

Full Length Research Paper

## Molecular characteristic of *Staphylococcus aureus* isolates in a Chinese teaching hospital

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The aim of this study is to investigate the genetic backgrounds of methicillin susceptible *Staphylococcus aureus* (MSSA), methicillin-resistant *Staphylococcus aureus* (MRSA) and heterogeneous vancomycin-intermediate *Staphylococcus aureus* (hVISA) isolates isolated from clinical specimens of the patients with verified infections in a Chinese teaching hospital. Macro E test (MET) was used to detect hVISA and multilocus sequence typing (MLST) was used to determine the STs of the selected isolates. The genotypes of SCCmec were determined by multiplex polymerase chain reaction (PCR) in MRSA isolates. Panton-valentine leucocidin (PVL) genes were also detected by PCR. Among 273 *S. aureus* isolates, hospital-acquired methicillin-resistant *Staphylococcus aureus* (HA-MRSA), community-associated methicillin-resistant *S. aureus* (CA-MRSA), hospital-acquired methicillin susceptible *Staphylococcus aureus* (HA-MSSA) and community-associated methicillin susceptible *Staphylococcus aureus* (CA-MSSA) isolates accounted for 55.6, 1.5, 36.3, and 6.6%, respectively. Nine isolates were confirmed as hVISA by MET. Among 60 HA-MRSA isolates, ST239-MRSA-III was the most prevalent clone accounting for 51.7%, followed by ST5-MRSA-II clone. Fifty percentage and 22.2% of CA-MSSA isolates were found to be ST121 and ST88. ST239-MRSA-III was the predominant clone in hVISA isolates. However, no predominant ST type was found in HA-MSSA isolates. Of 9 PVL-positive strains, ST88 was the most prevalent ST (50.0%; 4/8), followed by ST121 (33.3%; 3/9), ST5 (4.5%; 1/22) and ST239 (2.6%; 1/39). In conclusion, ST239- III was the major pandemic clone in hVISA and HA-MRSA and spread in China. ST5- emerging rapidly in China had remained stable viability.

**Key words:** *Staphylococcus aureus*, heterogeneous vancomycin-intermediate *Staphylococcus aureus*, molecular characteristic.

### INTRODUCTION

*Staphylococcus aureus* is one of the most virulent and common pathogens, accounting for a wide range of different serious infections from minor skin and soft tissue infections (SSTIs) to life threatening pneumonias and sepsis (Valentini et al., 2008). Since methicillin-resistant *S. aureus* (MRSA) first appeared in 1961 (Kowalski et al., 2005), MRSA has become a major cause of hospital- and community-acquired infections worldwide. MRSA is conferred by an acquired penicillin-binding protein encoded by the *mecA* gene located on a mobile genetic

element, the Staphylococcal cassette chromosome *mec* (SCCmec). Seven different SCCmec types with difference in size and construe (types I, II, III, IV, V, VII and VIII) have been found (Ito et al., 2004). SCCmec is regarded as an antibiotic-resistant island, as it can be integrated by other mobile elements and resistance genes. Thus, MRSA isolates are mostly multi-resistant to many antimicrobial agents, such as macrolides, aminoglycosides and fluoroquinolones. The prevalence of MRSA in China was about 50% in 1990s, and was close to 77.0% between 2005 and 2009 (Liu et al., 2010). Therefore, measures should be taken to control the spread of MRSA in both hospitals and communities.

Since initial reports of reduced susceptibility to vancomycin in clinical isolates of *S. aureus* from Japan in

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1997, vancomycin-intermediate *S. aureus* (VISA) and heterogeneous vancomycin-intermediate *S. aureus* (hVISA) have been reported in many countries all over the world (Hiramatsu et al., 1997). Intermediate resistance to vancomycin is due to increased thickness of the bacterial wall, but not acquisition of the *vanA* operon. hVISA show a vancomycin minimum inhibitory concentration (MIC) in the susceptibility range, but contain subpopulations of microorganisms for which the vancomycin MIC corresponds to the intermediate category (Jones, 2008). In China, 7.8% of MRSA was reported to be hVISA in the period 1997 to 1999, and 13 to 16% in 2005 to 2007 (Benquan et al., 2002; Sun et al., 2009). Three strains of VISA have been reported in China to date.

Recently, respectable cases about vancomycin treatment failures in serious infections caused by VISA and hVISA have been reported (Howden et al., 2004). The aim of this study was to investigate the genetic backgrounds of methicillin-susceptible *S. aureus* (MSSA), MRSA and hVISA isolates collected from a Chinese hospital located in central China using different methods to better survey molecular epidemiology of *S. aureus* clinical isolates in China.

## MATERIALS AND METHODS

### Bacterial isolates

From January to December, 2008, a total of 273 isolates of *S. aureus* isolates were isolated from clinical specimens in a teaching hospital, Anhui Province, centre China. *S. aureus* isolates were isolated from sputum (n=118, 43.2%), pus (n=57, 20.9%), wound secretion (n=48, 17.6%), blood (n=36, 13.2%), urine (n=10, 3.7%), and unknown sites (n=4, 1.5%). According to the criteria recommended by Centers for Disease Control and Prevention (CDC) of USA, the hospital-acquired isolates were isolated from the patients with infections detected after 48 h hospital admission and within a year before the present hospitalization had had any one of the following: hospitalization, surgery, dialysis, or residence in a long-term care facility and hemodialysis or peritoneal dialysis, or at the present admission had a permanent indwelling percutaneous medical devices or catheters. Community-associated methicillin-resistant *S. aureus* (CA-MRSA) infections were considered to be a MRSA infection without any of the above features. This information was based on medical record review. The *S. aureus* isolates were identified by Gram's stain, microscopic examination, coagulase test and Vitek-32 microbiology analyzer (bioMérieux, Marcy l'Etoile, France). MRSA was initially screened by oxacillin and cefoxitin discs (OXOID, Basingstoke, the United Kingdom), and then was confirmed by polymerase chain reaction (PCR) detecting *mecA*.

### Vancomycin agar screening test

Agar screening test was performed as described previously (Fitzgibbon et al., 2007). All isolates were screened on brain heart infusion (BHI) agar supplemented with 4 g/ml vancomycin (BHIV4) and 6 g/ml vancomycin (BHIV6) by using 10 l of bacterial suspensions of a 0.5 McFarland suspension. Plates were incubated for 24 h at 35°C, and growth was observed. The strain was considered as a possible VISA strain if one or more colonies had

grown within 24 to 48 h. According to the protocols provided by the Clinical and Laboratory Standards Institute (CLSI, 2009), the isolates with MICs for vancomycin 4 g/ml determined by Etest and broth dilution methods were confirmed as VISA isolates.

### The macro E test (MET)

hVISA was screened by MET described by (Fitzgibbon et al., 2007). Namely, 200 l of 2.0 McFarland suspensions was pipetted onto a BHI agar plate. Etest strips (AB Biodisk, Ltd., Solna, Sweden) with vancomycin and teicoplanin were applied and the plates were incubated for 48 h at 35°C. Strains with both vancomycin and teicoplanin MICs 8 g/ml or with teicoplanin MIC 12 g/ml alone were considered as hVISA. *S. aureus* strains Mu3 (hVISA), ATCC43300 (MRSA) and ATCC29213 (MSSA) were used as controls.

### DNA extraction

To prepare DNA templates, the isolates grew on blood agar, and were incubated at 37°C for 24 h. A total of three colonies were suspended and then incubated with lysostaphin and proteinase K at 37°C for 1 h, then boiled and centrifuged. The supernatants were used as DNA templates in the PCR tests.

### Staphylococcal cassette chromosome *mec* (SCC*mec*)

The SCC*mec* types were determined by a multiplex PCR method developed by Oliveira et al. (2006). Nontypeable (NT) types were recognized as strains showing unexpected fragments or lacking some fragments as identified by multiplex PCR. The reference strains used were *S. aureus* ATCC 25923, MRSA NCTC 10442 (SCC*mec*I), MRSA N315 (SCC*mec*II), MRSA 85/2082 (SCC*mec*III) and MRSA JCSC4744 (SCC*mec*IV).

### MLST and data analysis

Multilocus sequence typing (MLST) was performed as described previously (Enright et al., 2000). ST numbers were assigned according to the *S. aureus* database available on the MLST website (<http://saureus.mlst.net>) and the allelic number was determined for each sequence. The clustering of related STs, which were defined as clonal complexes (CCs), was determined by using the program eBURST (based on related STs).

### Detection of panton-valentine leucocidin (PVL) genes

The PVL genes were detected by PCR as described previously (Ghebremedhin et al., 2007). The identities of the PCR products were confirmed by sequencing using an ABI 3700 sequencer.

## RESULTS

### Prevalence of MRSA

Among 273 *S. aureus* isolates tested, 156 MRSA isolates (57.1%) were detected by phenotypic and molecular methods. Two hundred and fifty-one isolates (91.9%, 251/273) were hospital-acquired, of which MRSA and MSSA accounted for 60.6 and 39.4%, respectively.

Twenty-two isolates (8.1%, 22/273) were classified as community-acquired, of which 18 isolates belonged to CA-MSSA and 4 isolates belonged to CA-MRSA.

### Identification of VISA and h-VISA

None of *S. aureus* strains with vancomycin MICs  $\geq 4$  g/ml determined by Etest and broth dilution methods was found to be VISA. However, 9 HA-MRSA isolates were found to be hVISA determined by MET.

### SCCmec typing

Among 60 Hospital-associated methicillin-resistant *S. aureus* (HA-MRSA) isolates, SCCmecI, II, III and SCCmecIV were found in 2, 21, 35 and 2 isolates, respectively. Of 4 CA-MRSA isolates, 2, 1 and 1 isolate harbored SCCmecI, SCCmecII and SCCmecIII. Among 9 hVISA, 8 and 1 isolate harbored SCCmecIV and SCCmecV.

### MLST

Twenty-two different STs were found in 111 *S. aureus* (Table 1). The main STs were ST239, ST5, ST121, ST88, ST59 and ST398, accounting for 35.1, 19.8, 8.1, 7.2, 4.5 and 4.5%, respectively. Among 60 HA-MRSA isolates, ST239 and ST5 accounted for 51.7 and 30.0%. Fifty percentage (9/18) and 22.2% (4/18) of CA-MSSA isolates were found to be ST121 and ST88. Seven out of 9 hVISA isolates belonged to ST239. ST types showed diversiform in Hospital-associated methicillin-susceptible *S. aureus* (HA-MSSA). Two novel isolates were identified, ST 1226 and ST1296.

### PVL genes screening

Nine isolates (3.3%, 9/273) were positive for PVL genes. The prevalence of PVL genes in CA-MSSA, HA-MSSA, CA-MRSA, HA-MRSA and hVISA isolates were 27.8% (5/18), 5% (1/20), 25% (1/4), 3.3% (2/60) and 0% (0/9), respectively. Four STs were found among PVL-positive isolates, of which ST88 was the most prevalent ST (50.0%, 4/8), followed by ST121 (33.3%, 3/9), ST5 (4.5%, 1/22) and ST239 (2.6%, 1/39).

### Significant *S. aureus* clones

Of 60 HA-MRSA isolates, 31(51.7%) belonged to ST239-MRSA-III clone. One strain isolated from blood was PVL-positive. According to clinical record, this pneumonia patient in ICU, who was treated with vancomycin and

Linezolid, finally, the patient died. ST239-MRSA-III was also the most frequent clone in hVISA, accounting for 77.8% (7/9). One isolate was initially detected in the patient with intracranial infection in the department of infectious diseases. The patient had been treated with vancomycin for 24 days. Subsequently, this clone was found in 3 patients in the same ward. ST5-MRSA-II was another dominant clone in HA-MRSA, accounting for 30% (18/60). PVL gene was not detected in this clone. The first patient with ST5-MRSA-II was transferred from another hospital, and admitted to the ICU. Soon, this clone was found in other departments and spread in our hospital.

Of 18 CA-MSSA, 9 isolates were found to be ST121 (50%). 3 isolates recovered from wound secretion of the outpatients with skin and soft tissue infection were PVL-positive.

### DISCUSSION

*S. aureus*, especially MRSA, is one of the most common and dangerous pathogens. In China, the mean prevalence of MRSA was over 50%, while the prevalence was high to 80% in Shanghai, China (Wang et al., 2008). In the present study, the prevalence of MRSA was similar to the mean prevalence in China, but lower than that in Shanghai. Because vancomycin is a first selective drug for treatment of MRSA infections, the appearance of VISA and hVISA should be of concern. hVISA might be associated with treatment failure (Howden et al., 2004). In this study, 9 out of 156 MRSA isolates (5.9%) were identified as hVISA by MET. In USA, Italy, Canada, France, Israel, Belgium and Asia, the prevalence of hVISA in MRSA was 7.5, 2.8, 1.3, 0.21, 6, 0.7 and 4.3% (Rybak et al., 2008; Campanile et al., 2010; Adam et al., 2010; Reverdy et al., 2001; Maor et al., 2007; Nonhoff et al., 2005; Song et al., 2004), respectively. The disparity in the prevalence of hVISA in different areas may be due to the different detection methods, geographical differences and variable incidence.

The ST239-MRSA-III Brazilian clone was first found in Brazil and widely spread in the world. Subsequently, this clone spread to Australia (Dubin et al., 1992), the UK, and the USA (Pavillard et al., 1982) in the late 1970s and early 1980s and to Europe (Amorim et al., 2002) and South America (Arakere et al., 2005) in the 1980s and 1990s. Recently, ST239 was the epidemic clone in Asia (Hsu et al., 2005), and in China it accounted for 97% of nosocomial MRSA infections (Xu et al., 2009). A selective pressure created by unreasonable use of vancomycin would contribute to the outgrowth of hVISA, which is considered to be the precursors of VISA as a result of continued glycopeptide exposure (Loomba et al., 2010). One hVISA isolate was recovered from the patient who had been treated with vancomycin. In the present study, ST239-MRSA-III was found to be the most prevalent

**Table 1.** Molecular characterization of 111 isolates of *S. aureus*.

MLST-CC	SCCmec type				CA-MSSA	HA-MSSA	CA-MRSA	HA-MRSA	hVISA	PVL
	I	II	III	IV						
ST239-CC239			38			1		31	7	1
ST5-CC5		19		1	1	1	1	18	1	1
ST1-CC1				1				1		
ST1226-CC25				1				1		
ST399-CC121			1					1		
ST15-CC15		1						1		
ST182-CC182	1							1		
ST1296-CC8	1							1		
ST241-CC8			2			1		1	1	
ST88-CC88			2		4	2		2		4
ST398-CC398		2				2	1	2		
ST121-CC121					9					3
ST59-CC59			2		1	2	2			
ST221-CC5					3					
ST7-CC7						2				
ST188-CC1						1				
ST6-CC6						1				
ST20-CC20						1				
ST30-CC30						2				
ST8-CC8						1				
ST254-CC8						1				
ST240-CC239						2				
Total	2	22	45	3	18	20	4	60	9	9

clone in HA-MRSA and hVISA isolates. Interestingly, three hVISA isolates belonging to ST239-MRSA-III were detected in the same ward, indicating this clone disseminated in the hospital. Strategies to control and prevent emergence and dissemination of this clone in hospitals include effective laboratory screening to identify cases, using rationally antibiotics, decolonization, and routine infection prevention measures including disinfection, washing hands and wearing masks.

The second predominant clone in HA-MRSA isolates was ST5-MRSA-II, which belonged to the New York/Japan epidemic clone. This clone was initially found in the USA and Japan (Aires de Sousa et al., 2000), and spread to Europe and Asia (Soo et al., 2005; Stefani and Varaldo, 2003). In China, in the early 2000s, the New York/Japan emerged and rapidly increased, becoming another dominant clone in our country besides Brazilian clone (Chen et al., 2010). In this study, this clone was first detected in the patient from another hospital and subsequently found in our hospital. It is speculated that ST5-MRSA- was probably imported from abroad. The emergence of this clone shows the changing epidemiology of MRSA in our area. An active molecular epidemiological surveillance should be required.

The ST121 was one of the predominant lineages of PVL-positive MSSA in the world (Rasigade et al., 2010)

and the dominant clone in MSSA throughout Russia and Portugal (Vorobieva et al., 2008; Conceição et al., 2011). Moreover, this clone had been also reported in China, Nigeria and Germany (Chen et al., 2010; Conceição et al., 2011; Schefold et al., 2007). It is noteworthy that two PVL -positive ST 121-SCCmec V-MRSA isolates emerged in Cambodia (Chheng et al., 2009). In the present study, ST121 was the most common clone in CA-MSSA. Among CA-MSSA isolates, 3 PVL- positive isolates were associated with skin and soft tissue infection. To our best of knowledge, this was the first report of PVL-positive ST121-MSSA isolates in China. MRSA strains have emerged by acquisition of SCCmec, resulting in a small number of prevalent MRSA spread in the world (Robinson and Enright, 2003). PVL-positive MSSA strains are dynamically interrelated and as reservoirs of CA-MRSA reported by others (Rasigade et al., 2010). Therefore, a regular epidemiology investigation can contribute to detect and control the emergence and spread of PVL-positive MRSA infection in hospitals.

It is clear that MRSA have originated from the introduction of SCCmec into dominant MSSA backgrounds and disseminate worldwide (Robinson and Enright, 2003). However, the results showed that there was no consistence between the genetic backgrounds of the MSSA and MRSA strains, showing evidence of a high

genetic diversity among MSSA compared to MRSA. It is demonstrated that the introduction of SCCmec into MRSA may be a rare case compared to the local spread of MRSA clones.

The detection rate of the PVL gene among *S. aureus* was 3.3% in this study, which showed that the prevalence of PVL-positive *S. aureus* was still low in Chinese hospitals. The prevalence of PVL-positive *S. aureus* was 2.3% in Chinese hospitals, but 12.8% in Wenzhou, China (Liu et al., 2009; Yu et al., 2008). The prevalence of PVL-positive strains of *S. aureus* in Japan, Africa and the USA was 0.04, 14.7 and 48.3% (Kawaguchiya et al., 2011; Mesrati et al., 2010; Lowy et al., 2007), respectively. ST88 was the dominant clone among PVL-positive isolates. ST88 with PVL-positive isolates had been reported in Sweden, Brazil, Italy and Asia (Afroz et al., 2008; Fang et al., 2008; Reinert et al., 2008; Stefani et al., 2009). In China, this clone was the predominant lineages of PVL-positive clones and correlated to skin and soft tissue infection (Yao et al., 2010). One pediatric clone (ST-MRSA-VI) possessing PVL-gene had been detected in our hospital. This is a common clone in the United States, Poland, Portugal, and Colombia, but not in Asian countries such as Japan, China, and Taiwan (Robinson and Enright, 2003). MRSA with increased virulence is an issue of concern and could result in an occurrence of methicillin resistance and spread of staphylococcal fatal infections in both community and hospitals.

In summary, our study documented that ST239-III was the major pandemic clone in hVISA and HA-MRSA and spread in China. ST5- had rapidly emerged in China, had remained stable viability.

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