Association Between \textit{agr} Type, Virulence Factors, Biofilm Formation and Antibiotic Resistance of \textit{Staphylococcus aureus} Isolates From Pork Production

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Livestock-associated \textit{Staphylococcus aureus} colonization and/or infections exist in pigs and people in frequent contact with pigs. In this study, a total of 130 \textit{S. aureus} isolates obtained from different stages of pork production were subjected to antimicrobial susceptibility, biofilm formation, as well as PCR screening to identify virulence genes, and the accessory gene regulator alleles (\textit{agr}). Among all 130 \textit{S. aureus} isolates, 109 (83.8%, 109/130) isolates were positive for \textit{agr}. All swine farms isolates belonged to \textit{agr} IV, whereas \textit{S. aureus} isolated from slaughterhouse and retail indicated diverse \textit{agr} types. All isolates exhibited biofilm formation ability, and raw meat isolates (belonging to \textit{agr} I) exhibited a greater ability to form strong biofilms than swine farms isolates (belonging to \textit{agr} IV). \textit{agr}-positive isolates were associated with more virulence genes than \textit{agr}-negative isolates. Most biofilm-producing isolates were positive for microbial surface component recognizing adhesive matrix molecule (MSCRAMM), capsule type and \textit{ica} group genes. The results illustrate a significant association between the prevalence rate of MSCRAMM, capsule type and \textit{ica} group genes among isolates producing weak, moderate and strong biofilms. The high prevalence of resistance to ciprofloxacin, gentamicin, tetracycline, clarithromycin, clindamycin, and trimethoprim-sulfamethoxazole were mainly observed in moderate and weak biofilm producers. Our findings indicate that \textit{S. aureus} isolates from pork production displayed diverse molecular ecology.

Keywords: \textit{Staphylococcus aureus}, \textit{agr} typing, biofilm formation, virulence gene, antibiotic resistance, pork production

INTRODUCTION

\textit{Staphylococcus aureus} is an important zoonotic pathogen that is responsible for a variety of infectious diseases characterized by septicemia and sepsis (Crombe et al., 2013; Song et al., 2015). China is one of the world’s largest pork producers with more than 470 million pigs, accounting for ~50% of the total numbers in the world (Krishnasamy et al., 2015). Consecutively, several reports suggested transmission between pigs and humans causing livestock-associated \textit{S. aureus}...
swine farms, a contracted slaughterhouse for the designated farms, and a retail market in Xiamen, China to profile \textit{S. aureus} isolates along the production, processing and retail chain. The data enabled tracking of the spread of \textit{S. aureus} from pork production and a better understanding of the evolution of \textit{S. aureus}.

**MATERIALS AND METHODS**

**Bacterial Strains and Antibiotic Susceptibility**

From September – December 2014, three commercial swine farms with > 5000 pigs, one large slaughterhouse and several terminal markets were selected from Xiamen City, People’s Republic of China, and 501 samples were collected from these places for \textit{S. aureus} isolation. Pigs were born and raised in these three commercial swine farms with distance for more than 25 km from each other and then were sent to the slaughterhouse. These three swine farms and the slaughterhouse were vertically integrated pork processing plant, meaning pigs originated from these three swine farms contracted to sell hogs exclusively to the slaughterhouse. However, terminal samples from the markets did not totally originate from the slaughterhouse tested in the present study.

Briefly, a total of 501 non-duplicate samples were collected from the pork industry, including three commercial swine farms (sty door and soil, \(n = 71\); nasal swabs, \(n = 97\)) one slaughterhouse (pork, \(n = 173\)), and terminal markets (pork, \(n = 160\)). Isolation and identification of \textit{S. aureus} were performed according to China’s National Technical Standard GB4789.10-2010 and the special gene \textit{nuc} was targeted by PCR for identifying \textit{S. aureus} (Brakstad et al., 1992). Contamination with \textit{S. aureus} was detected in 26.0% (130/501) of the total samples, and the prevalence of \textit{S. aureus} was highest in the slaughterhouse (35.8%, 62/173) followed by the market (24.4%, 39/160) and the farm (17.3%, 29/168).

These isolates were assessed for antimicrobial susceptibility by the Kirby-Bauer disk diffusion method described by the Clinical and Laboratory Standards Institute (CLSI, 2012). The antibiotic disks used (Hangzhou Microbial Reagent Co., Ltd., Hangzhou) included ciprofloxacin (5 \(\mu\)g), penicillin (10 \(\mu\)g), gentamicin (10 \(\mu\)g), tetracycline (30 \(\mu\)g), clarithromycin (15 \(\mu\)g), clindamycin (2 \(\mu\)g), chloramphenicol (30 \(\mu\)g), sulfamethoxazole-trimethoprim (25 \(\mu\)g), nitrofurantoin (30 \(\mu\)g), rifampin (5 \(\mu\)g), cephalexin (30 \(\mu\)g), cefoxitin (30 \(\mu\)g) and oxacillin (1 \(\mu\)g).

\textbf{agr Genotyping}

Bacterial genomic DNA template was extracted from the isolates by a commercial DNA extraction kit (Biomed, Beijing, China). The \textit{agr} types (I–IV) were determined by a multiplex PCR assay as described by Gilot et al. (2002). In brief, multiplex PCR was performed with the following primers: Pan (5’-ATG CAC ATG GTG CAC ATG C-3’), \textit{agr}1 (5’-GTC ACA AGT ACT AIA AGC TGC GAT-3’), \textit{agr}2 (5’-TAT TAC TAA TTG AAA AGT GGC CAT AGC-3’), \textit{agr}3 (5’-GTA ATG TAA TAG CTT GTA TAA TAC CCA G-3’) and \textit{agr}4 (5’-CGA TAA TGC...
### TABLE 1 | Target genes, putative function of encoded protein, primer sequence, and PCR conditions.

| Target gene | Primer name | Putative function of encoded protein | Primer sequence (5′—3′) | Product size (bp) | Tm (°C) | Reference |
|-------------|-------------|--------------------------------------|--------------------------|--------------------|---------|-----------|
| clfA        | clfA-F      | Encoding Clumping factor, ClfA        | AAAACACGCAATTCGAAA      | 855                | 53      | Ote et al., 2011 |
|             | clfA-R      |                                       | GCGATTGAAATTACACCATTTAAGT |                    |         |           |
| clfB        | clfB-F      | Encoding Clumping factor, ClfB        | TGCGAATAAGCGAAATAG      | 505                | 49      | Ote et al., 2011 |
|             | clfB-R      |                                       | GGTGATGATTGCGTAAATC     |                    |         |           |
| bbp         | bbp-F       | Encoding bone sialoprotein-binding protein, Bbp | AACTCATCTGATCTACAACACAG | 575                | 55      | Tristan et al., 2003 |
|             | bbp-R       |                                       | ATGTGCTTGAAATAACACATCATCT |                    |         |           |
| ebpS        | ebpS-F      | Encoding cell surface elastin-binding protein | CATCCAGAACCAATCGAAGAC   | 186                | 55      | Tristan et al., 2003 |
|             | ebpS-R      |                                       | CTCAAATGTTACATCATGTTATCTTG |                    |         |           |
| cna         | cna-F       | Encoding collagen-binding protein     | GTCAAGCGTTATACACACAG    | 423                | 55      | Tristan et al., 2003 |
|             | cna-R       |                                       | AATCGAGTTGCAATTGTCACTG   |                    |         |           |
| eno         | eno-F       | Encoding laminin binding protein      | ATGCGCAACAGCTGACT       | 302                | 55      | Tristan et al., 2003 |
|             | eno-R       |                                       | CAACAGCATCCTACGTACCTTC   |                    |         |           |
| fib         | fib-F       | Encoding fibrinogen binding protein, Fib | CTACAACACATGCGTCACAG    | 404                | 55      | Tristan et al., 2003 |
|             | fib-R       |                                       | GCTCTGTGACACATTTCCTCAC   |                    |         |           |
| fnbA        | fnbA-F      | Encoding fibronectin-binding protein A | GTGAAGTTTTAAGGTGAAAGATAG | 643                | 55      | Tristan et al., 2003 |
|             | fnbA-R      |                                       | GCTCTGTGTAAGCACTTTTTCAC  |                    |         |           |
| fnbB        | fnbB-F      | Encoding fibronectin-binding protein B | GTAAAGCTTAATGCGTAATTTAC  | 524                | 55      | Tristan et al., 2003 |
|             | fnbB-R      |                                       | GCTCTGTGTAAGCACTTTTTCAC  |                    |         |           |
| cap5        | cap5-F      | Encoding CP5 synthesis enzyme         | ATGAGGTAGCGATGAAAA      | 518                | 49      | Ote et al., 2011 |
|             | cap5-R      |                                       | CCGCTCTTGATACGTCACTTTC   |                    |         |           |
| cap8        | cap8-F      | Encoding CP8 synthesis enzyme         | ATCGAAAGACATATCAAGG     | 834                | 46      | Ote et al., 2011 |
|             | cap8-R      |                                       | TCGCTCGTGCGACATATACACT   |                    |         |           |
| icaA        | icaA-F      | Encoding intercellular adhesion protein A | TCTGCTCGCCGAGCTAATAC   | 178                | 55      | Pereyra et al., 2016 |
|             | icaA-R      |                                       | CTGATGCAACAGACTTTTGA    |                    |         |           |
| icaC        | icaC-F      | Encoding intercellular adhesion protein C | CCGAGGTTATTTGACAAGCATT  | 209                | 55      | Pereyra et al., 2016 |
|             | icaC-R      |                                       | CCAACATGCAACAGACTTTTGA  |                    |         |           |
| icaD        | icaD-F      | Encoding intercellular adhesion protein D | GATCCTATGCGAGCTAATAC   | 184                | 55      | Pereyra et al., 2016 |
|             | icaD-R      |                                       | GCAATATGCGCCGACACCT     |                    |         |           |
| bap         | bap-F       | Encoding biofilm-associated protein, Bap | TCGCGAATAATGCGCCGATAGTT | 971                | 60      | Pereyra et al., 2016 |
|             | bap-R       |                                       | CCGCTATGCAAGGATGTAATGCGAC | 971                | 60      | Pereyra et al., 2016 |
| pvl         | pvl-F       | Encoding Panton-Valentine leukocidin   | GTCGTGTAAGGATAACTGTCCTGC | 423                | 48      | Ote et al., 2011 |
|             | pvl-R       |                                       | GTCGTGTAAGGATAACTGTCCTGC |                    |         |           |
| tst         | tst-F       | Encoding toxic shock syndrome toxin-1 | TTTTTATGCGAAAACACTTATTA | 550                | 51      | Ote et al., 2011 |
TABLE 1 | Continued

| Target gene | Primer name | Putative function of encoded protein | Primer sequence (5′→3′) | Product size (bp) | Tm (°C) | Reference |
|-------------|-------------|-------------------------------------|-------------------------|------------------|--------|-----------|
| hla         | hla-F       | Encoding alpha-haemolysin precursor | CACCCGGTTTATCGGCTTGAA   | 845              | 51     | Ote et al., 2011 |
|             | hla-R       |                                      | TGCCGCAGATTCTGATATA     |                  |        |           |
| hib         | hib-F       | Encoding beta-haemolysin precursor  | TTTCTGAAGAACGTCTGTCCA   | 524              | 49.4   | Ote et al., 2011 |
|             | hib-R       |                                      | GCCGTTGTGATCGATAATT     |                  |        |           |
| hld         | hld-F       | Encoding delta-haemolysin precursor  | GGGATGGCTTAATACTCATCTT   | 236              | 48     | Ote et al., 2011 |
|             | hld-R       |                                      | GGCGGCTGTGGATTCGATAAT   |                  |        |           |
| eta         | eta-F       | Encoding exfoliative toxin A         | CAGAGAATGTGAGAAAATAGTTGA | 544              | 49.4   | Ote et al., 2011 |
|             | eta-R       |                                      | TTGGAAAGGCAAAACAAGTGC   |                  |        |           |
| etb         | etb-F       | Encoding exfoliative toxin B         | TTCCAATACGACCAAAACCA    | 641              | 50     | Ote et al., 2011 |
|             | etb-R       |                                      | GGAAGATTGTGTTCCGCC      |                  |        |           |

CGT AAT ACC CG-3′). These primers yield a PCR product of 441, 575, 323, or 659 bp corresponding to agr group I, II, III, and IV, respectively. Each assay contained 2 μL of prepared DNA template, 2.5 μL of 10× Easy Taq Buffer [Takara Biomedical Technology (Beijing) Co., Ltd, China], 1 μL of 10 mM deoxynucleotide triphosphate [Takara Biomedical Technology (Beijing) Co., Ltd, China], 1 μL of upstream and downstream primers (10 μM), and 0.125 μL of DNA polymerase (5 U/μL) [Takara Biomedical Technology (Beijing) Co., Ltd, China], and the final system volume was adjusted to 25 μL with sterile ultrapure water. The PCR conditions were as follows: 1 cycle at 94°C for 5 min; 26 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and finally 1 cycle at 72°C for 10 min. All PCR products were analyzed by electrophoresis on a 1.5% (w/v) agarose gel.

Identification of Virulence Determinants

The nucleotide sequences of all PCR primers used in this study and their respective amplified products and specific Tm (°C) are listed in Table 1. All the oligonucleotide primers were synthesized by Sangon Biotech (Shanghai, China). Each assay contained 1 μL of prepared DNA template, 2.5 μL of 10× Easy Taq Buffer [Takara Biomedical Technology (Beijing) Co., Ltd, China], 1 μL of 10 mM deoxynucleotide triphosphate [Takara Biomedical Technology (Beijing) Co., Ltd, China], 1 μL of upstream and downstream primers (10 μM), and 0.125 μL of DNA polymerase (5 U/μL) [Takara Biomedical Technology (Beijing) Co., Ltd, China], and the final system volume was adjusted to 25 μL with sterile ultrapure water. The PCR conditions were as follows: an initial denaturation at 95°C for 5 min; 26 cycles of 94°C for 30 s, specific Tm for 30 s, and 72°C for 40–90 s depending on the PCR product length; and a final extension at 72°C for 10 min. Sequencing of the extracted PCR product was performed by Beijing Genomics Institute (Shenzhen, China) and the data were analyzed with the GenBank database using the BLAST algorithm at the National Center for Biotechnology Information web site 1.

Biofilm Formation

Quantification of biofilm formation was performed by spectrophotometry in microplates (Nest Biotechnology Co., Ltd. Wuxi, China) using crystal violet staining as previously described (Pereyra et al., 2016). Briefly, 20 μL of bacterial log phase culture was added to 200 μL of fresh 1% glucose BHI in 96-well flat-bottom microtiter plates. S. aureus ATCC25923 (biofilm-forming) and S. epidermidis ATCC12228 (not biofilm-forming) were used as positive and negative controls, respectively. BHI without bacteria served as the blank. The plates were incubated at 37°C for 24, 48, and 72 h under aerobic conditions. After each sampling time, wells were washed three times with 300 μL of sterile phosphate-buffered saline (PBS; pH 7.2) and drained by inversion. Subsequently, 200 μL of methanol was added to each well and the plates were dried for 15 min. The adherent cells were stained with 150 μL of 0.1% crystal violet solution for 15 min and then washed twice with sterile water. Bound crystal violet was dissolved by treatment with 150 μL of 95% ethanol for 10 min, and OD570 was measured for the stained bacteria and control wells. The experiment was performed in triplicate. An OD570 value of 0.3 was taken as the cutoff point to differentiate between biofilm producers and non-biofilm-producer strains [cut-off value (ODc) = average OD of negative control + 3× standard deviation (SD) of negative control] (Pereyra et al., 2016). The quantitative classification of biofilm production based on ODc and average OD values was carried out, resulting in four categories of strains: strong biofilm producers (OD > 4× ODc), weak biofilm producers (4× ODc > OD > 2× ODc), moderate biofilm producers (2× ODc > OD > 1× ODc), and weak biofilm producers (OD < 1× ODc) (Pereyra et al., 2016).

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biofilm producers (2 × ODc > OD > ODc), and no biofilm producers (OD < ODc) (Pereyra et al., 2016).

**Growth Rate Analysis**
The growth of 12 strong, 12 moderate and 12 weak biofilm formers were measured according to Qi et al. (2016). Briefly, isolates were cultured in BHI agar for 18–24 h and adjusted to 0.5 McFarland units with 0.85% NaCl medium, and diluted 1: 20 in BHI medium. The cultures were incubated for 24 h at 37°C with shaking at 200 rpm and the bacterial growth was monitored by measuring the OD600 values of the culture. All experiments include three independent replicates.

**Statistical Analysis**
Statistical analysis was performed with SPSS v.22.0 (SPSS Inc., Chicago, IL, United States). Differences groups were compared using the chi-squared test and a p-value of <0.05 was deemed to be significant. Spearman’s rank correlation test was used for comparison of biofilm formation ability and multi-drug-resistance (MDR).

**RESULTS**

**agr Genotyping**
By multiplex PCR, the agr types were successfully identified in 109 isolates, and 21 isolates were non-typeable for agr locus. As shown in Table 2, the agr I was most prevalent (39.2%; 51/130), followed by agr IV (32.3%; 42/130), agr II (9.2%; 12/130) and agr III (3.1%; 4/130). All swine farms isolates belonged to agr IV, whereas S. aureus isolated from slaughterhouse and retail indicated diverse agr types.

**Prevalence and Distribution of Virulence Genes**
As illustrated in Figure 1, nearly all isolates harbored the hla (95.4%), hlb (100%) and hld (98.5%) genes, encoding alpha-, beta-, and delta-hemolysins respectively. No isolate harbored bap, pvl, or tsst. It was found that the bhp, cna and cap8 genes were detected only in isolates obtained from slaughterhouse and terminal markets. As shown in Table 3, the most frequent numbers of toxin genes per isolate were 11–14 in all S. aureus isolates (Table 3). Notably, one isolate harbored 16 toxin genes and 5 isolates harbored 15 toxin genes, which were obtained from slaughterhouse (Table 3).

The average toxin gene number was also examined based on agr genotyping, and a higher average number of toxin genes was found in the agr-positive isolates compared to agr-negative isolates. The agr-positive isolates were associated with a high average number of toxin genes (averaging 13.2 for agr II, 12.6 for agr I, 12.6 for agr IV and 12.0 for agr III), whereas the agr-negative isolates were associated with a lower average number of toxin genes (averaging 9.9) (Figure 2). The distribution of virulence genes differed among the isolates according to the agr genotyping. Among the MSCRAMMs genes, the prevalence of 3 genes was significantly different between the agr-positive and agr-negative isolates: clfA (p < 0.01), clfB (p < 0.01) and fnbA (p < 0.05). The capsule multiple type (carriage of both capsule type 5 and 8) (p < 0.01) and icaC gene (p < 0.01) were positively associated with agr-positive isolates (Figure 3).

**Quantification of Biofilm Biomass and Growth Rate Analysis**
Biofilm formation was analyzed, and all the isolates were able to form biofilm. The biomass of biofilms formed by most isolates increased continuously during incubation for 72 h at 37°C (Table 4). Biofilm strong producers are mainly in slaughterhouse and biofilm biomass increase with time. No significant difference in the growth rates of the strong, moderate and weak biofilm formers was observed, indicating that the difference in biofilm formation was not due to the growth rate.

**Correlation Between Virulence Genes and Antibiotic Resistance in Biofilm Producing S. aureus**
The relationship between prevalence of biofilm-associated genes and biofilm formation ability (incubation for 24 h at 37°C) of S. aureus isolates was further analyzed (Figures 4, 5). Considering the studied gene status, 19 different gene patterns were observed (Table 5). The most prevalent gene pattern was clfA–clfB–ebpS–eno–fib–cap5–icaA–icaC–icaD which was identified in 13 (10.0%) of 130 isolates. However, there was only one strong biofilm producer, nine moderate biofilm producers and three weak biofilm producers in this genes pattern. Conversely,
among the genes patterns of clfA-clfB-ebpS-eno-fib-fnbB-cap5-cap8-icaA-icaC-icaD (3.8%, 5/130), clfB-eno-fib-fnbB-cap5-cap8-icaA-icaD (1.5%, 2/130), clfB-bbp-eno-fib-cap5-cap8-icaA-icaC-icaD (1.5%, 2/130), clfA-clfB-eno-fib-fnbB-cap5-cap8-icaA-icaD (1.5%, 2/130), and clfB-eno-fib-cap5-cap8-icaA-icaC-icaD (1.5%, 2/130), all isolates showed strong biofilm formation ability (Table 5). A comparison between the strong, moderate, and weak biofilm producers in the isolates showed a significant difference in the prevalence of virulence genes among these isolates.

To determine whether biofilm formation was correlated with resistance to any particular antibiotic(s), we compared the biofilm forming capacities (incubation for 24 h at 37°C) among isolates with different resistance profiles for the 14 antibiotics (Table 6). Resistance to ciprofloxacin, gentamicin,
tetracycline, clarithromycin, clindamycin and trimethoprim-sulfamethoxazole were significantly higher in moderate biofilm producers and weak biofilm producers than in strong biofilm producers (Table 6). Notably, resistance to nitrofurantoin was only found in strong biofilm producers (7.1%, 4/56) and moderate biofilm producers (1.8%, 1/56) (Table 6). Resistance to penicillin, cefoxitin and chloramphenicol showed no significant difference among strong biofilm producers, moderate biofilm producers and weak biofilm producers (Table 6). Regarding multidrug resistance, no significant association to strong, moderate or weak biofilm producers was observed (Table 7).

**DISCUSSION**

The **agr** (accessory gene regulator) system is a peptide quorum-sensing system present in all the Staphylococci and a dominant regulator of pathogenesis and biofilm development in *S. aureus* (Boles and Horswill, 2008; Paharik and Horswill, 2016). All the swine farms isolates were **agr** type IV, whereas the slaughterhouse and terminal markets isolates indicated diverse **agr** types. In addition, isolates belonging to **agr**-positive group had a higher number of toxin genes than those belonging to **agr**-negative group (*p* < 0.05), suggesting that **agr** profiles may be associated with the virulence potential of *S. aureus*, which is consistent with a previous finding (Cheung et al., 2011). Raw meat isolates (belonging to **agr** I) exhibited a great ability to form strong biofilms than swine farms isolates (belonging to **agr** IV). Previous studies have shown that biofilm formation in *S. aureus* isolated...
TABLE 4 | Biofilm phenotype of 130 S. aureus isolates at different time points.

| Strain source | No. of strains | 24 h | 48 h | 72 h |
|---------------|----------------|------|------|------|
|               | Weak | Moderate | Strong | Weak | Moderate | Strong | Weak | Moderate | Strong |
| Swine farms   | 29   | 6 | (20.7%) | 21 | (72.4%) | 2 | (6.9%) | 13 | (44.8%) | 16 | (55.2%) |
|               | (%)  | (13.8%) | (43.1%) | (43.1%) | (1.5%) | (23.1%) | (75.4%) | (6.2%) | (93.8%) |
| Slaughterhouse| 62   | 5 | (8.1%) | 27 | (43.6%) | 30 | (48.4%) | 1 | (1.6%) | 13 | (21.0%) | 48 | (77.4%) |
|               | (%)  | (8.1%) | (43.6%) | (48.4%) | (1.6%) | (21.0%) | (77.4%) | (3.2%) | (96.8%) |
| Terminal markets | 39 | 7 | (18.0%) | 8 | (20.5%) | 24 | (61.5%) | 1 | (2.6%) | 4 | (10.3%) | 34 | (87.2%) |
|               | (%)  | (18.0%) | (20.5%) | (61.5%) | (2.6%) | (10.3%) | (87.2%) | (10.26%) | (89.7%) |
| Total         | 130  | 18 | (13.8%) | 56 | (43.1%) | 56 | (43.1%) | 2 | (1.5%) | 30 | (23.1%) | 98 | (75.4%) | 8 | (6.2%) | 122 | (93.8%) |

*The number in parentheses represents the percentage of isolates with the corresponding number of biofilm phenotype for all S. aureus isolates of the same part in pork production.**Biofilm-forming ability was measured after 24, 48, and 72 h at 37°C in terms of biofilm biomass by crystal violet staining. The results are presented by optical density (OD) determination of three independent repeats and compared to ATCC 25923 (biofilm-positive) and ATCC12228 (biofilm-negative).

FIGURE 4 | Distribution of biofilm-associated genes of S. aureus isolates based on biofilm formation ability. Pearson’s chi-square test (two-tailed) was used to test the difference in the virulence determinants distribution among different ability of biofilm ability. *Demonstrates that the distribution of toxin genes was statistically significantly different (p < 0.05); **demonstrates that the distribution of toxin genes was more significantly different (p < 0.01).

from bovine mastitis with agr I is higher than those with other agr types (Bardiau et al., 2013; Bardiau et al., 2014; Khoramrooz et al., 2016).

The prevalence of virulence genes involved in biofilm formation and staphylococcal toxin genes were investigated. Most biofilm-producing isolates were positive for MSCRAMM, capsule type and ica group genes. The data show a significant association between the prevalence rate of MSCRAMM, capsule type and ica group genes among isolates producing weak, moderate and strong biofilms. Approximately 92.3% (120/130) of all isolates harbored icaA and icaD genes simultaneously, which were similar to those from previous studies (Szweda et al., 2012; Pereyra et al., 2016). Moreover, although both pvl and tst genes were not detected in the tested isolates, hemolysins and enterotoxin-producing genes (data not shown) were found. This suggests that these isolates exhibit pathogenic potential.

In the present study, all S. aureus isolates were biofilm producers. Biofilm formation is influenced by numerous factors, such as sugar content and concentration (glucose versus lactose), proteolytic enzymes and biofilm-associated genes, etc. (Coelho et al., 2008). In this study, biofilm production was higher for raw meat isolates compared to swine farms isolates. There was a difference in the prevalence of several genes involved in adhesion and biofilm production between raw meat and
swine farms isolates. However, further studies are required to quantify the expression of relevant genes. Moreover, biofilm biomass increased proportionally as biofilms aged, which is in accordance with previous findings (Akinbobola et al., 2017). High variability in biofilm biomass was found among isolates throughout the time course of biofilm formation (24 – 72 h), which is in accordance with previous findings (Marino et al., 2011; Vázquez-Sánchez et al., 2014). Moreover, our study demonstrated the potential association between antibiotic resistance and biofilm-forming ability of *S. aureus*. Apart from resistance to penicillin, the high prevalence of resistance to ciprofloxacin, gentamicin, tetracycline, clarithromycin, clindamycin and trimethoprim-sulfamethoxazole were mainly observed in moderate and weak biofilm producers. Together,
TABLE 6 | Biofilm formation and antibiotic resistance pattern of 130 S. aureus isolates from different stages of pork production.

| Antibiotic category | Antibiotic agent | Percentage of antibiotic-resistant strains in different biofilm phenotype |
|---------------------|-----------------|---------------------------------------------------------------|
| Strong biofilm producers (56) | Moderate biofilm producers (56) | Weak biofilm producers (18) |
| β-lactamase | Penicillin | 85.7% (48/56) | 98.2% (55/56) | 94.4% (17/18) |
| | Oxacillin | 17.9% (10/56) | 1.8% (1/56) | 11.1% (2/18) |
| | Cefoxitin | 19.6% (11/56) | 10.7% (6/56) | 27.8% (5/18) |
| | Cephalothin | 8.9% (5/56) | 1.8% (1/56) | 11.1% (2/18) |
| Fluoroquinolones | Ciprofloxacin | 17.9% (10/56) | 53.6% (30/56) | 66.7% (12/18) |
| Aminoglycosides | Gentamicin | 14.3% (8/56) | 35.7% (20/56) | 55.6% (10/18) |
| Tetracyclines | Tetracycline | 46.4% (26/56) | 58.9% (33/56) | 83.3% (15/18) |
| | Minocycline | 14.3% (8/56) | 0 | 16.7% (3/18) |
| Macrolides | Clarithromycin | 32.1% (18/56) | 60.7% (34/56) | 72.2% (13/18) |
| | Clindamycin | 30.4% (17/56) | 60.7% (34/56) | 83.3% (15/18) |
| Chloramphenicol | Chloramphenicol | 28.6% (16/56) | 14.3% (8/56) | 38.9% (7/18) |
| Sulfonamides | Trimethoprim-sulfamethoxazole | 21.4% (12/56) | 53.6% (30/56) | 66.7% (12/18) |
| Nitrofurans | Nitrofurantoin | 7.1% (4/56) | 1.8% (1/56) | 0 |
| Rifamycins | Rifampicin | 17.9% (10/56) | 1.8% (1/56) | 27.8% (5/18) |

The number in parentheses represents the corresponding number of biofilm phenotype for S. aureus isolates of antibiotic resistance. Biofilm phenotype was measured after 24 h at 37°C, and the number of strong biofilm producers, moderate biofilm producers and weak biofilm producers were 56, 56 and 18, respectively.

TABLE 7 | Occurrence of multidrug resistant pattern and their associations with biofilm phenotype in 130 S. aureus from different stages of pork production.

| Number of antibiotic category | Number of S. aureus biofilm phenotype | Total number of isolates |
|-----------------------------|--------------------------------------|-------------------------|
| Strong | Moderate | Weak |
| 9 | 6 (10.7%) | 4 (7.1%) | 1 (5.6%) | 1 (0.8%) |
| 8 | 6 (10.7%) | 16 (28.6%) | 6 (33.3%) | 28 (21.5%) |
| 7 | 3 (5.4%) | 9 (16.1%) | 4 (22.2%) | 16 (12.3%) |
| 6 | 1 (1.8%) | 1 (1.8%) | 2 (11.1%) | 4 (3.1%) |
| 5 | 1 (1.8%) | 3 (5.4%) | 1 (5.6%) | 5 (3.8%) |
| 4 | 3 (5.4%) | 1 (1.8%) | 4 (3.1%) |
| 3 | 15 (26.8%) | 7 (12.5%) | 0 | 22 (16.9%) |
| 2 | 13 (23.2%) | 14 (25.0%) | 1 (5.6%) | 28 (21.5%) |
| 1 | 8 (14.3%) | 1 (1.8%) | 1 (5.6%) | 10 (7.7%) |
| 0 | 56 (43.1%) | 56 (43.1%) | 18 (13.8%) | 130 (100%) |

Biofilm phenotype was measured after 24 h at 37°C.

Qi et al. (2016) reported that for Acinetobacter baumannii, there was a statistically negative correlation between antibiotic resistance and biofilm forming capacity, suggesting that biofilm-forming strains are less dependent on antibiotic resistance than no biofilm-forming strains for survival. Previous studies have demonstrated that biofilm resistance to antimicrobials is multifaceted, including reduced penetration of the agent into biofilms due to the presence of extracellular matrix, biofilm heterogeneity and biofilm-specific phenotypes such as expression of efflux pump and persister cells (Stewart and Costerton, 2001; Akinbobola et al., 2017). Moreover biofilm resistance is known to vary from one microorganism to another (Mah and O’Ttole, 2001). Thus our further study will focus on the enhancement in resistance of our Staphylococcus aureus after biofilm formation.

In summary, our study revealed agr type diversity, virulence potential, antibiotic multiresistance and high biofilm formation ability of S. aureus isolated from pork production. All swine farms isolates belonged to agr IV, whereas S. aureus isolated from slaughterhouse and retail indicated diverse agr types. Raw meat isolates (belonging to agr I) exhibited a great ability to form strong biofilms than swine farms isolates (belonging to agr IV). Most biofilm-producing isolates were positive for MSCRAMM, capsule type and ica group genes. The results illustrate a significant association between the prevalence rate of MSCRAMM, capsule type and ica group genes among isolates producing weak, moderate and strong biofilms. Clarifying these mechanisms could provide novel insights that would prevention against S. aureus biofilm-related infections.
AUTHOR CONTRIBUTIONS

HY and LS participated in the design of this study. RC, DX, LS, and CL provided assistance for concepts, design, literature search, data acquisition, and manuscript preparation. YZ collected important background information, carried out the study, and performed the statistical analysis. HY and YZ drafted the manuscript. HY and DX performed the manuscript review. All the authors have read and approved the content of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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