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spa typing and enterotoxin gene profile of *Staphylococcus aureus* isolated from bovine raw milk in Korea

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***Staphylococcus aureus* is a major etiological pathogen of bovine mastitis, which triggers significant economic losses in dairy herds worldwide. In this study, *S. aureus* strains isolated from the milk of cows suffering from mastitis in Korea were investigated by *spa* typing and staphylococcal enterotoxin (SE) gene profiling. Forty-four *S. aureus* strains were isolated from 26 farms in five provinces. All isolates grouped into five clusters and two singletons based on 14 *spa* types. Cluster 1 and 2 isolates comprised 38.6% and 36.4% of total isolates, respectively, which were distributed in more than four provinces. SE and SE-like toxin genes were detected in 34 (77.3%) isolates and the most frequently detected SE gene profile was *seg, sei, selm, seln*, and *selo* genes (16 isolates, 36.3%), which was comparable to one of the genomic islands, Type I νSaβ. This is a first report of *spa* types and the prevalence of the recently described SE and SE-like toxin genes among *S. aureus* isolates from bovine raw milk in Korea. Two predominant *spa* groups were distributed widely and recently described SE and SE-like toxin genes were detected frequently.**

Keywords: bovine mastitis, enterotoxin, mobile genetic elements, *S. aureus*, *spa* typing

Introduction

Staphylococcus aureus is a major etiological pathogen of bovine mastitis, which triggers significant economic losses in dairy herds worldwide. *S. aureus* produces a variety of virulence factors that are responsible for subclinical and persistent intra-mammary infections [12]. *S. aureus* associated mastitis is one of the most contagious diseases presently plaguing dairy herds [25]. Investigating distributions and the virulent factors of *S. aureus* provides important information for establishing infection control strategies.

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Among the various molecular typing methods for *S. aureus*, *spa* typing that targets the *spa* gene is an effective and rapid method [36]. The *spa* gene possesses a repetitive region called the short sequence repeat (SSR), which consists of a variable number of 21-27-bp nucleotide repeats. A *spa* typing is performed by analyzing the number and sequence of the repeats. Since results of *spa* typing agree with those of pulsed-field gel electrophoresis (PFGE), implicating *spa* typing as a useful alternative genotyping method [39]. Moreover, the clonal relation between isolates can be investigated by clustering related *spa* types using the Based Upon Repeat Patterns (BURP) algorithm [24].

Many mastitis associated *S. aureus* strains produce staphylococcal enterotoxin (SE) or SE-like toxin that are part of the superantigen (SAg) family [4,26]. SAgs manifest their virulence by ligation of the major histocompatibility complex class II molecules and the Vβ chain of the T-cell receptor from the outside. This leads to the stimulation of T-cell proliferation in a nonspecific manner, ultimately causing the host immune system to be suppressed [7]. There are 18 kinds of SE and SE-like toxin subtypes, which are defined based on the amino acid homology [23,29,40-43]. Each toxin subtype is assumed to be related with a host-specific immune reaction [8]. Thus, SE gene profiles, the detected SE and SE-like toxin genes of *S. aureus* isolates, can be used for investigating the role of those toxins in bovine mastitis.

Most of the genes encoding SE and SE-like toxin reside on the mobile genetic elements (MGE) including plasmids, prophages, staphylococcal pathogenic islands (SaPI) and genomic islands (νSa) [3,23,27,33,35]. Horizontal transfer of MGE is an important means for acquisition of virulence factors by *S. aureus* [3,14]. Several studies have predicted the MGE possessed by *S. aureus* isolates based on the SE gene profiles [16,28].

In this study, *S. aureus* strains isolated from the milk of cows suffering mastits in Korea were investigated by *spa* typing and SE gene profiling. From the *spa* typing, genotypes and geographical distributions of *S. aureus* in five provinces

were determined. Moreover, SE gene profiling revealed the prevalence of each subtype of SE or SE-like toxin genes and the associated MGE related with those genes.

Materials and Methods

Raw milk sampling

From 2006 to 2007, a total of 13,955 raw milk samples were collected from 922 dairy herds in Gyeonggi, Gangwon, Chung-cheong, Jeolla, and Gyeong-sang provinces. Samples were aseptically collected from individual quarters of the cows as previously described [26]. Somatic cell counts (SCC) of milk samples, which indirectly show the quantity of dead epithelial cells and neutrophils in the milk, were analyzed using a somatic cell counter (Bentley Somacount 300; Chaska, USA). Milk samples with over 500,000 cells/mL of SCC were selected for bacterial isolation [31].

Bacterial isolation and DNA extraction

S. aureus strains were isolated from the suspected mastitic milk. In brief, 10 μL of milk was inoculated onto 5% sheep blood agar (Promed, Korea), which was incubated at 37°C for 24 h. Colonies showing characteristic phenotype of *S. aureus* were sub-cultured on Baird-Parker agar (Becton Dickinson, USA). *S. aureus* was identified through Gram staining and catalase, oxidase, DNase, TNase and VP tests. Confirmation was provided by PCR targeting the *S. aureus*-specific *nuc* gene [5].

Genomic DNA was isolated using a DNeasy tissue kit (Qiagen GmbH, Germany) according to the manufacturer's instructions with a modification of the cell lysis step performed with 50 U/mL lysostaphin (Sigma-Aldrich, USA). The concentration of the isolated DNA was estimated using Specgene (Techne, UK) at A_{260}, and the DNA was diluted to a concentration of 50 ng/μL.

spa typing

The *spa* typing was preformed as previously described [15]. Briefly, the SSR region of the *spa* gene was amplified using primers 1095F (5′-AGACGATCCTTCGGTGAGC-3′) and 1517R (5′-GCTTTTGCAATGTCATTTACTG-3′). Sequence analyses of extracted PCR products were performed at Bionics (Korea). Sequence data were analyzed using Ridom StaphType software (Ridom GmbH, Germany) which automatically detects the *spa*-repeats. The *spa* repeats and *spa* types were determined and assigned a numeric code using a previously described method [15]. BURP clustering for the *spa* types were performed with Ridom StaphType software (Ridom GmbH, Germany).

Multiplex PCR detection of SE and SE-like toxin genes

Multiplex PCR was performed to detect 18 kinds of SE and SE-like toxin genes (*sea* to *see, seg* to *sej, selk* to *selr,* and *selu*) as previously described [17]. Each PCR reaction was performed with 1 μL of the prepared template DNA, 10 μL of ×5 primer mixture (0.5 μM of each primer), and 25 μL of ×2 Multiplex Master Mix (Seegene, Korea), with the final volume adjusted to 50 μL with distilled water. PCR was performed using the following steps: 94°C for 15 min, 30 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 2 min with a final extension at 72°C for 10 min. The amplified PCR products were resolved by electrophoresis in 2% agarose gel (Sigma-Aldrich, USA) at 100 V for 60 min.

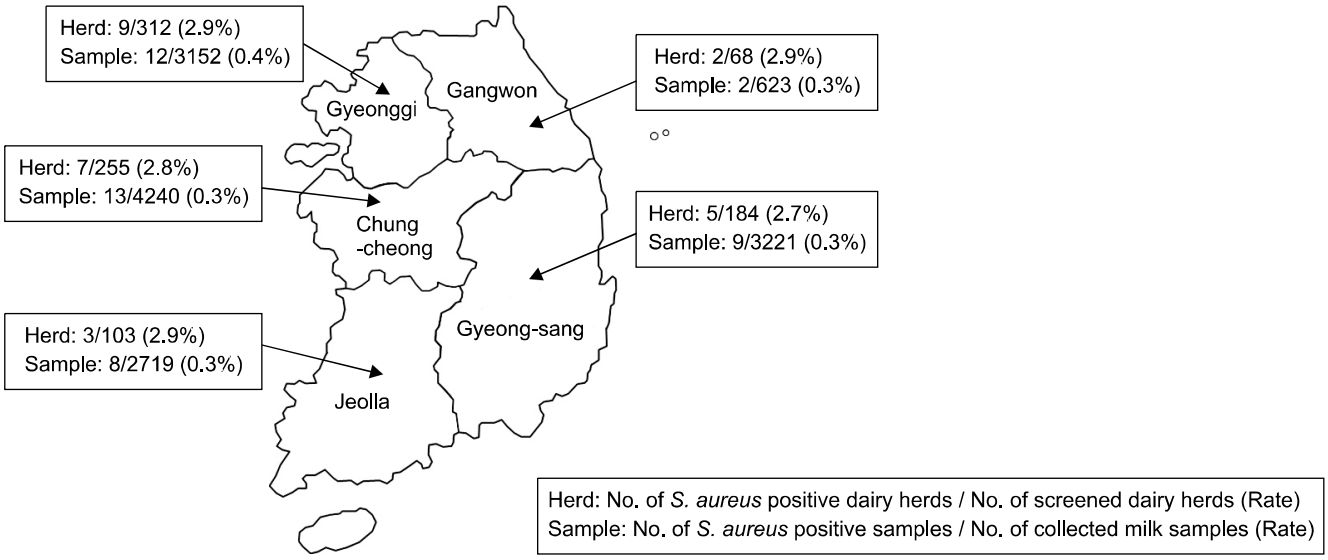

Fig. 1. The number of screened herds and collected samples from five provinces of South Korea. The detection rates of *S. aureus* are shown in boxes and arrows denote each province.

Results

spa types and geographical distribution of *S. aureus*

A total of 44 *S. aureus* strains were isolated from 26 herds in five provinces of Korea (Fig. 1). The positive rate of *S. aureus* detection over the regions was 2.82 ± 0.11% of screened dairy herds and 0.32 ± 0.02% of collected milk samples.

Table 1 shows the *spa* type and the geographical distributions of all isolates. A total of 14 different *spa* types were identified. All had 4-11 repeats and the numeric code of the *spa* types were assigned based on the repeat pattern. Several *spa* types showed similar repeat patterns, suggesting they had diverged from a common ancestor by the accumulation of point mutations or the rearrangement of *spa* repeats. These were grouped together into the same cluster using the BURP algorithm. The similar *spa* types were assorted into one cluster; each cluster displayed an evolutionary relationship between *S. aureus* isolates. For example, isolates t148 and t324, which are different only the r21 repeat, were included in the same cluster and it was assumed that those two isolates were propagated from the same ancestor. In total, the isolates were grouped into five clusters and two singletons based on their *spa* types. There was no herd where more than one *spa* type was detected. Seventeen isolates (38.6%) were included in cluster 1, distributed in all five provinces, and 16 isolates (36.4%) were included in cluster 2, distributed in four of the five provinces. The other minor clusters and singletons were distributed among one or two provinces.

SE gene profiling

SE and SE-like toxin genes were detected in 34 (77.3%) isolates among the 44 isolates (Table 2). Of the 18 kinds of SE and SE-like toxin genes, 10 genes were detected; *sea* (n = 2 isolates), *seb* (n = 1), *seg* (n = 17), *seh* (n = 16), *sei* (n = 17), *selk* (n = 2), *selm* (n = 17), *seln* (n = 17), *selo* (n = 17) and *selq* (n = 2). There was no detection of *sec, sed, see, sej, sell, selp, selr* and *selu* genes.

Six SE gene profiles were analyzed based on detected SE and SE-like genes in the *S. aureus* isolates (Table 2). The most prevalent SE gene profile was a composition of *seg, sei, selm, seln,* and *selo* genes, which was detected in 16 isolates (36.3%). Interestingly, this profile was the same as the SE and SE-like toxin gene composition of the Type I νSaβ genomic island [3]. Likewise, the other profiles were also comparable to the SE and SE-like toxin composition of previously reported MGE such as prophage ϕSa3mws (*sea, selk*, and *selq*) [3], pathogenic island SaPI3 (*seb, selk* and *selq*) [42] and prophage ϕSa3mu (*sea*) [23].

The SE gene profile and *spa* type of *S. aureus* isolates were also compared (Table 2). Several *spa* types showed more than two SE gene profiles. Among the eight isolates of the t164 type, seven isolates possessed *seg, sei, selm, seln* and *selo* genes, while the remaining isolates harbored none of the toxin genes. The eight t286 strains had two SE profiles; *seh* gene positive isolates (n = 6) and toxin negative isolates (n = 2). In the case of t127, seven isolates harbored the *seh* gene and one isolate harbored the *sea, selk, selq* and *seh* genes. The t189 strains displayed three kinds of SE gene profiles: three isolates were suspected to possess the *seg, sei, selm, seln* and *selo* genes, two isolates possessed the *seh* gene, and two isolates did not harbor the toxin genes.

Table 1. *spa* type and distribution of *Staphylococcus aureus* isolates

Cluster	*spa* type	*spa* repeats	No. of *S. aureus* isolates from provinces					
			Gyeonggi	Gangwon	Chungcheong	Jeolla	Gyeongsang	Total
1	t164	r07r06r17r21r34r34r22r34			3	3	2	8
1	t2094	r26r06r17r21r34r34r22	2					2
1	t1987	r07r06r17r21r34	4	2			1	7
2	t286	r07r23r13r34r16r34r33r13	3		3		2	8
2	t127	r07r23r21r16r34r33r13			6	1	1	8
3	t148	r07r23r12r21r12r17r20r17r12r12r17					1	1
3	t324	r07r23r12r12r17r20r17r12r12r17					1	1
3	t664	r07r23r12r12r17r20r17r12r17					1	1
4	t1151	r04r20r17r20r17r24r25r34				2		2
4	t519	r04r20r17r25				1		1
5	t034	r08r16r02r25r02r25r34r24r25	1			1		2
5	t1456	r08r16r02r25	1					1
Singletons	t002	r26r23r17r34r17r20r17r12r17r16			1			1
Singletons	t008	r11r19r12r21r17r34r24r34r22r25	1					1
Total								44

Table 2. Analyzed Staphylococcal enterotoxin (SE) gene profiles, associated mobile genetic elements (MGE) and *spa* types of *S. aureus* isolates

SE gene profiles*	MGE†	*Spa* types and no. of *S. aureus* isolates														
		t164	t2094	t1987	t286	t127	t148	t324	t664	t1151	t519	t034	t1456	t002	t008	Total
seg, sei, selm, seln, selo	Type I νSaβ	7	-	3	-	-	1	1	1	-	1	2	-	-	-	16
seh	-	-	-	2	6	7	-	-	-	-	-	-	-	-	-	15
sea, seh, selk, selq	ϕSa3mw	-	-	-	-	1	-	-	-	-	-	-	-	-	-	1
seb, selk, selq	SaPI3	-	-	-	-	-	-	-	-	-	-	-	1	-	-	1
sea, seg, sei, selm, seln, selo	ϕSa3mu + Type I νSaβ	-	-	-	-	-	-	-	-	-	-	-	-	1	-	1
None	-	1	2	2	2	-	-	-	-	2	-	-	-	-	1	10
																44

*SE gene profiles were investigated by detecting SE and SE-like toxin genes in *S. aureus* isolates. †Several MGE such as Type I νSaβ [3] (*seg, sei, selm, seln, selo*), ϕSa3mw [3] (*sea, selk, selq*), SaPI3 [43] (*seb, selk, selq*), and ϕSa3mu [23] (*sea*) were compared with the SE profile.

Discussion

Investigating distributions of *S. aureus* in dairy herds is important for establishing infection control strategies by providing basic information. Over the past decade, many epidemiological studies for *S. aureus* mastitis have been performed using various molecular typing methods. In the present study, the genotypes sand geographical distribution of *S. aureus* isolates were investigated in a rapid, unambiguously and cost-effective fashion using *spa* typing.

PFGE is the gold standard for genotyping because it is based on the whole genome of the isolate. Nonetheless, it is time-consuming to do and inter-lab comparison is onerous. Therefore, DNA sequence-based approaches have been suggested as an alternative typing method. Multilocus sequence typing (MLST), which is based on the sequences of seven housekeeping genes in *S. aureus*, has been successfully adapted to *S. aureus* [9]. However, MLST is not suitable for routine surveillance due to its high cost. In contrast, *spa* typing is more cost-effective because it targets only a single locus of repeat regions.

The results of *spa* typing can be easily compared between laboratories. A survey of Brazilian bovine *S. aureus* isolates revealed *spa* types of t359, t267 and t605 [1]; these isolates were not presently detected. On the other hand, *spa* types t127, t148, t324, t1151, t002 and t008 observed in this study have been reported human *S. aureus* isolates in Korea [32]. As a result, the distribution of *S. aureus* might be geographically dependent. The present prevalence of each bovine type differed from the Korean human *S. aureus* isolates. For example, t002, which is the predominant human type [32], was presently observed in only one isolate.

The other advantage of *spa* typing is the rapid clustering that can be accomplished using the BURP algorithm. The most significant epidemiological finding of this investigation was that predominant clusters were widespread over the provinces, although other minor clusters were only locally distributed. This agrees with a previous survey [26]. Several studies also revealed the distribution of only a few specialized clones among dairy herds [1,2,11,19,20]. The predominant strains may be subclones, which were a source of contagious transmission, or were host-adapted [34].

Moreover, the predominant clusters consisting of several *spa* types might have a lengthy history in bovine mastitis ecology [6], since considerable time is required for the accumulation of changes on *spa* gene repeat sequences [13, 22]. On the other hand, *spa* types classified as singletons may have been more recently introduced. These findings imply that country-wide control systems are needed to prevent the spreading of a contagious strain between herds. Additionally, genetic backgrounds and molecular mechanisms of these predominant *S. aureus* strains should be investigated.

In this study, 77.3% of the *S. aureus* strains possessed the SE or SE-like toxin genes. This supports the suggestion that SE and SE-like toxins may cause bovine mastitis by depressing the bovine immune system [10]. The most prevalent SE or SE-like toxin genes were *seg, sei, selm, seln* and *selo*, which were detected in 38.6% of the *S. aureus* isolates. This result agrees with a previous study [37] that reported the high prevalence rate of recently described SE or SE-like toxin genes among *S. aureus* strains isolated from bovine milk. The prevalence of those newly reported SE and SE-like toxin genes and their role in a bovine mastitis should be investigated further.

Among the classical SE genes, *sea* to *see, sea* showed the highest prevalence rate in the present study. This is a cause of concern as a potential health risk for humans, because most *S. aureus* strains that possess the *sea* gene produce the

SEA toxin, which is a major etiological factor of staphylococcal food poisoning [4]. On the other hand, it is interesting that no *sec* gene was detected, although *sec* gene detection from animal isolates has been described [21,30,38]. This may suggest a difference between geographical and periodical distributions of enterotoxigenic *S. aureus*.

The SE gene profile of *seg, sei, selm, seln,* and *selo* was always detected together in this study, and this composition exactly matched that of Type I νSaβ. This strongly suggests that *S. aureus* isolates possessing those genes might be associated with Type I νSaβ. The other presently-described SE gene profiles are also comparable to various MGE. Moreover, the SE gene profile differences within the same *spa* types support the possibility of MGE association. For example, ϕSa3mw might be associated with the t127 isolate possesses *sea, selk* and *selq* in addition to the *seh* gene, unlike the other seven t127 isolates. It is also possible, however, that the SE gene profile of *sea, seh, selk,* and *selq* might be associated with ϕSa3mu (*sea*) and that the *seh, selk,* and *selq* genes are encoded on a chromosome or other unknown type of MGE. More genetic investigations will be necessary to clearly reveal the association between SE profiles and MGE of *S. aureus* isolates.

This study is a first report of *spa* types and the prevalence of the recently described SE and SE-like toxin genes among *S. aureus* isolated from bovine raw milk in Korea. From the *spa* typing, it was shown that there were two predominant *S. aureus* clusters distributed widely in bovine husbandry: cluster 1 composed of t164, t2094 and t1987, and cluster 2 composed of t286 and t127. The transmission and pathogenesis of the predominant strains and the relationship between bovine mastitis and recently described SE and SE-like toxins will be investigated further.

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