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Original Articles

Antimicrobial Susceptibility Testing and Genotypic Characterization of *Staphylococcus aureus* from Food and Food Animals

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Abstract

Staphylococcus aureus is commonly present in humans and animals. The aim of this study was to investigate antimicrobial resistance and genetic characteristics of *S. aureus* from food and food animals in Shaanxi Province in China. A total of 332 nasal swabs, breast skin swabs, raw milk, and pork samples were collected from local pig, dairy farms, or local grocery stores and screened for the presence of *S. aureus*. *S. aureus* isolates were characterized using antimicrobial susceptibility, pulsed-field gel electrophoresis (PFGE) analysis, and polymerase chain reaction for detecting *pvl* and *mecA* genes. Methicillin-resistant *S. aureus* (MRSA) strains were additionally tested for SCC*mec* type and exfoliative toxin genes. The prevalence of *S. aureus* was 30.6% in pig nasal swabs, 32.5% in pork, 25.7% in cow nasal swabs, 30.8% in cow breast skin swabs, and 29.3% in milk samples. Resistances were common among isolates tested against erythromycin (65.7%), tetracycline (65.7%), ciprofloxacin (52.7%), followed by gentamicin (36.7%), chloramphenicol (23.1%), cefoxitin (8.3%), and oxacillin (7.7%), but no isolate was resistant to vancomycin, amikacin, or cefoperazone. *pvl* gene was found in the isolates from all types of samples except from cow nasal swabs. Fourteen isolates from pig nasal swabs contained *mecA* gene and were considered as MRSA. PFGE analysis showed that nasal isolates differed from food isolates, but isolates from the same animal source appeared to cluster closely. The PFGE patterns of MRSA isolates were different from other *S. aureus* isolates from pig nasal cavity even though they were from the same source. All the MRSA isolates belonged to SCC*mec* type IV_b. No isolates contained exfoliative toxin genes. These findings indicated that *S. aureus*, including multidrug-resistant *S. aureus*, are widely spread in food animals and animal-derived foods in Shaanxi Province, China. MRSA isolates from pigs may pose potential health risks for workers in swine farms and the community at large.

Introduction

Staphylococcus aureus frequently colonizes on the skin and mucous membranes of humans and animals. It is an opportunistic pathogen and could cause a wide range of infections including dermatitis, pneumonia, septicemia, osteomyelitis, and meningitis in humans and pigs, and mastitis in cattle (Hasman *et al.*, 2010). *S. aureus* is also commonly found in various food (especially of animal origin), and is an important foodborne pathogen worldwide (Normanno *et al.*, 2005, 2007). Important virulence factors implicated in the pathogenesis of *S. aureus* include enterotoxins, exfoliative toxins, toxic shock syndrome toxin-1, and Panton-Valentine Leucocidin, and other exoproteins are also responsible for particular clinical manifestations (Vancraeynest *et al.*, 2006).

Antimicrobial resistance has been an important public health concern and frequently reported in *S. aureus* (Lowy, 2003). Among resistant *S. aureus*, methicillin-resistant *S. aureus* (MRSA) is the most significant as it represents a leading cause of hospital-associated (HA) infections and has also emerged as an important cause of community infection (Ho *et al.*, 2008). The methicillin resistance of MRSA is mediated by the *mecA* gene that encodes a penicillin-binding protein with a low affinity for β-lactams (Pinho *et al.*, 2001). The gene resides in a mobile genetic element called staphylococcal cassette chromosome *mec* (SCC*mec*). Community-associated (CA) MRSA strains mainly harbor SCC*mec* IV or V, whereas HA MRSA strains tend to carry SCC*mec* I, II, and III (Kluytmans-VandenBergh and Kluytmans, 2006). In recent years, MRSA has been identified in food animals including pig, cattle,

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chicken, and foods of animal origin (Cuny *et al.*, 2010; Kluytmans, 2010). Therefore, livestock and foods of animal origin may become an important reservoir and source of community-acquired MRSA.

Several genotypic typing methods, such as multilocus sequence typing (MLST), ribotyping, pulsed-field gel electrophoresis (PFGE), and staphylococcal protein A (*spa*) typing, have been applied in the epidemiological investigation of *S. aureus* from different sources (Grundmann *et al.*, 2002). The MLST is thought to provide information about the phylogenetic clustering of *S. aureus* isolates and has been widely used in typing MRSA strains from animals and humans (Feil *et al.*, 2003). But it is relatively expensive for sequencing several house-keeping genes and has a lower discriminatory power compared with other typing methods. Although some strains are not typeable by restriction enzyme digestion possibly due to modification of restriction sites, PFGE is considered as the gold standard for typing *S. aureus* due to its discriminatory power (Struelens *et al.*, 2009). The single locus–based *spa* typing has a similar discriminatory power as PFGE and is extensively used currently, and it can be used together with PFGE for typing those isolates that are nontypeable by standard PFGE (Mellmann *et al.*, 2007).

There was a paucity of data regarding characteristics of *S. aureus*, especially MRSA, in food animals and foods of animal origin in China. The aim of this study was to determine the antimicrobial susceptibility and genetic characteristics of *S. aureus* in pig, cow, raw milk, and pork samples in Shaanxi Province in China. *S. aureus* isolated from these samples were analyzed by antimicrobial susceptibility testing and detection of *mecA* and *pvl* genes. The genetic relatedness among *S. aureus* isolates from different origin was evaluated by PFGE. In addition, MRSA isolates were further tested for SCC*mec* type and exfoliative toxin genes.

Materials and Methods

Isolation and identification of S. aureus

From January to August 2009, 332 samples including 173 pig nasal swabs, 35 cow nasal swabs, 26 cow breast skin swabs, and 58 raw milk samples from 10 farms, and 40 pork samples from five retail grocery stores were collected in Shaanxi Province, China. All swabs were incubated in trypticase soy broth (TSB) (Beijing Land Bridge Technology Co. Ltd., Beijing, China) containing 7.5% NaCl. A 50 mL aliquot of milk sample or pork rinse was enriched in an equal volume of double-strength TSB supplemented with 15% NaCl (Beijing Land Bridge Technology Co. Ltd.). After incubation at 35°C for 18–24 h with shaking, a loopful of the culture was inoculated onto Baird-Parker agar plates (Beijing Land Bridge Technology Co. Ltd.). Following incubation for 48 h, one to two colonies showing typical appearance (black colonies surrounded by 2 to 5 mm clear zones) of coagulase-positive staphylococci per sample were taken and confirmed as *S. aureus* by Gram staining, catalase test, coagulase test, and DNase production test on DNase Agar. All isolates were stored at −80°C in TSB plus 20% (v/v) glycerol for further use.

Polymerase chain reaction assays

Following the DNA extraction described previously (Xia *et al.*, 2010), presumptive *S. aureus* isolates were identified to the species level by polymerase chain reaction (PCR) detection of thermonuclease gene (*nuc*, *S. aureus* specific) (Brakstad *et al.*, 1992). *S. aureus* isolates were further tested by PCR for PVL-encoding gene (*pvl)* and methicillin resistance gene (*mecA*) (Zhang *et al.*, 2004; Wang *et al.*, 2009). All MRSA isolates were further examined by PCR for the presence of exfoliative toxin genes (*eta* and *etb* genes) as previously described (Noguchi *et al.*, 2006). SCC*mec* typing was performed by PCR as described previously (Zhang *et al.*, 2005). Primers for the PCR assays were listed in Table 1. The PCR products were resolved in 1.5% (w/v) agarose gel electrophoresis in 1 × TBE buffer.

Antimicrobial susceptibility testing

Antimicrobial susceptibility tests were performed using agar dilution method (CLSI, 2006). The isolates were tested with a panel of 10 antimicrobials: oxacillin (OXA), cefoperazone (CPZ), cefoxitin (FOX), erythromycin (ERY), gentamicin (GEN), chloramphenicol (CHL), ciprofloxacin (CIP), tetracycline (TET), amikacin (AMK), and vancomycin (VAN) according to the guidelines of the Clinical Laboratory Standards Institute (CLSI, 2006). *Escherichia coli* ATCC 25922 and *S. aureus* ATCC 29213 were included as quality control strains in each run.

Genomic DNA fingerprinting using PFGE

Genomic DNA fingerprints of *S. aureus* were determined using PFGE according to a standard protocol developed by PulseNet for *S. aureus* (McDougal *et al.*, 2003). Briefly, agarose-embedded DNA was digested with 50 U of *Sma*I for 4 h in a water bath at 30°C. DNA fragments were separated by electrophoresis in 0.5 × TBE buffer at 14°C for 18 h on a CHEF-III Mapper electrophoresis system with pulse time of 5–40 s. The gels were stained with ethidium bromide and images were taken under UV transillumination. The images were analyzed with BioNumerics Software (Applied Maths, Kortrijk, Belgium) by using Dice coefficients and unweighted pair group method with arithmetic averages to achieve dendrograms with a 1.5% band position tolerance. *Salmonella* serotype Branderup strain H9812 digested with *Xba*I was used as a molecular size marker.

Results

Isolation and identification of S. aureus

Of the 332 samples, 100 (30.1%) samples were positive for *S. aureus*, including 53 (30.6%) of the 173 pig nasal swabs, 13 (32.5%) of the 40 pork samples, 9 (25.7%) of the 35 cow nasal swabs, 8 (30.8%) of the 26 cow breast skin swabs, and 17 (29.3%) of the 58 raw milk samples (Table 2). A total of 169 *S. aureus* isolates were recovered from the 100 *S. aureus*–positive samples (1–2 isolates per sample), including 83 isolates from pig nasal swabs, 26 isolates from pork, 22 isolates from cow nasal swabs, 18 isolates from cow breast skin swabs, and 20 isolates from raw milk (Table 2).

pvl *and* mecA *detection*

Fourteen *S. aureus* isolates from pig swab samples showed positive for *mecA* gene, whereas none of the isolates from other samples were positive for *mecA*. Ninety-six *pvl*-positive

Table 1. Oligonucleotide Primers Used in Polymerase Chain Reaction

Gene	*Forward primer sequence (5′-3′)*	*Reverse primer sequence (5′-3′)*	*Product size (bp)*	*Reference*
nuc	GCGATTGATGGTGATACGGTT	AGCCAAGCCTTGACGAACTAAAGC	279	Brakstad *et al.* (1992)
mecA	GTAGAAATGACTGAACGTCCGATAA	CCAATTCCACATTGTTTCGGTCTAA	310	Zhang *et al.* (2004)
SCC*mec* I	GCTTTAAAGAGTGTCGTTACAGG	GTTCTCTCATAGTATGACGTCC	613	Zhang *et al.* (2005)
SCC*mec* II	CGTTGAAGATGATGAAGCG	CGAAATCAATGGTTAATGGACC	398	Zhang *et al.* (2005)
SCC*mec* III	CCATATTGTGTACGATGCG	CCTTAGTTGTCGTAACAGATCG	280	Zhang *et al.* (2005)
SCC*mec* IVa	GCCTTATTCGAAGAAACCG	CTACTCTTCTGAAAGCGTCG	776	Zhang *et al.* (2005)
SCC*mec* IVb	TCTGGAATTACTTCAGCTGC	AAACAATATTGCTCTCCCTC	493	Zhang *et al.* (2005)
SCC*mec* IVc	ACAATATTTGTATTATCGGAGAGC	TTGGTATGAGGTATTGCTGG	200	Zhang *et al.* (2005)
SCC*mec* IVd	CTCAAAATACGGACCCCAATACA	TGCTCCAGTAATTGCTAAAG	881	Zhang *et al.* (2005)
SCC*mec* V	GAACATTGTTACTTAAATGAGCG	TGAAAGTTGTACCCTTGACACC	325	Zhang *et al.* (2005)
pvl	ATCATTAGGTAAAATGTCTGGACATGATCCA	GCATCAAGTGTATTGGATAGCAAAAGC	433	Wang *et al.* (2009)
eta	ATATCAACCGTGAGGGCTCTAGTAC	ATGCAGTCAGCTTCTTACTGCTA	1155	Noguchi *et al.* (2006)
etb	CACACATTACGGATAATGCAAG	TCAACCGAATAGAGTGAACTTATCT	604	Noguchi *et al.* (2006)

S. aureus (56.8%) were identified among the 169 *S. aureus* isolates, including 23 (88.5%) *pvl*-positive *S. aureus* from pork, 18 (90%) from raw milk, 17 (94.4%) from cow breast skin swabs, and 38 (45.8%) from pig nasal swabs. No isolates from cow nasal swabs were tested positive for *pvl* gene (Table 2).

Antimicrobial susceptibility tests

The 169 *S. aureus* isolates were tested for susceptibility against 10 antimicrobials. The overall resistance to these antimicrobials was 65.7% to erythromycin, 65.7% to tetracycline, 52.7% to ciprofloxacin, 36.7% to gentamicin, 23.1% to chloramphenicol, 8.3% to cefoxitin, and 7.7% to oxacillin (Table 3). All *S. aureus* isolates were susceptible to vancomycin, amikacin, and cefoperazone. In addition, multidrug-resistant *S. aureus* isolates (resistant to ≥ 3 antimicrobials) were observed in 100% of the pig nasal isolates, 30.8% of the pork isolates, and 15% of the milk isolates, respectively. Resistance to β-lactam antimicrobials was most common among the pig nasal swab isolates, and oxacillin resistance (15.7%) and cefoxitin resistance (16.9%) were only observed among the pig nasal swab isolates. In general, *S. aureus* isolated from pig and pork showed a higher resistance rate than those from milk and cow.

Characteristics of MRSA isolates

All of the 14 MRSA isolates harbored SCC*mec* IV_b. None of the isolates were positive for exfoliative toxins genes (*eta* and *etb*). They were all resistant to erythromycin, cefoxitin, tetracycline, chloramphenicol, gentamicin, trimethoprim, and ciprofloxacin. All isolates except one were resistant to oxacillin. Eight PFGE types were identified using *Sma*I digestion among the MRSA isolates, which formed three clusters.

PFGE analysis of S. aureus *isolates*

There was a great genetic diversity among isolates from different sources (Fig. 1). Pig nasal isolates were generally different from pork isolates, except a few isolates (isolate Nos. 139, 138, 134, 131, and 132) showing a similarity of more than 80%. Most (9/12) of the isolates from milk samples tested were closely related (similarity of more than 80%) to cow breast skin isolates, and they were both separated from cow nasal isolates. However, the MRSA isolates clustered (similarity of 80%) together and differed from other nasal isolates and the pork isolates.

Discussion

Livestocks can carry *S. aureus* in nasal cavity and on skin. Foods derived from these animals can be contaminated during and after slaughtering, and processing. Studies on the prevalence and characteristics of *S. aureus*, especially MRSA from farm animals and foods derived from these animals in China, are very scarce. In addition, information on the relationship between food isolates and animal isolates is limited. Our findings showed that the contamination rate of *S. aureus* in the animal and food samples was between $\sim$25% and 32%, which was similar to the studies conducted in other countries. For example, *S. aureus* was detected in 24.2% of pork samples in Korea (Lee, 2003), and 39.2% in United States (Kluytmans, 2010), and 29.6% in raw milk in Korea (Lee, 2003), and 38.4% in Italy (Normanno *et al.*, 2005).

Table 2. Prevalence and Detection of *mecA* and *pvl* of *Staphylococcus aureus* from Food and Animals

Source	*No. of samples*	*No. (%) of samples positive for* Staphylococcus aureus	*No. of* S. aureus *isolates recovered*[a]	*No. (%) of* pvl-*positive isolates*	*No. (%) of* mecA-*positive isolates*
Pig nasal swabs	173	53 (30.6%)	83	38 (45.8%)	14 (16.7%)
Pork	40	13 (32.5%)	26	23 (88.5%)	0 (0%)
Raw milk	58	17 (29.3%)	20	0 (0%)	0 (0%)
Cow breast skin swabs	26	8 (30.8%)	18	17 (94.4%)	0 (0%)
Cow nasal swabs	35	9 (25.7%)	22	18 (90%)	0 (0%)
Total	332	100 (30.1%)	169	96 (56.8%)	14 (8.3%)

[a]One to two *S. aureus* isolates were collected from each positive sample.

Higher *S. aureus* contamination rates in pork samples were reported in the Netherlands (46%) (Kluytmans, 2010) and in Germany (57.7%) (Atanassova *et al.*, 2001). In Norway, 75% of tank milk samples were contaminated with *S. aureus* (Jorgensen *et al.*, 2005). Even though there are differences in hygienic control and processing on farms in different countries, the common presence of the pathogen in food indicates its ubiquitousness and the difficulty to control this pathogen.

Panton-Valentine Leukocydin is a leukotoxin associated with human clinical diseases (Chen *et al.*, 2009) and also with bovine mastitis (Zecconi *et al.*, 2006). *pvl* gene was identified at a high rate in *S. aureus* isolated from pork, cow breast skin, and milk, commonly found in pig nasal swab isolates, but not detected in isolates from cow nasal cavity. The rate of *pvl* in isolates from cow breast skin was high (94.4%) compared with the rate reported in another study (56% of bovine isolates) (Zecconi *et al.*, 2006). For food samples, only 3 *pvl*-positive *S. aureus* were detected from 219 *Staphylococcus* sp. isolated from various food samples in Turkey (Sudagidan and Aydin, 2010). In contrast, no *pvl*-positive *S. aureus* was found from milk samples in Brazil (Aires-de-Sousa *et al.*, 2007). In this study, *pvl* was commonly found in general *S. aureus* isolates as well as in MRSA isolates (13/14, 93%). The significance of the *pvl*-positive but methicillin-susceptible *S. aureus* and MRSA strains in foods and their contributions to infections in humans and animals need further elucidation.

There was a higher prevalence of antimicrobial resistance in pig isolates than in cattle isolates. These maybe due to the fact that a substantial amount of antimicrobials is used in pig farming for growth promotion and for prophylactic in addition to therapeutic purpose, while in cattle farm generally antimicrobials were mainly used for clinical purpose, such as treating mastitis. The high resistance (100%) to tetracycline in pig or pork isolates supported the selection effect of considerable use of this antimicrobial in pig farm, even though it has limited clinical significance today. These data underline the need for policies on the judicious use of antimicrobials on pig farms. Milk isolate differed from breast skin isolate with respect to resistance profiles, which, in contrast with similar PFGE patterns, indicates that sources other than cow breast might have contributed to the contamination of the milk samples. Swine nasal isolates showed greater resistance compared with pork isolates, together with different PFGE patterns, also suggesting that they were likely from different sources.

Oxacillin resistance was only detected in pig nasal isolate, which is consistent with another study by Cui *et al.* (2009) who reported that MRSA are only found in pig farm, but not in cattle farm in China. Among 14 *mecA*-positive isolates, only one isolate (isolate 61) was susceptible to oxacillin, but resistant to cefoxitin. It was suggested that cefoxitin has advantages over oxacillin for identifying low-level resistant MRSA

Table 3. Antimicrobial Resistance Phenotypes of *Staphylococcus aureus* Isolates Recovered from Food and Animals

Antimicrobial agent	*Resistance break point (μg/mL)*[a]	*% Resistance*					
		Pig nasal swab (n = *83*)	*Pork* (n = *26*)	*Cow nasal swab* (n = *22*)	*Cow breast swab* (n = *18*)	*Milk* (n = *20*)	*Total* (n = *169*)
Oxacillin	≥4	15.7	0	0	0	0	7.7
Amikacin	≥32	0	0	0	0	0	0
Cefoperazone	≥64	0	0	0	0	0	0
Cefoxitin[b]	≥8	16.9	0	0	0	0	8.3
Chloramphenicol	≥32	42.2	0	0	0	25	23.7
Ciprofloxacin	≥4	92.8	42.3	0	0	5	52.7
Erythromycin	≥8	100	100	0	0	10	65.7
Gentamicin	≥8	67.5	7.7	0	0	20	36.7
Tetracycline	≥16	100	100	0	0	10	65.7
Vancomycin	≥16	0	0	0	0	0	0
Multiresistance		100	100	0	0	35	68.6

[a]Minimum inhibitory concentrations (μg/mL) determined via agar dilution in accordance with CLSI.
[b]No CLSI breakpoint, the breakpoint as stated by Perez *et al.* (2008) was used.
CLSI, Clinical and Laboratory Standards Institute.

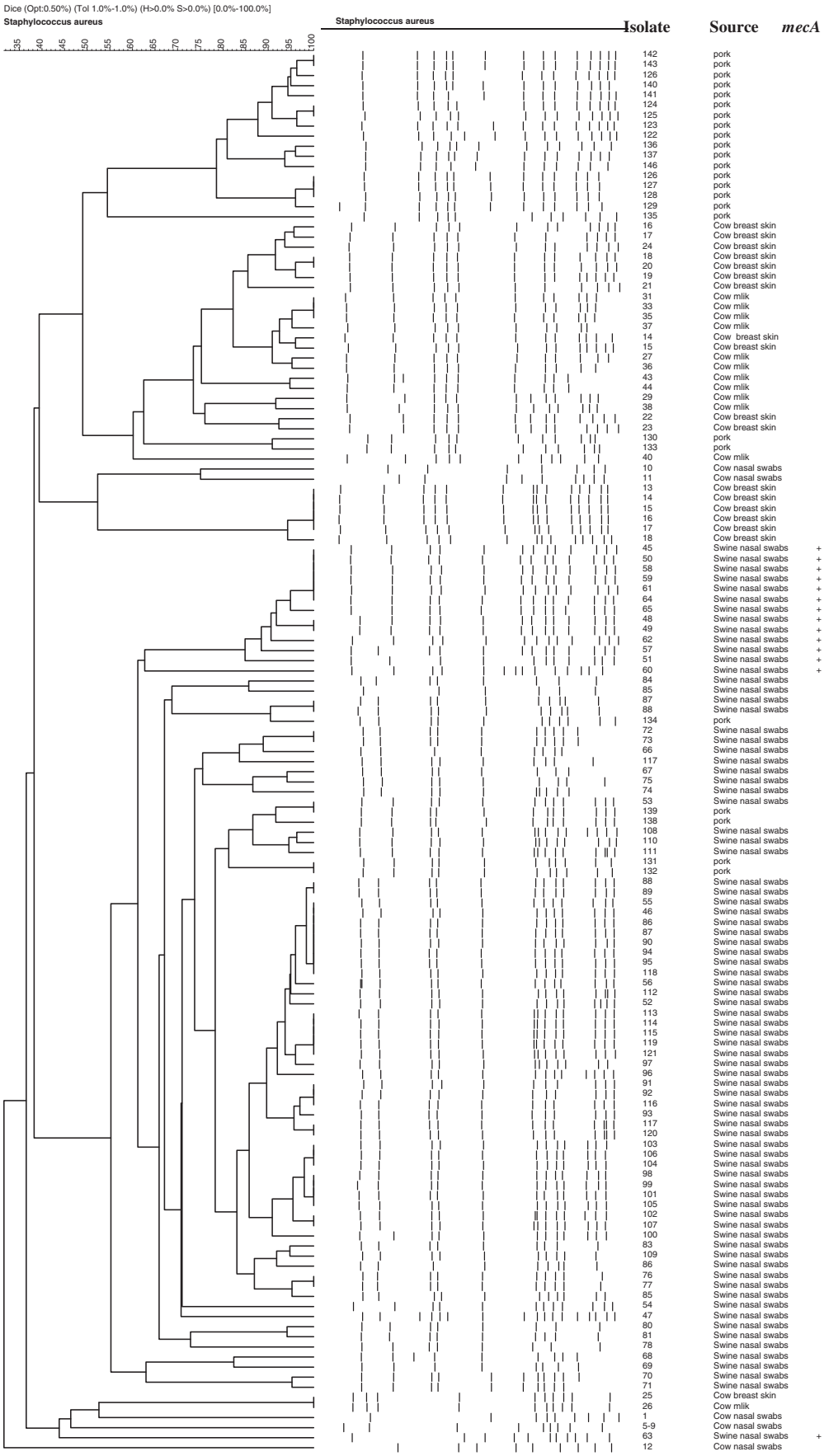

FIG. 1. Dendrogram of pulsed-field gel electrophoresis patterns of *Staphylococcus aureus* isolates from animals and food. The columns on the right show the identification number, the source, and *mecA* status of the isolates. Due to unavailability of some isolates during the experiment, only 139 out of 169 isolates were included in this part of study.

(Witte *et al.*, 2007). Thus this isolate was still considered as potential MRSA. All MRSA isolates in our study can be digested by *SmaI* enzyme. When compared with PFGE patterns of major MRSA clones in the United States (USA 100-900), none of the MRSA strains in this study matched with any U.S. types (data not shown), which indicates that MRSA in China was probably different from those in the United States and other countries, as suggested by MLST of pig MRSA strains in China (Cui *et al.*, 2009). In addition, all MRSA contained *pvl* gene, while no *pvl*-positive MRSA was reported by Cui *et al.* (2009) in China, de Neeling *et al.* (2007) in the Netherlands, and Huber *et al.* (2010) in Switzerland. All MRSA strains in this study belonged to SCC*mec* type IV_b, while only SCC*mec* type III in pig MRSA was reported in other provinces of China (Cui *et al.*, 2009). These differences demonstrated that different MRSA clones exist in various regions of China. In contrast to other studies that reported the presence of MRSA in pork (de Boer *et al.*, 2009), no MRSA was identified in pork samples in this study, possibly due to a limited number of samples taken and different methods used.

PFGE analysis revealed that most pork *S. aureus* were distant from pig nasal isolates, with only a few pork isolates (e.g., isolate Nos. 134, 139, and 131) exhibited >80% similarity to nasal isolates. They may come from contact with intestinal content during slaughtering and processing or from other sources (equipment, handlers, etc.) during processing. Most of the *S. aureus* isolates from milk tested showed similar PFGE pattern to isolates from breast skin, confirming the general conception that milk contamination is more related to the pathogen on breast skin of cows, and less connected with nasal carriage in cow. In addition, most MRSA isolates clustered together and differed from other pig nasal isolates, indicating that they may originate from sources other than pig itself, and their sources need further determination.

In summary, pork and milk samples were contaminated with *S. aureus* at similar rates compared with the carriage rates in pig and cow. *pvl* was detected frequently in isolates from all types of samples except cow nasal isolates. Antimicrobial resistance in cow isolates was less pronounced than that in pig isolates. MRSA were only found in pig nasal isolates. Further research into the origin of these MRSA is highly needed, and the possibility of their transfer to humans and their role to CA MRSA infection should also be elucidated in the future.

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Disclosure Statement

No competing financial interests exist.

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