ORIGINAL RESEARCH

Resistance and Molecular Characteristics of Methicillin-Resistant *Staphylococcus aureus* and Heterogeneous Vancomycin-Intermediate *Staphylococcus aureus*

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Objective: To investigate the molecular epidemiology of *Staphylococcus aureus* (*S. aureus*) isolated from patients in the Yellow River Delta region and to construct antibiotic resistance profiles in different genetic backgrounds.

Methods: Antibiotics susceptibility testing, staphylococcal protein A (*spa*) typing and accessory gene regulator (*agr*) typing were performed for all the 204 strains. Isolates with the positive *mecA* gene and heterogeneous vancomycin-intermediate *S. aureus* (hVISA) were subjected to multilocus sequence typing (MLST) and Staphylococcal chromosomal cassettes *mec* (SCC*mec*) typing.

Results: Thirty-nine MRSA strains were identified by *mecA* gene. Twenty-two hVISA isolates including 9 MRSA and 13 methicillin-sensitive *S. aureus* (MSSA) strains were confirmed, the rest isolates (n = 182) were vancomycin-susceptible *S. aureus* (VSSA). 94.9% of MRSA and 63.6% of hVISA belonged to *agr*I. Fifty-seven distinct *spa* types including 5 novel types were mainly t309 (30.9%), t078 (11.8%) and t437 (11.8%). Fourteen sequence types (STs) containing 3 new STs were classified into 3 clone complexes (CCs) and 7 singletons among MRSA and hVISA isolates. Most MRSA isolates (87.2%) belonged to type Ⅳ SCC*mec*.

Conclusion: The predominant genotype among MRSA population was ST59-t437-*agr*I-IVa (53.8%), followed by ST72-t2431-*agr*I-IVF (15.4%). ST72 and CC5 (ST5/965/7197) were the most common hVISA clones. Both CC59 (ST59/7437) and ST72 clones were resistant to erythromycin and clindamycin among MRSA population. Strains of MSSA with phenotypic hVISA (MS-hVISA) exhibited a striking genetic diversity accompanied by the diversification of drug resistance patterns.

Keywords: *Staphylococcus aureus*, heterogeneous vancomycin-intermediate *S. aureus*, *a*ccessory gene regulator, multilocus sequence typing, erythromycin

Introduction

For more than 140 years since its discovery, *Staphylococcus aureus* has remained a pathogen of clinical concern. Among the various models of *S. aureus*, the real challenge is the emergence of MRSA.[1] Over a long period of evolution, MRSA acquired the *mecA* gene encoding a penicillin-binding protein (PBP2a) that displays low affinity for most semisynthetic penicillins by horizontal transfer, resulting in resistance to β-lactams.[2] Due to the misuse of antibiotics in humans, the "cunning" MRSA has integrated antibiotic resistance genes into its genome in various ways, leading to the emergence of multidrug-resistant strains, making it more difficult to treat, and as a result, the spread of MRSA has become more unchecked worldwide. To understand the molecular epidemiology from different geographical distributions, researchers adopt typing methods including SCC*mec, spa, agr* and MLST as a means for the characterization and discrimination of isolates based on their genetic characteristics. However, the antibiotics susceptibility results of strains from the same background differed. ST22 lineage from a Kuwaiti hospital showed a high resistance rate of 69.2% and 84.6% to

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gentamicin and trimethoprim-sulfamethoxazole, respectively,[3] in contrast, the clone from China displayed no resistance activity to both antibiotics.[4] Strains ST8-IVa-t008 from China and Germany were both susceptible to clindamycin but differed in resistance to ciprofloxacin (100%, 37.5%).[5,6] ST59-IV-t437 from England was resistant to erythromycin, clindamycin and tetracycline, however, this lineage from the Chinese pediatric population was phenotypically resistant to trimethoprim-sulfamethoxazole, ciprofloxacin and chloramphenicol in addition to the above antibiotics.[7,8]

Gram-positive bacterial infections including MRSA are majorly treated using glycopeptide antibiotics, in which vancomycin is the first-line agent. Vancomycin binds the D-alanine-D-alanine terminus of the murein precursor, forming a complex that in turn blocks transpeptidation as well as transglycosylation, leading to incomplete or inefficient peptidoglycan cross-linking during cell wall synthesis. The resultant cell wall is weak and unable to adapt to changing osmotic pressures.[9] However, due to irrational use of vancomycin, hVISA/VISA strains which causes vancomycin treatment failure and prolonged hospitalization have emerged. The data suggested that the prevalence of hVISA/VISA in China (7.98%) is higher than the global average (3.39–3.68%), and causes the risk of persistent infection and higher treatment costs.[10–12] The aim of this study was to determine the molecular epidemiology of MRSA and hVISA/VISA strains in patients from the Yellow River Delta Regional Center (a tertiary care hospital) and to construct antibiogram under different molecular characteristics, thus providing a theoretical basis for clinicians to execute empirical drug administration well.

Materials and Methods
Collection of Bacterial Isolates
A total of 204 *S. aureus* strains were obtained from inpatients and outpatients from September 2019 to February 2021 which involved 25 sections as part of the routine laboratory procedures at the Binzhou Medical University Hospital. These strains were isolated from pus (n = 78, 38.2%), sputum (n = 40, 19.6%), secretions (n = 29, 14.2%), transudation (n = 24, 11.8%), blood (n = 17, 8.3%) and other seven sources (n < 10, <4.9% each). Strains were initially identified using matrix-assisted laser desorption ionization-time of flight mass spectrometry MALDI-TOF (Biomérieux, France) and stored at −70°C.

Antimicrobial Susceptibility Testing
All *S. aureus* isolates were subjected to antibiotic susceptibility testing using the automated VITEK 2 Compact system (Biomérieux, France). The antibiotics agents used were penicillin (PEN), oxacillin (OXA), cefoxitin (FOX), ceftarolin (CPT), gentamicin (GEN), levofloxacin (LVX), moxifloxacin (MFX), erythromycin (ERY), clindamycin (CLI), rifampin (RIF), trimethoprim-sulfamethoxazole (SXT), vancomycin (VAN), teicoplanin (TEC), daptomycin (DAP), and linezolid (LZD). In addition, we used the rapid E-test method to determine the MICs for 3 agents. Briefly, 50 μL of bacterial suspensions with turbidity adjusted to 0.5 McFarland's standard were evenly spread on BHI agar plates. E-test strips for amikacin (AMK), chloramphenicol (CHL) and minocycline (MNO) were placed on the plate. Susceptible, intermediate and resistant breakpoints for these antibiotics agents were determined using the Clinical and Laboratory Standards Institute (CLSI) interpretation criteria.[13] Strains that are resistant to 3 kinds or more of antibiotics are defined as MDR.

Identification of MRSA and hVISA Isolates
The criteria of resistance towards oxacillin (MIC ≥ 2 μg/mL) and/or cefoxitin (MIC ≥ 4 μg/mL) of phenotypic MRSA in *S. aureus* isolates was performed according to CLSI guidelines.[13] *mecA* was screened by PCR as a confirmatory experiment for MRSA. The primers were $mecA_{147}$-F 5' -GTGAAGATATACCAAGTGATT-3' and $mecA_{147}$-R 5' -ATGCGCTATAGATTGAAAGGAT-3'. Since there are no clear guidelines for identification of hVISA in CLSI, we performed "gold standard" test population analyses profile– area under curve (PAP/AUC) after positive primary screening to reduce the workload.[14,15] Briefly, randomly isolated colonies were diluted using 0.85% sodium chloride solution, and their density adjusted to be equivalent to that of 0.5 McFarland's standard (1.5×10^8 CFU/mL). Then, 10 uL of suspensions were spot inoculated onto Brain-Heart infusion agar (BHIA) plates containing casein digested by trypsin (16 g/L) and vancomycin (2, 3, 4 mg/L). The BHIA plates were incubated at 35°C for 48 h. If the number of colonies ≥1

within 48 h, the parental strain was designated as suspected hVISA. Positive primary parent bacterial cultures from overnight growth in 8 mL tryptone soy broth (TSB) liquid medium were suspended in 0.85% sterile saline to a density corresponding to 0.5 McFarland's standard. Then, the original suspensions were diluted to 10^{-3} (10^5 CFU/mL) and 10^{-6} (10^2 CFU/mL). Fifty uL aliquot of start cell suspension of each isolate and its serial dilution was spread on BHI agar plate containing increasing concentrations of vancomycin (0, 0.5, 1, 2, 3, 4, 6, 8, 12 mg/L). Plates were incubated at 35°C for 48 h. The hVISA reference strain (Mu3) was used as the positive control while the ATCC29213 strain was the negative control. The plate with the best growth of a single colony in three different concentrations was selected for manual counting, and the actual number of CFU determined as the dilution multiplied by the number of colonies in the plate under that dilution and expressed in log_{10} CFU/mL. The Graphpad Prism software was used to draw the curve of cell number versus vancomycin concentration and to calculate the area under the curve (AUC). The PAP/AUC ratio was calculated as AUC_{strain}/AUC_{Mu3}. The accepted criteria for confirmation was AUC_{strain}/AUC_{Mu3} <0.9 for VSSA, >1.3 for VRSA, and a ratio of 0.9 to 1.3 was considered as hVISA.

Accessory Gene Regulator (*Agr*) and Staphylococcal Protein a (*Spa*) Typing

All isolates were characterized by the combination of *spa* typing and *agr* locus analysis as previously described.[16,17]

Multi Locus Sequence Typing (MLST) Determination

The MLST scheme was followed using seven housekeeping genes (*arcC, aroE, glpF, gmk, pta, tpi*, and *yqiL*) on MRSA and hVISA isolates.[18] Allelic profiles containing 7 allele numbers for each strain were assigned a sequence type (ST) by the MLST database (https://pubmlst.org/saureus/). Phylogenetic tree construction was performed by the Neighbor-Joining method using mega7 software (https://www.megasoftware.net/) to cluster STs with close genetic backgrounds into identical clonal complexes (CCs).

Identification of the SCC*mec*

Multiplex PCR was performed to determine the SCC*mec* type and subtype as previously described.[19]

Statistical Analysis

The IBM SPSS version 22.0 software was used for analyses. The Pearson chi-square test or Fisher's exact test was used for categorical variables. $P < 0.05$ was the threshold for significance.

Results

Clinical Characteristics of *S. aureus*

Thirty-nine MRSA strains harboured the *mecA* gene based on PCR analysis. Fifty phenotypic methicillin-resistance strains were obtained, among which 49 resistance to cefoxitin, 43 resistance to oxacillin and 42 resistance to both agents. Among two *mecA*-positive strains, one was susceptible to cefoxitin and oxacillin and another was susceptible to oxacillin. Of the 12 strains absent methicillin-resistance determinant, *mecA*, six were resistant to cefoxitin, one to oxacillin, and the remaining five were tolerant to both.

Twenty-two isolates (9 MRSA and 13 MSSA exhibiting hVISA phenotypes, ie MR-hVISA and MS-hVISA) were categorized as hVISA with no vancomycin-intermediate *S. aureus* (VISA) strains. These hVISA isolates were significantly prevalent in MRSA than in MSSA (23.1% vs 7.9%, *p* = 0.014).

The distribution of isolates in each section was shown in Table S1. Strains from the thyroid unit accounted for most (11.8%) of *S. aureus*, but only three MRSA isolates were found, and none of the strains was hVISA. The pediatric department had the highest proportion of MRSA (50%) and the largest number of hVISA isolates (n = 8). None of the *S. aureus* from dermatology harboured *mecA* gene, but two cases were hVISA isolates (MSSA-6219 for 0.911 and MSSA-6550 for 0.95).

S.aureus from pus sources were the most abundant, accounting for 38.24% (data was not shown). MRSA strains were mainly isolated from pus and sputum (12/39, 30.8%, respectively), whereas, hVISA was more common in blood samples

(8/22, 36.4%). Notably, MR-hVISA was blood or sputum-borne (n = 5 and 4, respectively), while MS-hVISA was of wide origin (secretions, n = 4; blood, n = 3; sputum, n = 3; pus, n = 2; transudation, n = 1).

Molecular Types of *S. aureus*

The 204 *S. aureus* isolates included 57 different *spa* types that belonged to four *agr* groups which were not duplicated. The most common *spa* types were t309 (63/204, 30.9%). *agr*I had 37 *spa* types with the largest number of isolates (n = 165), among which 63 were divided into t309, 24 had t078, 22 exhibited t437, while the remaining 56 isolates exhibited 34 *spa* types. Seventeen isolates belonged to *agr*II were grouped into 8 *spa* types with the most abundant being t002. Six isolates covering 3 *spa* types belonged to *agr*III group. Sixteen isolates classified as *agr*Ⅳ yielded 9 *spa* types. Five novel *spa* types, namely t20226, t20227, t20228, t20229 and t20230 were observed.

t034, t309, t437 and t062 were distributed in both MRSA and MSSA groups, with t437 being more prevalent in the MRSA group (56.1% vs 1.2%, *p* = 0.001), whereas, t309 was more rich in the MSSA group (37.0% vs 5.1%, *p* = 0.001). All strains belonging to t078 and t002 were MSSA, in contrast, isolates classified as t2431 were all MRSA. There were no MRSA bacteria in group *agr* III and *agr* Ⅳ (Table S2).

A large number of *spa* types were distributed in both hVISA and VSSA, with t002, t062 and t2431 being more abundant in hVISA. None of the strains in the *agr* group Ⅳ was termed as hVISA.

MLST revealed 8 distinct STs for the MRSA isolates, of which ST59 (61.5%) was the most prevalent sequence type, followed by ST72 (20.5%), ST22 (5.1%), and other STs (ST9, ST965, ST1232, newly assigned ST7196 and ST7437). There were 10 different STs among hVISA isolates. Specifically, 9 MR-hVISA belonged to ST72 (n = 4), ST59 (n = 3), ST965 (n = 1) and novel ST7196 (n = 1). Thirteen MS-hVISA were assigned to ST25 (n = 4), ST5 (n = 2), while the remaining four STs had one isolate each. The phylogenetic tree based on alignment of 3186 single nucleotide polymorphisms of 7 housekeeping genes revealed that ST59/7437, ST15/5459 and ST5/965/7197 were from 3 clonal complexes (CC59, CC15 and CC5) in which the strains evolved in close evolutionary distance to each other (Figure 1).

SCC*mec* typing revealed that 87.2% of the MRSA isolates were type IV, which contained only two subtypes IVa (n = 24) and IVF (n = 10). Among the remaining isolates, 3 carried SCC*mec* III, one was SCC*mec* Ⅴ, and the last one failed to type. All the 9 MR-hVISA were classified as type IV.

A high correlation between the molecular typing of the strains was observed. ST-SCC*mec* combination showed that all ST72-positive isolates were grouped into SCC*mec* IVF. CC59 was determined as type IVa, except for one ST59 strain that was identified as SCC*mec* III. ST(CC)-*spa* combination indicated CC5 shared t002, t2431 strains belonged to ST72.
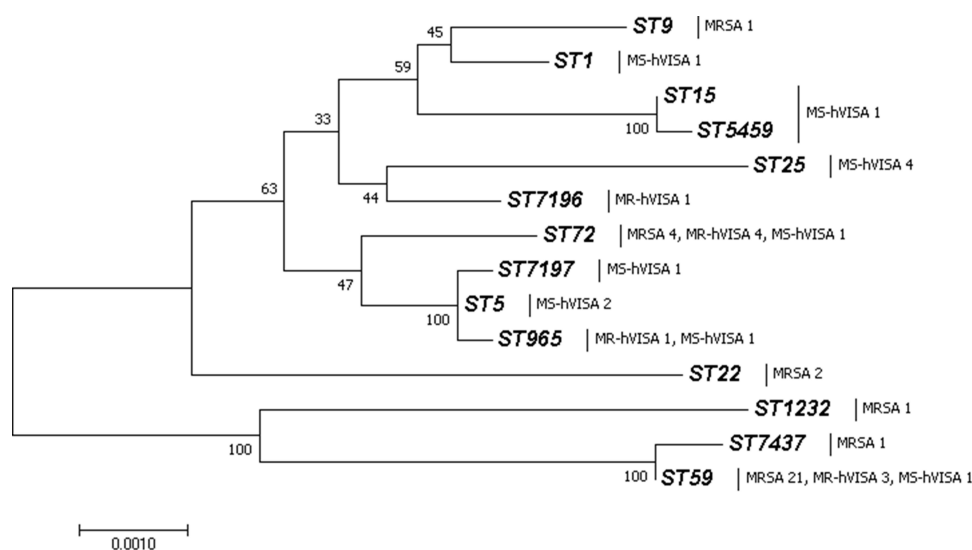

Figure 1 Molecular Phylogenetic analysis of all MRSA and hVISA isolate detected for MLST data by Neighbor-Joining method. ST59 and ST7437 belonged to CC59, CC15 contained ST15 and ST5459, ST965, ST7197 and ST5 grouped into CC5.

ST-*agr* combination revealed that ST1 was grouped into *agr* III. CC5, CC15 and monomeric ST9 were identified as *agr* II. The remaining STs or CCs were classified as *agr*I.

ST59-t437-*agr*I-IVa was the most prevalent genotype, occurring in 53.8% of the MRSA population (n = 21), followed by ST72-t2431-*agr*I-IVF (n = 6, 15.4%). Among the nine MR-hVISA isolates, the genotypes were ST59-t437-*agr*I-IVa (33.3%), ST72-t2431-*agr*I-IVF (33.3%) with ST72-t5155-*agr*I-IVF, ST72-t20226-*agr*I-IVF and ST965-t062-*agr*II-IVF (11.1% for each phenotype). Molecular typing analysis revealed a wide genetic diversity among the 13 MS-hVISA isolates, which included 9 different STs, 10 distinct *spa* types and 3 *agr* groups.

Antimicrobial Activities

All *S. aureus* isolates were sensitive to FOX, LZD, DAP, TEC, VAN and MNO. Resistance rates of MRSA and hVISA groups to PEN, ERY and CLI were above 75% (Table 1).

Compared to the MSSA group, the MRSA group was more resistant to FOX (97.44%), OXA (94.87%), AMK (5.13%) and CPT (92.31%), but exhibited low resistance to SXT (2.56% vs 26.06%). The MDR rate of MRSA isolates was significantly higher than that of MSSA isolates (92.3% vs 30.3%, $p < 0.001$). The most prevalent genotype among MRSA population ST59-t437-*agr*I-IVa (21/39, 53.8%) displayed resistance profile as FOX-OXA-PEN-ERY-CLI (17/21, 81.0%), consistent with other STs (Table 2).

Isolates from the hVISA group against FOX (50%), OXA (40.9%) and RIF (9.1%) were significantly higher than those from the VSSA group. Only 2 MS-hVISA strains were resistant to rifampicin while 2 MRSA (one MR-hVISA) strains were resistant to AMK. There were significant differences in MDR rate between the two groups (68.2% vs 39%, $p = 0.009$). 77.8% (7/9) of MR-hVISA strains expressed phenotypic resistance to FOX-OXA-PEN-ERY-CLI, while MS-hVISA isolates showed different resistance profiles. We found that in the MSSA population, MS-hVISA strains were more resistant to SXT than those without the hVISA phenotype (53.8% vs 23.7%, p = 0.040).

Table 1 Resistance Rate of *S.aureus*

Drugs	MRSA(n=39)		MSSA(n=165)		P	h-VISA(n=22)		VSSA(n=182)		P
	n(R)	R%	n(R)	R%		n(R)	R%	n(R)	R%	
FOX	38	97.44	11	6.67	0.001	11	50.00	38	20.88	0.003
PEN	38	97.44	159	96.36	1	21	95.45	176	96.70	0.761
OXA	37	94.87	6	3.64	0.001	9	40.91	34	18.68	0.033
CPT	0	0	0	0		0	0	0	0	
GEN	1	2.56	12	7.27	0.473	2	9.09	11	6.04	0.928
LVX	3	7.69	7	4.24	0.602	2	9.09	8	4.40	0.659
MFX	2	5.13	5	3.03	0.874	1	4.55	6	3.30	0.556
ERY	35	89.74	127	76.97	0.076	17	77.27	145	79.67	1
CLI	34	87.18	126	76.36	0.140	15	68.18	145	79.67	0.336
LZD	0	0	0	0		0	0	0	0	
DAP	0	0	0	0		0	0	0	0	
TEC	0	0	0	0		0	0	0	0	
VAN	0	0	0	0		0	0	0	0	
RIF	0	0	2	1.21	1	2	9.09	0	0	0.011
SXT	1	2.56	43	26.06	0.001	7	31.82	37	20.33	0.336
AMK	2	5.13	0	0	0.036	1	4.55	1	0.55	0.205
MNO	0	0	0	0		0	0	0	0	
CHL	3	7.69	2	1.21	0.075	1	4.55	4	2.20	0.438

Abbreviations: FOX, cefoxitin; PEN, penicillin; OXA, oxacillin; CPT, ceftaroline; GEN, gentamicin; LVX, levofloxacin; MFX, moxifloxacin; ERY, erythromycin; CLI, clindamycin; LZD, linezolid; DAP, daptomycin; TEC, teicoplanin; VAN, vancomycin; RIF, rifampin; SXT, trimethoprim-sulfamethoxazole; AMK, amikacin; MNO, minocycline; CHL, chloramphenicol.

Table 2 Antibiogram of MRSA and hVISA Isolates in the Context of Different Genotypes

Group		Molecular Type(%)	Antibiogram
MRSA		ST59-t437-*agr*I-IVa(53.8%)	FOX-OXA-PEN (n=1)
			[b]FOX-OXA-PEN-ERY-CLI (n=17)
			FOX-OXA-PEN-ERY-CLI-CHL (n=2)
			FOX-OXA-PEN-LVX-MFX-ERY-CLI (n=1)
		ST72-t2431-*agr*I-IVF (15.4%)	FOX-OXA-PEN-AMK (n=1)
			FOX-OXA-PEN-ERY-CLI (n=4)
			FOX-PEN-ERY-CLI-CHL (n=1)
		ST72-t20226-*agr*I-IVF (5.1%)	FOX-OXA-PEN-ERY-CLI (n=2)
		ST22-t309-*agr*I-III(5.1%)	FOX-OXA-PEN-ERY-CLI (n=2)
		ST59-t172-*agr*I-IVa (2.6%)	FOX-OXA-PEN-ERY-CLI (n=1)
		ST59-t3736-*agr*I-IVa (2.6%)	FOX-OXA-PEN-ERY-CLI (n=1)
		ST59-t3527-*agr*I-III(2.6%)	FOX-OXA-PEN-ERY-CLI (n=1)
		ST1232-t034-*agr*I-V (2.6%)	FOX-OXA-PEN-ERY-CLI (n=1)
		ST7196-t5155-*agr*I-IVF(2.6%)	FOX-OXA-PEN-ERY-CLI (n=1)
		ST7437-t437-*agr*I-IVa (2.6%)	FOX-OXA-PEN-ERY-CLI (n=1)
		ST965-t062-*agr*II-IVF (2.6%)	LVX-MFX (n=1)
		[a]ST9-t899-*agr*II-NT (2.6%)	[c]FOX-OXA-PEN-GEN-LVX-ERY-CLI-SXT (n=1)
hVISA	MRSA	ST59-t437-*agr*I-IVa (13.6%)	[b]FOX-OXA-PEN-ERY-CLI (n=3)
		ST72-t2431-*agr*I-IVF (13.6%)	FOX-OXA-PEN-ERY-CLI (n=3)
		ST72-t20226-*agr*I-IVF (4.5%)	FOX-OXA-PEN-ERY-CLI (n=1)
		ST7196-t5155-*agr*I-IVF (4.5%)	FOX-OXA-PEN-ERY-CLI (n=1)
		ST965-t062-*agr*II-IVF (4.5%)	LVX-MFX (n=1)
	[d]MSSA	ST25-t078-*agr*I (9.1%)	PEN-SXT (n=1)
			FOX-OXA-PEN-ERY-CLI (n=1)
		ST25-t167-*agr*I (4.5%)	PEN-SXT (n=1)
		ST25-t287-*agr*I (4.5%)	FOX-PEN-SXT (n=1)
		ST59-t1751-*agr*I(4.5%)	FOX-PEN-ERY-CLI-RIF-CHL (n=1)
		ST72-t148-*agr*I(4.5%)	PEN-ERY-CLI-SXT (n=1)
		ST5-t002-*agr*II (9.1%)	PEN-GEN-LVX-ERY-SXT (n=1)
			PEN-ERY-CLI-SXT (n=1)
		ST15-t084-*agr*II (4.5%)	PEN-ERY-CLI (n=1)
		ST965-t062-*agr*II (4.5%)	PEN (n=1)
		ST5459-t14014-*agr*II (4.5%)	PEN-ERY-CLI (n=1)
		ST7197-t002-*agr*II(4.5%)	PEN-GEN-ERY-CLI-SXT (n=1)
		ST1-t127-*agr*III (4.5%)	PEN-ERY-CLI-RIF (n=1)

Notes: a, SCC*mec* not typeable; b, isolate MRSA-42 resistant to amikacin; c, isolate MRSA-6221 intermediate resistance to moxifloxacin and chloramphenicol. d, hVISA isolated from MSSA strains could not be used for SCC*mec* typing as these organisms were failed to obtain the mobile genetic element SCC*mec* harbouring *mec*A gene.

Discussion

As sample sizes have increased, laboratory personnel have found an increasing discordance between cefoxitin and oxacillin assays, or in both phenotypic and genetic analyses.[20–22] Isolates MR-hVISA-33 and MRSA-6539 were mecA positive yet phenotypically susceptible to oxacillin (OS-MRSA), indicating the likely occurrence of frameshift mutations or single base substitutions in regions of nucleotide repeats within mecA, which have the ability to reverse into resistance when exposed to antibiotics.[23,24] The other remaining 12 strains, despite exhibiting phenotypic resistance to oxacillin or/and cefoxitin, were not identified by PCR for the *mecA* gene and had negative results in the SCC*mec* typing assay, so we did not treat them as MRSA.

The regional clone prevalent in the region belongs to ST59-IVa-t437 in agreement with the findings of Li and Wang.[25,26] Our findings are different from those of other national studies. ST8 strains were the most prevalent (59.8%) of PVL-positive MRSA in Japan, while ST59 clone consisted of 6 strains (6.5%).[27] The main MRSA type in the UK was

ST22,[28] which accounted for 5.1% in our findings. The most prevalent molecular types of MRSA in the United States in 2015 were ST5 and ST8.[29,30] Neither of which were not identified among our MRSA isolates; however, ST5 was present in 9.1% of hVISA isolates in our study.

The isolation rate of hVISA in *S. aureus* isolates accounted for 10.8%, which came from 23.1% of MRSA and 7.9% of MSSA. Most previous studies have focused on exploring hVISA from blood-borne MRSA isolates.[31–33] however, our current study demonstrated that hVISA can be identified from a wide range of clinical samples and that a certain percentage of MSSA strains also belonged to hVISA. Previous studies have demonstrated that hVISA can persist in blood for a long time, which may be the reason why hVISA is more common in blood-borne MRSAs in our study.[33] Also we suggest that the investigation of hVISA should be conducted from *S. aureus* from different sample sources. In our study, 22.7% of hVISA isolates belonged to ST72 and CC5 (ST5/965/7197), followed by CC59 (ST59) and ST25 (18.2%, respectively), which was different from some studies where ST5 clones were highly prevalent between hVISA strains (92.3%) in Latin American, but ST59 and ST72 were not found.[34] A systematic review and meta-analysis showed that ST239 was the most prevalent hVISA clone (58.62%), accompanied by ST5 (14.45%), while both ST72 and ST59 were less than 3.5%.[35] These studies imply the diversity of molecular genetic background of hVISA isolates.

97.4% and 92.3% of MRSA were susceptible to gentamicin and levofloxacin, respectively, which was significant when compared to findings by Riccobono et al (75%, 23.3%, with $p = 0.002$ and <0.001, respectively), but significantly low susceptibility to erythromycin (7.7% vs 29%, $p = 0.005$).[36] The reason for this difference may have been due to selective pressures of this bacteria with different geographical distribution to the drug.

Our study showed a multidrug resistance pattern of MRSA represented by ST59-t437 exhibiting β-lactams, macrolides (erythromycin) and lincosamides (clindamycin), while being sensitive to rifampicin, levofloxacin and moxifloxacin (100%, 92.3% and 92.3%, respectively). The lower cost of resistance adaptation gives this clone an advantage in terms of growth and competition. The previously dominant genotype ST239 was not identified in our study, as it was shown that this clone is resistant to rifampicin, levofloxacin, moxifloxacin and tetracycline, and the additional acquired resistance gene necessarily leads to its evolutionary burden resulting in the loss of its advantage to become dominant.[25,37] ST59 is only regionally prevalent, with major outbreaks in East Asia and sporadic occurrences in Australia, the Netherlands, and the United States.[38–41] In the United States, 72.1% of ST59-t437 strains were erythromycin resistant, but the resistance rate to clindamycin was low (11.8%), which was inconsistent with our study (Table 1), presuming that the genome of ST59 clones in the United States contained the erythromycin resistance gene *ermA*, but lacked the lincosamides resistance gene *ermB* present in the East Asian ST59 clones.[41] In addition to t437 and SCC*mec* IVa, the ST59 genealogy also contains t172, t3527, t3736 and type III SCC*mec*, but in low proportions, suggesting a diversity of gene structures in this clone; however, these did not alter the resistance phenotype of this clone, despite the presence of type III SCC*mec* carrying a higher number of resistance genes (plasmid pT181, transposon Tn554 and ψTn554).[42]

MRSA was common in pediatrics (50%), with the most frequently identified genotypes being ST72-IVF-t2431-*agr* I (6/11,54.5%), and secondly, ST59-IVa-t437-*agr*I(4/11,36.4%). Except for strain MRSA-110, which was resistance for only β-lactams and amikacin, the antibiotics susceptibility testing results of the two strain groups with very different molecular characteristics were almost identical, and the antibiogram was erythromycin-clindamycin-β-lactam, excluding MR-6539, which was additionally tolerant to chloramphenicol, indicating that the structural differences of resistance genes between the two lineages were not significant.

In addition, pediatrics was also the major source of hVISA in this study (31.8%). Notably, although MS-hVISA did not contain SCC*mec*, two of the three strains were more resistance to rifampicin or SXT than the five MR-hVISA strains with consistent resistance profiles, suggesting that mutations in the *rpoB* gene occurred during the evolution of the strain from methicillin-sensitive to heterogeneous intermediated resistance to vancomycin, or through the integration of other mobile genetic elements that confer resistance to erythromycin, clindamycin and SXT. This may also account for the higher resistance rate of MS-hVISA to trimethoprim-sulfamethoxazole than MSSA found in our study. Also, without considering the pediatric origin, a longitudinal view of the entire hVISA

and VSSA populations, the more widespread distribution of the *mecA* responsible for resistance to cefoxitin and oxacillin in hVISA and the identification of only two rifampicin-resistant isolates among 204 strains as hVISA do not suggest that *mecA* and mutations in the *rpoB* gene associated with reduced susceptibility to vancomycin (Table 1).[43,44]

ST72-IVF-t2431-*agr*I just became the children clone in this region, contrary to the findings of the recognized "pediatric clone" ST5-IV.[45,46] The results of antibiotics susceptibility testing revealed that the most common genotype ST59-MRSA-IV/V, representing the Chinese pediatric strain, had a resistance rate of more than 78.6% to erythromycin, but the resistance rate to clindamycin varies greatly (83.3% vs 14.3%, respectively).[47,48] ST72-IV has been isolated in a small amount among the MRSA population of Chinese children, showing sensitity to erythromycin and clindamycin.[47] However, sporadically ST72-IVc from Japan and Portugal were resistant to both agents.[49,50] There is no specific resistance spectrum for the dominant clone ST72-IV in the Korean pediatric population, but extensive data, including analysis of data from adult ST72-IV, demonstrated that this genotype is mostly sensitive to clindamycin, with varying rates of resistance to erythromycin.[51–53] All ST72-IVF identified in our study were resistant to erythromycin and clindamycin, except strain MRSA-110 (Table 2).

SCC*mec* type IV contains many subtypes (IVa to n), but we did not find any effect of subtypes on the resistance patterns, suggesting a weakened relationship between subtypes and resistance, Because (i) there is no SCC*mec* structure but still drug resistance (ii) no other resistance genes were found to be present in the type IV SCC*mec* structure except for *mecA*.[42] Although it has been shown that in adults, ST72-IV causes lower mortality than ST5-II,[52] nevertheless such a high number of MRSA and hVISA in pediatrics should be of concern to clinicians. Vancomycin and others still possess excellent antibacterial activity against MRSA and hVISA and can be used for anti-infective treatment caused by both (Table 1).

In summary, the frequency of MRSA and hVISA in the Yellow River Delta was 19.1% and 10.8%, respectively, with the predominant genotypes of MRSA were ST59-t437-*agr*I-IVa and ST72-t2431-*agr*I-IVF, and the majority were resistant to erythromycin and clindamycin. The primary clones of hVISA were CC5-*agr*II and ST72-*agr*I, but there was heterogeneity in *spa* typing. For MR-hVISA, phenotypic resistance was the same as MRSA. However, the resistance profiles of MS-hVISA to the antibiotics varies according to the diversity of molecular genetic background. Pediatrics as a primary source of MRSA, especially hVISA, is worrisome and highlights the necessity of rational use of drugs and improved management.

Ethical Statement

This study was approved by the Institutional Ethics Board of Binzhou Medical University Hospital (KYLL-2022-47).

Acknowledgments

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no conflicts of interest in this work.

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