compared the antibiotic resistance of the *S. aureus* strains isolated from pork *bulgogi* to that of the standard strains (KCCM 11593 and ATCC 25923) and the MRSA strains (KCCM 40510, KCCM 40511, and KCCM 40512) (Table 3). As expected the standard *S. aureus* strains demonstrated no antibiotic resistance. The KCCM 40510 strain was resistant to tetracycline, streptomycin, amoxicillin and clavulanic acid, ampicillin, penicillin, oxacillin, cefazoline, and cephalothin. The strains KCCM 40511 and KCCM 40512 were resistant to ampicillin, penicillin G, and oxacillin. The *S. aureus* isolates S10-2, S10-3, S12-2, and S13-2 strains were all resistant to tetracycline, whereas the S10-2 and S13-2 strains were resistant to ampicillin and penicillin G. Thus, the resistance profile of the isolated strains was similar to that of the MRSA strains, KCCM 40511 and KCCM 40512. Our findings are also consistent with those of Pereira *et al*. (2009), who reported that ampicillin and penicillin resistance was frequently founded at a ratio of 70 and 73%, respectively.

Detection of *mec*A and *fem*A genes

Table 2 shows results for detection of *mec*A and *fem*A genes in the *S. aureus* strains isolated from pork *bulgogi*. All the tested strains possessed the *fem*A gene. The MRSA strains KCCM 40510 and KCCM 40511 were positive for the *mec*A gene. Although the *mec*A gene was not detected in the *S. aureus* strain KCCM 40512, this strain was resistant to oxacillin. Among the strains isolated from pork *bulgogi*, only the strain S10-2 possessed the *mec*A gene; consequently, this strain was identified as a MRSA. Although the *mec*A gene was not detected in the S13-2 strain, antibiotic susceptibility testing revealed resistance to the β-lactam antibiotics, ampicillin, and penicillin. This may be due to a modified *mec*A gene, which was not detected by the primers used in this study. Antibiotic resistance can also be plasmid encoded rather than chromosomal (Lee and Lim, 2002). The MRSA strains isolated from pork *bulgogi* were resistant to a higher number of antibiotics than the methicillin-sensitive strains were. This is consistent with the finding that MRSA strains generally express genes that confer resistance to multiple antibiotics (Kumar *et al*., 2011).

The S10-2 and S13-2 strains were both resistant to ampicillin and penicillin G. However, the S10-2 stain expressed 6 enterotoxin genes (*seg*, *seh*, *sei*, *sej*, *selm*, and *seln*), while the S13-2 strain possessed only 3 enterotoxin genes (*sea*, *sed*, and *seh*). These results represents the potential occurrence of MRSA in pork *bulgogi*, and the need for a monitoring system for pork *bulgogi* in order to prevent an outbreak of staphylococcal food poisoning.

Acknowledgements

This work was supported by Priority Research Centers Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2009-0093824).

References

1. Ahn, S. H., Lee, Y. J., Lee, J. Y., and Paik, H. D. (2012) Microbiological investigation of ready-to-cook pork *bulgogi* on Korean market. *Korean J. Food Sci. An.* **32**, 441-447.
2. Andreja, R. (2012) Incidence, growth and enterotoxin production of *Staphylococcus aureus* in insufficiently dried traditional beef ham “govedja prsuta” under different storage conditions. *Food Control* **27**, 369-373.

3. Argudín, M. A., Mendoza, M. C., and Rodicio, M. R. (2010) Food poisoning and *Staphylococcus aureus* enterotoxins. *Toxins* **2**, 1751-1773.
4. Clinical Laboratory Standard Institute (CLSI) (2007) Performance Standards for Antimicrobial Susceptibility Testing; Seventeenth Informational Supplement. CLSI document M100-S17. Wayne, PA.
5. Hong, J. H., Yoon, E. K., Chung, S. J., Chung, L. S., Cha, M., O'Mahony, M., Vickers, Z., and Kim, K. O. (2011) Sensory characteristics and cross-cultural consumer acceptability of *bulgogi* (Korean traditional barbecued beef). *J. Food Sci.* **76**, S306-S313.
6. Jo, C., Lee, N. Y., Kang, H. J., Shin, D. H., and Byun, M. W. (2004) Inactivation of foodborne pathogens in marinated beef rib by ionizing radiation. *Food Microbiol.* **21**, 543-548.
7. Kataoka, S. (2005) Functional effects of Japanese style fermented soy sauce (shoyu) and its components. *J. Biosci. Bioeng.* **100**, 227-234.
8. Keun, S. B., Gwang, S. K., Han, N. C., Seong, C. P., Eun, C. K., Hyung, R. K., and Chi, N. S. (2011) Toxins and antibiotic resistance of methicillin-resistant *Staphylococcus aureus* isolated from clinical specimens. *J. Life Sci.* **21**, 257-264.
9. Kumar, R., Yadav, B. R., and Singh, R. S. (2011) Antibiotic resistance and pathogenicity factors in *Staphylococcus aureus* isolated from mastitic Sahiwal cattle. *J. Biosci.* **36**, 175-188.
10. Lee, H. S. and Lim, H. (2002) Evaluation of various methods for detection of methicillin-resistance *Staphylococcus aureus* (MRSA). *Korean J. Clin. Microbiol.* **5**, 105-110.
11. Ortega, E., Abriouel, H., Lucas, R., and Glvez, A. (2010) Multiple roles of *Staphylococcus aureus* enterotoxins: Pathogenic activity, and correlation to antibiotic resistance. *Toxins* **2**, 2177-2131.
12. Pereira, V., Lopes, C., Castro, A., Silva, J., Gibbs, P., and Teixeira, P. (2009) Characterization for enterotoxin production, virulence factors, and antibiotic susceptibility of *Staphylococcus aureus* isolates from various foods in Portugal. *Food Microbiol.* **26**, 278-282.
13. Yarwood, J. M., Mccormick, J. K., Paustian, M. L., Orwin, P. M., Kapur, V., and Schlievert, P. M. (2002) Characterization and expression analysis of *Staphylococcus aureus* pathogenicity island 3. Implications for the evolution of staphylococcal pathogenicity islands. *J. Biol. Chem*. **277**, 13138-13147.
14. Zschck, M., Kloppert, B., Wolter, W., Hamann, H. P., and Lämmler, C. (2005) Pattern of enterotoxin genes *seg*, *seh*, *sei*, and *sej* positive *Staphylococcus aureus* isolated from bovine mastitis. *Vet. Microbiol.* **108**, 243-249.