NOTE *Bacteriology*

Epidemiological Analysis of *Staphylococcus aureus* Isolated from Cows and the Environment of a Dairy Farm in Japan

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ABSTRACT. A case of *Staphylococcus aureus* intramammary infection (IMI) on a Japanese dairy farm was monitored for 9 months. *S. aureus* isolates from cows and environmental samples consisted of specific strains with sequence types 352 and 705, as determined by multilocus sequence typing. Clonal strains of these sequence types are isolated from cows worldwide, indicating that they are adapted to the bovine environment. These results explain why many IMI cases are persistent and lead to subclinical mastitis. The strain isolated from milk was identical to those isolated from the cows' bodies and cows carrying *S. aureus*, milking units, personnel, heifers and cats in the dairy barn. These locations and factors should be emphasized as sources and routes of strains causing IMI.
KEY WORDS: bovine mastitis, multilocus sequence typing, pulsed-field gel electrophoresis, *spa* typing, *Staphylococcus aureus*.

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The features of bovine mastitis caused by *Staphylococcus aureus* are that it is refractory, persistent, contagious and subclinical in nature, making preemptive care difficult to provide. It has long been suspected that *S. aureus* is an important causal bacterium of bovine mastitis [4]. Although the lactating mammary gland is regarded as the primary reservoir of *S. aureus* in intramammary infection (IMI) [5, 21], the bacterium has also been isolated from the dairy farm environment [17, 23]. Epidemiological studies are necessary to establish effective preventive measures. Genotyping of strains from bovine milk and the environment may provide information to help control and prevent the spread of the *S. aureus* involved in IMI.

Of the many methods for genotyping *S. aureus*, pulsed-field gel electrophoresis (PFGE) is regarded as the gold standard since it has great typability and reproducibility. Although it may be difficult to establish a precise database using PFGE [3], the method has advantages in epidemiological investigations and monitoring of infection outbreaks. Multilocus sequence typing (MLST) and typing of the X-region of protein A gene (*spa*) are also established techniques for which there are websites that contain very useful and abundant resources (http://www.mlst.net/ and http://www.ridom.de/spaserver/) for sharing and analyzing large genotype databases [7, 10]. Therefore, MLST and *spa* typing have advantages for examining and comparing the characteristics of genotypes. We carried out a 9-month epidemiological study of a case of *S. aureus* IMI on a Japanese dairy farm using these 3 genotyping methods.

The farm monitored in the study is located in Chiba Prefecture and houses approximately 50 milking cows in a tie-stall barn. Three staff members are employed at the farm to milk the cows twice daily using 4 milking units. The farm has purchased both cows and heifers. Collection of samples at the dairy farm was carried out a total of 4 times at 3-month intervals from April to December 2004. On each occasion, samples of bovine milk were collected aseptically, in addition to samples from the milking units and the dairy barn environment after milking. Samples from the bodies of the cows and their surroundings were collected before milking. Samples were also collected from all milk tubes and rubber liners in the milking units. Both the environmental and animal body samples were collected by wiping the area with a sterile cotton swab dampened with phosphate buffered saline. Samples of milk, body samples and samples from the surrounding area were collected from a total of 70 cows and their surroundings. Sampling locations and sites were as follows: teat orifices, mammary skin, hooves, abrasions, bedding, feeders and water cups, milking units, gloves and boot soles of the milking personnel, ventilators, dipping holders, aisles, air, nasal and oral mucosa of heifers, paw pads of cats and nasal and oral mucosa of dogs. Cats and dogs were kept as pets in the dairy barn; cats were left loose, and dogs were tethered in the corner of the dairy barn. We collected samples from the cats and dogs to check whether they are carriers of *S. aureus* involved in IMI. Coagulase (Eiken Chemical Co., Ltd., Tokyo, Japan), catalase and Voges-Proskauer test (Eiken)-positive cocci were identified as *S. aureus* by polymerase chain reaction testing for the species-specific 442-bp fragment or by sequencing of the 16S ribosomal RNA gene [6, 11, 16].

Preparation of genomic DNA plugs [13] and the conditions for PFGE [11], MLST [7] and *spa* typing [10] were performed as described previously. The PFGE pulsotypes were analyzed by visual inspection using the BioNumerics software (version 5.10) according to the criteria described previously [19]. Allele number and sequence type (ST) were determined by using the MLST website (http://

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www.mlst.net/). The founder and clonal complex (CC) of each ST were determined by the enhanced version of the Based Upon Related Sequence Types (eBURST) program [9], while numeric *spa* repeats and type codes were determined using the Ridom SpaServer website (http://www.ridom.de/spaserver/).

We collected a total of 234 *S. aureus* isolates from bovine milk and the bodies of the cows, milking units, personnel, bedding, water cup, heifers and cats. All of the isolates were classified as belonging to three pulsotypes (A1, A2 and B) by PFGE. Sixty-five representative isolates were genotyped by MLST and *spa* typing. The isolates with pulsotypes A1 and A2 were shown to be ST352 by MLST and t2844 by *spa* typing, while the isolates with pulsotype B were shown to be ST705 by MLST and t529 by *spa* typing (Fig. 1). According to eBURST, ST352 is a member of CC97, the founder of which is predicted to be ST97, and ST705 is member of CC705, the founder of which is predicted to be ST705. Of the 42 STs in CC97, 30 were found in isolates of bovine origin, with strains containing these STs having been isolated from bovine milk in Brazil, Canada, Chile, Italy, the Netherlands, Norway, Spain, the U.K. and the U.S.A. (Table 1). Isolates in CC705 were only obtained from bovine samples. Seventeen STs found in CC705 strains have previously been reported in isolates in Germany, Ireland, the Netherlands, Spain, the U.K. and the U.S.A. (Table 1). On the other hand, the ratio of STs of bovine isolates in most other CCs does not reach 10%. Moreover, the Canadian bovine strain, Newbould 305, and the Irish sequenced bovine strain, RF122, were shown to be ST115 of CC97 and ST151 of CC705, respectively (Table 1). Newbould 305 was isolated in 1958 from a clinical mastitis case in Canada, so it is likely that CC97 spread as a cause of bovine mastitis in the past [22]. These results and reports appear to support the hypothesis that strains of CC97 and CC705 are adapted to cows. Methicillin-resistant *S. aureus* (MRSA) strains of CC97 have been isolated from swine samples and so on. Many of these strains have been classified as SCC*mec* type IV, which appears to cause higher mobility than other SCC*mec* types [1]. Invasion and spread of SCC*mec* type IV strains in dairy barns may promote appearance of new MRSA strains adapted to cows. We should therefore take to prevent invasion of farms by SCC*mec* type IV strains.

Of the 70 cows tested, 15 were confirmed as carriers of *S. aureus* during the monitoring period. The majority of IMI cases were caused by a single strain, with superinfection by multiple strains not being observed in this study (Table 2). Of the 15 carrier cows, 8 (cows α, β, χ, δ, ε, φ, λ and μ) had persistent IMI for at least 3 months, with the longest cases of IMI persisting for at least 9 months in 2 cows (cow χ and φ). Whether long-term persistent infection is a characteristic of IMI caused by strains CC97 and CC705 is an interesting point for further investigation. Furthermore, many IMI cases resulting from *S. aureus* are subclinical; only two cases of IMI (cow γ and κ) showed clinical symptoms (Table 2). These findings also seem to support the hypothesis that the CC97 and CC705 strains have adapted to cows.

S. aureus was isolated not only from milk but also from the bodies and surrounding environments of the bovine carriers of *S. aureus*. Genotyping confirmed that the isolates from the body surface and environmental samples were identical to the milk isolates (Table 2). Moreover, the isolates from the bulk milk, milking units, hands of the personnel, heifers and cats were also shown to have an identical genotype to that of the isolates from the bovine milk (Table 2). Therefore, these locations and factors in the dairy barn should be recognized as potential sources and routes of infection for *S. aureus* strains involved in IMI. While it is well known that *S. aureus* spreads among cows during milking [20], it was not anticipated that strains involved in IMI would be isolated from heifers and pets in the dairy barn. Heifers at the monitored farm are routinely fed milk obtained from cows previously infected with *S. aureus*. Feeding of unpasteurized milk and close contact with carriers may be one reason for the spread of *S. aureus* to heifers, with this farming practice possibly leading to heifer mastitis. Furthermore, heifers purchased as replacements seem to be an important causal factor of IMI due to *S. aureus* [19, 23]. According to the MLST website and previous reports, *S. aureus* strains from pets have similar genotypes to those

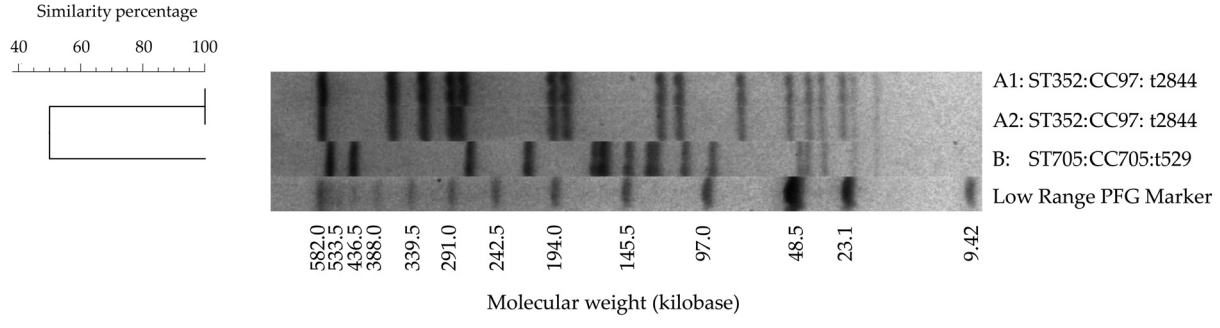

Fig. 1. Dendrogram based on pulsopatterns obtained by pulsed-field gel electrophoresis (PFGE) of *Sma*I-digested total DNA from *S. aureus* isolated from different samples in the dairy farm monitored. The genotype result of each pulsopattern is inscribed in the following order: pulsotype measured by PFGE, sequence type (ST) analyzed by multilocus sequence typing (MLST), clonal complex (CC) determined by MLST and *spa* typing.

Table 1. Information for the individual sequence types (ST) of clonal complexes (CC) 97 and 705

CC	ST	Origin	MSSA/MRSA	SCC*mec* type	References
97	70	Bovine milk (NED)	Unknown		[28], http://www.mlst.net/
97	71	Bovine milk (NED), unknown (FRA)	MSSA or unknown		[14, 28], http://www.mlst.net/
97	97	Bovine milk (BRA, CHI, ESP, ITA, JPN, NED, NOR), human (ITA, GBR), unknown (CAN, GRC, NED, GBR, GER, TUR)	MSSA, MRSA or unknown	IV	[8, 14, 15, 24, 26], http://www.mlst.net/
97	115	Bovine milk (CAN, USA), unknown (CAN)	Unknown		[26], http://www.mlst.net/
97	116	Bovine milk, unknown (GBR)	Unknown		[25], http://www.mlst.net/
97	117	Bovine milk, unknown (GBR)	Unknown		[25], http://www.mlst.net/
97	118	Bovine milk, unknown (GBR)	Unknown		[25], http://www.mlst.net/
97	124	Bovine milk (JPN, NED, USA), unknown (USA)	MSSA or unknown		[14, 26], http://www.mlst.net/
97	205	Unknown (GBR)	Unknown		http://www.mlst.net/
97	347	Bovine milk, unknown (USA)	Unknown		[26], http://www.mlst.net/
97	349	Bovine milk, unknown (USA)	Unknown		[26], http://www.mlst.net/
97	352	Bovine milk (ESP, JPN, NED, USA), unknown (USA)	MSSA or unknown		[26], http://www.mlst.net/, This paper
97	355	Bovine milk, unknown (CHI)	Unknown		[26], http://www.mlst.net/
97	358	Bovine milk, unknown (CHI)	Unknown		[26], http://www.mlst.net/
97	458	Unknown (CAN)	MRSA	Unknown	http://www.mlst.net/
97	742	Bovine milk (BRA)	MSSA		[24], http://www.mlst.net/
97	746	Bovine milk (BRA)	MSSA		[24], http://www.mlst.net/
97	747	Bovine milk (BRA), ostrich anus (BRA)	MSSA		[2, 24], http://www.mlst.net/
97	842	Unknown (GBR)	MSSA		http://www.mlst.net/
97	953	Unknown (AUS)	MRSA	IVa	http://www.mlst.net/
97	987	Unknown (USA)	MSSA		http://www.mlst.net/
97	1060	Unknown (NOR)	MSSA or unknown		http://www.mlst.net/
97	1072	Bovine milk (GBR)	Unknown		http://www.mlst.net/
97	1077	Bovine milk (GBR)	Unknown		http://www.mlst.net/
97	1119	Bovine milk (NED)	MSSA		[14], http://www.mlst.net/
97	1125	Bovine milk (NED)	MSSA		[14], http://www.mlst.net/
97	1126	Bovine milk (NED)	MSSA		[14], http://www.mlst.net/
97	1127	Bovine milk (NED)	MSSA		[14], http://www.mlst.net/
97	1128	Bovine milk (NED)	MSSA		[14], http://www.mlst.net/
97	1129	Bovine milk (NED)	MSSA		[14], http://www.mlst.net/
97	1174	Unknown (AUS)	MRSA	IVa	http://www.mlst.net/
97	1179	Unknown (MYS)	MRSA	IV	http://www.mlst.net/
97	1356	Unknown (NOR)	Unknown		http://www.mlst.net/
97	1366	Bovine milk (JPN)	MSSA		http://www.mlst.net/
97	1367	Bovine milk (JPN)	MSSA		http://www.mlst.net/
97	1379	Swine nose (ESP)	MRSA	Unknown	http://www.mlst.net/
97	1419	Unknown (CHN)	MRSA	Unknown	http://www.mlst.net/
97	1476	Swine (ITA)	MRSA	Unknown	http://www.mlst.net/
97	1527	Bovine milk (GBR)	Unknown		http://www.mlst.net/
97	1615	Bovine milk (ITA)	MSSA		http://www.mlst.net/
97	1623	Bovine milk (BRA)	MSSA		http://www.mlst.net/
97	1624	Bovine milk (BRA)	MSSA		http://www.mlst.net/
705	151	Bovine milk (ESP, GBR, IRL, NED), bovine invasive disease (GBR)	MSSA or Unknown		[12, 26], http://www.mlst.net/
705	351	Bovine milk, unknown (USA)	Unknown		[26], http://www.mlst.net/
705	504	Bovine milk (NED)	MSSA or unknown		[14], http://www.mlst.net/
705	705	Bovine milk (JPN, NED)	MSSA		http://www.mlst.net/, This paper
705	1074	Bovine milk (GBR)	Unknown		http://www.mlst.net/
705	1076	Bovine milk (GBR)	Unknown		http://www.mlst.net/
705	1078	Bovine milk (GBR)	Unknown		http://www.mlst.net/
705	1122	Bovine milk (NED)	Unknown		[14]
705	1123	Bovine milk (NED)	MSSA		[14], http://www.mlst.net/
705	1124	Bovine milk (NED)	MSSA		[14], http://www.mlst.net/
705	1230	Bovine milk (NED)	MSSA		http://www.mlst.net/
705	1248	Bovine milk (GBR)	MSSA		http://www.mlst.net/
705	1274	Bovine milk (GER)	MSSA		http://www.mlst.net/
705	1363	Bovine milk (JPN)	MSSA		http://www.mlst.net/
705	1364	Bovine milk (JPN)	MSSA		http://www.mlst.net/
705	1365	Bovine milk (JPN)	MSSA		http://www.mlst.net/
705	1520	Bovine milk (JPN)	Unknown		http://www.mlst.net/

Country abbreviations: AUS, Australia; BRA, Brazil; CAN, Canada; CHI, Chile; CHN, China; ESP, Spain; FRA, France; GBR, United Kingdom; GER, Germany; GRC, Greece; IRL, Ireland; ITA, Italy; JPN, Japan; MYS, Malaysia; NED, The Netherlands; NOR, Norway; TUR, Turkey; USA, United States. *S. aureus* strain Newbould 305 from clinical bovine mastitis in Canada was shown to be ST115 (http://www.mlst.net/), and sequenced Irish bovine strain RF122 was shown to be ST151 [12].

Table 2. Pulsotypes and sequence types (ST) detected in the cows and environmental samples at the dairy farm

Cow, heifer and milking unit	Origin	Pulsotypes and STs recoverd at month of visit			
		1	2	3	4
Cow α	Milk	A2: ST352	A2: ST352	Cull	
	Mammary skin	A2: ST352	*	Cull	
Cow β	Milk	A2: ST352	A2: ST352	A2: ST352	*
	Teat orifice	*	*	A2: ST352	*
	Mammary skin	*	*	*	A2: ST352
	Bedding	*	*	A2: ST352	*
Cow χ	Milk	A2: ST352	A2: ST352	A2: ST352	A2: ST352
	Teat orifice	A2: ST352	*	*	*
Cow δ	Milk	A2: ST352	A1/A2: ST352	A2: ST352	Cull
	Teat orifice	*	A2: ST352	A2: ST352	Cull
	Mammary skin	*	*	A2: ST352	Cull
	Bedding	*	*	A2: ST352	Cull
Cow ε	Milk	A2: ST352	A2: ST352	*	*
	Water cup	*	*	*	A2: ST352
Cow ϕ	Milk	A2: ST352	A2: ST352	A2: ST352	A2: ST352
	Teat orifice	*	*	*	A2: ST352
	Mammary skin	*	A2: ST352	*	*
Cow γ	Milk	–	A2: ST352, CM	*	Dry
	Teat orifice	–	*	A2: ST352	Dry
	Mammary skin	–	*	A2: ST352	Dry
Cow η	Milk	Dry	A2: ST352	Cull	
Cow ι	Milk	*	A2: ST352	Cull	
Cow φ	Milk	*	B: ST705	Dry	*
Cow κ	Milk	–	*	A2: ST352, CM	–
Cow λ	Milk	–	*	A2: ST352	A2: ST352
Cow μ	Milk	–	*	A2: ST352	A2: ST352
Cow ν	Milk	*	*	Dry	A2: ST352
Cow ο	Milk	*	*	*	A2: ST352
	Bulk Milk	A2: ST352	A2: ST352	A2: ST352	A2: ST352
Milking unit π	Milking unit	*	A2: ST352	A2: ST352	A2: ST352
Milking unit θ		*	A2: ST352	A2: ST352	A2: ST352
Milking unit ρ		A2: ST352	*	*	A2: ST352
Milking unit σ		A2: ST352	A2: ST352	A2: ST352	A2: ST352
	Personnel hands	A2: ST352	*	*	*
Heifer τ	Nasal and oral	A2: ST352	*	*	A2: ST352
Heifer υ	Cavity of heifer	*	*	*	A2: ST352
	Foot pads of cats	*	*	A2: ST352	*

Abbreviations and symbols: Cull, culled cow; Dry, dry period; CM, clinical mastitis case; *, No *S. aureus* isolated; –, No sample collected.

of human isolates [27]. ST1332 and ST1441 are STs that have been found in isolates obtained from cats in Japan, and their clonal strains, CC15, are often confirmed in human isolates. Furthermore, isolates from cats in Berlin contained ST22 and SCC*mec*IV, which have an identical genotype to that of epidemic strains of methicillin-resistant *S. aureus*-15 (EMRSA-15), an MRSA strain clonally spread among hospitals in that area [8, 27]. These reports support the concept that cats may easily carry *S. aureus* from other origins.

Specific *S. aureus* strains were confirmed to cause long-term IMI in the present study. We speculate that these strains have adapted to cows, so that IMI caused by these strains will cause mild symptoms, be persistent and spread easily. Immediate identification and elimination of these strains will be very difficult. Therefore, it is necessary to introduce farming practices that prevent invasion and spread

of *S. aureus*. In Japan, the majority of dairy farms purchase lactating cows and heifers as replacements or for expansion from the resale market or rearing farms. Middleton *et al.* reported that import of cattle leads to a higher prevalence of *S. aureus* mastitis and more invasion of new *S. aureus* strains into herds [18]. We should aim at management that includes little transfer of cows and heifers between farms. Furthermore, close contact with carrier cows and their surroundings, feeding milk obtained from *S. aureus* carriers and allowing pets in dairy barns should be avoided in order to prevent spread of *S. aureus* in these areas.

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