Molecular characterization of *Staphylococcus epidermidis* strains isolated from a teaching hospital in Shanghai, China

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*Staphylococcus epidermidis* is a leading cause of hospital-acquired infections, mostly associated with the use of medical devices in seriously ill or immunocompromised patients. Currently, the clonal characteristics of *S. epidermidis* in the hospital environment in China are unknown; neither is it known why these sequence types are easily disseminated in the hospital setting. In this study, multilocus sequence typing (MLST) was employed for the clonal analysis of 80 *S. epidermidis* isolates collected from patients with *S. epidermidis* infections. MLST revealed a total of 16 different sequence types among these isolates. ST2, which contained exclusively ica-positive, IS256-positive and biofilm-forming isolates, represented the majority of clinical strains tested. Of the *S. epidermidis* strains circulating in the hospital environment in China, as many as 96.25% are resistant to meticillin. Four staphylococcal chromosomal cassette mec (SCCmec) types were identified among the total 80 *S. epidermidis* isolates, none of the strains carried an SCCmec I cassette. All of the ST2 isolates carried the SCCmec type III cassette. Taken together, the combination of biofilm-forming ability and antibiotic resistance helps ST2 become successfully established within nosocomial environments, and promotes the device-related infection and bacteraemia.

**INTRODUCTION**

Staphylococci are one of the most important causes of nosocomial infections, from minor skin infections to life-threatening bacteraemia (Monk et al., 2008; Francois et al., 2008; Miragaia et al., 2008). The two major opportunistic pathogens of this genus, *Staphylococcus aureus* and *Staphylococcus epidermidis*, colonize a sizeable proportion of the human population. Because *S. aureus* can produce a greater variety of exotoxins and enterotoxins compared to *S. epidermidis* it is a more aggressive pathogen, in recent years, a lot of work has been focused on this bacteria (Diep et al., 2008; Diep & Otto, 2008; Otto, 2008). Compared to *S. aureus*, there is a scarcity of information concerning the molecular characteristics of *S. epidermidis*, especially in the hospital setting in China.

Biofilm formation is the most important factor for the establishment of *S. epidermidis* as a nosocomial pathogen (Hansen et al., 2007). Although the production of staphylococcal biofilm is dependent upon multiple regulatory proteins, an essential factor is the presence and expression of the four gene icaADBC operon (Li et al., 2005). It was reported recently that an insertion sequence element called IS256 has the capacity to influence expression of the ica operon, and subsequent biofilm formation, by reversible insertion into the ica operon and its regulatory genes, as well as by chromosomal rearrangement (Valle et al., 2007). *S. epidermidis* is a polymorphic species that was found to have a clonal population structure. With respect to the ica and IS256 operon, it is not currently known from where this genetic information originates, and there is uncertainty as to whether biofilm-forming strains disseminate clonally or by horizontal transfer of the ica and IS256 operon to different clonal lineages. Multilocus sequence typing (MLST), which has become established for the population analysis of many bacterial pathogens, including *S. aureus* and *S. epidermidis* (Enright & Spratt, 1999), is good method for addressing this lack of information. MLST is based on the comparison of the nucleotide sequences of seven housekeeping genes of a micro-organism. It can be used to elucidate relationships between strains and to identify ancestral genotypes, as well...

**Abbreviations**: CLSI, Clinical and Laboratory Standards Institute; CSF, cerebrospinal fluid; MLST, multilocus sequence typing; MRSE, meticillin-resistant *Staphylococcus epidermidis*; SCCmec, staphylococcal chromosomal cassette mec.

†These authors contributed equally to this work.
as to predict patterns of evolutionary divergence within groups of related genotypes. By using MLST, one sequence type, ST27, was identified in the hospitals of the USA, that contained exclusively ica-positive isolates and represented the majority of clinical strains (Ziebuhr et al., 2006).

Meticillin resistance is the other very important factor in the establishment of S. epidermidis as a nosocomial pathogen. Resistance to meticillin in staphylococci is known to be associated with the presence of the meca gene, which encodes a penicillin-binding protein with low affinity for β-lactam antibiotics (PBPs2A). The meca gene is carried by a genetic mobile element called the staphylococcal chromosomal cassette mec (SCCmec) (Ziebuhr et al., 2006; Goering et al., 2008). To date, five major SCCmec types have been identified, and SCCmec have been shown to be transferable among staphylococcal species. The predominant ica-positive S. epidermidis ST27 clone in the hospitals in the USA carried different SCCmec cassettes (Ziebuhr et al., 2006).

Because of the abuse of antibiotics, the status of antimicrobial resistance in bacteria in the hospitals of China is very complicated. We are not clear yet of the level of meticillin-resistant S. epidermidis (MRSE) isolates nor the types of SCCmec MRSE harboured in the hospital environment in China. ica-positive ST27 was detected in different hospitals both in Europe and in the USA; the sequence types of S. epidermidis and the molecular characteristics of these strains in China are unknown. In the present study, we examined diversity/clonality by characterizing the population structure of 80 S. epidermidis isolates that were collected from inpatients from Shanghai Huashan Hospital, China, with S. epidermidis infections, using both molecular (the improved MLST scheme, SCCmec typing and the presence of icaADBC gene and IS256 element) and phenotypic (antibiotic resistance and biofilm-forming ability) methods.

## METHODS

### Bacterial isolates.
A total of 80 S. epidermidis isolates were collected from January 2005 to December 2006 from different inpatients from Shanghai Huashan Hospital with S. epidermidis infections. This hospital is located in the centre of Shanghai, China, and is a large (1300 bed), teaching hospital that handles about 80,000 admissions per day. The isolates were recovered from blood (36.25%), catheters (33.75%) and cerebrospinal fluid (CSF) (30%). S. epidermidis ATCC12228 and S. epidermidis RP62A, two reference strains for S. epidermidis RP62A and ATCC12228 served as controls.

### Antimicrobial resistance profiles.
Antibiograms were determined by disc diffusion on Mueller–Hinton agar according to Clinical and Laboratory Standards Institute (CLSI) (formerly the National Committee for Clinical Laboratory Standards) guidelines. The antimicrobial agents tested included gentamicin (10 μg), penicillin (10 IU), cefoxitin (30 μg), ampicillin (10 μg) and sulfactam (10 μg), cefazolin (30 μg), vancomycin (30 μg), linezolid (30 μg), erythromycin (15 μg), clindamycin (2 μg), sulfamethoxazole (23.75 μg) + trimethoprim (1.25 μg), fosfomycin (200 μg), rifampicin (5 μg), teicoplanin (30 μg) and levofloxacin (5 μg). The MIC of vancomycin was determined by agar dilution method according to CLSI guidelines.

### Determination of SCCmec types.
SCCmec typing was performed by a multiplex PCR approach with subsequent visualization of the amplified DNA fragment patterns by agarose gel electrophoresis and ethidium bromide staining. To distinguish between SCCmec types I to V, primers and conditions identical to those described by Miragaia et al. (2007) were used.

### ica and IS256 specific PCRs.
Bacterial strains were tested for the presence of the ica gene and IS256 by PCR using the primers described by Kozitskaya et al. (2005). The PCR fragments were visualized by agarose gel electrophoresis and ethidium bromide staining. S. epidermidis RP62A and ATCC12228 served as controls.

### Semiquantitative biofilm assay.
Semiquantitative biofilm assays were performed as described elsewhere (Wang et al., 2008). Subsequently, cells were either directly stained with safranin or fixed by Bouin’s fixative. The fixative was removed after 1 h and wells were washed with PBS. Organisms in the wells were then stained with crystal violet, and the floating stain was washed off with slowly running water. After drying, the absorbance of the stained biofilm was measured with a MicroELISA autoreader (Bio-Rad) at 570 nm (crystal violet stain) or 490 nm (safranin stain).

### Evaluation of the expression level of icaB gene by RT-PCR.
For RNA isolation, cultures grown overnight were diluted 1:100 into 50 ml tryptic soy broth and incubated at 37 °C with shaking at 180 r.p.m. until the late exponential growth phase was reached. cDNA was synthesized from total RNA by using the SuperScript III first-strand synthesis system (Invitrogen) according to the manufacturer’s instructions. Oligonucleotide primers (icaB) as described by Wang et al. (2008) were used. The resulting cDNA and negative control samples were amplified by use of LC-Fast Start reaction mix SYBR Green I (Roche Diagnostics). Reactions were performed in a LightCycler apparatus (Roche Diagnostics). Standard curves were determined for each gene, by use of purified chromosomal template DNA at concentrations of 0.005–50 ng ml⁻¹. The experiments were performed in triplicate, with 16S rRNA used as a control.

### Statistical analysis.
Statistical analysis was performed using GraphPad Prism version 4.0.

## RESULTS

### MLST profiles and SCCmec typing of the invasive S. epidermidis isolates
From 2005 to 2006, 80 S. epidermidis isolates, which were recovered from blood, catheters and CSF from different...
inpatients with *S. epidermidis* infections, were collected. These isolates were considered to be invasive *S. epidermidis* isolates. The key characteristics of the 80 *S. epidermidis*-infected patients are provided in Table 1. All of the *S. epidermidis* isolates we investigated in this study were hospital-associated *S. epidermidis*. MLST revealed a total of 16 different sequence types among these isolates (Table 2), the majority of them (75%, 60/80) were clustered into 6 sequence types: ST2, ST5, ST66, ST23, ST6 and ST20. A total of 3 of these 16 STs corresponded to single isolates, while 7 STs included 2 or 3 isolates. The most represented sequence type was ST2, which comprised 31.25% of all isolates tested, 77 (96.25%) were *S. epidermidis* isolates we investigated in this study could be considered as clones circulating in the hospital. Isolates in *S. epidermidis* infections are an increasing cause of concern, due to the high distribution of meticillin resistance amongst the isolates and their persistence on indwelling devices, often resulting in replacement of the device, which causes more trauma and is costly (Hakim et al., 2000). In this investigation, 96.25% of isolates from hospitals in China were meticillin resistant. By using MLST, we examined the molecular characteristics of these clinical *S. epidermidis* isolates, which were recovered from blood, catheters and CSF of patients with *S. epidermidis* infections. In our study, the percentage of patients from whom *S. epidermidis* was isolated from within 48 h of admission was 10%, but all of these patients had been exposed to the healthcare setting before. So all of *S. epidermidis* isolates we investigated in this study could be considered as clones circulating in the hospital. Isolates

**Association of biofilm-forming ability and the presence of the *ica* gene and IS256**

By semiquantitative biofilm assay, 43 clinical isolates (53.8%) were found to be biofilm positive and displayed 14 different STs; 36 (45.0%) isolates were *ica* positive and displayed 6 different STs; 52 (65.0%) were IS256 positive and displayed by 9 STs (Table 2). The predominant clone, ST2, contained 68% (17/25) of the biofilm-forming and 100% of the *ica*- and IS256-positive, isolates. In our study, IS256-carrying strains were detected in four STs, which contained *ica*-positive and biofilm-forming isolates, and ST2 represented the group containing the most IS256-positive, *ica*-positive and biofilm-forming isolates. Biofilm-negative isolates were also found in 32% (8/25) of ST2 isolates. In the present study, *ica* operon expression was detected using RT-PCR in biofilm-positive and biofilm-negative isolates of ST2, but we did not find any difference in *ica* expression between two group of isolates (*P*> 0.05) (Fig. 1).

**Distribution of STs among clinical samples**

Of the 80 *S. epidermidis* isolates tested, 29 isolates were recovered from blood, 27 from catheters and 24 were from CSF. Fig. 2 shows the distribution of the 16 sequence types among these three infection sites. Interestingly, of the 25 ST2 isolates, 10 were from blood, 14 were from catheters and just 1 isolate was from CSF. This result suggests that the predominant sequence type, ST2, which contained exclusively *ica*-positive, IS256-positive and biofilm-forming isolates associated with device-related infection and bacteremia.

**DISCUSSION**

*S. epidermidis* epidemiological studies have been limited historically, due to the fact that *S. epidermidis* strains are often considered to be contaminants, as opposed to the disease-causing organism (Wang et al., 2003; Herwaldt et al., 1996). However, *S. epidermidis* infections are an increasing cause of concern, due to the high distribution of meticillin resistance amongst the isolates and their persistence on indwelling devices, often resulting in replacement of the device, which causes more trauma and is costly (Hakim et al., 2000). In this investigation, 96.25% of isolates from hospitals in China were meticillin resistant. By using MLST, we examined the molecular characteristics of these clinical *S. epidermidis* isolates, which were recovered from blood, catheters and CSF of patients with *S. epidermidis* infections. In our study, the percentage of patients from whom *S. epidermidis* was isolated from within 48 h of admission was 10%, but all of these patients had been exposed to the healthcare setting before. So all of *S. epidermidis* isolates we investigated in this study could be considered as clones circulating in the hospital. Isolates

<table>
<thead>
<tr>
<th>Sex/no. (%)</th>
<th>Age (years)/no. (%)</th>
<th>Sample collection day*/no. (%)</th>
<th>Infection site/no. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F/ 26 (32.5)</td>
<td>≤20/ 4 (15.4)</td>
<td>≤2/ 3 (11.5)</td>
<td>Blood/7 (26.9)</td>
</tr>
<tr>
<td>20–40/ 8 (30.8)</td>
<td>4–30/ 21 (80.8)</td>
<td>Catheter/10 (38.5)</td>
<td></td>
</tr>
<tr>
<td>40–60/ 10 (38.5)</td>
<td>30/ 2 (7.7)</td>
<td>CSF/ 9 (34.6)</td>
<td></td>
</tr>
<tr>
<td>60/ 4 (15.4)</td>
<td>≤20/ 8 (14.8)</td>
<td>30/ 9 (16.7)</td>
<td></td>
</tr>
<tr>
<td>M/ 54 (67.5)</td>
<td>20–40/ 8 (14.8)</td>
<td>≤2/ 5 (9.3)</td>
<td>Blood/22 (40.7)</td>
</tr>
<tr>
<td>40–60/ 25 (46.3)</td>
<td>4–30/ 40 (74.1)</td>
<td>CSF/ 15 (27.8)</td>
<td></td>
</tr>
<tr>
<td>60/ 13 (24.1)</td>
<td>30/ 9 (16.7)</td>
<td>Catheter/ 17 (31.5)</td>
<td></td>
</tr>
</tbody>
</table>

F, Female; M, male.

*The number of days after the patient’s admission to hospital that had elapsed when the samples were collected.

**Table 1. Key characteristics of *S. epidermidis*-infected patients in Shanghai Huashan Hospital, China**
that were recovered from sputum or urine were not included, because they were often considered to be contaminants. The isolates recovered from blood/catheters may also not represent infections. We should note here that because of the ubiquitous prevalence of *S. epidermidis* as a commensal bacterium, it is very difficult for a clinician to decide whether an isolate represents the causative agent of an infection. The MLST result showed that the most represented sequence type was ST2, which comprised 31.25% of all clinical isolates in our study. ST2 has also been detected in different hospitals in Europe, but this is believed to be the first report that the ST2 clone has spread in the hospital environment in China.

Why is the ST2 clone easy to disseminate in the hospital setting? As biofilm formation and meticillin resistance are the most important factors for the establishment of *S. epidermidis* as a nosocomial pathogen, in the present study, a semiquantitative biofilm assay was used to detect the biofilm-forming ability of all the clinical isolates; the presence of the *ica* operon, IS256 and different types of SCCmec cassette were tested by PCR. In previous studies, a correlation was shown between the presence of the *ica* operon, biofilm formation and the detection of the insertion sequence IS256 in clinical *S. epidermidis* isolates (Johansson et al., 2006; Spare et al., 2003; Kozitskaya et al., 2005). In our study, the predominant clone ST2, 100% *ica-* and IS256 positive, contained 68.0% of the biofilm-forming isolates, so ST2 represented the group containing the most IS256 positive, *ica*-positive and biofilm-forming isolates. These data suggest that *ica*, IS256 and biofilm-forming ability occur jointly in specific *S. epidermidis* clones and spread preferentially in the hospital environment. Recombination is the major mechanism for the divergence of *S. epidermidis* strains (Wisplinghoff et al., 2003). We hypothesized that by recombination, ST2 generates novel phenotypic and genotypic variants, such as *ica* and IS256, which makes it easily able to spread in the hospital environment, and causes device-related infection and bacteraemia. But our study was based on a limited number of *S. epidermidis* isolates, to get a firmer conclusion, further study will be needed.

**Table 2.** Characteristics of *S. epidermidis* clones isolated from patients with *S. epidermidis* infections in Shanghai Huashan Hospital, China

<table>
<thead>
<tr>
<th>MLST</th>
<th>MLST profile</th>
<th>No. of isolates</th>
<th>SCC mec type (no.)</th>
<th>Biofilm positive</th>
<th>IS256 positive</th>
<th><em>icaADBC</em> positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST2</td>
<td>7-1-2-2-4-1-1</td>
<td>25 II (25)</td>
<td>68.0% (17/25)</td>
<td>100% (25/25)</td>
<td>100% (25/25)</td>
<td></td>
</tr>
<tr>
<td>ST5</td>
<td>1-1-1-2-2-1-1</td>
<td>10 V (10)</td>
<td>50.0% (5/10)</td>
<td>70.0% (7/10)</td>
<td>0% (0/10)</td>
<td></td>
</tr>
<tr>
<td>ST66</td>
<td>12-3-5-7-7-11-14</td>
<td>7 V (6) MSSE (1)</td>
<td>14.3% (1/7)</td>
<td>85.7% (6/7)</td>
<td>0% (0/7)</td>
<td></td>
</tr>
<tr>
<td>ST23</td>
<td>7-1-2-1-3-1-1</td>
<td>7 II (7)</td>
<td>57.1% (4/7)</td>
<td>42.9% (3/7)</td>
<td>57.1% (4/7)</td>
<td></td>
</tr>
<tr>
<td>ST6</td>
<td>1-1-2-2-1-1-1</td>
<td>6 II (2) V (4)</td>
<td>66.7% (4/6)</td>
<td>33.3% (2/6)</td>
<td>33.3% (2/6)</td>
<td></td>
</tr>
<tr>
<td>ST20</td>
<td>1-1-2-2-1-1-3</td>
<td>5 III (5)</td>
<td>40.0% (2/5)</td>
<td>40.0% (2/5)</td>
<td>40.0% (2/5)</td>
<td></td>
</tr>
<tr>
<td>ST10</td>
<td>1-1-1-3-1-1-1</td>
<td>3 IV (3)</td>
<td>33.3% (1/3)</td>
<td>100% (3/3)</td>
<td>0% (0/3)</td>
<td></td>
</tr>
<tr>
<td>ST59</td>
<td>2-1-1-2-1-1-1</td>
<td>3 II (3)</td>
<td>66.7% (2/3)</td>
<td>0% (0/3)</td>
<td>0% (0/3)</td>
<td></td>
</tr>
<tr>
<td>ST71</td>
<td>3-1-5-3-1-1-1</td>
<td>3 II (2) MSSE (1)</td>
<td>33.3% (1/3)</td>
<td>66.7% (2/3)</td>
<td>0% (0/3)</td>
<td></td>
</tr>
<tr>
<td>ST130</td>
<td>1-1-1-2-1-1-1</td>
<td>2 II (2)</td>
<td>0% (0/2)</td>
<td>0% (0/2)</td>
<td>0% (0/2)</td>
<td></td>
</tr>
<tr>
<td>ST21</td>
<td>2-1-2-2-1-1-1</td>
<td>2 III (1) MSSE (1)</td>
<td>50.0% (1/2)</td>
<td>100% (2/2)</td>
<td>0% (0/2)</td>
<td></td>
</tr>
<tr>
<td>ST16</td>
<td>2-1-2-15-1-1-1</td>
<td>2 II (2)</td>
<td>100% (2/2)</td>
<td>0% (0/2)</td>
<td>100% (2/2)</td>
<td></td>
</tr>
<tr>
<td>ST110</td>
<td>1-1-1-6-1-1-1</td>
<td>2 V (2)</td>
<td>50.0% (1/2)</td>
<td>0% (0/2)</td>
<td>0% (0/2)</td>
<td></td>
</tr>
<tr>
<td>ST133</td>
<td>1-2-1-1-1-1-1</td>
<td>1 V (1)</td>
<td>0% (0/1)</td>
<td>0% (0/1)</td>
<td>0% (0/1)</td>
<td></td>
</tr>
<tr>
<td>ST89</td>
<td>1-1-2-2-1-1-1</td>
<td>1 V (1)</td>
<td>100% (1/1)</td>
<td>0% (0/1)</td>
<td>0% (0/1)</td>
<td></td>
</tr>
<tr>
<td>ST152</td>
<td>1-1-2-6-2-1-1</td>
<td>1 V (1)</td>
<td>100% (1/1)</td>
<td>0% (0/1)</td>
<td>100% (1/1)</td>
<td></td>
</tr>
</tbody>
</table>

*Fig. 1. Detection of *ica* operon expression level by RT-PCR. Eight isolates of ST2 MRSE with biofilm-forming ability and eight isolates without biofilm-forming ability were randomly selected for *ica* operon expression level detection.*
reason was the difference in the ica operon expression level in the biofilm-positive and biofilm-negative isolates of ST2, but we did not find any difference in the ica expression between the two groups of ST2. The biofilm formation of staphylococci is a very complicated process, and the real reason remains to be elucidated.

Four SCCmec types were identified amongst the total of 80 S. epidermidis isolates, none of the strains carried an SCCmec I cassette. All of the isolates that belonged to ST2 carried the SCCmec type III cassette. The result of the vancomycin MIC test showed that one isolate of ST2 MRSE was vancomycin intermediate (8 μg ml^{-1}), but this isolate was negative for the vanA/vanB gene by PCR. We found the biofilm formed by this isolate was thicker than most other S. epidermidis isolates. It is reported that bacteria organized in biofilms are more resistant to antibiotics (Ziebuhr et al., 2006). Maybe the biofilm thickening is responsible for the development of intermediate resistance to vancomycin.

It is reported that ST27, the sequence type represented in USA hospitals, and other ica-positive clones are rarely found outside of medical facilities (Ziebuhr et al., 2006). It is very likely that they are highly adapted to the hospital environment and differ from commensal S. epidermidis in the community. We are not clear yet if the molecular characteristics of S. epidermidis from the hospital setting in China are different to those of isolates from the community; future work needs to be done to clarify this.

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