

Molecular analysis and frequency of *Staphylococcus aureus* virulence genes isolated from bloodstream infections in a teaching hospital in Tianjin, China

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ABSTRACT. *Staphylococcus aureus* is an important cause of bloodstream infections worldwide. We examined the prevalence of genes that encode erythromycin ribosome methylase and bacterial toxins in *S. aureus* collected from bloodstream infections. Sixty different *S. aureus* isolates were obtained from blood cultures of patients who were admitted to a Teaching Hospital in Tianjin from January 2006 to August 2011. The susceptibility of the isolates to 16 antibiotics was tested. Methicillin-resistant *S. aureus* (MRSA) was identified using the disk diffusion method with cefoxitin. PCR was used to detect genes that encode the staphylococcal enterotoxins, Panton-Valentine leukocidin, toxic shock syndrome toxin 1 and erythromycin ribosome methylase. Molecular analysis of the MRSA strains was done using pulsed-field gel electrophoresis (PFGE) and staphylococcal cassette chromosome mec (SCCmec) typing. The positivity rates of *mecA*, *ermA*, *ermB*, and *ermC* in the isolates were 13/60, 10/60, 18/60, and 18/60, respectively. Among the

60 isolates, 30 harbored enterotoxin genes, with *sea* as the most frequent toxin gene (33%), followed by *sec* (15%), *sed* (12%), and *seb* (5%). The *see* and *tst* genes were not found in any of the isolates. The *pvl* gene was detected in four strains. Eleven MRSA isolates were of the SCCmec type III; two MRSA isolates could not be determined through SCCmec typing. PFGE analysis of the 13 MRSA isolates produced 8 distinct pulsotypes. Virulence genes and erythromycin ribosome methylase genes were highly prevalent in these isolates. The PFGE results demonstrated that the MRSA spread through cloning, mainly involving SCCmec type III.

Key words: *Staphylococcus aureus*; Bloodstream infections; Virulence gene; Erythromycin ribosome methylase gene

INTRODUCTION

Staphylococcus aureus is a leading cause of bloodstream infections (BSIs) worldwide (Malachowa et al., 2011). The 2008 Mohnarín Report from China showed that *S. aureus* (729/10519, 6.9%) is the 3rd most common cause of BSIs in China (Wang et al., 2010). In Italy and Brazil, *S. aureus* is also ranked as the most common cause of BSIs (Luzzaro et al., 2011; Marra et al., 2011). Methicillin-resistant *S. aureus* (MRSA) in particular has caused increasing concern in health systems throughout the world because of its high incidence and associated undesirable outcomes (Gasch et al., 2011).

The clinical importance of *S. aureus* is attributed to its high virulence and rapid development of drug resistance. *S. aureus* virulence factors include surface proteins, toxins, and enzymes (Arvidson and Tegmark, 2001). Thus far, studies on the virulence genes and epidemiology of *S. aureus* have been limited. Consequently, this study aimed to determine drug susceptibility patterns and identify the genes encoding staphylococcal enterotoxins (SEs) A to E, Pantón-Valentine leukocidin (PVL), toxic shock syndrome toxin (TSST-1), and erythromycin ribosome methylase (ERM) in 60 *S. aureus* isolates from BSIs patients.

MATERIAL AND METHODS

Bacterial isolates

A total of 60 *S. aureus* samples were isolated from January 2006 to August 2011 in the General Hospital of Tianjin Medical University, a Tertiary Care Teaching Hospital with 1600 beds in China. These isolates were collected from the blood cultures of patients admitted into the General Intensive Care Unit, the Departments of Hematology and Nephropathy and other wards. All strains were identified using the VITEK-2 compact automatic system (Biomérieux, Marcy l'Étoile, France). The isolates were classified as MRSA based on their resistance to oxacillin, which was confirmed by testing for *mecA*.

Antibiotic susceptibility tests

Antibiotic susceptibility test profiles for 16 antimicrobial agents (oxacillin, penicillin, gen-

tamicin, ciprofloxacin, linezolid, clindamycin, erythromycin, rifampin, tetracycline, moxifloxacin, teicoplanin, tigecycline, trimethoprim-sulfamethoxazole, vancomycin, ampicillin-sulbactam, and levofloxacin) were determined using a VITEK-2 compact automatic system (Biomérieux). Susceptibility to ceftiofur was determined using a disk diffusion test. The standards for antimicrobial susceptibility testing and interpretation were based on Clinical and Laboratory Standards Institute standard M100-S20 (National Committee for Clinical Laboratory Standards, 2010), and tigecycline minimum inhibitory concentration (MIC) results were interpreted according to the breakpoints approved by the U.S. Food and Drug Administration (susceptible ≤ 0.5 $\mu\text{g}/\text{mL}$). The *S. aureus* strain ATCC25923 was used as the quality control strain for the susceptibility test.

Detection of *mecA* and virulence and ERM genes

All isolates were cultured on blood agar and incubated overnight at 35°C. DNA for polymerase chain reaction (PCR) was extracted as previously described (Wu et al., 2011) and immediately used for PCR or stored at -20°C. The presence of *mecA* was determined via PCR using a primer, as described elsewhere (Oliveira and de Lencastre, 2002). PCR amplifications were performed using a thermal cycler (Perkin Elmer, Waltham, USA) as follows: initial denaturation for 5 min at 94°C; followed by 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 55°C, and extension for 60 s at 72°C, and a final extension for 7 min at 72°C.

PCR amplification was performed for genes encoding the SEs SEA (*sea*), SEB (*seb*), SEC (*sec*), SED (*sed*), SEE (*see*), PVL (*lukS-lukF*), TSST-1 (*tst*), and ERM (*ermA*, *ermB*, *ermC*) as previously described (Johnson et al., 1991; Lina et al., 1999a,b; Cao et al., 2010). All PCR products were analyzed through agarose gel electrophoresis on 1.5% agarose gels.

The PCR products were purified and then sequenced with BigDye terminator v3.1 using an ABI PRISM 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA). The DNA sequences obtained were compared with those in the National Center for Biotechnology Information database.

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

SCC*mec* typing and subtyping were performed as described previously (Zhang et al., 2005), and the amplification was performed in a GeneAmp PCR system 9700 system. PCR was conducted with an initial denaturation step at 94°C for 5 min followed by 10 cycles at 94°C for 45 s, 65°C for 45 s, and 72°C for 1.5 min. Afterward, another 25 cycles were conducted at 94°C for 45 s, 55°C for 45 s, and 72°C for 1.5 min, ending with a final extension at 72°C for 10 min and holding at 4°C.

Pulsed-field gel electrophoresis (PFGE)

Molecular analysis of the MRSA strains was accomplished through PFGE. Genomic DNA was prepared on agarose plugs as previously described (Goering and Winters, 1992). The DNA was digested with 30 U *Sma*I- (TaKaRa, Dalian, China) for 4 h at 30°C. Contour-clamped homogenous electric field electrophoresis of the digested chromosomal DNA was performed on 1.0% pulsed-field certified agarose (BioRad, Hercules, CA, USA) at 6 V/cm for 18 h at 14°C with pulse times of 4 to 40 s, using a contour-clamped homogenous electric field DRIII system

(BioRad). After electrophoresis, the gel was stained with 0.5 µg/mL ethidium bromide and photographed under ultraviolet illumination. The assessment and interpretation of the PFGE patterns were performed according to previously published criteria (Tenover et al., 1995).

RESULTS

Antimicrobial susceptibility

All *S. aureus* isolates were susceptible to teicoplanin, linezolid, tigecycline, and vancomycin. The majority of the isolates, that is, 55 (91.7%) and 39 (65.0%), were resistant to penicillin G and erythromycin, respectively. The resistance rates of *S. aureus*, MRSA, and methicillin-sensitive *S. aureus* (MSSA) are summarized in Figure 1. Up to 13 isolates were resistant to cefoxitin and harbored *mecA* (Figure 2A) (GenBank accession No. GU227428). Eleven (84.6%) of the 13 MRSA isolates were multi-drug resistant (i.e., resistant to more than 3 classes of the non-β-lactam antibiotics tested).

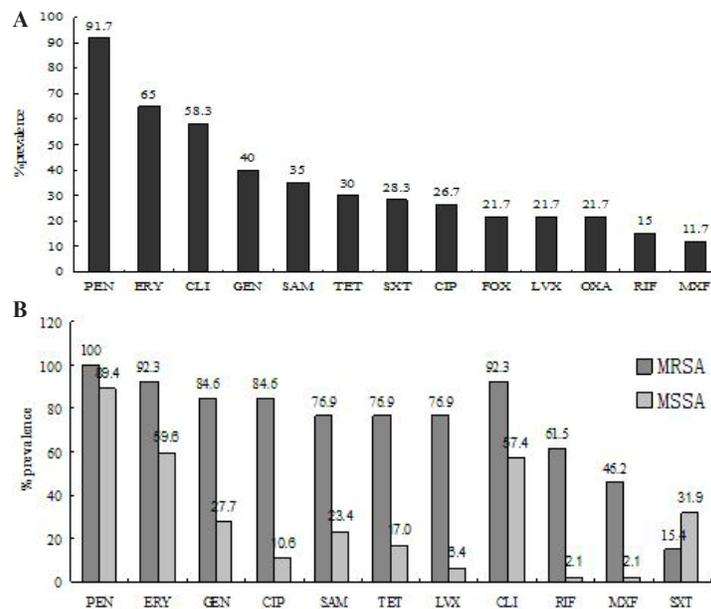


Figure 1. Antimicrobial resistance of *Staphylococcus aureus* isolates (A), antimicrobial resistance of methicillin-resistant *S. aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA) isolates (B). PEN = penicillin; ERY = erythromycin; CLI = clindamycin; GEN = gentamicin; SAM = ampicillin-sulbactam; TET = tetracycline; SXT = trimethoprim-sulfamethoxazole; CIP = ciprofloxacin; FOX = cefoxitin; LVX = levofloxacin; OXA = oxacillin; RIF = rifampin; MXF = moxifloxacin.

Distribution of virulence and ERM genes

The distribution of genes that encode SE, PVL, and ERM is summarized in Table 1. In total, 30 (50.0%) isolates were positive for SE genes (Figure 2B) (GenBank accession Nos. JN024684, AB479119, AB084256, and AY518388), 4 (6.7%) were positive for *pvl* (Figure

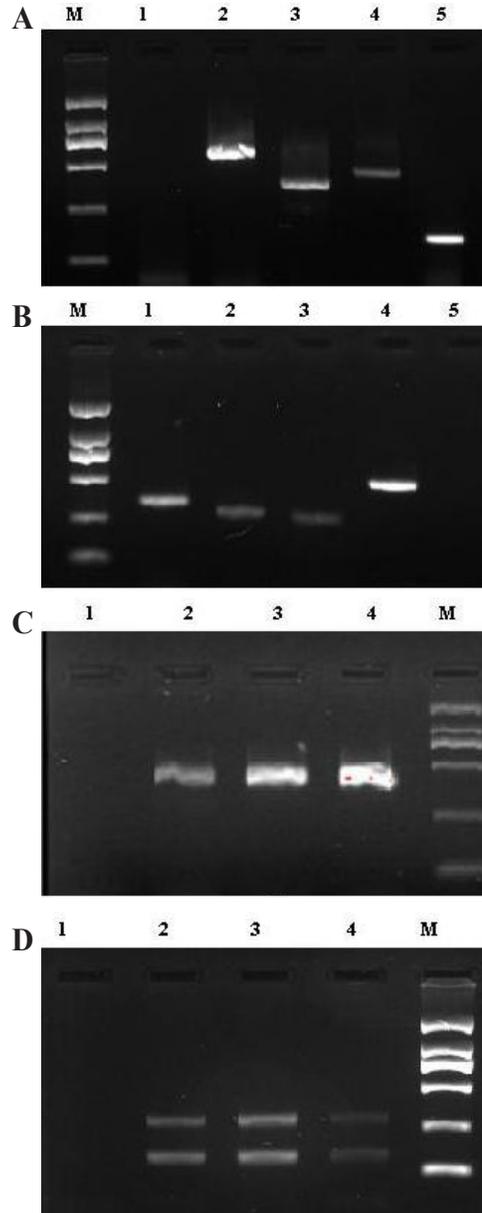


Figure 2. A. *mecA* and *erm* genes; B. staphylococcal enterotoxin (*se*) genes; C. Panton-Valentine leukocidin (PVL) genes, and D. SCCmec typing electropherogram. A. Lane 1 = negative control; lane 2 = *ermC* (572 bp)-positive; lane 3 = *ermB* (359 bp)-positive; lane 4 = *ermA* (421 bp)-positive; lane 5 = *mecA* (162 bp)-positive; lane M = DNA maker (up→down: 2000→100 bp). B. Lane 1 = *sea* (341 bp)-positive; lane 2 = *seb* (285 bp)-positive; lane 3 = *sec* (257 bp)-positive; lane 4 = *sed* (454 bp)-positive; lane 5 = negative control, lane M = DNA maker (up→down: 2000→100 bp). C. Lane 1 = negative control; lanes 2 to 4 = PVL (433 bp)-positive; lane M = DNA maker (up→down: 2000→100 bp). D. Lane 1 = negative control; lanes 2 to 4 = SCCmec III (280, 147 bp)-positive; lane M = DNA maker (up→down: 2000→100 bp).

2C) (GenBank accession No. AB532026), and 39, including all MRSA isolates, were positive for *erm* (see Figure 2A) (GenBank accession Nos. AF466413, HQ683763, Y17294). The gene that encodes SEA was the most common SE gene and was detected in 20 isolates. A total of 21 isolates contained single SE genes and 9 carried 2 SE genes; *see* and *tst* were negative in all strains. Four isolates that harbored *pvl* were MSSA, and *pvl* was not detected among the 13 MRSA isolates. Approximately 65.0% (39/60) of these isolates harbored ERM genes, with *ermB* and *ermC* being the most common (30.0%).

Table 1. Distribution of virulence genes and erythromycin ribosome methylase genes in 60 *Staphylococcus aureus* isolated from bloodstream infections.

	Number (%) of isolates positive for virulence genes and erythromycin ribosome methylase genes							
	<i>sea</i>	<i>seb</i>	<i>sec</i>	<i>sed</i>	<i>lukS-lukF</i>	<i>erma</i>	<i>ermB</i>	<i>ermC</i>
MRSA (13)	11 (84.6)	ND	ND	ND	ND	10 (76.9)	ND	9 (69.2)
MSSA (47)	9 (19.1)	3 (6.4)	9 (19.1)	7 (14.9)	4 (8.5)	ND	18 (38.3)	9 (19.1)
Total (60)	20 (33.3)	3 (5.0)	9 (15.0)	7 (11.7)	4 (6.7)	10 (16.7)	18 (30.0)	18 (30.0)

se = genes for staphylococcal enterotoxins; *lukS-lukF* = genes for Paton-Valentine leukocidin; *erm* = genes for erythromycin ribosome methylase; MRSA and MSSA = methicillin-resistant and -sensitive *S. aureus*, respectively; ND = not detected.

Molecular typing of MRSA isolates

SCCmec typing results showed that 11 (11/13, 84.6%) MRSA isolates were SCCmec type III (Figure 2D); however, typing in 2 MRSA isolates were undeterminable. Analysis of the 13 MRSA isolates produced 8 distinct pulsotypes designated A-H (Figure 3). The PFGE pattern types were as follows: type A (2 isolates), type B (2 isolates), type C (2 isolates), type D (1 isolate), type D1 (1 isolate), type E (2 isolates), type F (1 isolate), type G (1 isolate), and type H (1 isolate). No pattern predominated among the isolates, which were collected from patients in the General Intensive Care Unit, the Cadre Ward and Departments of Hematology, Nephrology, Rehabilitation Medicine, and Chinese and Western medicine.

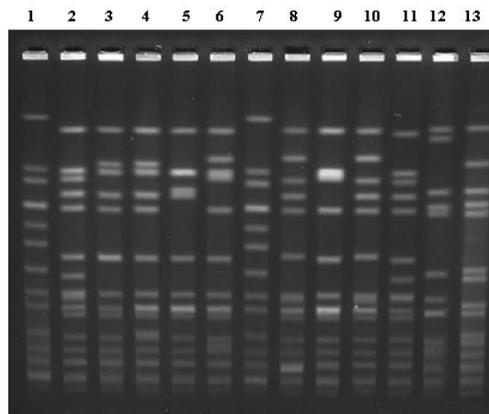


Figure 3. Pulsed-field gel electrophoresis of 13 methicillin-resistant *Staphylococcus aureus* from bloodstream infections. Lanes 1 and 7 = pulsed-field gel electrophoresis (PFGE) type A; lanes 2 and 11 = PFGE type B; lanes 3 and 4 = PFGE type C; lane 6 = PFGE type D; lane 9 = PFGE type D1; lanes 8 and 10 = PFGE type E; lane 5 = PFGE type F; lane 12 = PFGE type G; lane 13 = PFGE type H.

DISCUSSION

S. aureus is the causal pathogen of a wide range of infectious diseases ranging from skin and soft tissue infections to toxin-mediated diseases such as pneumonia and bacteremia (Holtfreter et al., 2007), many of which are caused by MRSA (Hesje et al., 2011). MRSA dissemination has become an important challenge at hospitals in all geographic areas owing to the emergence of isolates resistant to various antibiotic groups (Gomes et al., 2006). MRSA is a common cause of BSIs, which are often associated with invasive infections and high mortality rates (Moore et al., 2011). In this study, the antimicrobial susceptibility profile and distribution of the virulence and ERM genes of 60 *S. aureus* samples isolated from BSIs were determined. In addition, the molecular typing data for 13 MRSA strains were reported.

All of the isolates were susceptible to tigecycline, vancomycin, and linezolid. The resistance rates of the 60 isolates to penicillin and erythromycin exceeded 91.7 and 65.0%, respectively. In addition, all MRSA isolates were resistant to oxacillin and ceftiofur, which agrees with the results of previous experiments (Scazzocchio et al., 2011). Our data showed that the resistance rate of MRSA was higher than that of MSSA except with trimethoprim-sulfamethoxazole. Vancomycin has remained the mainstay therapy for serious Gram-positive infections, particularly MRSA BSIs; however, therapeutic failure with vancomycin is increasingly reported (Charles et al., 2004; Moore et al., 2011). During recent years, an increase in vancomycin MICs against *S. aureus* has been observed worldwide (Taj et al., 2010; Moore et al., 2012). Increased mortality, treatment failure, and length of hospital stay have been reported in patients treated with vancomycin for MRSA bacteremia when isolates have a vancomycin MIC of >1 µg/mL (Lubin et al., 2011). No vancomycin-resistant *S. aureus* isolates were detected in this study; however, 10 *S. aureus* isolates exhibited a vancomycin MIC of >1 µg/mL, which is often associated with treatment failure (Lubin et al., 2011). A recent study has revealed that daptomycin is associated with better outcomes than vancomycin for the treatment of BSIs caused by MRSA with higher vancomycin MICs, suggesting that switching to alternative agents may be an effective treatment strategy when the isolate has a high vancomycin MIC or when patients are not improving under conventional therapy (Moore et al., 2012).

The current study analyzed the genes that encode SE, PVL, and ERM. The results showed that the virulence gene profiles of 60 *S. aureus* isolates varied remarkably. Up to 84.6% of MRSA isolates harbored *sea*, whereas 19.1% of MSSA isolates harbored these genes. *seb*, *sec*, and *sed* were not detected in MRSA isolates but were present in MSSA isolates. These results differ from those of previous studies (Kim et al., 2011; Ho et al., 2012). The PVL genes, which encode a pore-forming cytotoxin and cause tissue necrosis and leukocyte destruction, are frequently present in community associated-MRSA (Vandenesch et al., 2003; Lo et al., 2008; Hesje et al., 2011). The prevalence of PVL genes in *S. aureus* from various samples is diverse, with 79.5% of *S. aureus* from recurrent furunculosis and 2.63% from lower respiratory tract infections harboring PVL genes (Li et al., 2011; Garbacz et al., 2011). The surveillance of *pvl* in *S. aureus* showed low occurrence. Only 4 MSSA strains were confirmed to have PVL genes (6.7%, 4/60), which was consistent with results from previous reports (Shallcross et al., 2010; Năstase et al., 2010; Li et al., 2011; Ho et al., 2012).

Macrolide, lincosamide, and streptogramin antibiotics are widely used in the treatment of staphylococcal infections (Lina et al., 1999b). Resistance to erythromycin was detected in more than 60% of *S. aureus* and more than 80% of MRSA isolates. Approximately 39

(100.0%) isolates resistant to erythromycin carried ERM genes. In 13 MRSA isolates, *ermA* (found only in MRSA) and *ermC* were prevalent. *ermB* was detected only in MSSA, and the coexistence of *ermA* and *ermC* was observed in 7 MRSA isolates.

Up to 11 MRSA isolates were SCCmec type III, whereas the typing of 2 isolates belonging to PFGE type A was indeterminable. The prevalence of SCCmec type III MRSA in this area is consistent with data in other reports (Chen et al., 2010). The 13 isolates belonged to 8 distinct PFGE patterns. In our hospital, the small-scale clonal spread of MRSA had been revealed between different wards or in one ward; however, no dominant isolates had been found.

S. aureus is one of the most frequent pathogens causing BSIs in China (Wang et al., 2010). The antimicrobial susceptibility profile and characteristics of virulence and ERM genes of BSI-causing *S. aureus* were explored in this study to enhance the current knowledge of these pathogens. Our results may ultimately help clinicians choose the most appropriate therapy for hospitalized patients.

Conflict of interest

The authors declare that they have no conflict of interest.

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