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REGULAR ARTICLES

Prevalence and characterization of *Staphylococcus aureus* isolated in raw milk from cows in Hokkaido, Japan

Sukanya Thongratsakul[1] · Masaru Usui[2] · Hidetoshi Higuchi[3] · Toshihiko Takahashi[4] · Tomomi Sato[2] · Chaithep Poolkhet[1] · Yutaka Tamura[2]

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Abstract

The aim of this study was to characterize the phenotypes and genotypes of *Staphylococcus aureus* isolated from raw bovine milk in Hokkaido, Japan. *S. aureus* isolates were identified in 135 of 436 milk samples from cows with and without signs of mastitis from three farms in Hokkaido. These clinical isolates were characterized for antimicrobial susceptibility patterns, molecular typing using phage-open-reading frame typing (POT), coagulase gene type, virulence genes, and biofilm-associated genes and were evaluated for biofilm-forming ability. Most isolates were susceptible to the antimicrobial agents tested. The highest rate of resistance was to ampicillin. Molecular typing of all *S. aureus* isolates indicated a predominance of coagulase type VI and 0–17–34 POT type, and virulence genes were highly prevalent in the isolates from all farms. Moreover, a high percentage of the 0-17–34 POT type isolates showed extensive formation of biofilm. These findings will help veterinarians and farmers to understand the epidemiology of *S. aureus* so that they can monitor the transmission and spread of this pathogen and control it more effectively.

Keywords Bovine mastitis · Antimicrobial resistance · Toxins · Biofilm · *Staphylococcus aureus*

Introduction

Bovine mastitis is a disease that causes substantial economic losses worldwide and can be classified as clinical or subclinical. Dairy cows with clinical mastitis show severe clinical signs (abnormal udder size and shape, abnormal milk secretion, and reduced milk production), whereas subclinical mastitis may not show any signs or symptoms and cause no visible changes in the milk (Seegers et al. 2003; Sinha et al. 2014). *Staphylococcus aureus* is the most common causative organism in bovine mastitis and can produce toxins that affect both animal and human health (Srinivasan et al. 2006). *S. aureus* produces a number of toxins that have public health implications, such as staphylococcal enterotoxin, which causes food poisoning, and toxic shock syndrome toxin; these toxins are often detected in *S. aureus* isolated from cows with bovine mastitis (Srinivasan et al. 2006). Such toxins may adversely affect consumers of food that has been contaminated during processing, such as use of raw material contaminated with the enterotoxin or poor hygiene practice.

S. aureus is a leading cause of bovine mastitis and can produce many types of cytolytic toxins, including hemolysin (induced by the *hla*, *hlb*, *hld*, and *hlg* genes) and leukocidin. Leukocidin can lyse white blood cells, particularly when induced by the *LukDE* and *LukM* genes (Vrieling et al. 2016). Virulent, gene-encoding, secreted leukocidin toxins are associated with the neutrophil-killing agent secreted in bovine mastitis and cause tissue necrosis by damaging the mammary epithelial cells in the udder (Vrieling et al. 2016). Moreover, leukocidin is an important toxin in cell-cell interactions and in the formation of an insoluble nucleoprotein matrix during formation of biofilm (Gogoi-Tiwari et al. 2017).

✉ Sukanya Thongratsakul
fvetskt@ku.ac.th

[1] Department of Veterinary Public Health, Faculty of Veterinary Medicine, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, Thailand

[2] Laboratory of Food Microbiology and Food Safety, Department of Health and Environmental Sciences, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido, Japan

[3] Laboratory of Animal Health, Department of Health and Environmental Sciences, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido, Japan

[4] Livestock Hygiene, Department of Sustainable Agriculture, College of Agriculture, Rakuno Gakuen University, Ebetsu, Hokkaido, Japan

Formation of biofilm is one of the antimicrobial resistance mechanisms used by *S. aureus*. This is an important characteristic of *S. aureus* and protects bacteria from the effects of antimicrobial agents, resulting in chronic infection in cows with mastitis. A biofilm is a cluster of bacterial cells that includes the extracellular matrix and water. The extracellular matrix is formed by intercellular adhesion of polysaccharides, which are synthesized at the locus of the *ica* gene. Other surface proteins that are also crucial in the formation of biofilm include biofilm-associated protein (Bap) (Latasa et al. 2006). Bap and polysaccharides are important in the intercellular adhesion step during formation of biofilm, which leads to increased microbial colonization and enhanced adhesion in the mammary gland (Cucarella et al. 2001).

Administration of an antimicrobial agent is important for the control and treatment of clinical and subclinical bovine mastitis. Therefore, the antimicrobial susceptibility of *S. aureus* is one of the factors used to determine the appropriate treatment for mastitis. The beta-lactam group of antimicrobials is widely used in the treatment of bovine mastitis. Similarly, penicillin is the drug of choice for treatment of *S. aureus* in human medicine; however, increased resistance to penicillin is now being reported (Rayner and Munckhof, 2005; Baba et al. 2012; Aslantas and Demir, 2016). Methicillin is a member of the beta-lactam group and is widely used to treat *S. aureus*, and bacteria that are resistant to methicillin are known as methicillin-resistant *S. aureus* (MRSA). Bovine mastitis caused by MRSA is resistant to most antimicrobial agents. Previous cases of bovine mastitis caused by MRSA have been of great concern in both human and animal health care systems and caused outbreaks in Japan and elsewhere (Wang et al. 2015; Hata, 2016; Guimaraes et al. 2017). The relationship between biofilm-forming ability and the genotype of *S. aureus* identified in raw bovine milk has not been well studied in Japan. The purpose of this study was to investigate the prevalence and phenotypic and genotypic characteristics of *S. aureus*, including MRSA, isolated in milk from cows with and without mastitis in Hokkaido, Japan.

Materials and methods

Sample collection and isolation of *S. aureus*

A cross-sectional study was performed in 436 raw milk samples collected from cows that were apparently healthy and cows with clinical mastitis on three farms in Hokkaido during 2016–2017. Farm A ($n = 124$) and farm B ($n = 254$) were private farms, and farm C ($n = 58$) was owned by a university. Raw milk samples were collected from more than 80% of the cows on each farm during routine farm service programs run by the Animal Health Laboratory, Department of Health and Environmental Sciences, School of Veterinary Medicine, Rakuno Gakuen University. All milk samples were collected into a sterile tube after cleaning the surface of the udder and teat using cotton swabs soaked in 75% alcohol and kept cool in an ice box while being transported to the laboratory for analysis. At the laboratory, the samples were cultured in tryptic soy broth (with 6% NaCl), incubated overnight at 37 °C, and then streaked onto Baird-Parker agar. After incubation at 37 °C for a second night, typical and suspected colonies of *S. aureus* were chosen for confirmation using the femA gene (Paule et al. 2004).

Antimicrobial susceptibility testing

An antimicrobial susceptibility test was conducted using the agar dilution method following the guidelines of the Clinical and Laboratory Standard Institute (CLSI, 2015). Briefly, serial twofold dilutions of a number of antibiotic agents were prepared in Mueller-Hinton agar for testing at concentrations of 0.125–512 μg/ml. The following antimicrobial agents sourced from Sigma-Aldrich (St. Louis, MO, USA) were tested: ampicillin (AMP), oxacillin (OXA), cefazolin (CEZ), enrofloxacin (ENR), ciprofloxacin (CIP), gentamicin (GEN), kanamycin (KAN), vancomycin (VAN), and erythromycin (ERY). Tetracycline (TET) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Reference strains of *S. aureus* (ATCC 25913) and *Enterococcus faecalis* (ATCC 25212) were used as quality controls. The breakpoints of these antimicrobial agents were determined according to the interpretation criteria of the Clinical and Laboratory Standard Institute (CLSI, 2015).

Molecular analysis

Genotyping by the POT and coagulase gene methods

A commercial multiplex polymerase chain reaction-based POT kit (Cica Geneus Staph POT, Kanto, Tokyo, Japan) was used for genomic characterization of *S. aureus*. In brief, this POT kit can detect the presence or absence of 22 target genes of *S. aureus*, and the results of typing are expressed as a triple POT score (Suzuki et al. 2006). The coagulase gene is one of the virulence factors in *S. aureus* and contributes to its pathogenicity. A coagulase typing (Cica Geneus Staph Coagulase Detection) kit was used for coagulase gene typing to investigate *coa* polymorphism.

Detection of virulence and biofilm-associated genes

The virulence genes that are important in human and animal health are the classical staphylococcal enterotoxins (*sea* to *see*), toxic shock syndrome toxin-1 (TSST-1) (Mehrotra et al. 2000), exfoliative toxin A (ETA; *eta*), exfoliative toxin B (ETB; *etb*), and leukocidin (*LukDE* and *LukM*) (Jarraud

et al. 2002). The presence of these genes and biofilm formation-associated genes (*ica* and *bap*), which are associated with increased severity of mastitis (Cucarella et al. 2004), was evaluated by polymerase chain reaction.

Biofilm-forming assay

A microtiter plate biofilm assay (Merritt et al. 2005) was used to investigate the biofilm-forming ability of the *S. aureus* strains isolated from the apparently healthy cows and those with clinical mastitis. In brief, the microtiter plate biofilm assay detects the ability of *S. aureus* to form a biofilm on a 96-well plate. After incubation overnight, the biofilms are stained using crystal violet dye and measured using spectrophotometry. All isolates were tested four times and the results were averaged. Samples of medium that was not inoculated were used as a negative control. The optical density (OD) of the microplate biofilm assay was measured at 595 nm using a spectrophotometer that reads 96-well plates (Sunrise Rainbow Thermo, Tecan Group Ltd., Männedorf, Switzerland). The cut point for the OD of biofilm production was identified using the classification devised by Christensen et al. (1985). All strains were classified into four categories: $OD_i \leq OD_C$ = non-adherent; $OD_C < OD_i \leq 2 \times OD_C$ = weakly adherent; $2 \times OD_C < OD_i \leq 4 \times OD_C$ = moderately adherent; and $4 \times OD_C < OD_i$ = strongly adherent (Yousefi et al. 2016).

Results

Positive ratio of *S. aureus* and characterization of antibiotic susceptibility testing

There was a detectable prevalence of *S. aureus* (28.2%, 123/436) in all samples (farm A, 31.5%, 39/124; farm B, 32.3%, 82/254; farm C, 3.4%, 2/58). One hundred and thirty-five isolates from the 123 positive samples were characterized as antibiotic-susceptible. Most *S. aureus* isolates were susceptible to the antimicrobial agents tested, and the highest resistance was to AMP (farm A, 76.1%; farm B, 89.7%). The next highest resistance rates were for OXA, CEZ, GEN, KAN, and ENR (2.2%) on farm A and for CEZ (1.1%) on farm B. One isolate showed multiple drug resistance (to AMP, OXA, CEZ, and KAN). No samples of *S. aureus* from farm C were resistant to antimicrobial agents (Table 1).

Molecular analysis

Results of genotyping by the POT and coagulase gene methods

The POT method could classify all of the *S. aureus* isolates in this study into six genotypic patterns, with 100% of the 0-17-34 POT type on farms A and C. There were six genotypes of *S. aureus* isolates on farm B and the POT type on farm B (Table 2). The most frequent type of POT on farm B was 0-17-34, with 52 isolates (59.8%, 52/87), followed by 0-1-0 (26.4%, 23/87) and 6-0-45 (6.9%, 6/87). Only the *coa* type VI genotype pattern was isolated from all *S. aureus* isolates on all farms.

Detection of virulence genes

One isolate was positive for the *seb* gene, and three were positive for the *sec* gene and co-harboring with the *tsst* gene. None of the *S. aureus* isolates from farm A or farm C were positive for the enterotoxin gene. Leukocidin (*LukDE* and *LukM* genes) was found in 100% (135/135) and 84.4% (114/135) of samples, respectively. Hemolysins (*hla* and *hld* genes) were found in 100% (135/135) and *hlb* was found in 95.6% (129/135) of the samples. No samples were positive for exfoliative toxin (*eta, etb*) or the *hlg* toxin genes. The percentages of toxin gene profile typing of *S. aureus* isolated from farms A, B, and C are shown in Table 2. No isolates tested were positive for MRSA.

Detection of biofilm-associated *ica* and *bap* genes and biofilm-forming assay

The detection rates for the biofilm-associated *ica* gene were 100.0% (46/46), 88.5% (77/87), and 100% (2/2) on farms A, B, and C, respectively. The isolates were positive for the *ica* gene in 92.6% (125/135) of cases and negative in 7.4% (10/135). None of the isolates was positive for the *bap* gene. *S. aureus* isolates on farm A were classified as strong biofilm producers (89.1%, 41/46) and those on farm B were classified as strong (16.1%, 14/87), moderate (5.7%, 5/87), weak (27.6%, 24/87), or not (50.6%, 44/87) biofilm producers. Two isolates from farm C were shown not to produce biofilm (100%, 2/2). The characteristics of the POT type are compared according to biofilm formation in all samples in Fig. 1.

Discussion

This study investigated the ratios, antimicrobial susceptibility, and genotypic characteristics of *S. aureus* in raw bovine milk in Hokkaido, Japan, using POT, the toxin profile, and biofilm-forming ability. The important finding of antimicrobial susceptibility testing in this study was the high rate of resistance to AMP in the samples. AMP is a member of the beta-lactam or penicillin group of antimicrobials, which are the drugs most commonly used to treat bovine mastitis (Pol and Ruegg, 2007). However, several reports from Japan and a number of other countries suggest that members of the penicillin group are the antibiotics

Table 1 Prevalence and antimicrobial susceptibility of *Staphylococcus aureus* isolated from raw bovine milk in Hokkaido, Japan

Farm	Positive samples, n	Positive isolates, n	Susceptibility interpretation*	Percentage antimicrobial agents									
				AMP	OXA	CEZ	GEN	KAN	ENR	CIP	TET	ERY	VAN
A ($n = 124$)	39 (31.5%)	46 (37.1%)	S	23.9	97.8	97.8	97.8	97.8	95.6	100	100	97.8	100
			I	0	0	0	0	0	2.2	0	0	2.2	0
			R	76.1	2.2	2.2	2.2	2.2	2.2	0	0	0	0
B ($n = 254$)	82 (32.3%)	87 (34.2%)	S	10.3	100	98.9	100	98.9	100	98.9	100	100	95.4
			I	0	0	0	0	1.1	0	1.1	0	0	4.6
			R	89.7	0	1.1	0	0	0	0	0	0	0
C ($n = 58$)	2 (3.4%)	2 (3.4%)	S	100	100	100	100	100	100	100	100	100	100
			I	0	0	0	0	0	0	0	0	0	0
			R	0	0	0	0	0	0	0	0	0	0

*Break point according to CLSI 2015 (*R* resistant, *I* intermediate, *S* susceptible). *AMP* ampicillin, *CEZ* cefazolin, *CIP* ciprofloxacin, *ENR* enrofloxacin, *ERY* erythromycin, *GEN* gentamicin, *KAN* kanamycin, *OXA* oxacillin, *TET* tetracycline, *VAN* vancomycin

most frequently resistant to *S. aureus* isolated in milk samples from cows with bovine mastitis, and our findings are consistent with these reports (Pitkälä et al. 2004; Rayner and Munckhof, 2005; Baba et al. 2012; Aslantas and Demir, 2016). The results of phenotypic testing in our study suggest that other antimicrobial agents, such as OXA, CEZ, ENR, CIP, GEN, KAN, VAN, ERY, and TET, can be used to control *S. aureus* infection, which is consistent with another report (Pitkälä et al. 2004). The results of antimicrobial susceptibility testing in this study can be used to predict the likelihood of *S. aureus* infection being cured in cows with mastitis and to assist veterinarians when selecting antibiotic therapy for this condition. *S. aureus* isolates detected in routine practice should also be monitored for resistance to beta-lactam agents (Barkema et al. 2006).

In this study, all *S. aureus* isolates were type VI, which is consistent with previous reports on the *coa* gene in milk from cows with mastitis in Japan, indicating that type VI is a common causative organism in cases of mastitis (Nagase et al. 2002; Hata, 2016). These studies indicate that *coa* typing is not sufficient for genomic classification of *S. aureus* derived from bovine milk in Japan because all isolates showed this type, whereas there were several types

Table 2 Genomic characterization of *Staphylococcus aureus* isolated from raw bovine milk in Hokkaido, Japan

Farm	POT type*	Positive isolates, n	Enterotoxin, %	Toxic shock syndrome, %	Leukocidin, %		Hemolysin, %			
					LukDE	*LukM*	*hla*	*hlb*	*hld*	*hlg*
A ($n = 46$)	0–17-34	46 (100%)	0	0	100 (46/46)	100 (46/46)	100 (46/46)	100 (46/46)	100% (46/46)	0
B ($n = 87$)	0–1-0	23 (26.4%)	0	0	30.4 (7/23)	100 (23/23)	100 (23/23)	95.6 (22/23)	100 (23/23)	0
	0–1-2	1 (1.1%)	0	0	0.0	100 (1/1)	100 (1/1)	0	100 (1/1)	0
	0–17-32	4 (4.6%)	0	0	75 (3/4)	100 (4/4)	100 (4/4)	75 (3/4)	100 (4/4)	0
	0–17-34	52 (59.8%)	0	0	96.1 (50/52)	100 (52/52)	100 (52/52)	96.1 (50/52)	100 (52/52)	0
	2–17-0	1 (1.1%)	100% (1/1)	0	0	100 (1/1)	100 (1/1)	0	100 (1/1)	0
	6–0-45	6 (6.9%)	50.0% (3/6)	50.0% (3/6)	100 (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	0
C ($n = 2$)	0–17-34	2 (100%)	0	0	100 (2/2)	100 (2/2)	100 (2/2)	100 (2/2)	100 (2/2)	0
Total					135/135	114/135	135/135	129/135	135/135	0

POT phage-open-reading frame typing

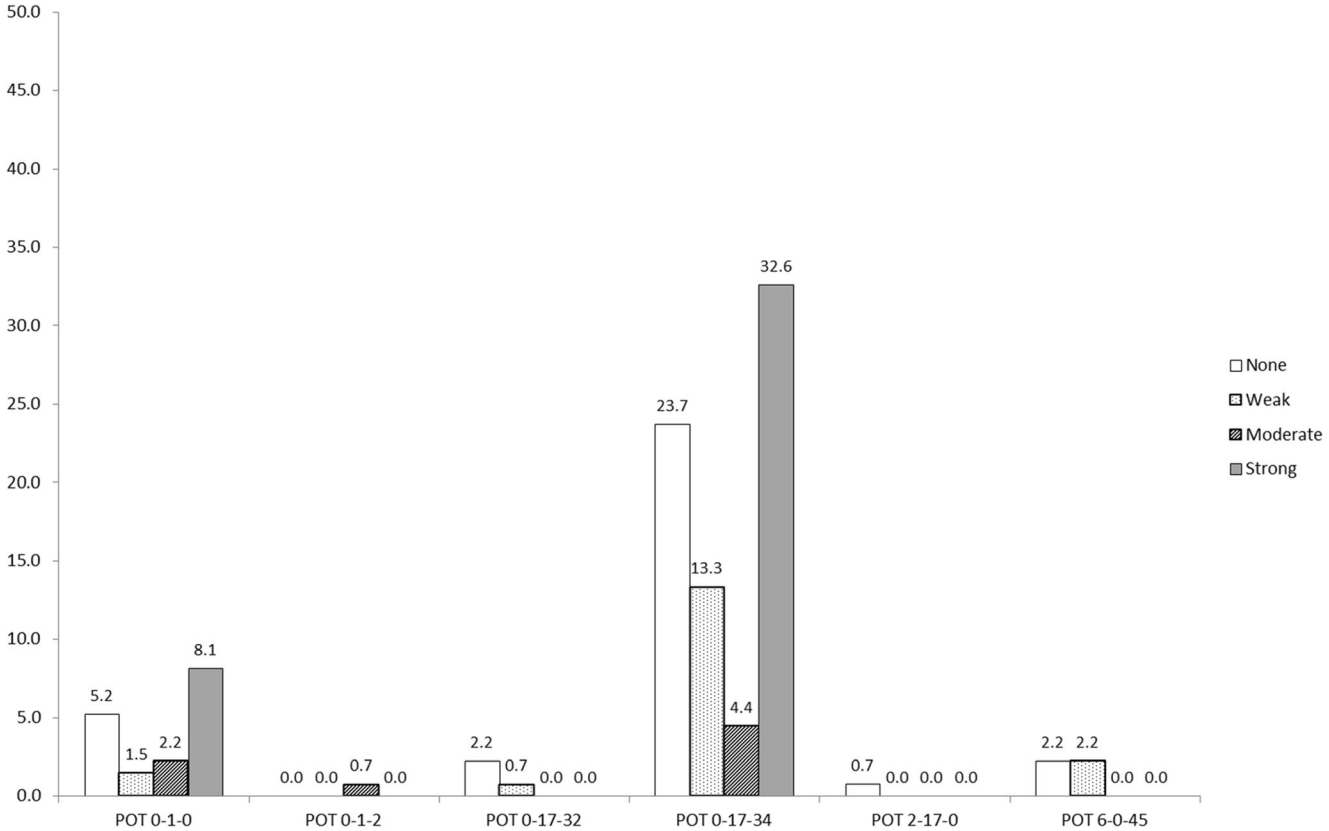

Fig. 1 Percentage of biofilm formation (none, weak, moderate, or strong) in different POT types from *Staphylococcus aureus*. *POT* phage-open-reading frame typing

of POT. We found that 0–17-34 was the most prevalent type of POT on all farms and showed a high percentage of leukocidin and hemolysin genes and strong formation of biofilm (32.6%). The high prevalence of leukocidin and hemolysin genes in this study is in line with that in a previous study of isolates from bovine and other domestic animals (Yamada et al. 2005). Leukocidin and hemolysin genes are located on a chromosome that potentially makes it easy for horizontal transmission to occur via bacteriophages in *S. aureus* (Yamada et al. 2005). Moreover, *sec*-producing strains are frequently found in milk from cows with mastitis in Japan and other countries (Nagase et al. 2002). The present study identified three isolates of POT type 6–0-45 that were *sec*-producing strains and co-harboring the *tsst* gene, which is again consistent with previous findings (Nagase et al. 2002). Furthermore, it has been suggested that *sec* promotes inflammation and chronic infection in the presence of mastitis (Ferens et al. 1998). However, all of the *S. aureus*-producing enterotoxin and toxic shock syndrome genes came from farm B. This indicates that a critical aspect for controlling the spread of virulent bacteria may be to limit the introduction of animals from high-risk sites. POT is useful in epidemiologic studies because of its rapid and highly discriminatory power (Nada et al. 2009). Moreover, other typing techniques, such as multilocus sequence typing, should also be used in genomic studies in order to understand the evolution and spread of resistant *S. aureus* strains and their role in build-up of biofilm (Bentley and Parkhill, 2015).

Conclusions

The findings of the present study suggest a considerable prevalence of *S. aureus* in raw bovine milk in Japan. Generally, different rates of *S. aureus* infection reflect differences in biosecurity measures, farm practices, and geographic locations. Furthermore, *S. aureus* strains that produce biofilm may cause mastitis to spread on farms and build up antimicrobial resistance because of treatment failure. In this study, the incidence of *S. aureus* resistance on farms A and B (both private farms) differed markedly from that on farm C (a university farm). An understanding of the differences in animal husbandry and biosecurity measures in place between privately owned farms, and government-funded farms may provide options for management of mastitis and for preventing its spread.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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