Genetic Polymorphisms of *Staphylococcus aureus* Isolated from Bovine Mastitis in China

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A B S T R A C T

In order to provide science-based information for the control of cow mastitis, to understand the genetic polymorphisms of dairy-sourced *Staphylococcus aureus*, we conducted genotyping of *Staphylococcus aureus* isolates from milk samples collected from cows with clinical mastitis using the random amplified polymorphic DNA (RAPD) method with three oligonucleotide primers. In total, 177 *Staphylococcus aureus* isolates were obtained from dairy farms in 5 different provinces in China, including Guizhou, Inner Mongolia, Shanghai, Sichuan, and Gansu. The results showed that the amplified product bands with three primers per isolate ranged from 1~9 and the product sizes from 240 to 4,500 bp. Primer OLP13 had the highest discriminant coefficient. The *Staphylococcus aureus* strains were divided into 11 genotypes by fingerprint with oligonucleotide primers OLP13. There were 12 strains in type 1, 8 in each of types 2 and 5, 22 in each of types 3 and 7, 13 in types 4, 11 in type 6, 25 in type 8, 28 in type 9, 8 in type 10 and 5 in type 11. Among the 11 types of *S. aureus* isolates, type3 (14/37) and type 7 (15/35) were the main prevalent strains in Guizhou and Inner Mongolia Province respectively. Type 9 (21/55) had the highest prevalence rate and type 8 (10/55) was higher than other types in Shanghai, both of them were closely related. The results showed that the genotype distribution in various regions was noticeably different. Differences in dairy farming practices, geographical environments, and the use of antibiotics may be the reasons for the varied distribution of different genotypes in those provinces.

INTRODUCTION

Bovine mastitis is a common disease of dairy cattle and an important cause of economic loss for dairy production. Additionally, bovine mastitis compromises milk quality and potentially affects public health and food safety (Freitas *et al*., 2018; Shirli *et al*., 2018). Bovine mastitis is caused by multiple types of non-specific pathogenic microorganisms, among which *Staphylococcus aureus* (*S. aureus*) is the most common one (Lundberg *et al*., 2016; Sarah, 2018). Reports on the isolation and identification of pathogenic bacteria causing bovine mastitis in recent years have shown that the detectable rate of *S. aureus* is as much as over 30% (MingMei *et al*., 2009). Genotyping pathogens associated with mastitis is important for understanding the epidemiological relationship of the pathogens, prevention and treatment of the disease, and the development of vaccines (Mostafa, 2013). With the advancement of molecular techniques, more and more genotyping methods have been applied to the molecular epidemiological study on pathogenic bacteria (Hamid *et al*., 2017; Tianming *et al*., 2017; Schmidt *et al*., 2017; Zhang *et al*., 2018). In 1990, based on the PCR technique, Williams *et al*. first proposed a new, simple, and sensitive genetic typing technique random amplified polymorphic DNA (RAPD) (Williams, 1990). The advantages of the RAPD-PCR technique are multiple. For example, it is simple and fast, and doesn't require prior knowledge of the genetic background of bacterial genome, Additionally, RAPD can be performed with a large number of usable primers, and it covers nearly the whole genome and provides large amount of polymorphism information (Adzitey *et al*., 2013). Rapidly after its introduction, this technique has been widely used for molecular typing and identification of microorganisms across the globe. In recent years, studies on the genotyping of *S. aureus* have been focused on strains isolated from humans (Boriollo, 2018; Kates *et al*., 2018; Martin *et al*., 2004), and few studies have been performed on strains isolated from livestock. In particular, there have rarely been any reports on the genotyping of *S. aureus* strains that cause bovine mastitis in China. To close this knowledge gap, we conducted RAPD-PCR genotyping of *S. aureus* isolates from bovine mastitis cases in Inner Mongolia, Guizhou, Shanghai, Sichuan and Gansu provinces, and analyzed the molecular epidemiological characteristics of *S. aureus* associated with mastitis in different regions.

The information should be useful for the prevention and treatment of bovine mastitis.

MATERIALS AND METHODS

Ethics

Milk samples were obtained on farms from dairy cows with naturally occurring clinical mastitis with consent from farm owners under the ethical approval by Lanzhou Institute of Husbandry and Pharmaceutical Sciences of CAAS. The collections were done by professional veterinarians and permitted by the owners of the dairy farms under investigation. Conventional milking methods were used and no invasive or pain-causing procedures were involved. This study did not involve endangered or protected species and did not use animals for experiments.

Bacterial strains

In total, 176 *S. aureus* strains isolated from clinical bovine mastitis samples were used in this study: 35 from the Inner Mongolia area (NY), 20 from the Sichuan area (CX), 27 from the Gansu area (HG, QY, KY), 56 from the Shanghai area (SH, SX), and 38 from the Guizhou area (ZY). These strains were all verified as *S. aureus* via biochemical and molecular biological identifications. The standard *S. aureus* strain CVCC2246 was purchased from the China Institute of Veterinary Drug Control.

Agents and instruments

The DNA ladder marker (molecular sizes at 4,500, 3,000, 2,250, 1,000, 750, 500 and 250 bp), proteinase K, lysozyme, and Premix EX Taq were all purchased from TaKaRa BIO Inc. (Dalian). The bacterial chromosome DNA extraction kit was purchased from TIANGEN Biological Product Inc. Other equipment used included Eppendorf high speed centrifuge, Biometra PCR thermal cycler, and DYY-8C electrophoresis apparatus (Beijing Liuyi, China).

Primer synthesis

Three random-sequence primers were selected according to the published literature (Naffa *et al.*, 2006; Neela *et al.*, 2005; Reinoso *et al.*, 2004). The selected sequences 4M (5′-AAGACGCCGT-3′), AP-7 (5'-GTG-GATGCGA-3') and OLP13 (5′-ACCGCCTGCT-3′) were used as the amplifying primers in three separate RAPD-PCR tests which was synthesized by TaKaRa BIO Inc. (Dalian, China).

RAPD reaction conditions

Each reaction consisted of 25 µl in volume with 12.5µl Premix mixture, 2µl DNA template, 2µl primer, and 8.5µl deionized water. The PCR cycling parameters were: 4 cycles of 94 °C 5 min, 37 °C 5 min, and 72 °C 5 min; 30 cycles of 94 °C 1 min, 37 °C 1 min, 72 °C 2 min; and 72°C extension for 10 min. Next, 5µl of the amplification product was subject to electrophoresis (8V/cm) in a 1.0% agarose gel. The electrophoresis buffer used was 1×TAE for a duration of 30~50 min. The DNA Ladder Marker was simultaneously subject to electrophoresis. Quantity One 1-D gel imaging system was used for UV imaging.

Data analysis

After the electrophoresis images were obtained using the Quantity One 1-D gel imaging system, BioNumerics software was used for data processing. The specific parameter settings were as follows: optimal value 0.5%, position tolerance 1.25%, and threshold 80%. A cluster analysis of the images was performed using Dice coefficient and an unweighted pair group method with the arithmetic mean (UPGMA).

RESULTS

RAPD amplification

RAPD was performed on 177 *S. aureus* strains (including the standard strain). Supplementary Figures S1-S3 show clear PCR products amplification the chromosome DNA of all strains produced clear, recognizable band spectra. The strains showed 2~9 bands with multiple patterns, and the sizes of the fragments were in the range of 240~4,500 bp. Table I shows a comparison of the three amplification results.

As shown in Table I, primer 4M amplified the largest number of bacteria, which had the highest typing rate and the lowest discriminant coefficient. Primer OLP13 amplified the highest discriminant coefficient and the lowest typing rate. Primer AP7 was between 4M and OLP13, but its strip size was the widest.

Genetic correlation analysis of RAPD products

A cluster analysis of the electrophoretograms of the RAPD products from the chromosome DNA of *S. aureus* strains was conducted by the Bio-Numerics software, and a genetic relationship clustering diagram was plotted afterwards (Figs. 1-3). Figures 1-3 show the result with primer 4M, AP7, and OLP13, respectively.

As illustrated in Figure 1, aside from the standard strain, the 174 clinically obtained *S. aureus* strains could be classified into 8 main clusters based on genetic diversity. Sixteen strains were of type 1, 37 of type 2, 37 of type 3, 15 of type 4, 18 of type 5, 14 of type 6, 13 of type 7 and 8 type 8. Type 2 was composed of 3 subtypes, and the rest were composed of 2 subtypes. Among the *S. aureus* isolates

Table I. Comparison of the amplified products by primers 4M, AP7 and OLP13.

Primer	No. of not ampli-fied strains	Classifica-tion rate	Amplifying band number	1~3 bands pro-portion %	1~4 bands pro-portion %	Bands size range	discrimination
4M	2	98.87	1~10	51.43	74.29	273~4,500bp	0.870
AP7	3	98.31	1~9	34.48	52.81	250~4,500bp	0.872
OLP13	5	97.18	1~10	50.00	63.37	345~4,500bp	0.891

Table II. Genotype distribution of *Staphylococcus aureus* as determined by primer 4M.

	Type 1	Type 2	Type 3	Type 4	Type 5	Type 6	Type 7	Type 8
Guizhou	3	18	7	3	0	2	3	0
Inner Mongolia	5	5	5	0	11	4	0	1
Sichuan	3	5	2	1	1	2	3	1
Shanghai	4	3	21	5	1	3	6	5
Gansu	1	5	2	6	5	3	1	1
Standard strain		1						
Percentage of the total isolates (%)	9.1	21.1	21.1	8.6	10.3	8	7.4	4.6

Table III. Genotype distribution of *Staphylococcus aureus* as determined by primer AP7.

	Type 1	Type 2	Type 3	Type 4	Type 5	Type 6	Type 7	Type 8	Type 9	Type 10	Type 11	Type 12
Guizhou	0	1	5	0	0	16	9	1	2	0	0	2
Inner Mongolia	13	0	1	2	1	2	0	3	1	0	1	3
Sichuan	10	0	0	2	1	1	1	1	0	1	1	0
Shanghai	7	6	0	8	1	19	2	2	4	1	1	4
Gansu	5	0	0	5	1	5	0	0	2	1	3	0
Standard strain					1							
Percentage of the total isolates (%)	20.1	4.0	3.4	9.8	2.9	24.7	6.9	4	5.2	1.7	3.4	5.2

Table IV. Genotype distribution of *Staphylococcus aureus* as determined by primer OLP13.

	Type 1	Type 2	Type 3	Type 4	Type 5	Type 6	Type 7	Type 8	Type 9	Type 10	Type 11
Guizhou	2	0	14	3	1	0	3	6	5	0	0
Inner Mongolia	1	2	1	2	1	4	15	1	1	6	1
Sichuan	0	1	3	1	4	3	3	2	0	2	0
Shanghai	7	5	3	2	0	1	1	10	21	0	4
Gansu	2	0	1	4	2	3	0	6	1	0	0
Standard strain				1							
Percentage of the total isolates	7.0	4.7	12.8	7.6	4.7	6.4	12.8	14.5	16.9	4.7	2.9

examined with primer 4M, type 2 (18/37), 3 (21/56) and 5 (11/34) had the highest prevalence in Guizhou, Shanghai and Inner Mongolia Province respectively (Table II).

Figure 2 indicated that, except the standard strain, the 173 clinically obtained *S. aureus* strains could be classified into 12 main clusters based on genetic diversity. Thirty-five strains were of type 1, 7 of type 2, 6 of type 3, 17 of type 4, 5 of type 5, 43 of type 6, 12 of type 7, 7 of type 8, 9 of type 9, 3 of type 10, 6 of type 11, and 9 of type 12. Type 1 and type 6 were both composed of 3 subtypes. Among the

S. aureus isolates generated with primer AP7, type 1 was the dominant type in Sichuan (13/20) and Inner Mongolia (13/34) Province, type 6 was mainly distributed in Guizhou (16/37) and Shanghai (19/56) Province (Table III).
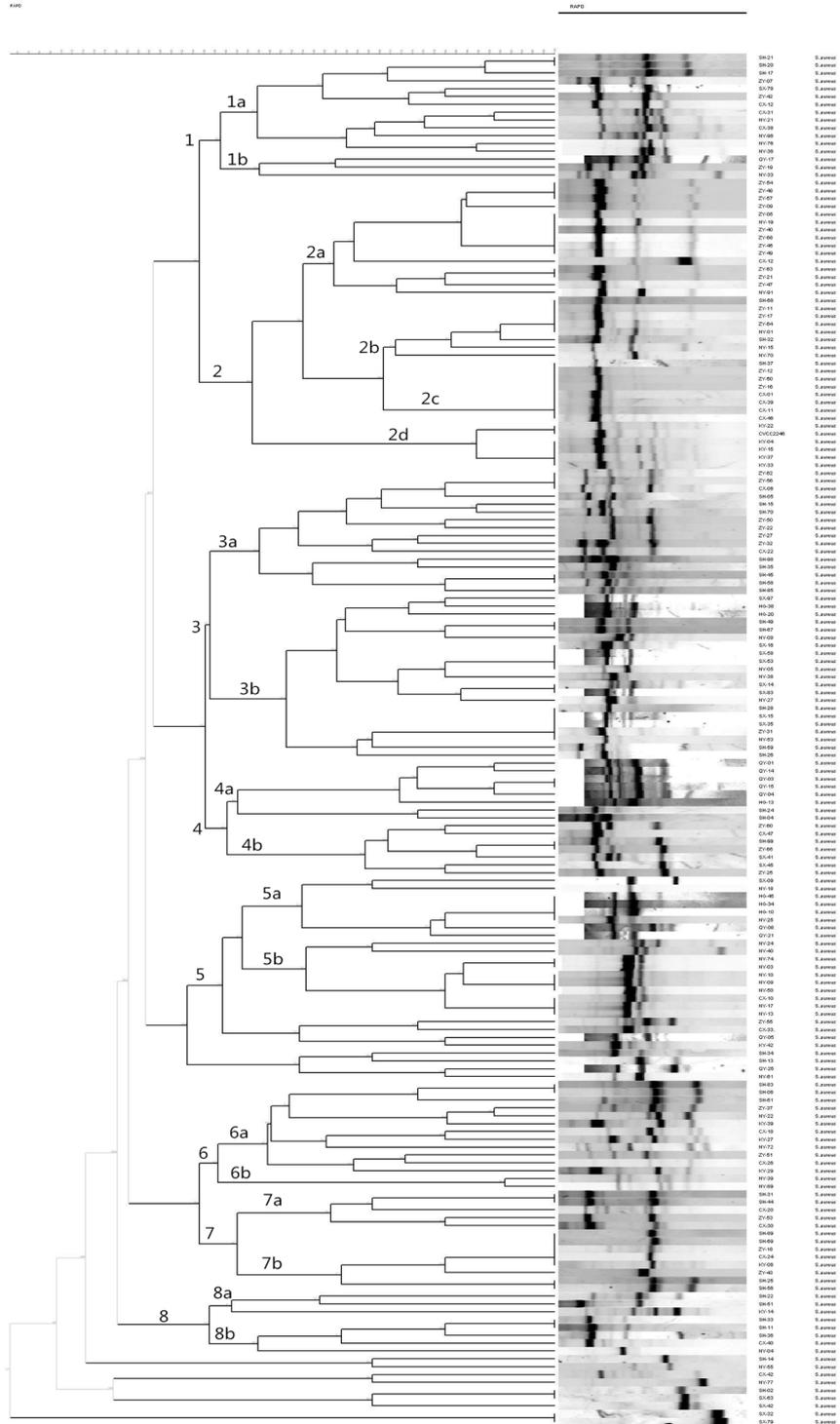

Fig. 1. Genetic relationship among the 175 *Staphylococcus aureus* isolates as estimated by clustering analysis of RAPD profiles obtained with primer 4M.
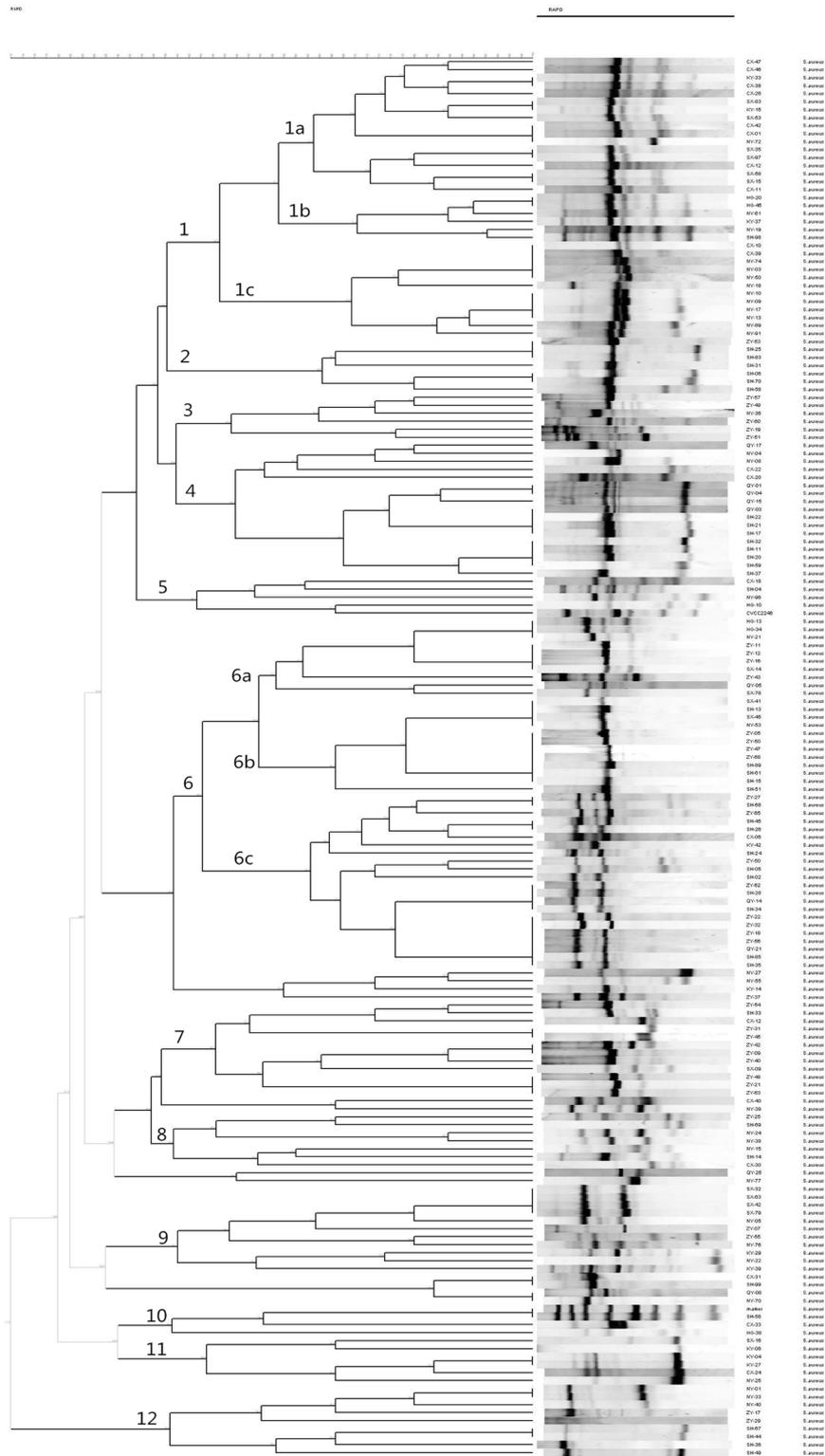
Fig. 2. Genetic relationship among the 174 *Staphylococcus aureus* isolates as estimated by clustering analysis of RAPD profiles obtained with primer AP7.
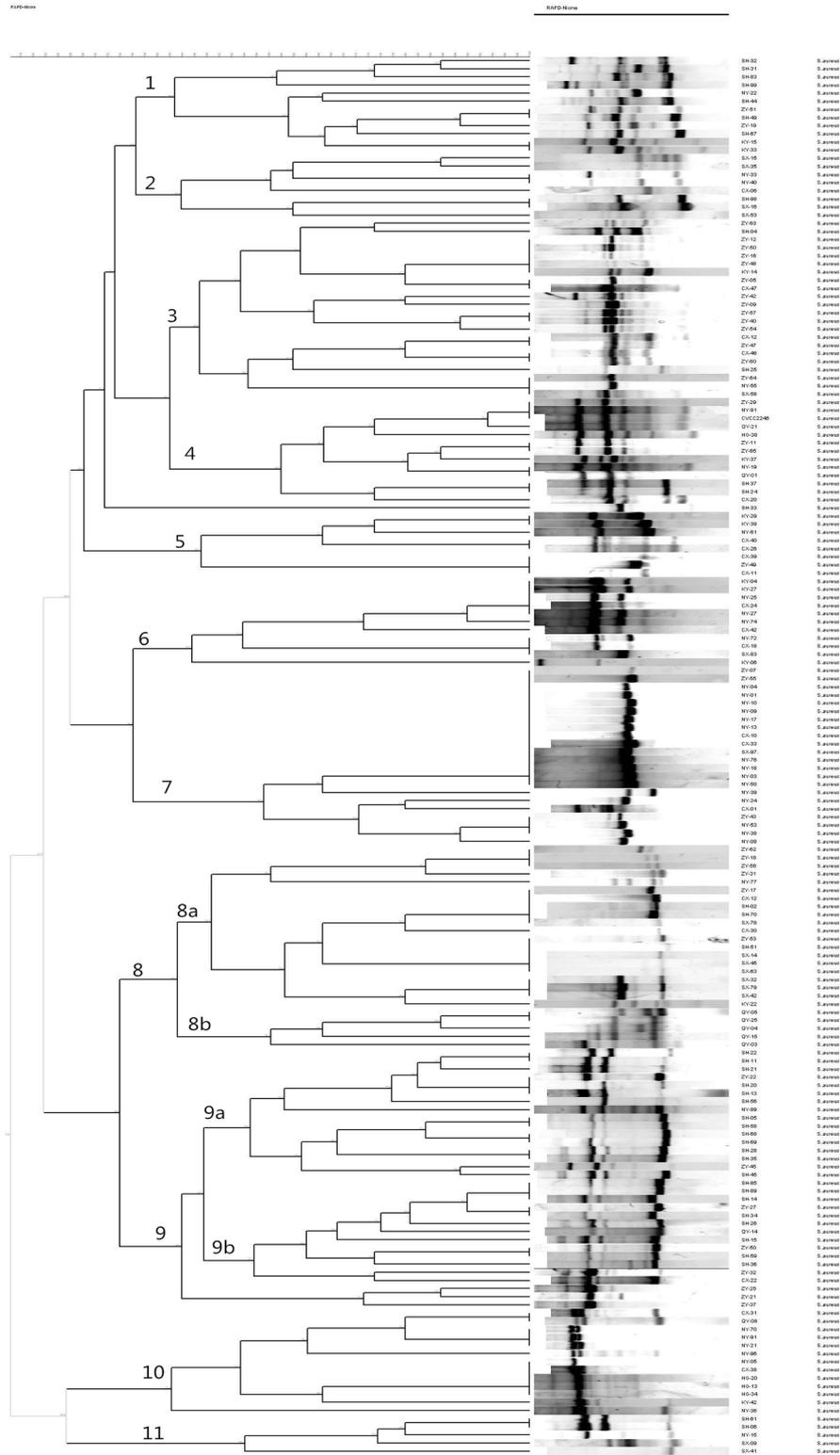
Fig. 3. Genetic relationship among the 172 *Staphylococcus aureus* isolates as estimated by clustering analysis of RAPD profiles obtained with primer OLP13.

Figure 3 displays that the 171 clinically obtained *S. aureus* strains were classified into 11 main clusters based on genetic diversity. Twelve strains were of type 1, 8 of type 2, 22 of type 3, 13 of type 4, 8 of type 5, 11 of type 6, 22 of type 7, 25 of type 8, 28 of type 9, 8 of type 10, and 5 of type 11. Type 8 and type 9 were both composed of 2 subtypes. Among the *S. aureus* isolates generated with primer OLP13, type3 (14/37) and type 7 (15/35) were the main prevalent strains in Guizhou and Inner Mongolia Province respectively. Type 9 (21/55) had highest prevalence rate and type 8 (10/55) was higher than other types in Shanghai, both of them were closely related.

From the above results, Guizhou, Inner Mongolia and Shanghai Provinces were dominated by one main type, while others are not.

DISCUSSION

S. aureus is one of the main pathogenic bacteria that cause bovine mastitis, which is a common disease in dairy cattle and results in significant economic losses. With the increasing prevalence of methicillin-resistant *S. aureus* (MRSA) strains, treatment of bovine mastitis caused by *S. aureus* has become more and more difficult, a significant problem for the dairy cattle farming industry worldwide (Ronco *et al.*, 2018; Zhang *et al.*, 2018). In recent years, advances in molecular biology and immunology have greatly facilitated the development of a new generation of effective vaccines. However, many vaccines against bovine mastitis reported in recent years have not been widely used due to a lack of effectiveness, high production costs, and instability under certain circumstances. Thus, in order to develop convenient, highly effective, and safe vaccines against *S. aureus* infection, the genotypes of the pathogenic strains in different areas and the relations between them must be deciphered first.

RAPD is a PCR-based genotyping method that reveals genetic polymorphisms among bacterial isolates. The randomness of RAPD is mainly determined by primer selection. The number and intensity of bands in the RAPD fingerprint are associated with the selected primer, the quality of the DNA template, and the PCR reaction system and cycling conditions (Cui *et al.*, 2007). Therefore, in order to obtain clear and highly recognizable bands, and to reduce non-consistent amplification, it is extremely important to select an appropriate primer, optimize reaction conditions, and repeat the experiments.

From the results obtained in this study, it was found that RAPD typing of 177 *S. aureus* strains with primers 4M, OLP13, and AP7 produced clear, recognizable band patterns which allowed the genotyping of the *S. aureus* isolates. The number of bands varied between 2 and 9,

and the size of the products varied between 240 and 4,500 bp, demonstrating genetic polymorphisms between the strains. From the classification rate, band size, and number of the amplified bands, primer 4M performed better than other primers. However, based on the clustering map, primer OLP13 yielded more even distribution of the analyzed strains in the clusters, and its differentiation coefficient was the highest among the three primers. Belkum *et al.* (1995) suggested that primer AP7 had lower discrimination than other primers. Rodriguez-Calleja *et al.* (2006) showed a discrimination of 0.877 with primer 4M. Naffa *et al.* (2006) found that after amplification with primers OLP13, the number of bands varied between 2 and 9, and the size of the products varied between 240 and 4,500 bp. Results from this study showed certain similarities to the previously reported findings. Based on these results, it appears that primer OLP13 has advantages in RAPD-based typing of *S. aureus*.

The 172 *S. aureus* strains were classified into 11 main clusters by primer OLP13 (Table IV). Within each cluster, the genetic distances among the isolates were either similar or close, reflecting the close relationship of the strains in the 11 clusters. Regarding the distribution of genotypes in each region, the strains from Guizhou were mainly concentrated in type 3, and there were no strains in the genotypes 2,6,10 and 11. The limited diversity among the strains from Guizhou may be explained by the fact that the samples collected from Guizhou were from remote areas, where dairy farms were small (less than 800 cows) and there were limited transmission opportunities of *S. aureus* isolates between farms due to geographical reason. There are mountains barrier between every living area, the infectious disease in Guizhou are not easy to spread. Even when an outbreak occurs, the pathogenic bacteria can only spread in a limited area. In the Inner Mongolia area, type 7 was the dominant genotype, and the proportions of the strains of the other genotypes were relatively even. The Inner Mongolia area is the main region for national milk supply, where the dairy cattle farming industry is large (>10,000 cows each) and advanced, and there are frequent movement between farms in terms of personnel, animals and vehicles. Thus, the transmission of pathogenic microorganisms between dairy farms is more frequent, leading to distribution of multiple genotypes in the Inner Mongolia area. The strains in Shanghai were mainly concentrated in types 8 and 9, accounting for 56.4% of the total, which were the dominant genotype strains. Three genotypes were absent in the isolates from Sichuan, and the number of the isolates in the remaining genotypes was similar. For the isolates from Gansu, the number of isolates in each genotype was small, but the isolates were widely distributed across different genotypes,

with high similarity observed with the isolates from the same dairy farm. In general, the similarity of the strains from the same region shared higher similarity, while strains from different regions were genetically diverse, indicating geographic separation more or less affected the mingling of the bacterial strains. However, the presence of genetically distant strains in the same region suggests cross-transmission or mutations occurred in the pathogen.

CONCLUSIONS

In summary, this work represents the first RAPD typing study on *S. aureus* strains from different areas in China. It also confirmed that primer OLP13 is more suitable than other primers for RAPD typing of the pathogen. Using primer OLP13, the 170 *S. aureus* strains were separated into 11 main clusters. The results showed that the genotype distribution in various regions were noticeably different. This may be explained by the geographical separation, climate variation, and environmental difference of the dairy farms in different regions as well as differences in dairy cattle farming practices. Our study provides timely information for molecular epidemiology of *S. aureus* and useful information for the treatment and prevention of bovine mastitis as well as the development of cross-protective vaccines.

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Supplementary material
There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20200109070125

Statement of conflicts of interest
The authors have declared no conflict of interest.

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