High Incidence of Oxacillin-Susceptible \textit{mecA}-Positive \textit{Staphylococcus aureus} (OS-MRSA) Associated with Bovine Mastitis in China

WanXia Pu*, Yang Su, JianXi Li, ChunHui Li, ZhiQiang Yang, HaiPing Deng, ChunXia Ni

Key Laboratory of New Animal Drug Project, Gansu Province, Key Laboratory of Veterinary Pharmaceutical Development, Ministry of Agriculture, Lanzhou Institute of Husbandry and Pharmaceutical Sciences of CAAS, Lanzhou, China

Abstract

\textit{Staphylococcus aureus} is a main cause of bovine mastitis and a major pathogen affecting human health. The emergence and spread of methicillin-resistant \textit{Staphylococcus aureus} (MRSA) has become a significant concern for both animal health and public health. This study investigated the incidence of MRSA in milk samples collected from dairy cows with clinical mastitis and characterized the MRSA isolates using antimicrobial susceptibility tests and genetic typing methods. In total, 103 S. aureus isolates were obtained from dairy farms in 4 different provinces in China, including Gansu, Shanghai, Sichuan, and Guizhou. Antimicrobial susceptibility testing of these isolates revealed that the resistance rates to penicillin and sulfamethoxazole were high, while the resistance rates to ciprofloxacin and vancomycin were low. Among the 103 isolates, 49 (47.6\%) were found to be \textit{mecA}-positive, indicating the high incidence of MRSA. However, 37 of the 49 \textit{mecA}-positive isolates were susceptible to oxacillin as determined by antimicrobial susceptibility assays and were thus classified as oxacillin-susceptible \textit{mecA}-positive \textit{S. aureus} (OS-MRSA). These isolates could be misclassified as methicillin susceptible \textit{Staphylococcus aureus} (MSSA) if genetic detection of \textit{mecA} was not performed. Molecular characterization of selected \textit{mecA}-positive isolates showed that they were all negative with Panton-Valentine leukocidin (PVL), but belonged to different \textit{spa} types and SCC\textit{mec} types. These results indicate that OS-MRSA is common in bovine mastitis in China and underscore the need for genetic methods (in addition to phenotypic tests) to accurately identify MRSA.

Introduction

\textit{Staphylococcus aureus} causes infections in both people and animals. Methicillin-resistant \textit{S. aureus} (MRSA) is a prominent pathogen in nosocomial and community acquired infections, and is a major threat to human health worldwide due to its antimicrobial resistance, infectivity and possession of virulence factors [1]–[5]. Methicillin resistance in \textit{S. aureus} is mainly mediated by the expression of the \textit{mecA} gene, which is located on a mobile genetic element, staphylococcal cassette chromosome \textit{mec} (SCC\textit{mec}), and encodes an altered penicillin-binding protein (\textit{PBP}2\textit{a}) with an extremely low affinity to \textit{\beta}-lactam antibiotics, making it possible for \textit{S. aureus} to survive the treatment of \textit{\beta}-lactam antibiotics [6]. \textit{S. aureus} that either have the \textit{mecA} gene or show a minimum inhibitory concentration (MIC) of oxacillin higher than 4 \textit{mg}/ml are defined as MRSA. However, \textit{S. aureus} that are positive for \textit{mecA} and \textit{PBP}2\textit{a}, but phenotypically susceptible to oxacillin have been reported [7]–[9]. It is generally accepted that such \textit{S. aureus} isolates should be defined as oxacillin-susceptible \textit{mecA}-positive \textit{S. aureus} (OS-MRSA). Identification of OS-MRSA has clinical implications as precautions have been proposed for the treatment of OS-MRSA infections. Although OS-MRSA is phenotypically susceptible to oxacillin, it may be prone to the development of highly resistant MRSA under antibiotic selection due to the possession of \textit{mecA} [10] [11].

China has recently experienced significant growth in dairy industry and the total milk production in China reached to the third place worldwide in 2012 [12]. In China, bovine mastitis is a serious problem for dairy industries and the average incidence rate is about 33\%, incurring considerable economic losses [13]. For example, it is estimated that 5–10\% cows are culled annually in the Shanghai region due to mastitis [14]. Although multiple pathogens are associated with bovine mastitis, \textit{S. aureus} is a common and important cause of the disease [15]. Antimicrobial treatment is often used to decrease the incidence or shorten the duration of bovine mastitis; however, treatment failure occurs due to development of antibiotic resistance [16].

Despite the fact that \textit{S. aureus} is commonly associated with bovine mastitis, MRSA isolates have been infrequently reported with the disease. There have been a few reports of MRSA colonization and/or infections in dairy cattle since the very first report of MRSA in mastitis in 1972 [17]–[21]. Recently, a highly divergent \textit{mecA} gene (now named \textit{mecC}) in a typeXI SCC\textit{mec} was found in bovine mastitis \textit{S. aureus} [22] [23]. Mastitic MRSA strains from different continents may share similar or different molecular characteristics. For example, reports from some European...
countries indicated that ST398 MRSA with SCCmec type IV or V played an important part in clinical or subclinical bovine mastitis, although it was not the only clonal lineage associated with mastitis [24]. Several genotypes including ST1/t226 MRSA with SCCmec type IVa, ST72/t324 MRSA with SCCmec type IV or IVa, and ST72/untypeable spa-type with SCCmec type IV were reported in Korea [25]. The majority of reported MRSA isolates in Turkey belonged to ST329/spa-type t30 with SCCmec type III, while others belonged to ST8/spa-type t190/SCCmec type IV, or ST329/spa-type t30/SCCmec type III [26]. These data indicated that various MRSA clones or genotypes were associated with bovine mastitis in different countries.

In China, there have been a few reports on MRSA of animal origin. Cui et al. reported presence of MRSA from swine and swine farm workers in four Chinese provinces, all of which belonged to ST9 and spa type t899, contained a type III SCCmec element, and lacked the Panton-Valentine Leukocidin (PVL) gene [27]. There was a report on MRSA from pet animals and veterinary staff in China, in which 22 MRSA isolates were identified by using the API Staph Ident System, MIC tests and staff in China, in which 22 MRSA isolates were identified by using the API Staph Ident System, MIC tests and genetic methods. The objective of this study was to characterize the MRSA isolates using various methods.

3. Phenotypic identification of MRSA

S. aureus isolates were tested for methicillin resistance using the cefoxitin and oxacillin disk diffusion methods outlined by the Clinical and Laboratory Standards Institute [32]. Cefoxitin disk (30 µg, Oxoid) and oxacillin disk (1 µg, Oxoid) were used in this study. The zones of inhibition were measured after 18–20 hours incubation at 35°C. Isolates with zone sizes less than 21 mm for cefoxitin and 10 mm for oxacillin were considered methicillin resistant according to the criteria of CLSI [32].

4. Antimicrobial susceptibility tests

The mecA-positive MRSA isolates were tested for susceptibility to various commonly used antimicrobial agents by using two different methods. The disk diffusion test was performed with penicillin (10 IU/disk), gentamicin (10 µg/disk), tetracycline (30 µg/disk), erythromycin (15 µg/disk), ciprofloxacin (5 µg/disk), sulfamethoxazole (300 µg/disk), cefazolin (30 µg/disk) and clindamycin (2 µg/disk). The agar dilution method was used to measure the MICs of vancomycin and oxacillin. Both disk diffusion and agar dilution were performed according to the recommendations of CLSI [32]. The breakpoints of CLSI for the tested antibiotics (for both disk diffusion and agar dilution) were used to determine the susceptibility profiles. All antimicrobial susceptibility testing assays were repeated at least 3 times.

5. Genotypic identification of MRSA

A single colony of S. aureus was inoculated into LB culture medium, and the culture was shaken overnight at 37°C. Then the culture was used for preparation of genomic DNA using the TIANamp Bacteria DNA Kit [TIANGEN BIOTECH (Beijing) CO., LTD] according to the manufacturer’s instructions. The genomic DNA was used as template for PCR for typing of SCCmec and spa and for detection of mecA and the PVL toxin gene ( lukS-PV).

The primers used to amplify the mecA gene (310 bp) of MRSA were P1: 5’-TGGGATCCTTGTTGACGAG-3’ and P2: 5’-CTGGAACTTGTTGAGCAGAG-3’ as previously described [33]. Each PCR mixture was composed of 2 µl DNA template, 0.5 µl of each primer (10 µM), 12.5 µl ExTaq buffer mix [TaKaRa Biotechnology (Dalian) Co., Ltd], and 9.5 µl sterile distilled H2O. PCR program began with an initial denaturation step at 94°C for 4 min followed by 34 cycles of 92°C for 1 min, 55°C for 50 seconds, and 72°C for 1 min, and ended with a final extension step at 72°C for 10 min. The mecA-positive strain ATCC43300 and the mecA-negative ATCC25923 were included as positive and negative controls, respectively. The amplified PCR products were electrophoresed in 2% agarose gel at 120 V for...
In detail, all 17 spa online spa and sequenced using the same PCR primers at Sangon Biotech graphed under UV light.

6. Spa-typing

Using the Ridom StaphType standard protocol (www.ridom.com), the MRSA strains from Gansu and Shanghai were PCR amplified for analyzing the polymorphic X-region of Staphylococcus protein A (spa) gene. The amplicons were purified using a TIANgel Midi Puriﬁcation Kit [TIANGEN BIOTECH (Beijing) CO., LTD] and sequenced using the same PCR primers at Sangon Biotech (Shanghai) Co., Ltd. The spa types were assigned by using an online spa database [34].

7. SCCmec typing

SCCmec types were determined using the primers as described by Zhang et al [35]. A combination of different PCR reactions was performed to type the SCCmec elements. For specific SCCmec types, the SCCmec M-PCR typing assay contained 2 pairs of primers including one pair speciﬁc for the mecA gene and another pair speciﬁc for SCCmec types and subtypes I, II, III, IVa, IVb, IVc, IVd and V. Then a single PCR reaction was performed to identify the related mec and cer gene complexes using speciﬁc primers. In addition, the PCR amplicon of SCCmec of isolate B5 was sequenced and compared with the standard SCCmec sequences in the NCBI database.

8. Detection of the Panton-Valentine Leukocidin gene lukF-lukS

PCR was performed to determine the presence of the PVL toxin gene lukF-lukS as previously described by Lina et al [36].

Results

1. The overall antimicrobial susceptibility proﬁles of the bovine S. aureus isolates

The antibiotic susceptibility of the analyzed S. aureus isolates were shown in Table 1. The overall resistance rates were high with penicillin (97.1%) and sulfa furazole (83.5%), while the overall resistance rates were generally low with gentamicin (11.7%), ciprofloxacin (2.9%), cefazolin (6.8%), vancomycin (0%), and oxacillin (12.6%). The resistance rates were at moderate levels to tetracycline (35%), erythromycin (31.1%), and clindamycin (29.1%). Although there were variations in the resistance rates among the 4 regions, it was not obvious that one region showed more incidence of resistance than others (Table 1).

2. Identiﬁcation of mecA by PCR

Using primers P1 and P2, we analyzed the presence of mecA in the S. aureus isolates. As shown in Fig. 1, the positive control strain ATCC43300 showed a distinct 310-bp band, while the negative control strain ATCC 25923 did not show a PCR product, indicating the speciﬁcity of the PCR assay. Among the S. aureus isolates examined in this study, 8, 20, 11, and 10 mecA-positive strains were identiﬁed for Gansu, Shanghai, Sichuan, and Guizhou, respectively (Fig. 1). The overall detection rate of mecA was 47.6% (49 out of 103), indicating the high prevalence of MRSA in S. aureus isolates derived from bovine mastitis in China.

3. Identiﬁcation of OS-MRSA

According to the oxacillin disk diffusion tests, only 12.6% of the isolates were resistant to this antibiotic (Table 1), but 47.6% of the isolates were mecA-positive, suggesting the presence of OS-MRSA. In detail, all 17 S. aureus isolates from Gansu were susceptible to the antibiotic, but 8 (47.06%) of them were found carrying the mecA gene by PCR (Fig. 1; Table 2) and were classiﬁed as OS-MRSA. Four of the eight mecA-positive isolates were from farm A, and the other 4 from farm B. Among the 52 isolates from Shanghai, 11 were resistant to oxacillin. However, the PCR assay revealed that 20 of the 32 isolates were positive for mecA (Fig. 1). These mecA-positive isolates included 11 oxacillin-resistant strains and 9 oxacillin-susceptible strains (Table 3). Thus the 9 mecA-positive but oxacillin-susceptible isolates were classiﬁed as OS-MRSA. Among the 16 S. aureus isolates from Sichuan, 2 were resistant to oxacillin, but 11 were positive for mecA by PCR (Table 4), which included 1 oxacillin-resistant strain and 10 oxacillin-susceptible isolates (classiﬁed as OS-MRSA). Thus OS-MRSA accounted for 62.5% of the isolates from Sichuan. For the 18 isolates from Guizhou, none was resistant oxacillin, but 10 were positive for mecA and were considered OS-MRSA (Table 5). Among the 49 mecA-positive isolates, 37 were susceptible to oxacillin, indicating that the majority (75.5%) of the bovine MRSA are OS-MRSA. In total, OS-MRSA accounted for 35.9% (37 out of 103) of the total S. aureus isolates, indicating the high prevalence of OS-MRSA in clinical bovine mastitis cases in China.

4. Antimicrobial susceptibility patterns of the mecA-positive S. aureus isolates

Antibiotic susceptibility patterns of the mecA-positive isolates from different provinces are shown in Tables 2–5. For the mecA-positive isolates from Gansu, their oxacillin MICs were all lower than 2 μg/ml, consistent with the result from the disk diffusion test. They were also all susceptible to gentamicin, ciprofloxacin, cefazolin, and vancomycin, and most of them were non-resistant to tetracycline, erythromycin, and clindamycin (Table 2). However, the isolates were generally resistant to penicillin and sulfa furazole.

For the mecA-positive isolates from Shanghai (Table 3), 11 were resistant and 9 were susceptible to oxacillin, consistent with the result from the disk diffusion test. All of the 20 isolates were resistant to penicillin and sulfa furazole, but were susceptible to vancomycin. Most of them were also resistant to clindamycin. However, there were major differences in the resistance to other antibiotics between the isolates from farm SX and those from farm SH (Table 3). For example, all of the SX isolates were resistant to gentamicin and cefazolin, while all of the SH isolates were susceptible to the two antibiotics. Additionally, the SX isolate were more resistant to tetracycline, erythromycin, and ciprofloxacin than the SH isolates. These results suggest farm-to-farm variations in the antimicrobial susceptibility patterns.

Among the 11 mecA-positive isolates from Sichuan, 10 were OS-MRSA (oxacillin MICs<2 μg/ml) (Table 4). High resistance rates were observed with penicillin and sulfa furazole, but the isolates were generally susceptible to other tested antibiotics. The 10 mecA-carrying S. aureus from Guizhou were all susceptible to oxacillin (Table 5) and are considered OS-MRSA. Similar to the isolates from Sichuan, the Guizhou isolates were generally resistant to penicillin and sulfa furazole, but susceptible to other examined antibiotics (Table 5).

5. Molecular characterization of selected MRSA isolates

Molecular typing analysis was done with the mecA-positive isolates from Gansu and Shanghai. The ones from Gansu were spa-type t267 (allelic proﬁle: 07-23-12-21-17-34-34-33-34), SCCmec-type V, cer complex 5, and PVL negative (Table 2). The sequence of the SCCmec amplicon of isolate HG5 was determined and it was found that it shared 99% identity to SCCmec-type V sequences (GenBank Accession No. AB505629.1), further
| Region  | Number of isolates | R   | S   | R   | S   | R   | S   | R   | S   | R   | S   | R   | S   | R   | S   | R   | S   | R   | S   | R   | S   |
|---------|-------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Shanghai(52) | 52    | 0   | 11  | 37  | 23  | 29  | 17  | 35  | 24  | 21  | 3   | 45  | 49  | 3   | 6   | 46  | 0   | 52  | 11  | 41  |
| Percentage (%) | 100  | 21.2 | 71.2 | 44.2 | 32.3 | 67.3 | 46.9 | 39.5 | 5.7 | 92.3 | 94.2 | 5.8 | 11.5 | 88.5 | 0   | 100 | 21.2 | 78.8 |
| Sichuan(16) | 16    | 0   | 0   | 15  | 6   | 10  | 3   | 12  | 2   | 14  | 0   | 15  | 12  | 4   | 1   | 15  | 0   | 16  | 2   | 14  |
| Percentage (%) | 100  | 0   | 0   | 93.75 | 37.5 | 62.5 | 18.75 | 75  | 14.3 | 85.7 | 0   | 93.75 | 75  | 25  | 6.67 | 93.33 | 0   | 100 | 12.5 | 87.5 |
| Guizhou(18) | 18    | 0   | 0   | 18  | 4   | 14  | 7   | 11  | 2   | 16  | 0   | 18  | 12  | 6   | 0   | 18  | 0   | 18  | 0   | 18  |
| Percentage (%) | 100  | 0   | 0   | 100  | 22.22 | 77.78 | 38.89 | 61.11 | 12.5 | 87.5 | 0   | 100  | 66.67 | 33.33 | 0   | 100  | 0   | 100  | 0   | 100  |
| Gansu(17) | 14    | 1   | 1   | 16  | 3   | 13  | 5   | 6   | 2   | 10  | 0   | 17  | 13  | 3   | 0   | 17  | 0   | 17  | 0   | 17  |
| Percentage (%) | 82.4 | 5.88 | 5.88 | 94.1 | 17.7 | 76.5 | 29.4 | 35.3 | 11.8 | 58.8 | 0   | 100  | 76.5 | 17.7 | 0   | 100  | 0   | 100  | 0   | 100  |
| Total(103) | 100   | 1   | 12  | 86  | 36  | 66  | 32  | 64  | 30  | 61  | 3   | 98  | 86  | 16  | 7   | 96  | 0   | 103 | 13  | 90  |
| percentage (%) | 97.1 | 0.97 | 11.7 | 83.5 | 34.95 | 64.1 | 31.1 | 62.1 | 29.1 | 59.2 | 2.9 | 95.1 | 83.5 | 15.5 | 6.8 | 93.2 | 0   | 100 | 12.6 | 87.4 |

*R: resistant; S: susceptible.

The number in parenthesis indicate the total number of isolates from a given region.

doi:10.1371/journal.pone.0088134.t001

*OS-MRSA Associated with Bovine Mastitis*
Figure 1. PCR detection of mecA-positive S. aureus isolates. The results were analyzed by Agarose gel electrophoresis. (A) mecA-positive isolates from Gansu province. Lane M: DNA size Marker. Lane 1: positive control strain ATCC43300. Lane 2: negative control strain ATCC25923. Lanes 3–10: isolate QY4, QY6, QY8, QY10, HG2, HG3, HG4 and HG5, respectively. (B) mecA-positive isolates from Shanghai. M: DNA size Marker. Lane 1: positive control strain ATCC43300. Lane 2: negative control strain ATCC25923. Lane 3–8: isolate SX5, SX6, SX10, SX11, SX13 and SX15, respectively; and lanes 9–22: isolates SH1, SH2, SH3, SH4, SH7, SH8, SH9, SH10, SH13, SH14, SH16, SH17, SH18 and SH20, respectively. (C) mecA-positive isolates from Guizhou. M: DNA size Marker. Lane 1 positive control strain ATCC43300. Lane 2: negative control strain ATCC25923. Lanes 3–12: isolates zy1, zy2, zy4, zy5, zy6, zy8, zy11, zy12, zy14, and zy15, respectively. (D). mecA-positive isolates from Sichuan. M: DNA size Marker. Lane 1 positive control strain ATCC43300. Lane 2: negative control strain ATCC25923. Lanes 3–14: isolates cx1, cx2, cx5, cx6, cx8, cx9, cx10, cx13, cx14, cx17, cx18, and cx19, respectively.

doi:10.1371/journal.pone.0088134.g001

Table 2. Genotyping and antibiotic susceptibility patterns of the mecA-positive S. aureus isolates from Gansu Province.

| Isolate  | mecA | OXA MIC (µg/mL) | PVL | spa complex | SCCmec | Resistance profileb |
|----------|------|-----------------|-----|-------------|--------|---------------------|
| QY4      | +    | ≤ 0.5           | –   | t267        | 5      | V R S R R I S R S S |
| QY6      | +    | ≤ 0.5           | –   | t267        | 5      | V R S S R S S S S S |
| QY8      | +    | 0.5             | –   | t267        | 5      | V R S S I I S S S S |
| QY10     | +    | ≤ 0.5           | –   | t267        | 5      | V R S S I I S S S S |
| HG2      | +    | ≤ 0.5           | –   | t267        | 5      | V S S S I S R S S S |
| HG3      | +    | ≤ 0.5           | –   | t267        | 5      | V R S S I S R S S S |
| HG4      | +    | ≤ 0.5           | –   | t267        | 5      | V R S S S S S R S S |
| HG5      | +    | ≤ 0.5           | –   | t267        | 5      | V R S S S S R S S S |

*QY and HG represent isolates from two different farms.

bR, resistant; S, susceptible.

OXA, Oxacillin; PEN, penicillin; GENTA, gentamicin; TETR, tetracycline; ERYTH, erythromycin; CLIN, clindamycin; CIP, ciprofloxacin; SULF, sulfamethoxazole; CEFA, cefazolin; VAN, vancomycin.

doi:10.1371/journal.pone.0088134.t002
confirming the PCR result. For the MRSA from Shanghai, all were PVL negative; spa types included t1234 (8 isolates), t267 (10 isolates), and two non-typeable; and their SCCmec types were II and V (Table 3). The isolates from Sichuan and Guizhou were not typed. These results suggest the genetic diversity of the mecA-positive isolates from cases of bovine mastitis.

**Table 3.** Genotyping and antibiotic susceptibility profiles of the mecA-positive isolates identified in Shanghai.

| Isolates* | mecA (µg/mL) | PVL | spa | SCCmec | PEN | GENTA | TETR | ERYTH | CLIN | CIP | SULF | CEFA | VAN |
|-----------|--------------|-----|-----|--------|-----|-------|------|-------|------|-----|------|------|------|
| SX5       | +            | ≥8  | –   | t1234  | II  | R     | R    | R     | R    | R   | R    | R    | S    |
| SX6       | +            | ≥8  | –   | t1234  | II  | R     | R    | R     | R    | R   | S    | R    | S    |
| SX10      | +            | ≥8  | –   | NT     | V   | R     | R    | R     | S    | R   | R    | R    | S    |
| SX11      | +            | ≥8  | –   | NT     | II  | R     | R    | R     | S    | R   | R    | R    | S    |
| SX13      | +            | ≥8  | –   | t267   | V   | R     | R    | R     | S    | R   | S    | R    | S    |
| SX15      | +            | ≥8  | –   | t267   | V   | R     | R    | R     | R    | S   | R    | R    | S    |
| SH1       | +            | ≤1  | –   | t267   | V   | R     | S    | S     | S    | R   | S    | R    | S    |
| SH2       | +            | ≥8  | –   | t267   | V   | R     | S    | R     | R    | S   | R    | S    | S    |
| SH3       | +            | ≥8  | –   | t267   | V   | R     | S    | R     | R    | S   | R    | S    | S    |
| SH4       | +            | ≤1  | –   | t267   | V   | R     | S    | S     | S    | I   | S    | R    | S    |
| SH7       | +            | ≤1  | –   | t267   | V   | R     | S    | S     | S    | I   | S    | R    | S    |
| SH8       | +            | ≥8  | –   | t267   | V   | R     | S    | S     | R    | S   | R    | S    | S    |
| SH9       | +            | ≤1  | –   | t267   | V   | R     | S    | S     | S    | R   | S    | R    | S    |
| SH10      | +            | ≤1  | –   | t267   | V   | R     | S    | S     | S    | R   | S    | R    | S    |
| SH13      | +            | ≤1  | –   | t1234  | II  | R     | S    | S     | S    | R   | S    | R    | S    |
| SH14      | +            | ≤1  | –   | t1234  | II  | R     | S    | S     | R    | S   | R    | S    | S    |
| SH16      | +            | ≥8  | –   | t1234  | II  | R     | I    | S     | R    | R   | S    | R    | S    |
| SH17      | +            | ≥8  | –   | t1234  | II  | R     | I    | S     | R    | R   | S    | R    | S    |
| SH18      | +            | ≤1  | –   | t1234  | II  | R     | S    | S     | S    | R   | S    | R    | S    |
| SH20      | +            | ≤1  | –   | t1234  | II  | R     | S    | S     | S    | R   | S    | R    | S    |

*SX and SH represent isolates from two different farms. Bold indicates OS-MRSA.

**Table 4.** Antibiotic susceptibility profiles of the mecA-positive isolates identified in Sichuan.

| Isolates | mecA (µg/mL) | OXA MIC | Resistance profile* |
|----------|--------------|---------|---------------------|
| CX1      | +            | <0.5    | R       | S       | S       | S       | I       | S       | R       | S       | S |
| CX2      | +            | <0.5    | R       | S       | S       | S       | S       | S       | S       | S       | S |
| CX5      | +            | ≤1      | R       | S       | R       | R       | R       | S       | R       | S       | S |
| CX6      | +            | ≤2      | R       | S       | S       | S       | I       | S       | R       | S       | S |
| CX8      | +            | <0.5    | S       | S       | R       | S       | S       | S       | R       | S       | S |
| CX9      | +            | ≥8      | R       | I       | S       | I       | S       | S       | R       | R       | S |
| CX10     | +            | ≤2      | R       | S       | S       | S       | S       | S       | R       | S       | S |
| CX13     | +            | ≤2      | R       | S       | R       | S       | S       | S       | S       | S       | S |
| CX14     | +            | ≤2      | R       | S       | R       | S       | S       | S       | S       | S       | S |
| CX17     | +            | ≤2      | R       | S       | R       | S       | S       | S       | S       | S       | S |
| CX19     | +            | ≤2      | R       | S       | S       | R       | R       | S       | S       | S       | S |

*R, resistant; S, susceptible.

OXA, Oxacillin; PEN, penicillin; GENTA, gentamicin; TETR, tetracycline; ERYTH, erythromycin; CLIN, clindamycin; CIP, ciprofloxacin; SULF, sulfamethoxazole; CEFA, cefazolin; VAN, vancomycin.

doi:10.1371/journal.pone.0088134.t003
**Discussion**

In this study, we characterized *S. aureus* isolates from bovine mastitis milk samples collected from 4 different province/regions in China and identified the high prevalence of OS-MRSA. To our best knowledge, this is the first comprehensive investigation of OS-MRSA of bovine origin, following our initial report [37] on the presence of OS-MRSA on dairy farms in the Inner Mongolia region of China. Although *S. aureus* is a major cause of bovine mastitis, previously published reports revealed low prevalence of bovine MRSA, implying that MRSA was not commonly associated with mastitis [38]. However, most of previous studies were based on genotypic tests for identifying MRSA, which may misidentify OS-MRSA as MSSA and underestimate the true prevalence of MRSA. In this study, 47.6% (49 out of 103) of the *S. aureus* isolates were found carrying *mecA*, which is unexpectedly higher than the highest reported incidence (17.5%) of MRSA from mastitic milk samples [39]. Presence of *mecA* is generally recognized as the most reliable method for detection of methicillin resistance, and *mecA*-positive *staphylococcal* strains are considered to be resistant to all β-lactam antibiotics [32]. Nevertheless, *S. aureus* that carry the *mecA* gene but appear phenotypically susceptible to oxacillin and vice versa have been reported recently [40]–[42] [37]. Thus, combination of genotypic and phenotypic tests is necessary to avoid false positive or false negative results in identifying MRSA.

All of the OS-MRSA isolates had an oxacillin MIC<2 μg/ml (Tables 2–5), indicating that presence of the *mecA* gene did not confer a high-level resistance to oxacillin. The reason for this phenotype remains to be elucidated. A recent study suggested that amino acid mutations in the FemXAB proteins (involved in cell wall synthesis) might contribute to the OS-MRSA phenotype [43], but the association of the mutations with the phenotype has not been formerly proven. Without testing the *mecA* gene, these isolates could be misclassified as MSSA based on the result of conventional antimicrobial susceptibility tests. OS-MRSA that carry *mecA* may result in the emergence of highly resistant MRSA under treatment with β-lactam antibiotics, which underscores the need for precautions when treating OS-MRSA infections [10] [42]. Due to this risk, treatment of OS-MRSA should avoid β-lactam antibiotics. As shown in Tables 2–5, the identified MRSA were generally susceptible to other classes of antibiotics, such as gentamicin, ciprofloxacin, kanamycin, and vancomycin. Therefore, treatment of mastitis caused by OS-MRSA using non-β-lactam antibiotics may prevent the unwanted consequence of antibiotic resistance development.

Based on the results of molecular characterization, all the OS-MRSA isolates from Gansu belonged to *spa*-type t267, *SCCmec*-type V, and *ccr* complex 5, and were *PVL* negative (Table 2), suggesting that these isolates may be a single lineage that was common in the surveyed region. However, the MRSA isolates from Shanghai belonged to different *spa* and *SCCmec* types (Table 3), suggesting their genetic diversity. Among the previously reported MRSA isolates associated with bovine mastitis, lineages with *spa*-type t267 and *SCCmec*-type V have not been identified [24]. However, a clinical MRSA isolate with the group V *SCCmec* element and t267 polymorphic X-region of *spa* was recovered in American University of Beirut Medical Center (AUB-MC) by Tokajian et al [44]. Another *mecA*-positive oxacillin-resistant *S. aureus* carrying *SCCmec* type V and *spa* type t267 was identified from an inpatient in England [45]. These reports plus findings from this study suggest that this lineage of MRSA may cause infections in both human and bovine and can be either susceptible or resistant to oxacillin. On the other hand, OS-MRSAs belonging to *SCCmec* types I, II, III, IV, and IV were reported previously [10] [11], suggesting that MRSA of different genetic backgrounds could be phenotypically susceptible to oxacillin.

None of the OS-MRSA isolates identified in this study harbored the *PVL* gene. This finding is similar to that reported by Turkylmaz et al. and Aires-de-Sousa et al. [26] [46]. In contrast, over 50% of bovine *S. aureus* isolates were determined to carry the *PVL* gene in another study [47]. *PVL* is a known virulence factor in *S. aureus* and is involved in the development of soft tissue infections [48]. The absence of *PVL* in the identified OS-MRSA isolates and their susceptibility to certain antimicrobial agents suggest that they may be eliminated from infected animals by appropriate antibiotic treatments.

**Conclusions**

This study demonstrates a high incidence of OS-MRSA on dairy farms in different regions of China. These OS-MRSA
isolates carried the mecA gene, were susceptible to oxacillin, and could be mistakenly identified as MSSA if testing of the mecA gene were not conducted. These results suggest that MRSA is more commonly associated with bovine mastitis than previously realized, which is the case at least in China. Findings from this study and the increasing reports of OS-MRSA in clinical settings underscore the need for genetic tests as well as phenotypic assays to accurately identify MRSA.

Author Contributions
Conceived and designed the experiments: WXP YS. Performed the experiments: YS JXL CHL. Wrote the paper: WXP YS.

References
1. Klevenos RM, Morrison MA, Nadle J, Petri S, Gershman K, et al. (2007) Invasive methicillin-resistant Staphylococcus aureus infections in the United States. J Am Med Assoc 298: 1763–1771.
2. Lowy FD (2003) Antimicrobial resistance: the example of Staphylococcus aureus. J Clin Invest 111: 1265–1273.
3. Cosgrove SE, Sakoulas G, Perencevich EN, Schwaber MJ, Karchmer AW, et al. (2003) Comparison of mortality associated with methicillin-resistant and methicillin-susceptible Staphylococcus aureus bacteremia: a meta-analysis. Clin Infect Dis 36: 53–59.
4. Tienmersu EW, Bronzaer SLAM, Lyutyikainen O, Degener JE, Schrijnemakers P, et al. (2004) Methicillin-resistant Staphylococcus aureus in Europe, 1999–2002. Emerg Infect Dis 10: 1627–1633.
5. Chambers HF (2003) Community-associated MRSA—resistance and virulence converge. New Engl J Med 352: 1485–1487.
6. Hiramatsu K, Cui LZ, Kuroda M, Is T (2001) The emergence and evolution of methicillin-resistant Staphylococcus aureus. TRENDS Microbial 9: 486–493.
7. Sakoulas G, Gold HS, Verkaztaranan L, DeGreilou PC, Eliopoulos GM, et al. (2001) Methicillin-resistant Staphylococcus aureus: comparison of susceptibility testing methods and analysis of mecA-positive susceptible strains. J Clin Microbial 39: 3986–3991.
8. Labrou M, Michal G, Ntokou E, Pittaras TE, Pourmaras S, et al. (2012) Activity of oxacillin versus that of vancomycin against oxacillin-susceptible mecA-positive Staphylococcus aureus clinical isolates evaluated by population analyses, time-kill assays, and a murine thigh infection model. Antimicrob Agents Chemother 56: 3383–3391.
9. Saced K, Dryden M, Parvaby R (2010) Oxacillin-susceptible MRSA, the emerging MRSA clone in the UK. J Hosp Infect 76: 267–268.
10. Hiramatsu K, Endo H, Sumiki Y, Nagamma S, et al. (2007) Characterization of oxacillin-susceptible mecA-positive Staphylococcus aureus: a new type of MRSA. J Infect Chemother 13: 79–86.
11. Ikononimithi M, Michael G, Vasdeki M, Labrou M, Karavasilis V, et al. (2008) In vitro and in vivo evaluations of oxacillin efficiency against mecA-positive oxacillin-susceptible Staphylococcus aureus. Antimicrob Agents Chemother 52: 3905–3908.
12. Feedtrade website (2013). Available: http://www.feedtrade.com/cn/whr/milk/ market/2013-01-17/2013257.html.Accessed 2013 Jan 17.
13. Bai L, Hao M, Deng J, Qing JH (2013) Research advances in the treatment of dairy mastitis and sequence analysis of their mecA genes. Acta Vet Scan 50: 28.
14. Jin YZ, Wan SP, Jiang FM, Gong ZL, Cao J, et al. (2011) Isolation and characterization of methicillin-resistant Staphylococcus aureus from pet animals and veterinary staff in China. Vet J 190: e125–e129.
15. Wang DF, Duan XH, Wu JY, Yang XY, Li JH, et al. (2011) The current status of coagulase-negative Staphylococcus aureus and MRSA isolates from bovine of China. Acta Veterinaria Et Zootecnica Sinica 42: 1416–1425.
16. Banerjea TL (2003) Staphylococcus, Micrococcus, other catalase-positive cocci that grow aerobically. In Manual of Clinical Microbiology, pp 384–404. Edited by Murray PF, Baron EJ, Jorgensen JH, Paller MA, Sokken RH. Washington, DC: ASM Press.
17. Turk DC, Porter DA (1973) A Short Textbook of Medical Microbiology, 4th edn. London: Hodder and Stoughton.
18. Clinical and Laboratory Standards Institute (2010) Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals: Informational Supplement. CLSI Document M100-S20. Clinical and Laboratory Standards Institute, Wayne PA.
19. Gallardo S, Lago G, Isanto MD, Damiano N, Sommese L (2003) Distribution of mecA among methicillin-resistant clinical staphylococcal strains isolated at hospitals in Naples, Italy. Eur J Epidemiol 10: 139–145.
20. Sherlock (2003) Available: http://www.sphereassembler.com/staphylococcus/.
21. Zhang K, Green SE, Slevay S, Long J, Conly JM (2003) Novel Multiplex PCR assay for characterization and concomitant subtyping of Staphylococcus aureus, Staphylococcus intermedius, and Staphylococcus hominis using mecA, nuc, mecC2, and lgtA genes. J Clin Microbial 41: 3905–3908.
22. Lina G, Pie´mont Y, Godail-Gamot F, Bes M, Peter MO, et al. (1999) Involvement of Panton-Valentine Leukocidin-Producing Staphylococcus aureus in primary skin infection and pneumonia. Clin Infect Dis 29: 1128–1132.
23. Su Y, Pu WX, Chen ZH, Deng HP (2012) Antimicrobial resistance analysis and detection of methicillin-resistant Staphylococcus aureus (MRSA) among Staphylococcus aureus strains isolated from bovine mastitis. Scientia Agricuila Sinica 36: 3602–3607.
24. Hendriksen RS, Mewis DJ, Schroeter A, Teale C, Meinert D, et al. (2008) Prevalence of antimicrobial resistance among bacterial pathogens isolated from cattle in different European countries: 2002–2004. Acta Vet Scand 50: 56–20.
25. Turutoglu H, Ezerlik S, Ozturk D (2006) Antibiotic resistance of Staphylococcus aureus and coagulase-negative Staphylococci isolated from bovine milk. Bull Vet Inst Pulawy 50: 31–39.
26. Turutoglu H, Haso˘luz M, Ozturk D, Yildirim M, Sagnak S (2009) Methicillin and aminoglycoside resistance in Staphylococcus aureus isolates from bovine milk and sequence analysis of their mecA genes. Vet Res Commun 33: 945–956.
27. Jannati E, Arzannal M, Habibzadeh S, Mohammadi S, Ahadi P, et al. (2013) Nasal colonization of mecA-positive, oxacillin-susceptible, methicillin-resistant Staphylococcus aureus isolates among nursing staff in an Iranian teaching hospital. Am J Infect Control 41: 1125–1128.
28. Sharif KA, Moncek S, Skead N, Forrest G, Pfeiffer C, et al. (2012) Genotypic resistance testing creates new treatment challenges: two cases of oxacillin-susceptible methicillin-resistant Staphylococcus aureus. J Clin Microbial 50: 4153–4153.
29. Giannouli S, Labrou M, Kyrkysi A, Ikononimithi A, Pourmaras S, et al. (2010) Detection of mutations in the FmXAB protein family in oxacillin-susceptible mecA-positive Staphylococcus aureus clinical isolates. J Antimicrob Chemother 65: 626–633.
44. Tokajian S, Haddad D, Andraos R, Hashwa F, Araj G (2011) Toxins and antibiotics resistance in Staphylococcus aureus isolated from a major hospital in Lebanon. ISRN Microbiol 812049: 9 pages.
45. Ellington MJ, Yearwood L, Garner M, East C, Kearns AM (2008) Distribution of the ACME-arcA gene among methicillin-resistant Staphylococcus aureus from England and Wales. J Antimicrob Chemother 61: 73–77.
46. Aires-de-Sousa M, Parente CESR, Vieira-da-Motta O, Bonna ICF, Silva DA, et al. (2007) Characterization of Staphylococcus aureus isolates from Buffalo, Bovine, Ovine, and Caprine milk samples collected in Rio de Janeiro State, Brazil. Appl Environ Microbiol 73: 3845–3849.
47. Zecconi A, Cesaris I, Liandri E, D’Aprà V, Piccinini R (2006) Role of several Staphylococcus aureus virulence factors on the inflammatory response in bovine mammary gland. Microb Pathogenesis 40: 177–183.
48. Boyle-Vavra S, Daum RS (2007) Community-acquired methicillin-resistant Staphylococcus aureus: the role of Panton-Valentine leukocidin. Lab Invest 87: 3–9.