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Differences in Virulence Genes and Genome Patterns of Mastitis-Associated *Staphylococcus aureus* Among Goat, Cow, and Human Isolates in Taiwan

Chishih Chu,[1] Yajiun Wei,[2] Shih-Te Chuang,[3] Changyou Yu,[2] Chih-Hsuan Changchien,[4] and Yaochi Su[2]

Abstract

A total of 117 mastitis-associated *Staphylococcus aureus* isolates from cow, goat, and human patients were analyzed for differences in antibiotic susceptibility, virulence genes, and genotypes using accessory gene regulator (*agr*) typing, pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). Multidrug-resistant (MDR) *S. aureus* were commonly found in all sources, though they were predominantly found in human and goat isolates. Almost 70% of the isolates were resistant to ampicillin and penicillin. Host-associated virulence genes were identified as follows: *tst*, a gene encoding toxic shock syndrome toxin, was found in goat isolates; *lukED* and *lukM,* genes encoding leukocidin, found in cow isolates; *lukPV*, a gene encoding leukocidin, found in human isolates; and *eta*, a gene encoding for exfoliative toxin, found in both human and cow isolates. All four types of hemolysin, α, β, γ, and δ, were identified in human isolates, three types (α, γ, and δ), were identified in cow isolates, and two types (α and δ) were identified in goat isolates. *Agr*-typing determined *agr*1 to be the main subtype in human and cow isolates. PFGE and MLST analysis revealed the presence of diverse genotypes among the three sources. In conclusion, mastitis-associated, genetically diverse strains of MDR *S. aureus* differed in virulence genes among human, cow, and goat isolates.

Introduction

Staphylococcus aureus is an opportunistic zoonotic pathogen that causes mastitis in cattle and dermatitis, pneumonia, septicemia, osteomyelitis, and meningitis in humans and swine (Quinn *et al*., 2000). *S. aureus* tends to infect humans through contaminated foods (Phillips *et al*., 2004). Antibiotics are routinely administered to food-producing animals for disease prevention and growth enhancement, which has led to the development of antimicrobial-resistant bacteria (Barber *et al*., 2003). Recently, the increase in prevalence of multidrug resistant (MDR) *S. aureus* has affected our ability to control *S. aureus* infections in humans and animals (Enright, 2003; Spohr *et al*., 2011; Kreausukon *et al*., 2012).

Since the discovery of methicillin-resistant bacteria in 1941 (Barrett *et al*., 1968), it has been shown that methicillin-resistant *S. aureus* (MRSA) isolates emerge from methicillin-susceptible *S. aureus* isolates via introduction of SCC*mec* elements, including the *mecA* gene (Jarraud *et al*., 2002). Methicillin-resistant *S. aureus* ST398/CC398, which originates from livestock, has recently increased in prevalence in many countries (Lewis *et al*., 2008). As a zoonotic pathogen, MRSA can cause mastitis in cows (Devriese *et al*., 1972) and accounts for 53–83% of *S. aureus* isolated from hospitals in Taiwan (Hsueh *et al*., 2001). Priority by prime characterized MDR *S. aureus* and MRSA isolates from dairy goats in 2006–2008 (Chu *et al*., 2010; Chu *et al*., 2012). The emergence of MDR MRSA has becomes a public health problem of prime concern.

Though it is normally a species of commensal bacteria found in nasopharyngeal mucosa and on the skin of humans and animals, *S. aureus* can become pathogenic through acquisition of virulence factors (e.g., toxins) that damage the host cell (Shopsin *et al*., 1999). *S. aureus* can cause a variety of symptoms in the host through various virulence factors, including toxic shock syndrome toxin (TSST), which stimulates the massive release of multiple cytokines resulting in toxic shock syndrome; exfoliative toxin, which causes skin abnormalities; and leukocidin and hemolysins, which destroy both leukocytes and erythrocytes. As a regulatory protein, accessory gene regulator (*agr*) can regulate the expression of cell surface proteins and extracellular virulence factors (Moodley *et al*., 2006). Previous studies that analyzed *agr* types found

[1]Department of Microbiology, Immunology, and Biopharmaceuticals and [2]Department of Veterinary Medicine, National Chiayi University, Chiayi, Taiwan, China.
[3]Department of Veterinary Medicine, National Chung Hsing University, Taichung, Taiwan, China.
[4]Department of Plastic and Reconstructive Surgery, Chiayi Christian Hospital, Taiwan, China.

that *agr1* was associated with mastitis in cows (Buzzola *et al.*, 2007) and *agr* type IV was associated with skin infection in humans (Garbacz *et al.*, 2009).

S. aureus belongs to a genetically diverse group of bacteria. The genomic variations of *S. aureus* and the traceability of outbreak isolates from humans and several animal species have been investigated using various methods, such as multilocus sequence typing (MLST), ribotyping, pulsed-field gel electrophoresis (PFGE), and staphylococcal protein A typing (Melles *et al.*, 2007; Moneke *et al.*, 2007; Milheirico *et al.*, 2011). Of these methods, PFGE and MLST generally provide the most reliable information for phylogenetic analysis of *S. aureus* isolates (Feil *et al.*, 2003; Turner and Feil, 2007). In this study, we investigated the differences in genomic patterns, virulence genes, and antimicrobial resistance of 117 mastitis-associated *S. aureus* isolates from humans, cows, and goats.

Methods

Samples and isolates

Twenty herds of dairy cows and 20 dairy goat farms were randomly selected from Taichung, Chunghua, Yunlin, Chiayi, and Tainan counties from January 2006 to December 2007. Milk sample collection was performed using the method of the National Mastitis Council, with some modifications, under aseptic conditions (Hogan *et al.*, 1999). A total of 1372 cows and 3427 goats milk samples were randomly collected. For each sample, 1 mL out of a 10-mL milk sample was mixed with 9 mL of tryptic soy broth and then incubated for 8 to 12 h at 37°C. *Staphylococcus aureus* isolates were initially identified by colony shape, colony hemolytic type, Gram stain, and coagulase test and further confirmed by the Analytical Profile Index method (Bio-Merieux, France). A total of 101 *S. aureus* isolates were identified from the milk of cows and goats with mastitis. Additionally, 16 isolates were kindly provided to us from female patients with mastitis by Chiayi Christian Hospital, Taiwan.

Antibiotic susceptibility testing

An overnight-grown bacterial culture was adjusted to 0.5 McFarland (approximately 10^8 CFU/mL) and then plated on Mueller-Hinton agar. The disc diffusion method and the guidelines of the Clinical and Laboratory Standards Institute standards (CLSI, 2006) and of the manufacturer were used to determine the susceptibility of each isolate to ampicillin (10 μg), bacitracin (10 units), oxacillin (1 μg), cefuroxime (30 μg), cephalothin (30 μg), cloxacillin (5 μg), enrofloxacin (5 μg), gentamicin (10 μg), neomycin (30 μg), oxytetracycline (30 μg), penicillin G (10 units), streptomycin (10 μg), sulfamethoxazole/trimethoprim (Sxt; 23.75 μg for S and 1.25 μg for t), tetracycline (30 μg), and vancomycin (30 μg). The results of the antibiotic susceptibility test were also validated using *Escherichia coli* (ATCC No. 25922). Discs were purchased from BD (Bacto™, Becton, Dickinson and Company, Sparks, MD). Finally, the minimum inhibitory concentration to oxacillin of each oxacillin-resistant *S. aureus* isolate was determined by Etest® (AB® Biodisk, Solna, Sweden) using 5% sheep blood Mueller-Hinton agar (Bacto™, Becton, Dickinson and Company).

Table 1. Primers and Their Sequences Used for Detecting Virulence Genes

Gene	*Primer*	*Sequence (5′– 3′)*	*Size (bp)*	*Accession no.*	*Reference*
tst	TST-1	TTCACTATTTGTAAAAGTGTCAGACCCACT	180	J02615	Jarraud *et al.*, 2002
	TST-2	TACTAATGAATTTTTTTATCGTAAGCCCTT			
eta	mpETA-1	ACTGTAGGAGCTAGTGCATTTGT	190	M17347	
	mpETA-3	TGGATACTTTTGTCTATCTTTTTCATCAAC			
etb	mpETB-1	CAGATAAAGAGCTTTATACACACATTAC	612	M17348	
	mpETB-2	AGTGAACTTATCTTTCTATTGAAAAACACTC			
lukS-lukF	PVL-1	ATCATTAGGTAAAATGTCTGGACATGATCCA	433	AB006796	
	NPVL-2	GCATCAASTGTATTGGATAGCAAAAGC			
lukE-lukD	LUKDE-1	TGAAAAAGGTTCAAAGTTGATACGAG	269	Y13225	
	LUKDE-2	TGTATTCGATAGCAAAAGCAGTGCA			
lukM	LUKM-1	TGGATGTTACCTATGCAACCTAC	780	D42144	
	LUKM-2	GTTCGTTTCCATATAATGAATCACTAC			
hla	HLA-1	CTGATTACTATCCAAGAAATTCGATTG	209	M90536	
	HLA-2	CTTTCCAGCCTACTTTTTTATCAGT			
hlb	HLB-1	GTGCACTTACTGACAATAGTGC	309	S72497	
	HLB-2-2	GTTGATGAGTAGCTACCTTCAGT			
hld	HLD-1	AAGAATTTTTATCTTAATTAAGGAAGGAGTG	111	AF288215	
	HLD-2	TTAGTGAATTTGTTCACTGTGTCGA			
hlg	mpHLG-1	GTCAYAGAGTCCATAATGCATTTAA	535	L01055	
	mpHLG-2	CACCAAATGTATAGCCTAAAGTG			
hlg-2	mpHLG2-1	GACATAGAGTCCATAATGCATTYGT	390	D42143	
	mpHLG2-2	ATAGTCATTAGGATTAGGTTTCACAAAG			
agr 1	AGR1-F	ATGCACATGGTGCACATG C	441	M21854	Gilot *et al.*, 2002
	AGR1-R	GTCACAAGTACTATAAGCTGCGAT			
agr 2	AGR2-F	ATGCACATGGTGCACATGC	575	AF001782	
	AGR2-R	TATTACTAATTGAAAAGTGGCCATAGC			
agr 3	AGR3-F	ATGCACATGGTGCACATGC	323	AF001783	
	AGR3-R	GTAATGTAATAGCTTGTATAATAATACCCAG			
agr 4	AGR4-F	ATG CAC ATG GTG CAC ATG C	659	AF288215	
	AGR4-R	CGATAATGCCGTAATACCCG			

PCR detection of toxin genes and agr *typing*

Chromosomal DNA templates were purified using the QIAamp® DNA Mini Kit (Qiagen GmbH, Hilden, Germany). Table 1 lists the primers used to detect the toxin genes *tst*, *eta*, *etb*, *lukE-lukD*, *lukM*, *lukS*-PV–*lukF*-PV, *hla*, *hlb*, *hld*, *hlg*, and *hlg-2* (Jarraud *et al*., 2002) and for *agr* typing (Gilot *et al*., 2002). PCR was performed in a 50-μL reaction volume containing 5 μL of the DNA template, 0.2 μmol/L of each primer, 200 μmol/L of dNTPs, 1x polymerase chain reaction (PCR) buffer, and 1.4 units of Taq DNA polymerase. The PCR procedure was as follows: 5 min of pre-denaturation at 94°C, followed by 30 cycles of 30 s of denaturation at 94°C, 30 s of annealing at 53–55°C, and 40–60 s of extension at 72°C. All PCR products were separated on 2% agarose with 0.5 × Tris-acetate (TAE) buffer at 100 V.

PFGE and MLST analysis

PFGE analysis of *Sma*I-digested genomic DNA was performed to determine the genotype of each isolate according to the method of Bannerman *et al.* (1995). Briefly, restriction endonuclease *Sma*I was used to digest genomic DNA in whole-cell embedding agarose plugs, and DNA fragments were separated using a CHEF DR-III apparatus (Bio-Rad). After staining with ethidium bromide and under ultraviolet illumination, DNA banding patterns were recorded and analyzed using an IS-100 Digital System running Gelcompare Version l.3.1b (Applied Math, Kortrijk, Belgium). The MLST types of the isolates were determined according to the method of Enright *et al*. (2000) using MLST databases (http://saureus.mlst.net/-SA).

Results

S. aureus was isolated from milk with a prevalence of 2% (2.0%, 28/1372) and 2.1% (2.1%, 73/3427) in cows and goats, respectively.

Antibiotic susceptibility

All strains were sensitive to bacitracin, cephalothin, enrofloxacin, oxacillin, and vancomycin. Of the remaining 11 antimicrobials tested, susceptible strains (14.5%) were only identified in the cow and goat isolates; 79.5% of the strains were resistant to at least two of the antimicrobials tested (Table 2). The prevalence of antimicrobial resistance was approximately 70% for ampicillin and penicillin, 48.7% for

Table 2. Antimicrobial Resistance of 117 *Staphylococcus aureus* Isolates from Different Sources

	Resistance to:													
Resistance number	*AMP*	*CLO*	*CXM*	*GEN*	*NEO*	*OXY*	*PEN*	*STR*	*SxT*	*TET*	*Number of isolates*	*Human*	*Cow*	*Goat*
0											17		4	13
1				R							1			1
							R				1			1
								R			5	1		4
2	R						R				24	2	18	4
						R				R	8			8
3	R					R				R	1			1
	R						R			R	1			1
						R	R			R	1			1
				R	R			R			1			1
4	R						R		R		6	1	5	
	R	R		R			R				1	1		
	R				R		R	R			2	2		
	R					R	R			R	28	1	1	26
						R	R	R		R	2			2
				R	R	R		R			1			1
5	R			R		R		R		R	1			1
	R	R			R		R	R			1	1		
	R	R				R	R			R	1			1
	R				R	R	R			R	1			1
	R					R	R	R		R	1			1
6	R			R	R	R		R		R	1			1
	R			R		R	R	R		R	1			1
	R				R	R	R	R		R	2	2		
7	R	R	R			R	R	R		R	1	1		
	R	R			R	R	R	R		R	1	1		
	R			R	R	R	R	R		R	1			1
8	R	R	R		R	R	R	R		R	2	2		
9	R			R	R	R	R	R	R	R	2			2
	R	R	R	R	R	R	R	R		R	1	1		
Total	80	8	4	11	16	57	81	26	8	57	117	16	28	73
	68.4	6.8	3.4	9.4	13.7	48.7	69.2	22.2	6.8	48.7				

Ampicillin (AMP), cloxacillin (CLO), cefuroxime (CXM), gentamicin (GEN), neomycin (NEO), oxytetracycline (OXY), penicillin G (PEN), streptomycin (STR), sulfamethoxazole/ trimethoprim (SxT), tetracycline (TET).

oxytetracycline and tetracycline, and lower than 25% for streptomycin (22.2%), neomycin (13.7%), gentamicin (9.4%), cloxacillin (6.8%), sulfamethoxazole/trimethoprim (6.8%), and cefuroxime (3.4%). The human and goat isolates were more resistant to the tested antimicrobials than cow isolates. Furthermore, 31.2% (5/16) of the human and 39.7% (29/73) of the goat isolates were resistant to four antimicrobials, and 64.3% (18/28) of the cow isolates were resistant to two antimicrobials.

Variations in toxin genes

Analysis of 10 virulence genes revealed absence of the *etb* gene and varying of the other genes in isolates (Table 3). Four hemolysin genes (*hla*, *hlb*, *hld*, and *hlg-2*) differed in prevalence among sources from 83.6% in the goat isolates to 100% in the human isolates for α-hemolysin (*hla*); from 38.4% in the goat isolates to 75% in the human isolates for *β*-hemolysin (*hlb*); from 95.9% in the goat isolates to 100% in the human and cow isolates for *δ*-hemolysin (*hld*); and from 50.7% in the goat isolates to 96.4% in the cow isolates for *γ*-hemolysin (*hlg-2*) (Table 3). Additionally, we assessed host-associated virulence genes and found *lukPV* (13.8%), *lukM* (25%) and *eta* (25%) for human isolates; *lukM* (3.6%), *eta* (21.4 %), and *lukED* (100%) for cow isolates, and *tst* (28.8%) and *lukED* (27.4%) for goat isolates.

Genomic and genetic variations

Using *agr* typing, the isolates were separated into four types: *agr*1, *agr*2, *agr*3, and nontypeable, the last of which was only found in the human (25%) and goat (52.1%) isolates (Table 4). As the most prevalent type, *agr1* isolates were identified in almost all of the cow strains (96.4%) and most of the human strains (62.5%), but was less prevalent in the goat strains (30.1%). PFGE analysis separated all of the isolates into two clusters, with each cluster containing three subclusters (Fig. 1). The pulsotype number was three for the cow isolates (pulsotypes C1-C3), six for the human isolates (pulsotypes H1-H6), and eight for the goat isolates (pulsotypes G1-G8) (Fig. 1). The human isolates were found to be predominantly in cluster I, and the goat isolates were predominantly found in cluster II. Additionally, cow pulsotype C1 and goat pulsotype G6 were found to be identical.

Table 3. Prevalence of Virulence Genes in *Staphylococcus aureus* Isolates from Different Sources

Genes[a]	*Human (%)*	*Cow (%)*	*Goat (%)*
Tsst	0 (0/16)	0 (0/28)	28.8 (21/73)
Eta	25.0 (4/16)	21.4 (6/28)	0 (0/73)
Etb	0 (0/16)	0 (0/28)	0 (0/73)
lukED	0 (0/16)	100 (28/28)	27.4 (20/73)
lukPV	13.8 (7/16)	0 (0/28)	0 (0/73)
lukM	0 (0/16)	3.6 (1/28)	0 (0/73)
α-*hemolysin*	100 (16/16)	96.4 (27/28)	83.6 (61/73)
β-*hemolysin*	75.0 (12/16)	46.4 (13/28)	38.4 (28/73)
δ-*hemolysin*	100 (16/16)	100 (28/28)	95.9 (70/73)
γ-*hemolysin*	87.5 (14/16)	96.4 (27/28)	50.7 (37/73)

[a]*Tsst* stands for toxic shock syndrome toxin, *eta* and *etb* stand for exofoliative toxin, and *lukED, lukPV,* and *lukM* stand for leukocidin.

Table 4. Prevalence of *agr* Types of *S. aureus* Strains from Different Sources

Agr *type*	*Human (%)*	*Cow (%)*	*Goat (%)*
*agr*1	62.5 (10/16)	96.4 (27/28)	30.1 (22/73)
*agr*2	0 (0/16)	3.6 (1/28)	15.0 (11/73)
*agr*3	12.5 (2/16)	0 (0/28)	2.7 (2/73)
Nontypeable	25 (4/16)	0 (0/28)	52.1 (38/73)

The MLST analysis of 10 isolates determined that the ST types were ST59 for the human isolates; ST188 and ST 705 for the cow isolates; and ST1, ST133, and ST1027 for the goat isolates (Table 5). Although the strains shared the *agr*1 type, the ST type differed among sources, with, for example, ST59 found in the human strains, ST188 found in the cow strains, and 133 or 133-like found in the goat strains.

Discussion

S. aureus is generally considered to be a contagious udder pathogen, which mainly spreads within and between cows or goats at milking; the udder is the main source of infection. Therefore, control measures are primarily aimed at improving milking hygiene and milking routines such as milking order and teat dipping. Recently, MDR pathogenic *S. aureus* has been frequently isolated from cows and goats with mastitis (Dung, 2004). Normally, MRSA are MDR with various toxins (Tang *et al*., 2007; von Eiff *et al*., 2008), highly pathogenic and infectious in humans (Strommenger *et al*., 2003), canines (Rao *et al*., 1987), geriatric patients (Scott *et al*., 1988) and cattle (Rao *et al*., 1987; Lee, 2003). Although our previous study discovered genetically divergent MRSA isolates from goats with mastitis (Chu *et al*., 2012), in this study, we were only able to isolate MDR *S. aureus* from goats, not MRSA (Table 2). Furthermore, our *S. aureus* isolates were more resistant to ampicillin, penicillin, tetracycline, and oxytetracycline, which are drugs that are often used to treat bacterial infections in goats.

Pathogenic *S. aureus* strains have differing prevalence of various virulence genes. Our present study identified host-associated virulence genes in mastitis-related *S. aureus*. In goats, *S. aureus* toxins consist mostly of TSST (Adesiyun *et al*., 1992). Indeed, we confirmed the presence of *TSST-1* in a few goat isolates, but found none in the cow or human isolates (Table 3). Furthermore, the virulence gene *eta* was only detected in the human and cow isolates and not in the goat isolates. Further investigation is needed to determine whether these two genes are related to host-associated mastitis. The virulence factor ETA differs in prevalence among geographical regions, mainly distributed in Africa, Europe, and North America (Ladhani, 2001; Rabello *et al*., 2007). In Taiwan, the major breed of dairy cattle is the Holstein-Friesan, imported mainly from Australia and the United States. It is possible that the *S. aureus* strains with *eta* are from imported cows.

Leukocidins are bicomponent toxins, which include Panton-Valentine leukocidin (LukS-PV + LukF-PV), *LukM* (LukM + LukF'-PV), and *LukED* (LukE + LukD) (Holmes *et al*., 2005; Clark, 2008). Previous reports frequently identified *lukM* in sheep and goat isolates and infrequently in cow isolates (Rainard *et al*., 2003). Leukocidin *E* and *lukD* were found to be predominant in human isolates from blood (82%) (von Eiff *et al*., 2008). In the present study, we observed *lukPV* and

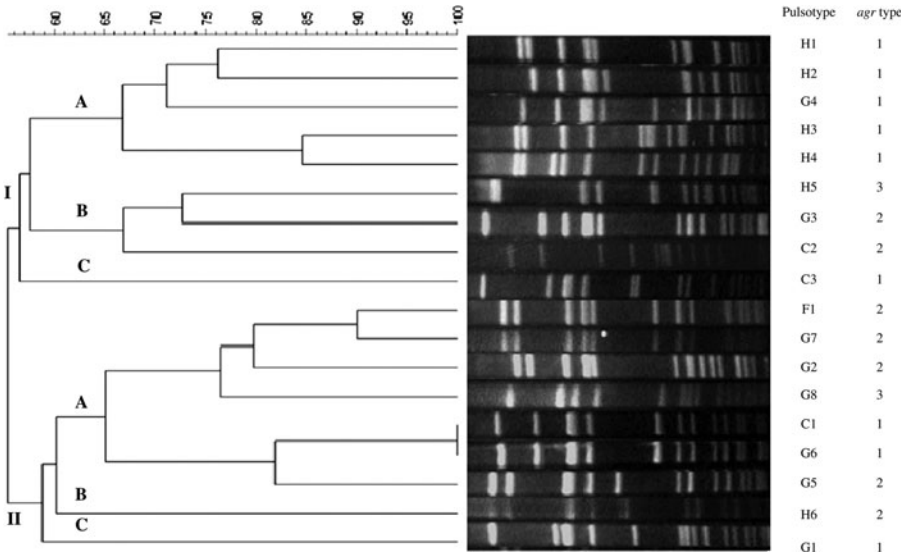

FIG. 1. Phylogenetic distance tree of pulsed-field gel electrophoresis typing. C1–C3, cow isolates; H1–H6, human isolates; F1, food isolate; G1–G8, goat isolates. Most human pulsotypes belonged to cluster I, whereas most goat pulsotypes were grouped into cluster II. Additionally, cow pulsotype C1 and goat pulsotype G6 were found to be identical.

lukM in isolates from humans (13.8%) and cows (3.6%), and *lukED* in isolates from cows (100%) and goats (27.4%) (Table 3). Additionally, genes encoding hemolysins, which can destroy cell membranes (Ikawaty *et al*., 2009), were found to be present in our isolates: 75–100% of human isolates had α, β, δ, and γ-hemolysin genes; 96.4–100% of bovine isolates had α, δ, and γ-hemolysin genes; and 83.6–95.9% of caprine isolates had α- and δ-hemolysin genes (Table 3). These results suggest that mastitis-associated virulence genes include α-, δ-, and γ-hemolysin genes, *eta, lukED*, and *lukM* for cows; α- and δ-*hemolysin* genes, *tst* and *lukED* for goats; and all four hemolysin genes, *eta* and *lukPV* for humans.

The analysis of the *agr* types demonstrated that the major type was *agr*1 in the MRSA isolates. Previous studies have indicated that *agr* type IV is the predominant type for human dermatitis isolates (Garbacz *et al*., 2009; Ho *et al*., 2010); *agr*1 and *agr*2 for bovine mastitis-associated isolates (Buzzola *et al*., 2007); and *agr*1 for penicillin-resistant strains (Melchior *et al*., 2009; van den Borne *et al*., 2010). In this study, although *agr*1 was the major type in human and cow isolates, nontypeable isolates were predominant in the goat isolates and absent in the cow isolates (Table 4), demonstrating the possible diverse origins of *S. aureus* in human and goat isolates. Pulsotype analysis also confirmed the different origins of the human and goat isolates. However, we encountered identical pulsotypes for certain goat and human isolates, suggesting the possibility of transfer of MDR *S. aureus* between humans and goats. Diverse genomic variations of *S. aureus* generally limit the capacity to perform PFGE analysis in epidemiological studies. Instead, MLST is highly effective for epidemiological research on *S. aureus* (Enright *et al*., 2000; Denis *et al*., 2004). Recent MLST analysis of mastitis-associated *S. aureus* isolates identified 11 ST types for goats and 10 ST and nontypeable types for sheep in Spain (Concepción Porrero *et al*., 2012). A similar study identified four ST types in Brazil (de Almeida *et al*., 2011). Our ST types differed among the three sources, and identical ST types (ST133 and 1027) were found in different goat isolates (Table 5), demonstrating clonal dissemination in goats. When comparing our ST types with those reported in Spain and Brazil, we found only ST133 to be common to both Spain and Taiwan; ST types were otherwise diverse and distinct (de Almeida *et al*., 2011; Concepción Porrero *et al*., 2012), suggesting that mastitis-associated *S. aureus* strains are genetically diverse and that clonal dissemination may have occurred in goats.

Table 5. Multilocus Sequence Typing and *agr* Analysis of Representative *Staphylococcus aureus* Isolates from Different Sources

Isolates	*Sources*	*Allele profile*	*Sequence types*
O122	Human	*agr*1	59
1556C	Cow	*agr*1	188
5171		*agr*2	705
1C	Goat	*agr*1	133-like
99Q		*agr*1	133-like
13LV		*agr*1	133
1E		*agr*2	1027
2E		*agr*2	1027-like
6E		*agr*2	1027
9F		*agr*3	1

In conclusion, MDR *S. aureus* was commonly isolated from humans, cows, and goats with mastitis. Ten virulence genes differed in prevalence and appeared to be host-associated. These isolates revealed the presence of diverse genetic variations among the three host species and indicated a possible clonal dissemination in goats.

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Yaochi Su, PhD
Department of Veterinary Medicine
National Chiayi University
No. 580, Sing Ming Road
Chiayi, 60004
Taiwan, China

E-mail: shu@mail.ncyu.edu.tw