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Molecular epidemiological characteristics and genetic evolutionary relationships of methicillin-resistant *Staphylococcus aureus* of different avian origins in Qingdao, China, using whole-genome sequencing

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Abstract

Introduction: To understand the prevalence of avian methicillin-resistant *Staphylococcus aureus* (MRSA) and the current status of drug resistance in Qingdao, a comprehensive molecular epidemiological investigation and analysis of evolutionary relationships of MRSA isolates from broiler and layer chickens and waterfowl was conducted. **Material and Methods:** One hundred and two avian MRSA strains were identified by multi-locus sequence typing, staphylococcal protein A (*spa*) and staphylococcal cassette chromosome *mec* (SCCmec) typing, and whole-genome sequencing. **Results:** The sequence type (ST) 9-t899-SCC*mec* IVb type represented the highest proportion of avian-derived MRSA strains (71.57%), with ST398 type strains occasionally observed in broilers and waterfowl. The poultry-derived MRSA strains were all resistant to eight or more antimicrobials. Avian-derived MRSA strains carried 20 resistance genes, 109 virulence genes and 10 plasmids. Strains carrying the *cfr* oxazolidinone resistance gene were occasionally seen in broiler- and layer-derived MRSA. Single nucleotide polymorphism (SNP) core genome evolution and locus difference analysis showed that the closest strains were all of ST9-t899 type (to which also affiliated the highest number of strains) and this type occurred on all three kinds of poultry farm, but the SNP difference loci between strains of the same type ranged from 0 to 1472. **Conclusion:** The dominant type of MRSA from different poultry sources in Qingdao is ST9-t899-SCC*mec* IVb, which is commonly resistant to a variety of antimicrobial drugs and carries a variety of resistance genes and a large number of virulence genes. Sequence type 9-t899 type is widely spread among the three kinds of poultry investigated, but there are differences in affiliations.

Keywords: MRSA, poultry, whole genome sequencing, SNP.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) has important public health implications and significantly higher drug resistance than methicillin-sensitive *S. aureus*, and is characterised by high infection and lethality rates, posing a potential public health threat. Several papers have reported that domestic animals can carry livestock-associated MRSA (LA-MRSA) (1, 2), but most studies have focused on strains of porcine origin and few concern other livestock. Infection with LA-MRSA is also capable of spreading between animals and humans (3, 4). *Staphylococcus aureus* can be molecularly typed according to multi-locus sequence typing (MLST), staphylococcal protein A (*spa*), staphylococcal cassette chromosome *mec* (SCC*mec*) typing and whole-genome sequencing. The LA-MRSA identified in China belongs mainly to clonal complex (CC)9 (5), while LA-MRSA CC398 is predominant in Europe (6). Whole-genome sequencing

is a new technology widely used in epidemiological investigations of various pathogenic bacteria, which can not only provide data on the molecular epidemiological characteristics of pathogenic bacteria and accurately distinguish the relatives of isolates, but also systematically and comprehensively detect various drug-resistance and virulence genes carried by drug-resistant bacteria. This guides clinical drug use in treating resistant bacteria and provides early warning of highly virulent strains to allow their prevention and control. In this study, analysis was undertaken of the molecular prevalence and drug resistance of MRSA from three poultry sources in Qingdao, namely laying hens, broilers and waterfowl. Analysis was also undertaken of the virulence factors and drug-resistance genes carried by the strains using whole-genome sequencing, to gain insight into their prevalence and the drug-resistance characteristics of avian MRSA in the region. As the third aspect, single nucleotide polymorphisms (SNPs) in the core genome of the strains were identified by comparative genomics, and an evolutionary tree was constructed based on the core genomic SNP information. These methods achieved kinship analysis of the prevalent clones that could not be distinguished by traditional molecular typing methods, provided information on the potential transmission relationship between different poultry sources of the bacteria, and set out a basis for effective prevention and control of avian MRSA and rational clinical use of drugs.

Material and Methods

Strains. A total of 102 MRSA strains of avian origin were isolated and obtained from August to October 2021. Among them, 45 strains were from broilers on eleven farms, 34 strains were from layers on eight farms, and 23 strains were from ducks and geese on six farms. For the experiment, the frozen strains were inoculated on trypticase soy agar (TSA) medium and placed in a 37°C incubator for 18–20 h to resuscitate them. The quality control strain ATCC 29213 was provided by the Pathogenic Microbial Surveillance Unit of the China Animal Health and Epidemiology Center.

Reagents and instruments. The TSA medium was purchased from Beijing Luqiao Technology Co. (Beijing, China). Ninety-six-well drug sensitivity plates coated with penicillin, amoxicillin/clavulanic acid, erythromycin, clindamycin, enrofloxacin, ofloxacin, ceftiofur, cefoxitin, sulfisoxazole, benzocillin, vancomycin, co-trimoxazole, doxorubicin, florfenicol, tiamulin, temsirolimus, gentamicin, linezolid, cephalexin, doxycycline, and tilmicosin were supplied by Shanghai Xingbai Biotechnology Co. (Shanghai, China). A Type II A2 biosafety cabinet was obtained from Shanghai Lishen Scientific Instrument Co. (Shanghai, China), and an LT-LBX45N thermostatic incubator was used manufactured by Shanghai Ailang Instrument Co. (Shanghai, China). A PTC200 thermal cycler, Powerpac Basic power supply and XR gel imaging analyser were needed and were products of Bio-Rad (Hercules, CA, USA). An F-TC2015 turbidimeter was utilised (Oxoid, Basingstoke, UK). The vortex mixer for the experiment was a model HVM1 (Shenzhen Tiannanhaibei Co., Shenzhen, China). Finally, GoTaq Green Master Mix and DL1000 DNA marker were supplied by TaKaRa Bio Beijing Baori Medical Biology (Beijing, China), nucleic acid dye was procured from Rayborschenko (Shanghai, China) and EEO 015 agarose electroendosmosis gel was sourced from Biowest (Barcelona, Spain).

MRSA drug sensitivity test. The minimal inhibitory concentration (MIC) of 18 antimicrobial drugs from 13 categories was determined by the micro broth dilution method recommended by the Clinical and Laboratory Standards Institute (CLSI) using a 96-well drug sensitivity plate. The strains' susceptibility, intermediate susceptibility or resistance was established according to the CLSI criteria, and was validated if the quality control strain was within the quality control range. The positive control was ATCC 29213, and the negative control was normal saline. If the quality control strain was within the quality control range, and the positive control had bacterial growth and the negative control had aseptic growth, interpretation of the results was effective. The standard definition of multiple resistance was adopted – simultaneous resistance to three or more classes of antimicrobials.

MRSA strain *spa* typing, MLST typing and SCC*mec* typing. Bacterial DNA was crudely extracted by boiling to provide a PCR template. The primer sequences, PCR reaction system and amplification procedure were as described in the literature (7–9). The primers were synthesised by Beijing Prime Tech Biotechnology Co. (Beijing, China) and the amplification products were subjected to gel electrophoresis in 1.5% agarose gel in 1× tris acetate ethylenediaminetetraacetic acid at 130 V for 25 min, and the results were observed with the XR gel imager. The amplified products were sent to Beijing DynaScience Biotechnology Co for forward sequencing.

Multi-locus sequence typing was performed by comparing the sequencing results with standard sequences and clipping them into the PubMLST website (https://pubmlst.org) for the sequence number of each allele. The ST of the strain was determined from the seven sequence numbers. Staphylococcal protein A sequencing results were submitted to the *spa* typing database (http://www.ridom.de/spaserver/) for typing analysis. Staphylococcal cassette chromosome *mec* sequencing results were compared with known SCC*mec* types through the BLAST function of the NCBI web page (www.ncbi.nlm.nih.gov).

Whole-genome sequencing. Thirty representative strains (12 from broilers, 11 from laying hens and 7 from waterfowl) were selected, and each strain was Illumina sequenced at the Institute of Microbiology of the

Chinese Academy of Sciences in Beijing. The genomic samples were interrupted, and after purification, the interrupted sticky ends were repaired with T4 DNA polymerase, Klenow DNA polymerase and T4 polynucleotide kinase to form flat ends. The DNA fragments were joined to the special junction with the T base at the 3′ end by adding an "A" base at the 3′ end. The DNA-seq library was enriched with high-fidelity PCR enzymes, and the quality-checked library was double-end Illumina sequenced with the Hiseq system. The resultant data were saved in double-end (Paired-end) FASTQ format and subjected to corresponding bioinformatic analysis.

Drug resistance and virulence gene comparison. The ResFinder database (https://cge.cbs.dtu.dk/services/) and VFDB: Virulence Factor Database (http://www.mgc.ac.cn/VFs/search_VFs.htm) were used to find and compare drug resistance genes and virulence genes, respectively. The following parameter settings were selected: threshold ID = 90% and minimum length = 80%.

MRSA core genome evolutionary tree construction. The core genome evolutionary tree was constructed using BioNumerics v7.6 software (Applied Maths, Sint-Martens-Latem, Belgium) for the 30 MRSA strains selected in this study, and the constructed core genome evolutionary tree was embellished and visualised in the Evolview online tool (https://www.evolgenius.info/evolview/#/). Single nucleotide polymorphism analysis of the 30 MRSA strains was performed through the BacWGSTdb database (http://bacdb.cn/BacWGSTdb/index.php), and the BA01611_CP019945_ST9 strain in the database was selected as the reference strain, which was isolated from bovine-derived MRSA in China in 2014.

Data processing and analysis. A one-way analysis of variance and chi-squared test were used to analyse the significance of differences using SPSS 26.0 software (IBM, Armonk, NY, USA), with significance thresholds of $P \leq 0.001$ (extreme significance), $P \leq 0.01$ (high significance), $0.01 < P \leq 0.05$ (significance) and $P > 0.05$ (no significance).

Results

Drug sensitivity test results. The resistance of 102 MRSA strains to 18 drugs applying the CLSI criteria is shown in Table 1. A total of 102 MRSA strains showed potent resistance to penicillin, macrolides, quinolones, clindamycin and florfenicol, with the resistance rate reaching 100%. The resistance rate to cephalosporins, benzocillin, tiamulin, and gentamicin was over 90%, and the resistance rate to amoxicillin/clavulanic acid was 77.45%. Strains showed susceptibility to linezolid and doxycycline, with resistance rates below 5%. The resistance rate to sulfonamides ranged from 10% to 50%. Vancomycin-resistant strains were not isolated. Four of the eighteen antimicrobial drugs, namely tiamulin ($P < 0.001$), amoxicillin/clavulanic acid ($P = 0.009$), sulfisoxazole ($P = 0.01$) and gentamicin ($P = 0.04$) showed significant differences in the resistance rates between the three kinds of poultry farm (Table 1).

Table 1. Resistance rates of 102 methicillin-resistant (MRSA) strains of different avian origins to 18 antimicrobials

Drug Class	Antibacterial drugs	MRSA (n = 102)			P-value	Overall drug resistance rate
		Layers	Broilers	Waterfowl		
Penicillin	PEN	100%	100%	100%	ns	100%
	OXA	97.10%	97.80%	95.50%	0.87 (ns)	97.06%
Quinolones	ENR	100%	100%	100%	ns	100%
	OFL	100%	100%	100%	ns	100%
Cephalosporins	CFX	97.10%	97.80%	95.50%	0.87 (ns)	97.06%
	FOX	100%	97.80%	100%	0.53 (ns)	99.02%
Sulfonamides	SF	28.60%	4.40%	13.60%	0.01**	14.71%
	SXT	20.00%	24.40%	13.60%	0.59 (ns)	19.33%
Macrolides	ERY	100%	100%	100%	ns	100%
	TIL	100%	100%	100%	ns	100%
β-lactamase inhibitor class	A/C	77.10%	95.60%	68.20%	0.009**	77.45%
Lincosamides	CLI	100%	100%	100%	ns	100%
Glycopeptides	VAN	0.00%	0.00%	0.00%	ns	0.00%
Tetracycline	DOX	2.80%	11.11%	0%	0.18 (ns)	4.63%
Amide alcohols	FFC	100%	100%	100%	ns	100%
Pleuromutilin	TIA	100%	100%	77.30%	<0.001***	95.10%
Aminoglycosides	GEN	100%	100%	90.90%	0.04*	98.04%
Azolidinones	LZD	2.80%	2.20%	0.00%	0.74 (ns)	1.96%

PEN – penicillin; OXA – benzocillin; ENR – enrofloxacin; OFL – ofloxacin; CFX – cephalexin; FOX – cefoxitin; SF – sulfisoxazole; SXT – cotrimoxazole; ERY – erythromycin; TIL – temsirolimus; A/C – amoxicillin/clavulanic acid; CLI – clindamycin; VAN – vancomycin; DOX – doxorubicin; FFC – florfenicol; TIA – tiamulin; GEN – gentamicin; LZD – linezolid; ns – non-significant ($P > 0.05$); * – $0.01 < P \leq 0.05$, significance threshold; ** – $P \leq 0.01$, high significance threshold; *** – $P \leq 0.001$, extreme significance threshold

Table 2. Multi-locus sequence, staphylococcal protein A and staphylococcal cassette chromosome *mec* (SCC*mec*) typing results of 102 different avian-derived methicillin-resistant *Staphylococcus aureus* strains (number of strains/percentage %)

Type	ST9-t899 (n = 97)			ST9-t1939 (n = 2)	ST398-t034 (n = 2)		ST398-t1250 (n = 1)
	SCC*mec* III	SCC*mec* IVb	Not typed	SCC*mec* IVb	SCC*mec* III	SCC*mec* V	SCC*mec* III
Layers	10 (9.80)	22 (21.57)	2 (0.98)	0	0	0	0
Broilers	5 (4.90)	37 (36.27)	0	2 (1.96)	0	0	1 (0.98)
Waterfowl	3 (2.94)	13 (12.75)	5 (4.90)	0	1 (0.98)	1 (0.98)	0
Total	17.65%	70.59%	6.86%	1.96%	0.98%	0.98%	0.98%

ST – sequence type

All 102 strains of MRSA were multi-resistant strains, impervious to more than five antimicrobials. The most resistant group of strains resisted nine or ten of them. The results of multi-drug resistance testing are shown in Fig. 1. Summarising them by bird type, broiler and laying hen strains' multi-drug resistance was relatively serious, with all resisting eight or more drugs, and the proportions of strains resisting nine or more were as high as 91.43% and 97.77%, respectively, while waterfowl strains' multi-drug resistance was significantly lower at 77.27%.

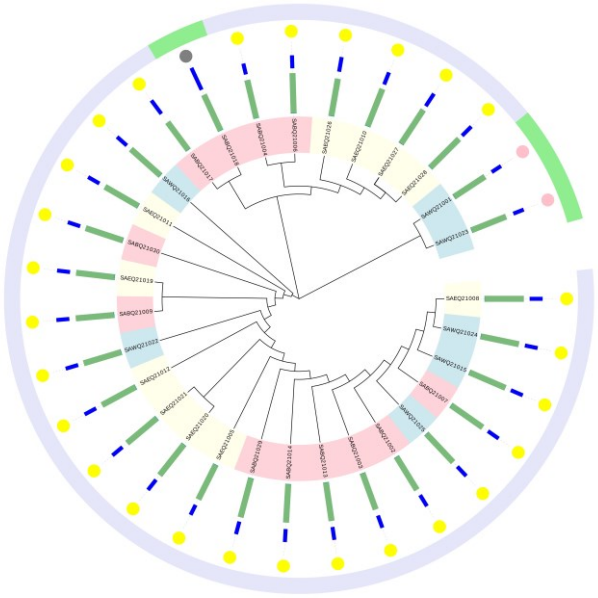

Fig. 1. Evolutionary tree of the core genome of methicillin-resistant *Staphylococcus aureus* (n = 30) from different avian sources.
From the outside to the inside, the order is ST type (light purple is ST9, green is ST398), *spa* type (yellow is t899, pink is t034 and dark grey is t1250), number of drug resistance genes, number of virulence genes, quantity, and source (red is from broilers, yellow is from layers and blue is from waterfowl)

***Spa*, MLST, and SCC*mec* typing results.** According to MLST, *spa*, and SCC*mec* typing, there were six types in the 102 MRSA strains, among which ST9-t899-SCC*mec*IVb was the most prevalent, accounting for 70.59% of resistant strains overall. Another strain which was notably prevalent was ST9-t899-SCC*mec*III. The six phenotypes were distributed in the different poultry farms as follows: from laying hens no strains other than ST9-t899 were isolated, from broilers two strains of ST9-t1939-SCC*mec*IVb and one strain of ST398-t1250-SCC*mec*III were yielded, and from waterfowl one strain of ST398-t034-SCC*mec*V and one strain of ST398-t034-SCC*mec*III were identified (Table 2).

MRSA core genome evolutionary tree analysis. The phylogenetic tree was drawn including the whole-genome sequencing results of 30 strains and is mainly divided into three evolutionary branches, among which the one closest to the root node and with the largest number of strains is for ST9-t899, including seven strains of broiler origin, eight strains of laying hen origin and five strains of waterfowl origin. Particularly close relationships emerged between SAEQ21019 and SABQ21009, SAEQ21020 and SAEQ21021, and SAEQ21008 and SAWQ21024. The branch farthest from the root node contained only two MRSA strains of waterfowl origin, SAWQ21001 and SAWQ21023, which were also highly related to each other and both of ST398-t034 type. The other branch containing four broiler-derived and four laying hen-derived MRSA strains was closer to the root node, with seven strains of ST9-t899 and one strain of ST398-t1250 (Fig. 1).

Analysis of MRSA core genomic locus differences. From Fig. 2, it can be seen that there are varying site differences among strains from different sources. In general, strains from the same source had few differences: for example, SABQ21014 and SABQ21029 had only three different loci and were highly related. However, strains from the same source also occasionally had large differences, such as SAEQ21020 and SAEQ21028. These strains are the same sequence type, ST9, and the difference between them was 1,472 mutation loci, which made them more distantly related. In addition, there were strains from different sources that differed only by a small number of loci, such as SAEQ21008 and SAWQ21024, which differed by only two loci and were highly related to each other.

Drug resistance genes. Thirty sequenced MRSA strains carried multiple drug resistance genes, and the number in each strain ranged from 6 to 14. Twenty resistance genes were detected in all MRSA strains, including the aminoglycoside resistance genes *aac(6')-aph(2")*, *aadD*, *ant(6)-la*, *ant(9)-la* and *aph(3')-III*; the tetracycline resistance genes *tet(L)* and *tet(S)*; the methicillin resistance gene *dfrK*; the β-lactam resistance gene *mecA*; the macrolide resistance genes *erm(A)*, *erm(B)*, *erm(C)* and *erm(T)*; the pleuromutilin resistance gene *lsa(E)*; the quaternary ammonium disinfectant resistance

gene *qacG*; the oxazolidinone resistance gene *cfr*; the amide alcohol resistance genes *fexA*, *cat(pC221)* and *cat(pC233)*; and the fosfomycin resistance gene *fosB6*.

The carriage rate of different drug resistance genes varied among the different kinds of poultry (Fig. 3). The *aac(6')-aph(2")*, *aadD*, *ant(6)-la*, *mecA*, *tet(L)*, *erm(C)*, *and lsa(E)* genes all had higher carriage rates in layers, broilers and waterfowl, these rates ranging from 70% to 100%. The carriage rates of *erm(B)*, *cfr* and *fexA* ranged from 0.08% to 0.17%, and although the carriage rates were not high, the genes were found in a variety of poultry. The *dfrk*, *qacG* and *fosB6* genes were only distributed in strains of waterfowl origin, *cat(pC221)* was distributed only in strains of layer origin, and *cat(pC233)*, *aph(3')-III*, *erm(T)*, *erm(A)*, *tet(S)* and *ant(9)-Ia* were only found in strains of broiler origin.

The results from the box plot (Fig. 4) show that the mean numbers of resistance genes carried by broiler, layer and waterfowl isolates were 7.1, 7.1 and 6.7, respectively, with no significant difference between the three (P = 0.351).

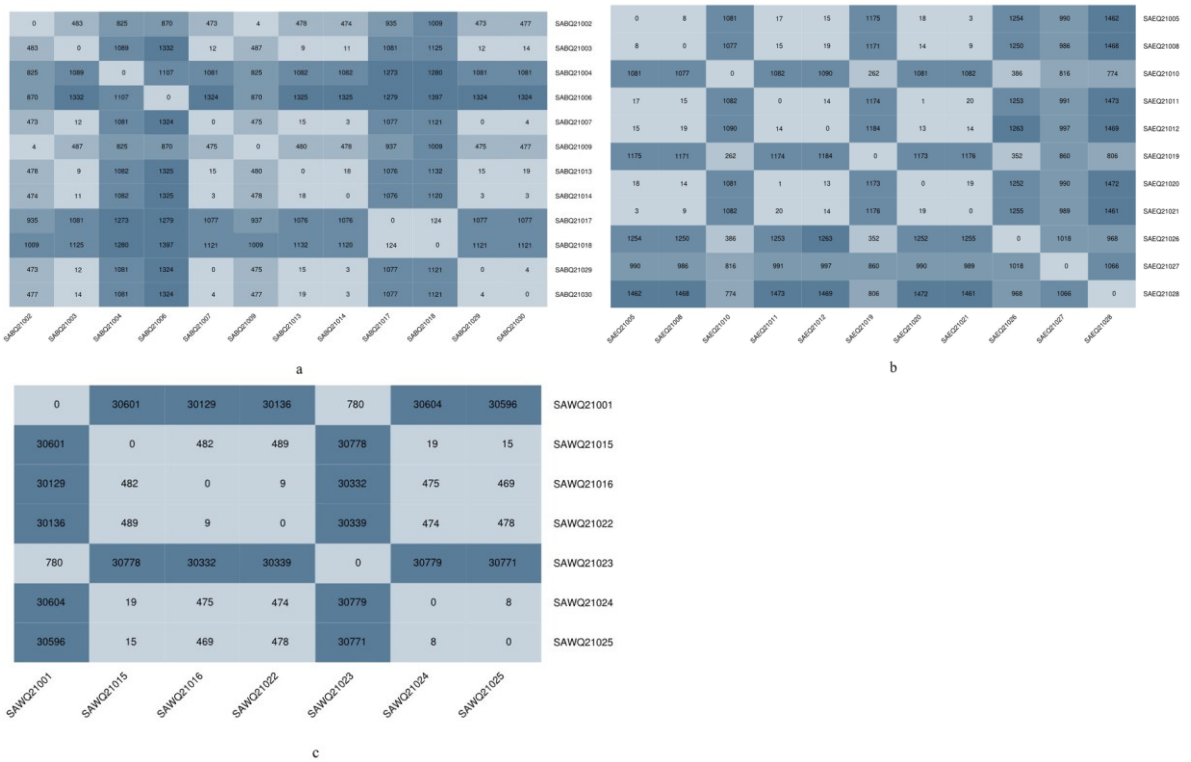

Fig. 2. Genome-wide single nucleotide polymorphism locus difference analysis of methicillin-resistant *Staphylococcus aureus* from: a – broiler sources; b – egg sources; c – waterfowl sources. Darker colour indicates more differential loci. SABQ – *Staphylococcus aureus* isolated from broilers; SAEQ – *Staphylococcus aureus* isolated from egg-layers; SAWQ – *Staphylococcus aureus* isolated from waterfowl

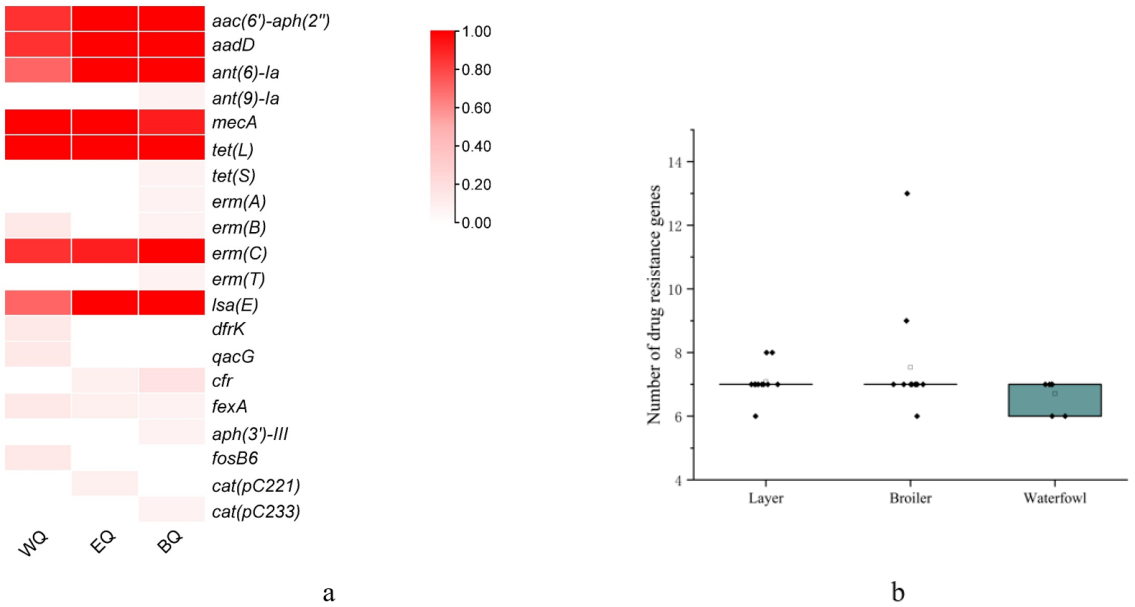

Fig. 3. Carriage rate (a) and number (b) of drug resistance genes in 30 strains of methicillin-resistant *Staphylococcus aureus* of different avian sources. EQ – layer source; WQ – waterfowl source; BQ – broiler source. Intensity of red in the left part of the figure represents carriage and 0.00–1.00 equates to 0–100% carriage

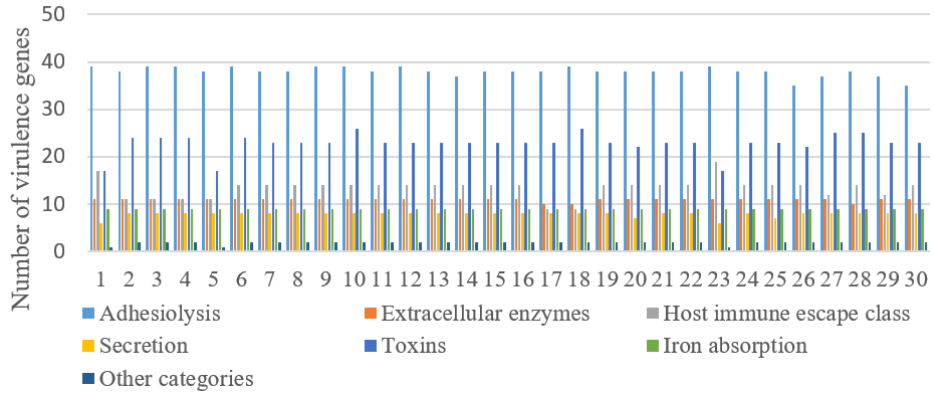

Fig. 4. Types and numbers of methicillin-resistant *Staphylococcus aureus* virulence genes of different avian origins

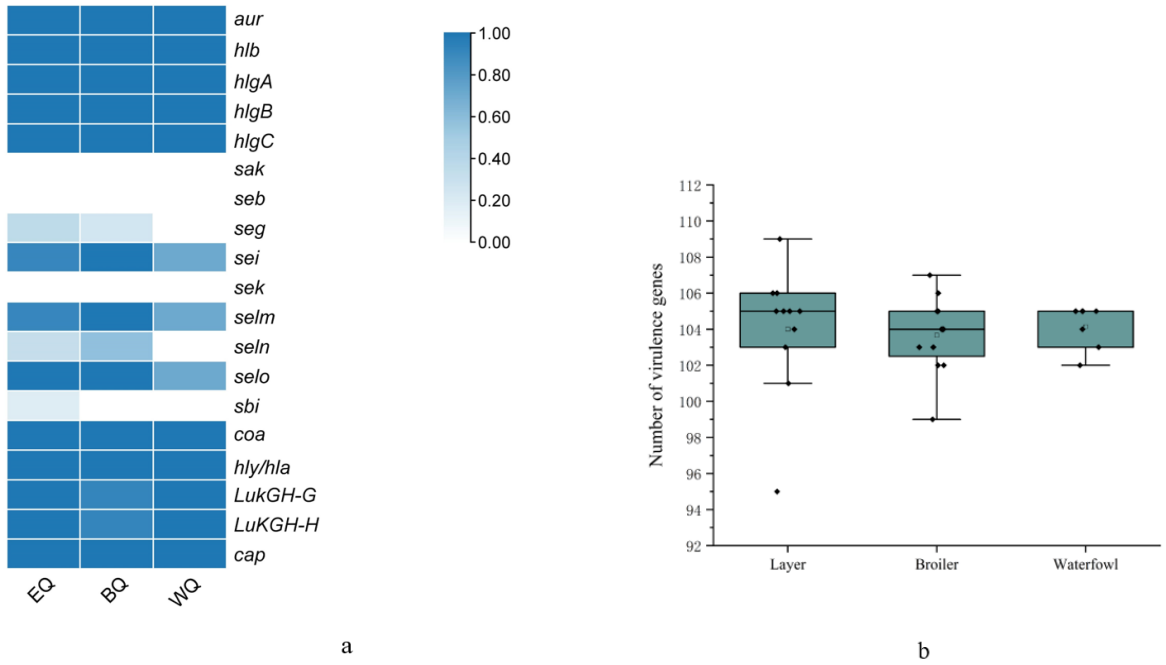

Fig. 5. Carriage rate (a) and number (b) of partial virulence genes in 30 strains of methicillin-resistant *Staphylococcus aureus* from different avian sources. EQ –layer source; WQ – waterfowl source; BQ – broiler source. Intensity of blue in the left part of the figure represents carriage and 0.00–1.00 equates to 0–100% carriage

Toxicity genes. A total of 109 virulence genes were detected in all MRSA strains, and each strain contained 95 to 109 pathogenic virulence genes with a broad classification, mainly including the adhesion, extracellular enzyme, host immune escape, secretion, toxin, and iron uptake classes (Fig. 4).

All strains carried the *atl*, *eap/map*, *fnbA*, *clfB*, *icaA/B/C/R*, *spa*, *sdrC/D/E*, *vwbp*, *srap*, *emp*, *sfaB/C/D*, *htsA/B/C*, *sbi*, *sbnA/B/C/D/E/F/G/H/I* and *sasc* genes, which are related to bacterial adhesion function; the *coa*, *hysA*, *nuc*, *sspA/B/C, Geh*, *lip* and *aur* extracellular enzyme–related genes; the *capA/C/D* host immune escape related gene; the *esaA/C*, *essB/C* and *esxA* secretory class genes; the *eta*, *hly/hla*, *hld*, *hlb*, *set17/26/30* and *hlgA/B/C* toxin genes; and the *isdA/B/C/D/E/F/G* iron uptake–related genes. There were also a number of genes which, while not in all strains, had high carriage rates, such as the *sei*, *selm* and *seln* enterotoxin genes, the *lukG* leukocidin gene, the *srtb* gene encoding the specific sorting enzyme, the *adsA* gene encoding adenosine synthase A, the *ebh* gene encoding cell wall binding protein and the *ebp* gene encoding cell surface elastin binding protein, all of which had carriage rates above 90%. The carriage rates for some of the common and pathogenicity-related virulence genes are shown in Fig. 5.

Most virulence genes were present in all three poultry sources of MSRA, *seg* and *seln* were carried in layer and broiler isolates, and *sbi* was carried only in layer isolates. Waterfowl isolates carried fewer virulence genes and had a lower carriage rate. The box plot results showed that the mean number of virulence genes carried by broiler, layer and waterfowl isolates were 103.7, 104.0 and 104.1, respectively, with no significant difference between the three isolate sources ($P = 0.918$).

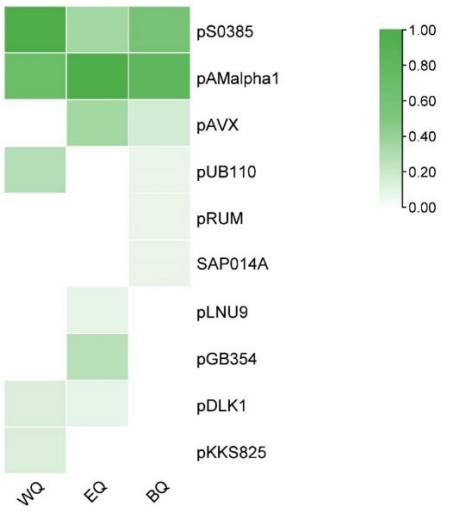

Fig. 6. Plasmid carriage of 30 methicillin-resistant *Staphylococcus aureus* strains from different poultry sources. EQ – layer source; WQ – waterfowl source and BQ – broiler source. Intensity of green represents carriage and 0.00–1.00 equates to 0–100% carriage

Plasmids. A total of 10 plasmid types were detected in the 30 MRSA strains, namely *pS0385*, *pAMalpha1*, *pAVX*, *pUB110*, *pRUM*, *SAP014A*, *pLNU9*, *pGB354*, *pDLK1* and *pKKS825*. The first two of these had the highest carriage rates in all types of poultry and were the most common plasmid type. Notably, the *pS0385* plasmid had a 100% carriage rate in waterfowl and *pAMalpha1* had the same rate in layers. The third in the list, *pAVX*, was distributed in layers and broilers, *pUB110* was distributed in waterfowl and broilers, and *pDLK1* in layers and waterfowl. In addition, *pLNU9* and *pGB354* were only distributed in layer strains, *pRUM* and *SAP014A* in broiler strains, and *pKKS825* in waterfowl strains (Fig. 6).

Discussion

Methicillin-resistant *S. aureus* (MRSA) is ranked by the World Health Organization as one of the most important antimicrobial-resistant bacteria worldwide because of its high virulence and the lack of effective targeted drugs. This study showed that 102 strains of MRSA from different poultry sources were highly resistant to seven drugs, including penicillin and macrolides, with a rate reaching 100%. The resistance rates to cephalosporins, benzocillin, tiamulin, gentamicin and amoxicillin/clavulanic acid ranged from 70% to 100%. All MRSA isolates were multi-drug resistant strains against which eight or more antimicrobials were ineffective. Isolates of MRSA from diseased chicken samples in Sichuan Province had high resistance rates to 11 antibiotics ranging from 33.33% to 100% and had a 100% multi-drug resistance rate, with which the results of this study are consistent (10). Methicillin-resistant *S. aureus* isolated from broiler nasal swabs and cecum swabs samples from Quebec in a Canadian study were also multi-drug resistant (11). Notably, all the MRSA strains in this study had a high resistance rate of 82.35% to doxycycline, which should alert us to the possibility of a greater increase in the resistance rate of MRSA strains in the future. Overall, various studies in China and abroad have shown prevalent MRSA resistance to a variety of antimicrobials, which poses a serious threat to the healthy development of the farming industry and to public health. Therefore, to control the spread of drug resistance, the frequency of prescribing drugs to which susceptibility is low, such as penicillin, macrolides, quinolones and clindamycin, should be reduced when antibiotics must be used clinically.

The results of drug-resistance gene screening showed that *mecA*, *erm(C)*, *lsa(E)*, *aac(6')-aph(2")*, *aadD* and *ant(6)-la* had high carriage rates in poultry from all three kinds of farm, and drug-resistance phenotype testing also showed that the MRSA strains from all three bird farming operation types were highly resistant to β-lactams. The presence of these resistance genes is presumed to be the reason for the serious resistance to four classes of antimicrobials in poultry-derived MRSA strains in Qingdao.

Notably, one strain of MRSA in laying hens and one strain in broilers were found to carry the *cfr* gene for resistance to oxazolidinones, and the corresponding linezolid resistance was also observed in the *cfr* resistance gene carriers. Oxazolidinones are potent drugs against Gram-positive infections in human medicine and are prohibited in farming and veterinary practice. Li *et al.* (12) also isolated four (3.1%) *cfr*-positive MRSA strains from chicken and duck sources. Although the *cfr* carriage rate in this study was not high, it has been observed in two poultry species and high priority should be given to preventing the widespread transmission of *cfr* resistance genes between humans and poultry, which would affect poultry farming safety and public health.

The seven pairs of housekeeping genes of *S. aureus* are highly conserved, with the maximum number of alleles and sufficient discriminatory features to distinguish genetic diversity among populations. Typing by *spa* is based on amplification and sequencing of the X region of the *S. aureus*-specific protein A gene (*spa*). The X region genes show high polymorphism, good reproducibility and stability (13, 14). According to the SCC*mec* characteristics of MRSA, 11 types have been reported, among which I, II, III, IV and V are better known (15).

After MLST-spa-SCC*mec* typing experiments, the prevalent MRSA strains of different avian origins in Qingdao were determined mainly to belong to the CC9 clone complex group, with ST9-t899-SCC*mec*IVb as the dominant phenotype. He *et al.* (8) found that the predominant MRSA clone was ST9-t899SCC*mec*IVb/PFGE A (70.0%, 14/20) after a survey of relevant chicken products in Shandong Province, and the current study was consistent with that finding. Sixty-nine percent of MRSA isolates from retail fresh chicken meat in Germany belonged to the clonal complex CC398 (16). The predominant type of MRSA isolated from

Portuguese food quails belonged to ST398-SCC*mec*V. Ribeiro *et al.* (17) isolated MRSA from 15 broiler farms in Germany, half of which were of the t011 type and the other half of which were t1430 and t034. In turkey meat, the most common *spa* types were t011, t034, t1430 and t899. The prevalent type of avian-derived MRSA isolate in Sichuan, China was SCC*mec* type III (10), and in this the Sichuan study differed from the 19.61% prevalence of SCC*mec* type III in the present study. Another study demonstrated that hospital-associated MRSA mainly carried SCC*mec* types I–III, while livestock-associated MRSA tended to carry smaller SCC*mec* components, such as SCC*mec* type IV or V (18, 19), which could explain the typing results in this study. In summary, the prevalence of molecular typing of avian-derived isolates varies between countries and regions.

Another study on human-derived MRSA in a hospital in Shandong (21) found multiple ST types among 65 human-derived MRSA strains, with ST59 (33.85%, 22/65) and ST239 (21.54%, 14/65) being the prevalent types of MRSA in the region. However, ST9 (1.54%, 1/65) and ST398 (4.62%, 3/65) did not represent high proportions of human-derived MRSA in the region, suggesting that these two types of strains are not yet widely transmitted between humans and poultry, but that there is a potential risk.

The closest to the root node and the largest number of strains in the evolutionary tree circle diagram were the strains of ST9-t899, including seven broiler-derived, eight layer-derived and five waterfowl-derived MRSA strains, with close affinities between strains. In addition, the ST9 type was present in all poultry isolates, suggesting that ST9-t899 type strains have been widely distributed among a variety of farmed birds. The two ST398 isolates of waterfowl origin were more distantly related to ST9 in the core genome evolutionary tree, suggesting that ST398 may be spreading to a small extent within the waterfowl population. In a related study (21), ST9-t899 did not appear in human-derived MRSA, indicating that the MRSA strain did not undergo large-scale transmission between avian and human sources. The presence of ST398 in a small number of human-derived MRSA in some areas and the common presence of this type in both humans and poultry deserve attention.

From the analysis of SNPs, it can be seen that most avian-derived MRSA strains are closely related to each other, but the closeness of the relationship does not correlate with commonality of strain origin. For example, SAEQ21020 and SAEQ21028 are both ST9 strains and both isolates from layers, but differ by 1,472 mutation loci. In contrast, SAEQ21008 and SAWQ21024 are one isolate from layers and one from waterfowl, but differ by only two loci, and SABQ21007 and SAWQ21025 came from a broiler and waterfowl but differ by only one locus, suggesting that there is a certain transmission relationship between MRSA in different poultry.

Previous studies have demonstrated that MRSA strains have higher adherence and invasion rates compared to methicillin-susceptible *Staphylococcus aureus* strains, with a large number of virulence genes being an important determinant (20, 22). The MRSA strains in this study were all sequenced to carry a variety of virulence genes associated with pathogenicity, with numbers of such genes ranging from 95 to 109. The main categories of function of these genes are adhesion, extracellular enzyme encoding, immune evasion, secretion, toxin production and iron uptake. The presence of virulence genes can help bacteria survive the host's immune response or other extreme conditions, and play an important role in pathogenic bacteria invading tissues and developing drug resistance (21). For example, the *fnbA* and *fnbB* genes, which encode the FnBPA and FnBPB fibronectin-binding proteins, promote bacterial adhesion to form biofilms, thereby exacerbating drug resistance. In the present study, *fnbA* and *fnbB* virulence genes were widely detected in poultry MRSA strains from all three kinds of farm and were presumed to be an important cause of severe drug resistance. The widespread carriage of virulence genes suggests that alertness be maintained to the prevalence and spread of highly pathogenic and virulent MRSA strains.

Plasmids are important for the epidemiology and evolution of drug resistance in bacteria (22). Ten plasmid types were detected in this study, of which the most abundant were pS0385 and pAMalpha1, which were distributed in poultry from all three farming operations. All waterfowl-derived MRSA carried ps0385 and pAMalpha1, and all layer-derived MRSA carried pAMalpha1. Bosch *et al.* (2) confirmed that the pS0385 plasmid could carry *tek* and *aadD*. This may be the reason for the high detection rate of *aadD* resistance genes in avian-derived MRSA strains in Qingdao, which bestow a high level of resistance to aminoglycosides. Plasmid pAMalpha1 improved its structure by binding to other replicons from plasmid pUB110, and the new conjugate significantly increased the yield and stability of the plasmid in *S. aureus*. The latter plasmid, which was also detected in this study, is a shuttle plasmid vector with wide host range and is reported in *Staphylococcus*, *Bacillus* and *Streptococcus* isolates (23); it is important for the horizontal transmission of drug resistance genes between different strains of bacteria.

The prevalent strains of avian MRSA in Qingdao primarily belonged to the CC9 clonal complex, with ST9-t899-SCC*mec*IVb as the dominant clonal transmission. These prevalent strains were generally resistant to eight or more antimicrobial drugs. The ST9 type has become widely distributed among the three types of poultry investigated and there were extensive differences at some loci.

In summary, epidemiological investigations and analyses of drug-resistant strains in poultry farming should be broadened and intensified, and veterinary antimicrobials should be more strictly regulated to alleviate the serious drug resistance situation. Alertness should be maintained to the prevalence of some highly resistant, highly pathogenic and virulent MRSA strains

and to their transmission between humans and poultry to mitigate or avoid their impact on poultry breeding and public health.

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