

Genetic Variation among *Staphylococcus aureus* Strains from Bovine Milk and Their Relevance to Methicillin-Resistant Isolates from Humans[∇]

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In genetic analysis of bovine *Staphylococcus aureus* isolates that are recognized as an important pathogenic bacterium in bovine mastitis, multilocus sequence typing (MLST) showed strong correlation to the results of pulsed-field gel electrophoresis, *coa* PCR-restriction fragment length polymorphism (RFLP), *spa* typing, and the coagulase serotyping method. According to MLST results, strains derived from sequence type 97 (ST97) and ST705 were suggested as not only dominant bovine *S. aureus* lineages in Japan but also pandemic bovine *S. aureus* lineages. Although both lineages seem to be distantly related to each other by phylogenetic analysis, both had common characteristics, i.e., *lukM/lukF'-PV* and coagulase serotype VI. These characteristics were very rare among minor bovine strains and human strains and may contribute to the host specificity of these lineages. Four methicillin-resistant *S. aureus* (MRSA) isolates were first confirmed from bovine milk in Japan; these isolates showed geno- and serotypes that were identical or similar to those of human MRSA isolates in Japan (ST5, staphylococcal cassette chromosome *mec* type II [SCC*mec* II], *Spa* type t002 or t375, and coagulase serotype II, and ST89, SCC*mec* IIIa, *Spa* type t5266, and coagulase serotype I). ST5 and ST89 are uncommon among bovine isolates in the world, whereas these STs are common among human MRSA isolates in Japan.

Staphylococcus aureus is a major causal bacterium in contagious bovine mastitis. Although *S. aureus* has been isolated from heifer body sites and in the dairy farm environment, the lactating mammary gland is the primary reservoir of *S. aureus* involved in bovine mastitis (35, 36). Analyzing the genetic variation among *S. aureus* isolates from bovine milk is essential in bovine mastitis studies such as the identification of protective antigens for vaccine development and elucidation of the mechanism of pathogenesis. Furthermore, investigation of the relationship with isolates from environmental and other origins in dairy farms will lead to the identification of the sources of contagion on dairy farms and ways to prevent contagion from occurring.

There have been reports of the isolation of methicillin-resistant *S. aureus* (MRSA) from bovine milk for a long time (7). The emergence of MRSA infection on dairy farms is of great concern for animal and public hygiene. MRSA-contaminated livestock products, including bovine milk, may become causal agents for human MRSA infection (27). In addition, the spread of MRSA in herds will cause a delay in the treatment of not only mastitis but also other bovine diseases, because most MRSA strains in Japan exhibit multidrug resistance (37). Therefore, screening for MRSA from bovine milk and genetic analysis of isolates are indispensable, and these analyses will lead to elucidation of the mechanisms of MRSA emergence or invasion in dairy farms.

Many different typing methods have been developed for epidemiological studies or the analysis of genetic characteristics and relationships, but each method has drawbacks and advantages, so it is important that an optimal typing method be selected depending on the purpose. The correlation among different genotyping results, the relations with the phenotype, and the discriminatory power become reference points to select a genotyping method. Pulsed-field gel electrophoresis (PFGE), which shows high discriminatory power, is considered “the gold standard” for the typing of *S. aureus* isolates, but it is difficult to establish a precise database with PFGE (3). However, multilocus sequence typing (MLST) and X region of protein A gene (*spa*) typing are established and have websites with very useful resources (www.mlst.net and www.spaserver.ridom.de) for sharing and analyzing substantial databases of genotypes (9, 14). Other than these methods, multiple-locus variable-number tandem-repeat analysis (MLVA), which is based on the number of direct repeats of staphylococcal interspersed repeat units (SIRUs), has demonstrated high discriminatory power for human *S. aureus* isolates (19). Furthermore, staphylococcal cassette chromosome *mec* (SCC*mec*) typing is indispensable for the full characterization of MRSA (31).

This study analyzed the genetic variation among *S. aureus* isolates from bovine milk in Japan using different typing methods. Consequently, the genetic characteristics of bovine milk isolates were evaluated along with their relationship with foreign bovine milk isolates and human MRSA isolates. Furthermore, the discriminatory powers of the different typing methods for bovine *S. aureus* isolates were evaluated and compared.

MATERIALS AND METHODS

***S. aureus* isolates.** A total of 363 *S. aureus* isolates were collected from bovine milk from 260 Japanese dairy farms located in 21 prefectures, and 359 methi-

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cillin-susceptible *S. aureus* (MSSA) isolates and 4 MRSA isolates were obtained between May 1998 and May 2005. Furthermore, 9 previously reported human MRSA isolates (11) were added to evaluate the relationship between bovine isolates and human MRSA isolates. Species identification was demonstrated by detection of a species-specific 442-bp fragment using Voges-Proskauer test-positive and coagulase test-positive staphylococcal isolates (28). The sequence of the 16S rRNA gene was confirmed for isolates that could not be identified (8). MRSA isolates were confirmed in accordance with the Clinical and Laboratory Standards Institute method (6), and the possession of the penicillin-binding protein 2a gene (*mecA*) was confirmed by PCR with the primers described previously (21).

Genotyping and serotyping methods. All of the isolates were analyzed by PFGE, which was performed as described by Ichijama et al. (17). Chromosomal DNA included in the thinly sliced agarose gel was digested with 10 U of SmaI or XhoI for 18 h and then electrophoresed through a 1% pulsed-field certified agarose (Bio-Rad) gel in TBE buffer (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA [pH 8.0]) at 10°C using a CHEF-DR11 system (Bio-Rad). The conditions for electrophoresis for SmaI digestions were 5.7 V/cm for 25 h, with pulse times ranging from 5 to 40 s, and for XhoI digestions were 6.0 V/cm for 24 h, with pulse times ranging from 0.1 to 8 s. Low Range PFG marker (New England Biolabs) was used as the size standard. The PFGE pulsotypes were analyzed by visual inspection using BioNumerics software (version 5.10) according to the criteria described previously (30, 38). Briefly, a dendrogram was created using the Dice coefficient with optimization and a position tolerance setting of 1% based on the banding patterns of each pulsotype, and the pulsotypes that showed more than 80% similarity in this dendrogram were defined as the same cluster (30). MLST was performed with the primers described previously (9), and the products were purified using a QIAquick PCR purification kit (Qiagen) and sequenced on a 3130 genetic analyzer (Applied Biosystems) using a BigDye Terminator (version 3.1) ready reaction cycle sequencing kit (Applied Biosystems). Allele number and sequence type (ST) were determined using the MLST website (www.mlst.net). The founder and clonal complex (CC) of each ST were determined by using the enhanced version of Based Upon Related Sequence Types (eBURST) (10). A phylogenetic tree was constructed using the ClustalW website (<http://clustalw.ddbj.nig.ac.jp/top-e.html>), based on the 3,198 bp of the seven target loci sequences, which were concatenated in the order *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yiiL*. *spa* typing was performed with the primers described previously (14), and numeric *spa* repeats and Spa type codes were determined using the Ridom SpaServer website (www.spaserver.ridom.de). MLVA typing was performed with the primers (13) that amplified the 6 SIRU loci (SIRU01, -05, -07, -13, -15, and -21). PCR-based restriction fragment length polymorphism (RFLP) analysis of the coagulase gene (*coa*) was performed with the primers described previously (16), and representative PCR products of each *coa* PCR-RFLP type were sequenced for determination of the precise restriction fragment lengths. The SCC_{mec} types were determined by multiplex PCR with the primers described previously (29, 31, 41). The coagulase serotype was confirmed using a Seiken coagulase typing kit (Denka Seiken, Japan).

Detection of staphylococcal exotoxin genes. The detection of gamma hemolysin, LukE/LukD, LukM/LukF'-PV, and the Panton-Valentine leukocidin genes (*hlg*, *lukE/lukD*, *lukM/lukF'-PV*, and *pvl*) was performed with the primers described previously (20). The following primers were used for the detection of the streptolysin-associated protein SagD-like protein gene (*sagD*) and the SagB-like protein gene (*sagB*): SagD-F (5'TTG AAT AAT CAA AAA AGT AAT3'), SagD-R (5'TTA AAG GTT ATC ATT TTC TAA3'), SagB-F (5'GTG GCT CAA AAG GAC ATA AAC3'), and SagB-R (5'CTA TTC CTT CCC ACA TAT A3'). The sizes of the PCR products were 564 bp and 309 bp, respectively.

Evaluation of the discriminatory power and correlation among different typing methods. The discriminatory power of the typing methods and correlation among typing methods were calculated by using EpiCompare version 1.0 (Ridom GmbH, Wurzburg, Germany), as was the determination of Adjusted Rand's index and Wallace's coefficients (19).

RESULTS

Genetic and serological analysis of dominant *S. aureus* strains. The DNA of the 372 *S. aureus* isolates after digestion with SmaI showed 144 pulsotypes in 23 clusters (A to W). Furthermore, the tested isolates were segmented into 191 pulsotypes by adding PFGE results following XhoI digestion. Eighty-four isolates (22.6%) from 16 prefectures showed 38 pulsotypes in cluster N, and 178 isolates (47.8%) from 21

prefectures showed 58 pulsotypes in cluster O (Table 1). A total of 191 representative *S. aureus* isolates from individual pulsotypes were analyzed by further typing.

Representative isolates were grouped into 34 STs in 15 CCs and, independently, into 3 STs by MLST (Table 2). All of the representative isolates belonging to cluster O showed 5 STs (ST97, ST124, ST352, ST1366, and ST1367) that are members of CC97, the predicted ancestral ST of which is ST97 (Table 1). Two isolates belonging to clusters L and P also showed ST124 of CC97. All of the representative isolates belonging to cluster N showed 4 STs (ST705, ST1363, ST1364, and ST1365) that are members of CC705, the predicted ancestral ST of which is ST705 (Table 1).

According to *spa* typing, the representative isolates were grouped into 59 Spa types (Table 2). Representative isolates that are members of CC97 showed 15 Spa types (t189, t203, t224, t267, t359, t458, t521, t1109, t1234, t2101, t2453, t2844, t3782, t5265, and t5352); many of these Spa types consist of repeat units of shared or analogous sequences, and the order of repeat units is also similar (www.spaserver.ridom.de) (Table 1). The representative isolates that are members of CC705, however, showed an identical Spa type (t529) (Table 1).

Representative isolates produced *coa* PCR amplification products of 352, 514, 595, 676, 757, 838, 919, or 1,000 bp. Furthermore, these *coa* PCR products were segmented into 28 genotypes by AluI digestion (Table 2). The genotypes and their respective restriction fragments (by length [bp]) from *coa* PCR-RFLP were as follows: 352A, 214-138; 352B, 352; 514A, 214-162-138; 514B, 300-214; 595A, 381-214; 595B, 214-162-138-81; 595C, 376-138-81; 595D, 595; 595E, 300-295; 595F, 300-214-81; 676A, 457-219; 676B, 295-243-138; 676C, 381-214-81; 676D, 243-219-214; 676E, 300-214-162; 676F, 381-295; 676G, 676; 676H, 295-162-138-81; 757A, 457-219-81; 757B, 757; 757C, 381-376; 757D, 381-214-162; 757E, 324-295-138; 838A, 381-295-162; 838B, 381-295-81-81; 919A, 214-138-81-81-81-81-81-81; 919B, 214-162-162-138-81-81-81; and 1000A, 243-219-214-162-81-81. Representative isolates that are members of CC97 showed 7 genotypes (352B, 595E, 676F, 757C, 757D, 838A, and 838B), and representative isolates that are members of CC705 showed an identical genotype (595D) (Table 1).

Representative isolates showed 91 MLVA profiles (Table 2), and members of CC97 and CC705 showed 28 and 6 profiles, respectively (Table 1).

By coagulase serotype, representative isolates showed 8 serotypes (Table 2), and members of CC97 and CC705 showed coagulase serotype VI (Table 1). Furthermore, most of the CCs other than CC15 and CC8 showed a single coagulase serotype (Table 2).

As for the staphylococcal exotoxin gene profiles, 9 combinations were confirmed in representative isolates (Table 1). *lukM/lukF'-PV* was only detected in members of CC705 and CC97 (Table 1). *sagB* and *sagD* were detected in all of the representative isolates that are members of CC705, ST789, and ST1361 (Table 1). Additionally *sagB* was also detected in representative isolates of ST89 and ST1359 (Table 1). Of the bovine milk isolates, there were only 3 isolates, ST1, ST81, and ST243, detected by *pvl* (Table 1). On the other hand, *hlg* was detected in all representative isolates from bovine milk, and *lukE/lukD* was also detected in all representative isolates from

TABLE 1. Genetic and serological characteristics of *Staphylococcus aureus* isolates from bovine milk and humans^a

MLST CC	ST	Spa type	Coagulase serotype	coa RFLP genotype	PFGE pulsotype(s)	Toxin gene profile	No. of RUS ^b in SIRU locus:							SCC _{mec} type	No. of locations where found ^c	Prefecture(s) where found	Source(s)
							1	5	7	13	15	21					
5	5	t001	II	676H	B6	hlg, lukE/lukD	2	30<	1	3	999	9	9	II	1	Tochigi	Human wound
5	5	t002	II	676B	D	hlg, lukE/lukD	2	3	1	3	1	9	9	II	1	Unknown	Bovine milk
5	5	t179	II	676B	B1, B2, B3	hlg, lukE/lukD	2	3	1	3	1	9	9	II	3	Fukuoka	Bovine milk
5	5	t375	II	676B	E1, E2	hlg, lukE/lukD	2	2	1	3	999	9	9	II	1	Unknown	Bovine milk
5	5	t375	II	676B	E3	hlg, lukE/lukD	2	2	1	3	999	9	9	II	1	Ibaraki	Human pus
5	5	t5259	II	676B	B4, B5	hlg, lukE/lukD	2	3	1	3	1	6	6	II	1	Fukuoka	Bovine milk
6	1362	t701	IV	676D	O11	hlg, lukE/lukD	2	7	999	4	1	9	9	II	1	Ishikawa	Bovine milk
6	6	t2360	IV	676D	O10	hlg, lukE/lukD	2	7	999	4	1	10	10	II	1	Tottori	Bovine milk
7	789	t091	III	757B	H1, H2	hlg, lukE/lukD, sagB, sagD	999	999	3	2	3	9	9	II	2	Iwate, Saga	Bovine milk
8	8	t008	III	595B	G2	hlg, lukE/lukD	3	3	1	0	1	9	9	II	1	Iwate	Bovine milk
8	8	t008	III	595C	G6	hlg, lukE/lukD	3	3	2	0	1	9	9	II	1	Hokkaido	Bovine milk
8	8	t008	III	352A	G3	hlg, lukE/lukD	3	3	3	0	1	9	9	II	1	Ishikawa	Bovine milk
8	8	t4133	III	514A	G1	hlg, lukE/lukD	3	3	3	0	1	9	9	II	1	Gunma	Bovine milk
8	72	t4359	V	676F	M	hlg, lukE/lukD	3	9	999	3	7	4	4	II	1	Ishikawa	Bovine milk
8	630	t160	VII	595B	G4, G5	hlg, lukE/lukD	1	7	1	3	999	6	6	II	1	Chiba	Bovine milk
12	12	t160	VII	676D	R5	hlg, lukE/lukD	1	7	1	3	1	6	6	II	1	Saga	Bovine milk
12	12	t160	VII	676D	R2	hlg, lukE/lukD	1	7	1	4	1	5	5	II	1	Tochigi	Bovine milk
12	12	t3418	VII	676D	R3	hlg, lukE/lukD	1	7	1	4	1	5	5	II	1	Tochigi	Bovine milk
12	12	t160	VII	676D	R4	hlg, lukE/lukD	1	7	1	2	999	6	6	II	1	Saitama	Bovine milk
12	12	t160	VII	676D	R1	hlg, lukE/lukD	1	7	1	2	1	6	6	II	1	Saitama	Bovine milk
15	1	t1775	VII	514B	O7	hlg, lukE/lukD, pvl	1	999	3	1	2	9	9	II	1	Tochigi	Bovine milk
15	81	t127	VII	514B	O3	hlg, lukE/lukD	1	999	2	0	2	6	6	II	1	Ishikawa	Bovine milk
15	81	t127	VII	514B	O8	hlg, lukE/lukD	1	999	3	1	6	6	6	II	1	Iwate	Bovine milk
15	81	t127	VII	595F	O6	hlg, lukE/lukD, pvl	1	999	2	0	1	6	6	II	1	Hokkaido	Bovine milk
15	81	t127	VII	595F	O1, Q5	hlg, lukE/lukD, pvl	1	999	2	1	1	6	6	II	1	Hokkaido	Bovine milk
15	81	t127	VII	595F	O2	hlg, lukE/lukD	1	999	2	1	2	6	6	II	1	Kochi	Bovine milk
15	81	t127	VII	595F	O4	hlg, lukE/lukD	1	999	3	1	1	6	6	II	1	Fukuoka	Bovine milk
15	81	t127	VII	595F	O9	hlg, lukE/lukD	1	999	3	1	2	6	6	II	1	Nara	Bovine milk
15	188	t189	V	676A	K6	hlg, lukE/lukD	1	999	2	1	4	5	5	II	1	Tochigi	Bovine milk
15	188	t189	V	676E	K11	hlg, lukE/lukD	1	999	1	2	4	5	5	II	1	Hokkaido	Bovine milk
15	188	t189	V	676E	K1, K2, K4, K5, K7, K8, K9, K10, K12, K13	hlg, lukE/lukD	1	999	3	2	4	5	5	II	8	Hokkaido, Iwate, Saitama, Shizuoka, Gifu, Fukuoka	Bovine milk
15	188	t1858	V	676E	K3	hlg, lukE/lukD	1	999	3	1	4	6	6	II	1	Tottori	Bovine milk
20	20	t164	VIII	595E	U1, U4, U7	hlg, lukE/lukD	3	10	2	1	4	7	7	II	3	Iwate	Bovine milk
20	20	t164	VIII	595E	U11	hlg	3	10	2	1	4	7	7	II	1	Hiroshima	Bovine milk
20	20	t164	VIII	595E	U5	hlg, lukE/lukD	3	11	2	0	4	7	7	II	1	Gunma	Bovine milk
20	20	t164	VIII	595E	U10	hlg	3	11	2	1	4	7	7	II	1	Tokyo	Bovine milk
20	20	t693	VIII	595E	U9	hlg, lukE/lukD	3	10	2	1	4	0	0	II	1	Tokyo	Bovine milk
20	20	t2109	VIII	595E	U15	hlg, lukE/lukD	3	10	2	1	4	3	3	II	1	Ishikawa	Bovine milk
20	20	t3277	VIII	595E	U2, U18	hlg, lukE/lukD	3	10	2	1	4	6	6	II	2	Hokkaido, Ishikawa	Bovine milk
20	20	t3332	VIII	595E	U3	hlg, lukE/lukD	3	10	2	1	4	5	5	II	1	Hokkaido	Bovine milk
20	20	t3929	VIII	514B	U13, U14	hlg, lukE/lukD	3	8	2	1	4	3	3	II	1	Hyogo	Bovine milk
20	20	t4542	VIII	595E	U8	hlg, lukE/lukD	3	10	2	1	4	3	3	II	1	Saitama	Bovine milk
20	20	t5267	VIII	595E	U6	hlg, lukE/lukD	3	10	2	1	4	10	10	II	1	Niigata	Bovine milk
20	20	t5412	VIII	595E	U12	hlg, lukE/lukD	3	10	2	1	4	11	11	II	1	Hokkaido	Bovine milk
20	1368	t3277	VIII	595E	U17	hlg, lukE/lukD	3	10	2	1	4	6	6	II	1	Hokkaido	Bovine milk
20	1370	t881	VIII	595E	U16	hlg, lukE/lukD	3	10	2	0	4	6	6	II	1	Ishikawa	Bovine milk
25	25	t078	II	595A	V5	hlg, lukE/lukD	3	5	3	2	4	8	8	II	1	Hokkaido	Bovine milk
25	25	t258	II	514B	V4	hlg, lukE/lukD	3	5	3	2	3	9	9	II	1	Fukuoka	Bovine milk
25	26	t287	II	595A	V1, V2	hlg, lukE/lukD	3	5	2	2	4	2	2	II	1	Tokyo	Bovine milk
25	1372	t258	II	514B	V3	hlg, lukE/lukD	3	3	3	2	2	3	3	II	1	Gunma	Bovine milk
30	243	t021	IV	676C	J	hlg, pvl	2	3	2	2	2	8	8	II	1	Hokkaido	Bovine milk
45	508	t050	VII	676G	T1, T3	hlg	999	999	2	0	1	9	9	II	2	Gunma, Fukuoka	Bovine milk
45	508	t362	VII	676G	T4	hlg	999	999	2	0	1	1	1	II	1	Kochi	Bovine milk
45	508	t630	VII	676G	T2	hlg	999	999	2	0	1	1	1	II	1	Fukuoka	Bovine milk
59	59	t216	VII	757E	S	hlg	2	999	4	1	4	7	7	II	1	Iwate	Bovine milk
88	88	t1028	III	757A	F3, F4	hlg, lukE/lukD	2	999	3	2	0	3	3	II	1	Shizuoka	Bovine milk
88	88	t1028	III	757A	F5	hlg, lukE/lukD	2	999	3	3	0	3	3	II	1	Shizuoka	Bovine milk

TABLE 1—Continued

MLST CC	ST	Spa type	Coagulase serotype	<i>coa</i> PCR- RFLP genotype	PFGE pulsotype(s)	Toxin gene profile	No. of RUS ^b in SIRU locus:							SCC _{mec} type	No. of locations where found ^c	Prefecture(s) where found	Source(s)
							1	5	7	13	15	21					
705	705	t529	VI	595D	N3, N8, N19, N37, N38	<i>hlg, lukE/lukD, lukM/lukF'- PV, sagB, sagD</i>	1	999	2	999	999	1	5	Tokyo, Ishikawa, Gifu, Saga	Bovine milk		
705	705	t529	VI	595D	N25	<i>hlg, lukE/lukD, lukM/lukF'- PV, sagB, sagD</i>	1	999	3	2	0	1	1	Hokkaido	Bovine milk		
705	705	t529	VI	595D	N6	<i>hlg, lukE/lukD, lukM/lukF'- PV, sagB, sagD</i>	1	999	4	999	0	1	1	Ishikawa	Bovine milk		
705	1363	t529	VI	595D	N21, N23	<i>hlg, lukE/lukD, lukM/lukF'- PV, sagB, sagD</i>	1	999	999	999	0	1	2	Tochigi, Okinawa	Bovine milk		
705	1364	t529	VI	595D	N26	<i>hlg, lukE/lukD, lukM/lukF'- PV, sagB, sagD</i>	1	999	999	999	0	1	1	Tochigi	Bovine milk		
705	1365	t529	VI	595D	N35	<i>hlg, lukE/lukD, lukM/lukF'- PV, sagB, sagD</i>	1	999	999	999	999	1	1	Hokkaido	Bovine milk		
1359	1361	t5263 t5260	II Untypeable	919A 919B	C I	<i>hlg, sagB</i>	3	999	1	2	3	8	1	Hyogo Iwate	Bovine milk		
1371	1371	t5261	IV	1000A	W	<i>hlg</i>	3	999	3	999	3	8	1	Hiroshima	Bovine milk		

^a MRSA isolates are highlighted in boldface. The major bovine lineages were CC97 and CC705.
^b “999” indicates that there was no amplification of SIRUs.
^c Locations were dairy farms or hospitals.

TABLE 2. Simpson’s index of diversity and 95% confidence interval values of the methods used to characterize the 191 *S. aureus* isolates from bovine milk and humans

Typing method	No. of different types found	Discriminatory index	95% Confidence interval
MLVA (SIRU1, -5, -7, -13, -15, -21)	91	0.971	0.96–0.982
<i>spa</i> typing	59	0.928	0.906–0.95
MLST (STs)	37	0.903	0.879–0.926
<i>coa</i> PCR-RFLP	28	0.88	0.855–0.905
MLST (CCs)	15	0.83	0.796–0.863
Coagulase serotype	8	0.699	0.636–0.763

bovine milk, except for the 12 isolates of ST20, ST59, ST89, ST243, ST508, ST1359, ST1361, and ST1371 (Table 1).

Comparison of different typing methods. The discriminatory powers of the five typing methods (MLST, *spa* typing, coagulase serotype, *coa* PCR-RFLP, and MLVA) other than PFGE were determined by calculating the Simpson’s index of diversity with 95% confidence intervals of the isolates typed by these methods (Table 2). MLVA showed the highest discriminatory power (0.971) of the five typing methods. In addition, *spa* typing and MLST also showed high discriminatory power, with a discriminatory index of 0.9 or higher (0.928 and 0.903, respectively). Adjusted Rand’s and Wallace’s coefficients were calculated to explore the correlation between typing methods (Tables 3 and 4). MLST (CCs) showed high adjusted Rand’s coefficient (0.941) and Wallace’s coefficients (0.911 and 0.993) with PFGE (clusters). Furthermore, MLST (CCs) showed comparatively high adjusted Rand’s coefficients and Wallace’s coefficients with *coa* PCR-RFLP (0.721, 0.658, and 0.903), *spa* typing (0.546, 0.425, and 0.977), and coagulase serotyping (0.597, 0.959, and 0.553). MLVA showed high Wallace’s w_1 coefficients for every typing method. *spa* typing also showed high Wallace’s w_1 coefficients for every typing method except for MLVA.

Genetic characteristics of major lineages from bovine milk in Japan, and relationship with foreign bovine milk isolates. *S. aureus* strains of CC97 and CC705 are dominant lineages from bovine milk in Japan. Using many genotyping methods (MLVA, *coa* PCR-RFLP, and *spa* typing), the CC97 strains showed more genetic variation than the CC705 strains, as previously noted (Table 1). Newbould 305 is the strain that was isolated in 1958 from a clinical mastitis case in Canada (32), and this strain shows ST115, which is the ST of CC97 (Fig. 1). All isolates in CC705 showed an identical genetic background (t529 for *Spa* type, 595D for *coa* PCR-RFLP, and detection of *hlg, lukE/lukD, lukM/lukF'-PV, sagB, and sagD*) and coagulase serotype VI (Table 1). Moreover, the sequenced Irish bovine *S. aureus* strain RF122 (ET3-1) also shows ST151, which is a member of CC705 (Fig. 1) (15).

According to the MLST website and eBURST, 30 of 42 STs of CC97 (ST70, ST71, ST97, ST115, ST116, ST117, ST118, ST124, ST347, ST349, ST352, ST355, ST358, ST742, ST746, ST747, ST1072, ST1077, ST1119, ST1125, ST1126, ST1127, ST1128, ST1129, ST1366, ST1367, ST1527, ST1615, ST1623, and ST1624) have been confirmed in bovine isolates, while all 17 STs of CC705 (ST151, ST351, ST504, ST705, ST1074,

TABLE 3. Adjusted Rand's coefficients for the methods used to characterize the 191 *S. aureus* isolates from bovine milk and humans^a

Typing method	MLVA	<i>spa</i> typing	MLST (STs)	<i>coa</i> PCR-RFLP	PFGE clusters	MLST (CCs)	Coagulase serotype	Staphylococcal exotoxin gene profile
MLVA (SIRU1, -5, -7, -13, -15, -21)								
<i>spa</i> typing	0.540							
MLST (STs)	0.413	0.663						
<i>coa</i> PCR-RFLP	0.289	0.543	0.576					
PFGE clusters	0.286	0.589	0.733	0.719				
MLST (CCs)	0.262	0.546	0.705	0.721	0.941			
Coagulase serotype	0.131	0.297	0.404	0.418	0.585	0.597		
Staphylococcal exotoxin gene profile (<i>hlg</i> , <i>lukE/lukD</i> , <i>lukM/lukF'-PV</i> , <i>pvl</i> , <i>sagB</i> , <i>sagD</i>)	0.115	0.249	0.350	0.257	0.345	0.358	0.129	

^a The methods are listed in order of discriminatory power, with the highest to lowest discriminatory powers from left to right and top to bottom.

ST1076, ST1078, ST1122, ST1123, ST1124, ST1230, ST1248, ST1274, ST1363, ST1364, ST1365, and ST1520) have only been confirmed in bovine isolates. CC97 strains have been isolated from bovine milk in Brazil, Canada, Chile, Italy, Norway, Netherlands, Spain, the United Kingdom, and the United States (Fig. 1). CC705 strains have been isolated from bovine milk in Chile, Germany, Ireland, Netherlands, Spain, the United Kingdom, and the United States (Fig. 1).

Genetic analysis of MRSA isolates from bovine milk. Four bovine MRSA isolates were obtained from different bovine mastitis cases from 1999 to 2005 (Table 1). Of 4 bovine MRSA isolates, 3 isolates exhibited ST5-SCCmec II (Table 1). Two bovine MRSA isolates with pulsotypes E1 and E2 and a human MRSA isolate with pulsotype E3 showed identical genotype results (t375 for *Spa* type, 676B for *coa* PCR-RFLP, 2-2-1-3-999-9 in the MLVA profile, and the detection of *hlg* and *lukE/lukD*) and coagulase serotype II (Table 1). One bovine MRSA isolate with pulsotype D also showed similar genotype results (t002 for *Spa* type, 676B for *coa* PCR-RFLP, 2-3-1-3-1-9 in the MLVA profile, and the detection of *hlg* and *lukE/lukD*) and coagulase serotype II (Table 1). One remaining bovine MRSA isolate with pulsotype A1 exhibited ST89-SCCmec IIIa (Table 1). The ST89-SCCmec IIIa isolate had a different genotype from hospital-acquired MRSA (HA-MRSA) pandemic clones, such as the New York/Japan clone, pediatric clone (ST5-SCCmec IV), Berlin clone (ST45-SCCmec IV), Iberian clone (ST247-SCCmec Ia), Brazilian-Hungarian clone

(ST239-SCCmec III), epidemic MRSA strain 15 (EMRSA-15) (ST22-SCCmec IV), and EMRSA-16 (ST36-SCCmec II). However, ST89-SCCmec IIIa is confirmed in a human MRSA isolate with pulsotype A3 (Table 1). MRSA isolates which are members of CC509 are members of PFGE cluster A and showed an identical *coa* PCR-RFLP genotype (676A) and coagulase serotype I. Moreover, they showed analogous *Spa* types (t375 and t5226) and MLVA profiles (2-999-1-1-1-7 and 2-999-1-0-1-6) (Table 1).

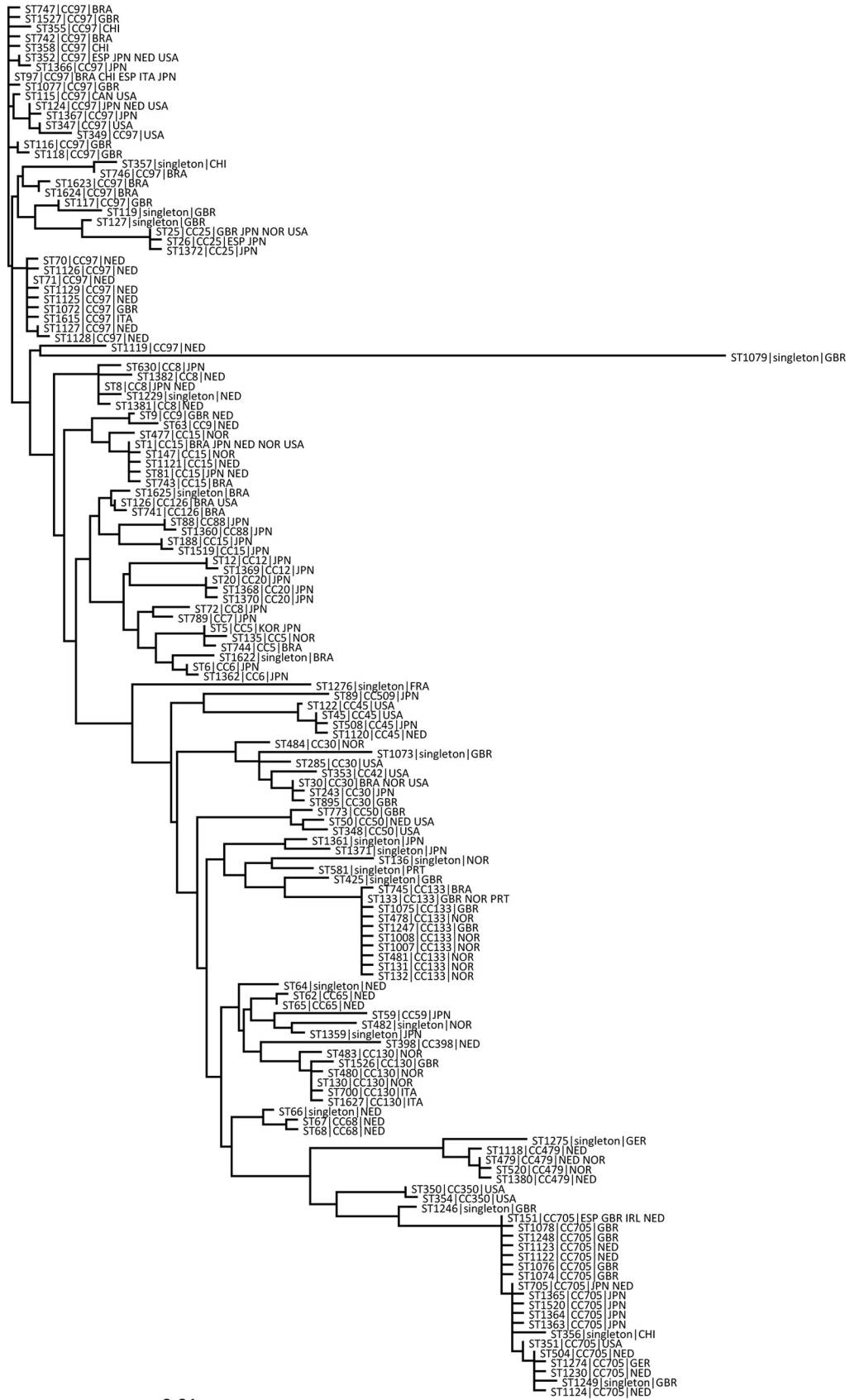
DISCUSSION

Weighing the results of various typing methods for bovine *S. aureus* isolates, three typing methods (MLVA, *spa* typing, and MLST) showed comparatively high discriminatory power. The discriminatory power of MLVA was the highest among these methods. Furthermore MLVA and *spa* typing showed high Wallace's w_1 coefficients for various typing methods. These results show that both methods have high discriminatory power and that there is strong correlation between them. When there is a need to complement more elaborate genotyping results, both methods will be useful. On the other hand, MLST showed high adjusted Rand's coefficients and Wallace's coefficients with various typing methods for *S. aureus*, especially PFGE. This suggests that the genotyping results of MLST almost accord with the genotyping results of PFGE. As for correlation with other typing methods, isolates belonging to the same CC

TABLE 4. Wallace's coefficients for the methods used to characterize the 191 *S. aureus* isolates from bovine milk and humans^a

Typing method	MLVA	<i>spa</i> typing	MLST (STs)	<i>coa</i> PCR-RFLP	PFGE clusters	MLST (CCs)	Coagulase serotype	Staphylococcal exotoxin gene profile
MLVA (SIRU1, -5, -7, -13, -15, -21)								
<i>spa</i> typing	0.393	0.971	0.956	0.829	0.994	1	1	0.939
MLST (STs)	0.285	0.6	0.813	0.781	0.977	0.977	0.977	0.886
<i>coa</i> PCR-RFLP	0.2	0.467	0.562	0.694	0.976	1	1	0.926
PFGE clusters	0.191	0.464	0.628	0.679	0.854	0.903	0.903	0.685
MLST (CCs)	0.176	0.425	0.59	0.658	0.911	0.993	0.993	0.723
Coagulase serotype	0.097	0.235	0.327	0.364	0.504	0.553	0.959	0.711
Staphylococcal exotoxin gene profile (<i>hlg</i> , <i>lukE/lukD</i> , <i>lukM/lukF'-PV</i> , <i>pvl</i> , <i>sagB</i> , <i>sagD</i>)	0.089	0.207	0.294	0.268	0.356	0.385	0.39	0.4

^a Each value in the body of the table is Wallace's w_1 coefficient of the typing method given in the first column to the typing method given in the column head and is also Wallace's w_2 coefficient of the typing method given in the column head to the typing method given in the first column.



showed genotypes that were identical or analogous to each other. Moreover, there are established, useful websites for sharing and analyzing substantial databases of MLST genotypes (9), so MLST may become the main genotyping method for cluster analysis of bovine *S. aureus* isolates.

According to the results of various genotyping and coagulase serotyping methods, *S. aureus* strains as a causal bacterium of bovine mastitis in Japan mainly consist of specific lineages, CC97 and CC705. These results supported our previous speculation that two major *S. aureus* lineages spread as bovine mastitis causal strains (12). Both lineages have also been isolated from bovine milk worldwide. In particular, CC97 strains have become the dominant lineage in Chile (36), Brazil (2, 33), Japan, Netherlands (18), and the United States (36). CC705 strains have become the dominant lineage in Japan, Netherlands (18), and the United Kingdom (36). In addition to CC97 and CC705 strains, the CC126, CC133, and CC479 lineages are dominant in Brazil (33), Norway (22), and the Netherlands (18), respectively. Strains of these CCs have never been confirmed in Japan but have been widely isolated in South and North America and Europe and may spread to Asia in the future with the movement of cattle. According to the MLST website, CC97, CC126, CC133, CC479, and CC705 strains have been isolated mainly from ruminant samples, but strains of other CCs have not. As for CC705 and CC479 strains, these strains have only been isolated from bovine samples.

The CC97 strains showed multiple genotypes with the *coa* PCR-RFLP and *spa* typing, whereas the CC705 strains showed an identical genotype. Moreover, a phylogenetic analysis based on the seven locus sequences of MLST seems to suggest that the CC97 lineage emerged earlier than the CC705 lineage, and bovine strains worldwide seem to derive from CC97. Newbould 305, a member of the CC97 lineage, was isolated from bovine mastitic milk in 1958 (32). These facts led us to predict that CC97 is a bovine mastitis causal lineage that has existed for at least 50 years. However, CC705 seems to be the most newly established bovine lineage (Fig. 1), and the identical genotype results of CC705 strains seemed to support this speculation.

As this study elucidated, certain hypotheses have been proposed to explain why specific *S. aureus* lineages are frequently isolated from bovine samples. Herron-Olson et al. advocate the idea that host specialization of *S. aureus* depends on gene mutation, transfer, and decay. As noted previously, specific gene variations may play an important role in *in vivo* bacterial pathogenicity and colonization (15). According to phylogenetic analysis based on MLST data, CC97 and CC705, which are dominant lineages from bovine milk, are distantly related to each other (Fig. 1). However, *lukM/lukF'-PV* and coagulase serotype VI were nominated as common characteristics of both strains by our study. *lukM/lukF'-PV* is associated with horizontal gene transmission by temperate bacteriophages of bovine

origin (40), so *lukM/lukF'-PV* will be widely found in these strains. *LukM/LukF'-PV* has highly active cytotoxicity on bovine neutrophils in comparison with that of other *S. aureus* leukocidins (4), and *LukM/LukF'-PV* may contribute to the neutralization of immunity by neutrophils in mammary organs. Moreover, *LukM/LukF'-PV* is a family of pore-forming toxins which may be involved with escape from the phagosome after phagocytosis and intracellular survival, which is an important capability for the maintenance of *S. aureus* intramammary infection (23). Intracellular survival is considered to be related to rebelliousness, which is a characteristic mark of bovine mastitis due to *S. aureus*, because most antibiotics cannot act in a cell, except for macrolides. Judging from the viewpoint of iron acquisition, obligate intracellular conditions will be favorable for the survival of *S. aureus* (15). Many of the biological activities of *LukM/LukF'-PV* have never been elucidated, including its participation in bovine mastitis, so further analysis of this toxin will be required in the future.

Coagulase, which is recognized as one of the virulence factors of *S. aureus*, causes coagulation of plasma. The antigenic diversity of coagulase is a major phenotypic determinant and is used as a characteristic marker, with 12 types having been discriminated so far (24). Coagulase serotype VI is dominant only in bovine isolates and seems to be extremely rare in human isolates (11, 24). The antigenic variation in coagulase may result from adaptation to prothrombin in different animal species in order to elude the host immune response (24). Thus, the antigenicity of coagulase may be an important factor for evading the host defense system, as well as for adapting to animals that are infected by *S. aureus*.

Staphylococcus protein A is a virulence factor participating in humoral immunity restraint. According to *spa* typing, CC705 strains showed an identical genotype (t529), and CC705 strains in the Netherlands also showed the same genotype (18). CC97 strains showed multiple genotypes, but many of these genotypes (t189, t203, t224, t267, t359, t521, t1109, t1234, t2453, and t3782) are closely related to each other (Table 1). Of these genotypes, t224 and t521 were confirmed in CC97 strains in Netherlands (18) and t267 and t359 were confirmed in CC97 strains in Brazil (2). *spa* of bovine strain RF122 contains stop codons and is, therefore, a pseudogene (15). The relationship between point mutations or mutations resulting in a pseudogene of *spa* and the pathogenicity of *S. aureus* strains is an interesting point for further examination.

Herron-Olson et al. suggested several genetic characteristics of bovine strain RF122. The streptolysin homolog genes (*sagB* and *sagD*), pyrogenic toxin super antigen TSST-1 gene (*tst*), and the bovine variant of the staphylococcal enterotoxin C gene (*sec*) are genetic characteristics of bovine strain RF122 (15). Previous studies also suggested that not only leukocidin but also superantigens are important during mastitis pathogen-

FIG. 1. Phylogenetic tree based on the seven locus sequences (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqiL*) that have been reported from bovine *S. aureus* isolates worldwide by the MLST website and previous reports (2, 15, 18, 22, 26, 33, 35, 36). The sequence type (ST), clonal complex (CC), and country or countries that each ST was detected from are shown. Country abbreviations: BRA, Brazil; CAN, Canada; CHI, Chile; ESP, Spain; FRA, France; GBR, United Kingdom; GER, Germany; IRL, Ireland; ITA, Italy; JPN, Japan; KOR, Republic of Korea; NED, Netherlands; NOR, Norway; PRT, Portugal; USA, United States. *S. aureus* strain Newbould 305 from clinical bovine mastitis in Canada shows ST115, and the sequenced Irish bovine strain RF122 shows ST151 (15). The scale bar indicates the rate of nucleotide substitution.

esis due to their immunomodulatory effects (34). However, this study and the previous studies suggested that these genes are rarely present in CC97 strains, so these exotoxins will not be fundamental in bovine mastitis (11, 12).

As for dominant foreign lineages from bovine milk, such as CC126, CC133, and CC479, the bacteriological characteristics of these CCs have not yet been sufficiently elucidated. Further analysis of these CCs will be indispensable for examining the universal characteristics of dominant bovine strains.

The New York/Japan MRSA clone has mainly spread as HA-MRSA in Japan and Korea, and ST5-SCC*mec* II is the specific genotype of this clone (25). Our study indicated that bovine MRSA isolates in Japan showed the specific genotype of the New York/Japan MRSA clone. Of them, 2 bovine MRSA isolates showed genotypes identical to the human MRSA isolate, and one bovine MRSA isolate also showed similar genotype results. One remaining bovine MRSA isolate, with ST89-SCC*mec* IIIa, is not identical to genotypes of the human MRSA isolate with ST89-SCC*mec* IIIa; however, both isolates showed analogous genotype results. Therefore, we cannot be sure how this bovine MRSA isolate arrived at the bovine mammary organ. However, the fact that this bovine MRSA isolate shows genotype results similar to those of a human MRSA isolate is interesting. Moreover ST5 and ST89 are uncommon among bovine isolates in the world, whereas both STs are common among human MRSA isolates in Japan. These analytical results and information lead us to guess that bovine MRSA emerged elsewhere than in the dairy environment. In Korea, Kwon et al. reported MRSA isolation from bovine milk and performed genetic analysis of these isolates (26). In this report, all 14 isolates showed ST5-SCC*mec* IVg. As to the origin of these isolates, pediatric clone ST5-MSSA and isolates with ST5-SCC*mec* II were the expected source. In any case, the dominant bovine MRSA isolates CC97 and CC705 observed in the present study have never been confirmed from bovine samples until now. In the first place, MRSA isolation from bovine samples is uncommon; this paper is the first report of MRSA isolation from bovine milk in Japan. However, eight STs of CC97 (ST97, ST458, ST953, ST1174, ST1179, ST1379, ST1419, and ST1476) have been confirmed in human or swine MRSA isolates, and it will therefore be necessary to take measures to prevent the appearance of MRSA strains that may be suitable for the *in vivo* environment of cattle. According to the comparative analysis of genomes and mobile genetic elements between bovine isolate RF122 and MRSA252, horizontal gene transfer (HGT) between the two isolates has been suggested, because RF122 and MRSA252 shared multiple genes in related genomic positions. Moreover, MRSA252 also shared enzyme genes with *Listeria monocytogenes* and *Staphylococcus saprophyticus* plasmid pSSP2 (5). Bovine MSSA strains may also acquire methicillin resistance by HGT from human MRSA or other bacteria. Because of their relatively small size, SCC*mec* types IV and V seem to have higher mobility than the other SCC*mec* types, i.e., I, II, and III (1). This feature may be responsible for these SCC*mec* types being found among community-acquired MRSA isolates worldwide (39). Moreover, SCC*mec* type IV was also detected among four STs of CC97 (ST97, ST953, ST1174, and ST1179). To prevent the coexistence and contact of bovine MSSA and MRSA strains, measures should be taken

to prevent MRSA from spreading to dairy farms. Unauthorized persons and ambulatory patients who can be assumed to be sources of MRSA should be prohibited from entering dairy farms. Moreover, personnel are important sources or carriers of *S. aureus* on dairy farms, and thus hand washing and changing into clean clothing should be enforced as routine control measures (35, 36).

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