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Prevalence, genetic diversity, and antimicrobial susceptibility profiles of *Staphylococcus aureus* isolated from bovine mastitis in Zhejiang Province, China*

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Abstract: This study was conducted to determine genetic diversity and antimicrobial susceptibility profiles of *Staphylococcus aureus* recovered from bovine mastitis in Zhejiang Province, China. Out of 3178 quarter milk samples from 846 lactating cows, among which 459 cows (54.3%) were found HMT positive, 890 quarters (28%) were found having subclinical mastitis. From 75 representative *S. aureus* isolates, 16 distinct types were identified by pulsed-field gel electrophoresis (PFGE). Four major PFGE types (A, B, C, and D) accounted for 82.7% of all isolates, and type A (41.3%) was observed in multiple herds across the studied areas. Each region was found to have a predominant type: Hangzhou type A (64.1%), Ningbo type C (34.5%) and type B (23.1%), Jinhua type D (53.3%), and Taizhou type C (62.5%). Results of antimicrobial susceptibility tests showed that 90.7% of the isolates were resistant to at least one antimicrobial. Resistance to penicillin and ampicillin (77.3%), tetracycline (60.0%), or erythromycin (48.0%) was observed. The bacteria resistant to multiple antibiotics such as penicillin, ampicillin, tetracycline, and erythromycin were commonly found. The information obtained from this study is useful for designing specific control programs for bovine *S. aureus* mastitis in this region.

Key words: *Staphylococcus aureus*, Bovine mastitis, Antimicrobial resistance, Genetic diversity, Pulsed-field gel electrophoresis
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INTRODUCTION

Bovine mastitis is a complex and economically important infectious disease for dairy cattle throughout the world, which can result in substantial losses due to reduced milk yield, and increase culling rates and veterinary expenses (Miles *et al*., 1992). Subclinical mastitis, without any signs of inflammation compared with clinical mastitis, is the main form of the disease, and accounts for the majority of bovine mastitis cases in dairy herds (Oliver *et al*., 2004). One of the most important causes of bovine subclinical mastitis is the intramammary infection (IMI) caused by *Staphylococcus aureus*, which is difficult to eradicate (Østeras *et al*., 2006; Ferguson *et al*., 2007).

Genetic characterization of mastitis-causing *S. aureus* isolates is vital for an effective mastitis control program, especially for developing a vaccine against *S. aureus*. With the development of molecular biology techniques, microbiologists have more choices for epidemiologic typing of *S. aureus* isolates at the present time. A discriminatory and reliable molecular typing method for the identification of bacterial isolates at strain level is the pulsed-field gel electrophoresis (PFGE) (Tenover *et al*., 1995; Peacock *et al*.,

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2002), which has successfully been used by many researchers in the field of molecular subtyping of mastitis-causing *S. aureus* (Fueyo *et al*., 2005; Haveri *et al*., 2007; Rabello *et al*., 2005; Sabour *et al*., 2004). So far, however, there is little literature available on this field in China.

As a remedy antimicrobials are used very frequently for infectious diseases on dairy farms (Kaneene and Miller, 1992; Sandgren *et al*., 2008), but the therapeutic outcome of *S. aureus* causing disease is poor, in part due to the versatile ability of the pathogen (Archer, 1998; Owens *et al*., 1988; Sol *et al*., 2000). Therefore, selection of a more effective drug for staphylococcal mastitis therapy by the evaluation of antimicrobial resistance may become a reality to clinical veterinarians.

Dairy cattle are economically important animals in Zhejiang Province, China. There are four major dairy farming regions that constitute over 80% of the dairy cow population in this province. To our knowledge, no study has been performed to investigate genetic diversity and antimicrobial susceptibility profiles among *S. aureus* isolates recovered from bovine mastitis in this area. The objectives of the present study, therefore, were to estimate the prevalence of bovine mastitis, compare genetic relatedness and antimicrobial susceptibility profiles of *S. aureus* isolated from mastitic milk from different regions of Zhejiang Province in order to provide a useful guide for veterinary practice in the treatment of *S. aureus* mastitis, and design mastitis control strategies directed to specific strains.

MATERIALS AND METHODS

Prevalence of subclinical mastitis and *Staphylococcus aureus*

The study was conducted in the period of April 2007 to June 2008 in four major dairy farming regions of Zhejiang Province, namely, Hangzhou, Jinhua, Ningbo, and Taizhou. To achieve representative distribution of parameters concerning size, location, and milking mode across the province, data were collected from 30 dairy farms. The herd structure was characterized by large-scale state-owned dairy farms and smallholder farms varied from 1200 to 20 cows per herd and the Chinese Holstein cow breed comprised the majority of cows. A total of 3178 quarter milk samples from 846 lactating cows without clinical signs of mastitis were investigated.

Analysis of milk samples

The incidence of subclinical mastitis was determined at cow and quarter levels based on indirect tests (Hangzhou mastitis test (HMT)). Mastitic paddle wells were used to collect milk from individual quarters, ensuring that the premilk was discarded. The procedures and the interpretation were performed using HMT test according to Hu *et al*.(1990). Samples with an HMT score of 0 or 1 were considered negative. Cows with an HMT score ≥1+ in one quarter but without clinical signs of mastitis were considered to have subclinical mastitis. Aseptic collection of quarter milk samples with HMT score of 2 or 3 were carried out for bacteriological assay.

Identification of *S. aureus* was done according to the standard routine (National Mastitis Council, 1999) and confirmed by the API STAPH® identification system.

After the investigation, 2~10 *S. aureus* strains for a herd were randomly chosen for antimicrobial susceptibility test and genotyping according to epidemiological distribution across the province, taking the size, location, and milking mode of the farms into consideration.

Antimicrobial susceptibility test

The disk diffusion method, an in vitro antimicrobial susceptibility test of the *S. aureus* isolates, was performed according to the guideline of the National Committee for Clinical Laboratory Standards (NCCLS, 2002). The antimicrobial agents tested were selected based on two factors, the recommendation of NCCLS and the actual veterinary practice, and included ampicillin (10 μg/disk), cefoxitin (30 μg/disk), cefalotin (30 μg/disk), chloramphenicol (30 μg/disk), erythromycin (15 μg/disk), gentamicin (10 μg/disk), oxacillin (1 μg/disk), penicillin G (10 IU/disk), tetracycline (30 μg/disk), clindamycin (2 μg/disk), ciprofloxacin (5 μg/disk), and vancomycin (30 μg/disk). Results were recorded as sensitive and resistant. Antimicrobial disks were purchased from Tianhe (Tianhe microbial reagent Co., Ltd., Hangzhou, China). The strain *S. aureus* ATCC 25923 was used as the control strain.

Molecular typing by pulsed-field gel electrophoresis

Molecular typing of *S. aureus* isolates was performed using restriction enzyme *Sma*I (TaKaRa Corp., Japan) and subsequent PFGE according to the method of McDougal *et al*.(2003). PFGE was run on the CHEF Mapper (Bio-Rad, Hercules, CA, USA) using 1% (w/v) SeaKem Gold agarose (Cambrex Bio Science, Rockland, ME, USA) with 0.5× Tris-borate-ethylenediaminetetraacetic acid (EDTA) (1 mol/L Tris, 0.01 mol/L EDTA, and 1 mol/L boric acid) as running buffer. A *Salmonella* serotype Braenderup strain (H9812) digested with *Xba*I was used as size standards, which were run in the first, middle, and last lanes of each gel. The electrophoresis conditions were as follows: 14 °C, 120° ramp angle, 5 s to 40 s pulse time, 19 h at 6 V/cm.

Macrorestriction bands were analyzed by using visual judgment according to criteria proposed by Tenover *et al*.(1995) and using the BioNumerics software (Applied Maths, Kortrijk, Belgium) with Dice coefficients. Similarity was calculated on a dendrogram using the unweighted-pair group method with arithmetic means. Position tolerance and optimization were set at 1.2% and 1.0%, respectively. A similarity coefficient of 80% was selected to define the pulsed-field type clusters.

RESULTS

Incidence of subclinical intramammary infections and frequency of *Staphylococcus aureus* infection in four different regions

From a total of 846 clinically healthy lactating cows, 459 (54.3%) were found with 1 or more quarters HMT positive. Out of 3178 quarters, 890 (28.0%) were HMT positive. The highest prevalence in cow and quarter levels was found to be 69.7% and 36.4%, respectively in Ningbo. The prevalence of subclinical mastitis at cow and quarter levels in different regions is presented in detail in Table 1.

Rates of *S. aureus* detected from quarters with HMT score of 2 or 3 varied greatly in different regions (Table 1). The overall prevalence of this pathogen was 12.2% ranging from 3.3% to 27.9% in different regions. *S. aureus* IMIs were identified in 23 (76.7%) out of the 30 farms investigated. A total of 108 *S. aureus* isolates were recovered from mastitic milk. Subsequently, 75 representative *S. aureus* isolates were submitted to antimicrobial susceptibility test and genotyping.

Antimicrobial susceptibility

The test was performed in a satisfactory manner when the quality control strains produced values within the established ranges. Antimicrobial susceptibility of 12 antimicrobial agents against 75 representative *S. aureus* isolates from 4 different regions is presented in Table 2. Seven isolates (9.3% of total) were susceptible to all drugs tested. The remaining 68 isolates (90.7%) were resistant to at least 1 antimicrobial class. *S. aureus* isolates were observed mostly resistant to penicillin and ampicillin (77.3%), while it appeared non-resistant to cefalotin and vancomycin throughout the region. The proportion of *S. aureus* isolates resistant to gentamicin, chloromycetin, clindamycin, tetracycline, and erythromycin varied greatly among the four regions. Nevertheless, oxacillin and cefoxitin exhibited minimum antibacterial-resistance to all the strains tested. Resistance to multiple antibiotics such as penicillin, ampicillin, tetracycline, and erythromycin was common among the bacterial isolates. Six major resistance patterns were observed among the isolates: no resistance (pattern I: 9.3%), penicillin/ampicillin resistance (pattern II: 21.3%), penicillin/ampicillin/tetracycline/

Table 1 Prevalence of subclinical mastitis based on Hangzhou mastitis test (HMT) at the cow and quarter levels in four regions in Zhejiang Province, China

Region	Number of farms	Cow level		Quarter level		Prevalence of *S. aureus* (%)
		n	%	*n*	%	
Jinhua	8	340	51.8	1235	24.5	9.7
Hangzhou	5	168	54.8	645	29.3	27.9
Ningbo	7	208	69.7	797	36.4	3.3
Taizhou	10	130	40.8	501	21.8	8.4
Total	30	846	54.3	3178	28.0	12.2

Table 2 Percentage of resistant isolates for *Staphylococcus aureus* isolated from subclinical mastitis in the four regions of Zhejiang Province, China

Antimicrobial	Percentage of resistant isolates (%)				
	Hangzhou (*n*=39)	Jinhua (*n*=15)	Ningbo (*n*=13)	Taizhou (*n*=8)	Total (*n*=75)
Penicillin	78.9	88.9	60.0	75.0	77.3
Ampicillin	78.9	88.9	60.0	75.0	77.3
Oxacillin	2.6	0.0	0.0	0.0	1.3
Cefalotin	0.0	0.0	0.0	0.0	0.0
Cefoxitin	2.6	5.6	0.0	0.0	2.7
Ciprofloxacin	5.3	22.2	0.0	12.5	8.0
Gentamicin	18.4	44.4	20.0	50.0	28.0
Chloromycetin	13.2	61.1	20.0	62.5	29.3
Clindamycin	15.8	72.2	0.0	62.5	29.3
Tetracycline	68.4	94.4	20.0	12.5	60.0
Erythromycin	36.8	88.9	40.0	50.0	48.0
Vancomycin	0.0	0.0	0.0	0.0	0.0
Main resistance patterns	Patterns II, III, IV	Patterns II, III, V	Patterns I, II	Patterns III, IV	

erythromycin resistance (pattern III: 27.5%), penicillin/ampicillin/erythromycin/tetracycline/gentamicin resistance (pattern IV: 15.7%), penicillin/ampicillin/erythromycin/tetracycline/chloromycetin/clindamycin resistance (pattern V: 8.3%), and tetracycline resistance (pattern VI: 6.2%). Most of the staphylococci isolates belonged to patterns II and III. In addition, 2 or 3 predominant resistance patterns were noticeable in each of the regions analyzed.

Pulsed-field gel electrophoresis

Of the 75 representative *S. aureus* isolates collected from 23 herds in the 4 regions, PFGE can type all of the isolates, and 39 distinct PFGE patterns were identified (Fig.1). According to genetic relationships, the 39 PFGE patterns were assigned to 16 lineage groups, designated as pulsed-field types with a capital letter, and patterns within these lineages were represented by designations with numeric suffixes. The dendrogram in Fig.1 demonstrates the banding patterns and genetic relationships. Four major types represented 82.7% of all isolates. Being the largest group among the 75 isolates, type A accounted for 31 (41.3%) of all of the isolates, which were distributed in multiple herds across the 4 regions. Different types predominated in each region, observed as: Hangzhou type A (64.1%), Ningbo type C (34.5%) and type B (23.1%), Jinhua type D (53.3%), and Taizhou type C (62.5%).

Nearly two thirds of the herds with *S. aureus* infection exhibited a unique type, whereas type A was observed in multiple herds (*n*=9, 47.4%) for all of the area (Table 3). No farm was found to have more than three types.

Table 3 Distribution of *Staphylococcus aureus* in farms based on PFGE types

Farms	Distribution	
	n	%
One type only	10	43.5
Two types	7	30.4
Three types	6	26.1
More than three types	0	0

DISCUSSION

Subclinical mastitis is hard to detect and is the main form of mastitis (Oliver *et al*., 2004; Pitkala *et al*., 2004). In China, many researchers reported that 40%~80% of lactating dairy cows in most farms suffer from subclinical mastitis (Pan *et al*., 1996; Zhang *et al*., 2005). The present study shows a high prevalence of subclinical mastitis in dairy cows in Zhejiang Province identified by HMT, a qualitative measurement of the somatic cell count in milk that can be easily used at cow side. The overall prevalence of subclinical mastitis in this study was 54.3% on cow basis and 28.0% on an udder quarter basis. Among different regions, the prevalence ranged from 40.8% to 69.7% on cow basis and 21.8% to 36.4% on an udder quarter basis. The results are consistent with the

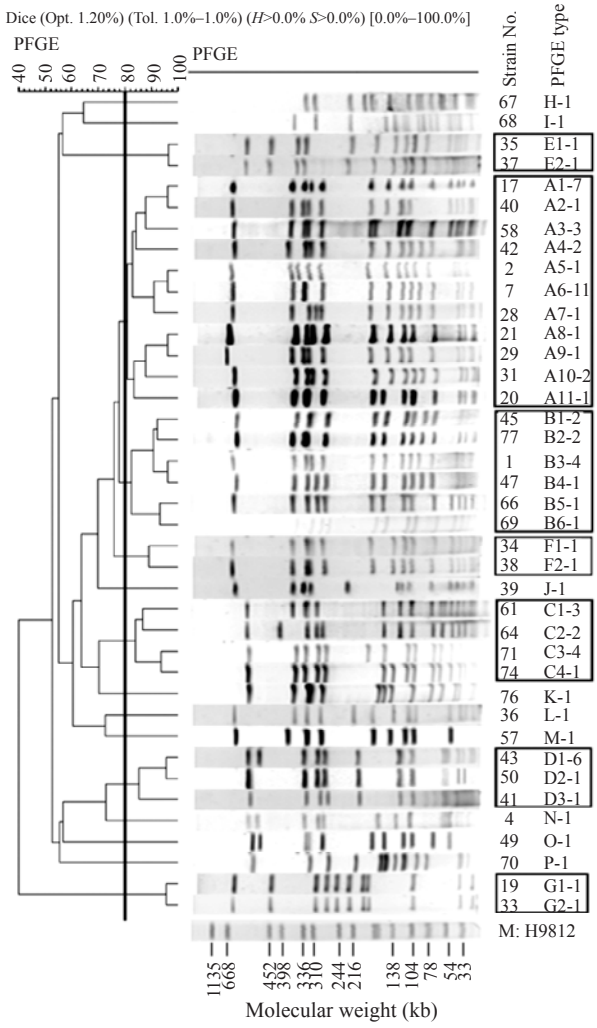

Fig.1 Dendrogram showing the level of similarity among *Sma*I macrorestriction patterns of 75 strains of *Staphylococcus aureus* recovered from bovine subclinical mastitis in Zhejiang Province, China

The pulsotype and the capital letter of isolates assigned to each pulsotype are indicated to the right of the banding patterns

previous findings of Zhang *et al*.(2005), who reported a prevalence of 48.1% and 22.4% in cow and quarter in Jinhua, a region of Zhejiang Province. The variation in prevalence of mastitis observed among the four regions in the present investigation may be due to differences in husbandry condition and management, as well as a lack of a mastitis-control program in certain areas.

As a major contagious pathogen associated with bovine mastitis, *S. aureus* frequently causes subclinical chronic infections of the mammary gland in dairy cows (Østeras *et al*., 2006; Wilson *et al*., 1997). In United Kingdom, with the implementation of the Five-Point Plan there has been a dramatic decrease in the prevalence of contagious mastitis pathogens, especially for *S. aureus*, from 67 quarter case/100 cows per year in 1967 to 2.2 quarter case/100 cows per year in 1998 (Bradley, 2002). In China, however, *S. aureus* isolates have been reported to be involved in about 10%~50% of mastitic milk samples from dairy cows (Hu *et al*., 1998; Liu *et al*., 2007). In the current study, our results demonstrate that 76.7% of the examined herds suffered from *S. aureus* infection, and the overall prevalence of this pathogen is 12.2% in quarter milk samples with HMT score of 2 or 3. It is worth to note, however, that the prevalence of *S. aureus* can vary greatly among different regions, ranging from 27.9% to 3.3% in the present investigation.

Bacterial resistance has been reported for more than 30 years and *S. aureus* is the most extensively studied pathogen in dairy diseases (Lowy, 2003). Many authors had reported resistant strains in *S. aureus* isolated from bovine mastitis in different countries (Moroni *et al*., 2006; Gentilini *et al*., 2000; de Oliveira *et al*., 2000). In our study, the rate of resistant strains was high (90.7%), and resistance to penicillin G and ampicillin (77.3%) was the most frequently observed. This is in a good agreement with the results reported by Wang *et al*.(2007) and Liu *et al*.(2007), regarding *S. aureus* isolated from mastitis infected cows in China, but much higher than those reported for *S. aureus* strains isolated from bovine mastitic milk in Europe and the United States (de Oliveira *et al*., 2000). Intensive, empirical or inappropriate use of antimicrobial drugs as a treatment for dairy diseases may be a plausible explanation for the higher antibiotics resistance in *S. aureus* in China. This is further confirmed by the lower levels of resistance reported in Nordic countries, such as Denmark and Norway, where strict antimicrobial management policy is existing in veterinary practices (Pitkala *et al*., 2004). To our knowledge, clindamycin and chloromycetin are rarely used to treat bovine mastitis in Zhejiang Province, but, surprisingly, resistance to these antimicrobial agents was observed. Perhaps, intensive use of these antimicrobial agents as a therapy for other infectious diseases in dairy farms can be attributed to this resistance; therefore, considerable attention is needed in the future.

The emergence of multi antimicrobial-resistance is usually observed among bovine isolates (Gentilini *et al*., 2000; Waage *et al*., 2002). In Shitandil and Sternesjö (2004)'s study, 34.3% and 18.0% *S. aureus* isolates recovered from small and large farms, respectively, developed resistance to two or more antimicrobial drugs. Resistance to penicillin and ampicillin was also common in other countries (de Oliveira *et al*., 2000). The present study shows that more than 80% of *S. aureus* strains were resistant to more than one antimicrobial agent tested, and the predominant pattern was simultaneously resistant to penicillin, ampicillin, tetracycline, and erythromycin, which is consistent with the findings of Wang *et al*. (2007). Those antimicrobial drugs are the largest group involving the treatment of infectious diseases in dairy farms in China. The current study shows that variation for antimicrobial susceptibility pattern of *S. aureus* also exists in different regions, which may be associated with complicated reasons, such as different habits of clinical veterinary in selection of therapeutic drugs. These results may provide important knowledge for the development of effective control strategies for *S. aureus* mastitis in Zhejiang Province.

PFGE is considered to be the 'gold standard' for molecular typing of pathogenic bacteria regarding its discriminatory ability, reproducibility, and typeability, which has been recommended to type *S. aureus* isolates (Weller, 2000). In this study, genetic diversity of 75 representative *S. aureus* isolates recovered from different regions in Zhejiang Province was examined by PFGE. This may be the first time that PFGE is used in this field in China. For the genetic diversity of mastitis-causing *S. aureus*, some investigations revealed that some predominant strains can exist in different dairy farms, even in different countries (Fox *et al*., 1991; Smith *et al*., 2005). On the contrary, Joo *et al*.(2001) reported that *S. aureus* types associated with IMI were much more likely to be unique to a herd than to be found in multiple herds. In the current study, type A was represented by 41.3% of isolates analyzed and was recovered from a number of herds, which is consistent with the findings of Smith *et al*. (2005). Accordingly, it can be inferred that some strains prevailing among herds, even in different regions, have some advantages of infecting due likely to special combination of virulence factors. On the other hand, nearly two thirds of herds with *S. aureus* infection had a unique type, which is in agreement with the results reported by Joo *et al*.(2001). Therefore, both situations may exist simultaneously. Geographical variation in the distribution of major prevalent types was also noted among different regions in the current investigation. So, comparison of predominant and minor strains in different regions or herds can be an important preliminary study for a successful herd autogenous *S. aureus* vaccination program. Further studies are needed to elucidate potential combination of virulence factors that favor the advantages within particular geographical environment.

An association between bovine mastitis-causing *S. aureus* PFGE type and antimicrobial susceptibility phenotypes was reported by Anderson *et al*.(2006). However, in our study agreement between antimicrobial resistance patterns and particular *S. aureus* PFGE types was not observed for most isolates, except that tetracycline resistance was not found in type C. Disparate results may be derived from a much higher degree of resistance of *S. aureus* isolates in our study.

In conclusion, the present study describes the *S. aureus* genotypes responsible for the mastitis cases in different dairy regions of Zhejiang Province, China. This information might help in formulating strategies to alleviate *S. aureus* mastitis. Furthermore, antimicrobial susceptibility test of *S. aureus* could be used as a guide to select antimicrobials for the therapy of mammary infections.

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