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ORIGINAL RESEARCH

Community-associated *Staphylococcus aureus* PVL$^+$ ST22 predominates in skin and soft tissue infections in Beijing, China

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Nan Xiao[1,*]
Jianghui Yang[2,*]
Ning Duan[1]
Binghuai Lu[3]
Lijun Wang[4]

[1]Department of Laboratory Medicine, Beijing Tsinghua Changgung Hospital, Tsinghua University, Beijing 102218, People's Republic of China; [2]Department of Pathology, Beijing Tsinghua Changgung Hospital, Tsinghua University, Beijing 102218, People's Republic of China; [3]Laboratory of Clinical Microbiology and Infectious Diseases, Department of Pulmonary and Critical Care Medicine, China-Japan Friendship Hospital; National Clinical Research Center of Respiratory Diseases, Beijing 100029, People's Republic of China; [4]Clinical Research Center, Beijing Tsinghua Changgung Hospital, School of Clinical Medicine, Tsinghua University, Beijing 102218, People's Republic of China

*These authors contributed equally to this work

Purpose: Community-associated *Staphylococcus aureus* (CA *S. aureus*) is the most common causative pathogen of the skin and soft tissue infections (SSTIs). This study aims to determine clonal distribution, virulence factors of CA *S. aureus* clinical isolates from purulent SSTIs in Beijing, China.

Materials and methods: CA-*S. aureus* isolates were collected from 115 outpatients with purulent SSTIs from the department of dermatology from April 2015 to April 2017. Multilocus sequence typing and Staphylococcus cassette chromosome mec typing were performed to explore molecular characteristics. Phylogenetic analysis of 16S rRNA of dominant *S. aureus* isolates was performed using MEGA-X software. Virulence genes were detected by PCR, while biofilm formation was evaluated by a microtiter plate method. The antimicrobial susceptibility was tested by an automatic VITEK system.

Results: Forty-four CA-*S. aureus* isolates identified from SSTIs contain 9 methicillin-resistant *S. aureus* (MRSA) isolates (20.4%) and 35 methicillin-susceptible *S. aureus* isolates (MSSA) (79.6%). The dominant sequence types (STs) were ST22 (40.9%) and clonal complex 59 (CC59; 77.8%) in Community-associated methicillin resistant methicillin-resistant *S. aureus*. 27.8% of ST22 isolates were homologous to the epidemic ST22 EMRSA-15 in Europe. The prevalence of virulence genes *lukS/lukF, tst-1, etA, edinA, icaA*, and *icaD* was 50%, 93.2%, 4.5%, 4.5%, 100%, and 100%, respectively. All CC59 isolates exhibited stronger biofilm-forming capability than ST22 clones. Among the MSSA subgroup, the poor biofilm producers had significantly higher sensitivity to sulfamethoxazole/Trimethoprim.

Conclusion: The dominant epidemic clone PVL$^+$ ST22 MSSA containing *tst-1* occurs in Beijing, indicating that a PVL$^+$ ST398 clone which was previously predominant in this district had been replaced by a new clone.

Keywords: community-associated *Staphylococcus aureus*, skin and soft tissue infection, biofilm formation, MLST-genotyping

Introduction

Skin and soft tissue infections (SSTIs) are common in both the outpatient and inpatient settings. *Staphylococcus aureus* is the most common causative pathogen of the SSTIs,[1] particularly in purulent infections such as furuncles, carbuncles, cutaneous abscesses, and impetigo.

Community-associated methicillin-resistant *S. aureus* (CA-MRSA) has emerged as an important pathogen worldwide. The prevalence of CA-MRSA in SSTIs varies from region to region, 2.6% in China and 23% in the Middle East.[2,3] In addition, the global distribution of CA-MRSA clones is heterogeneous and often characterized by a

Correspondence: Lijun Wang
Clinical Research Center, Beijing Tsinghua Changgung Hospital, School of Clinical Medicine, Tsinghua University, No.168 Litang Road, Changping District, Beijing 102218, People's Republic of China
Tel +86 1 381 037 0450
Email wlj7878@163.com

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regional predominant clonal lineage.[4] ST121 was previously the most common community-associated *Staphylococcus aureus* (CA-*S. aureus*) clone in SSTIs in China.[2] The ST398 was then reported as the predominant clone in Jiangsu Province and Beijing, China.[5,6] Since the dominant CA-MRSA clones are likely to have evolved from locally circulating community-associated methicillin-sensitive *S. aureus* (CA-MSSA) genotypes that are strongly associated with SSTIs,[7,8] it is important to investigate the CA-*S. aureus* clonal structure within SSTIs around the local district.

Furthermore, virulence factors are highly associated with the pathogenesis of *S. aureus* invasive infections such as toxic shock syndrome and staphylococcal scalded skin syndrome (SSSS). Panton–Valentine leukocidin (PVL), for instance, is a key virulence factor. Previous studies have shown the association between PVL and severe invasive infections.[9,10] Thus, it is important to characterize the virulence factors of CA-*S. aureus* for improving therapeutic approaches.

This study is focused on investigating purulent SSTIs cases in Beijing and characterizing *S. aureus* by multilocus sequence typing (MLST) types, SCCmec types, and key virulence factors. To our knowledge, it is the first study to report PVL+ ST22 CA-MSSA, an epidemic lineage in Europe,[11] as a predominant clone in China.

Methods

Staphylococcus aureus isolates

From April 2015 to April 2017, pathogen detections were performed for all outpatients with purulent SSTIs in the department of dermatology in a hospital located in Beijing. The bacterial identification was performed by Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry. All *S. aureus* clinical isolates were maintained with tryptic soy broth (TSB) at −70°C. And they were revived by streaking onto Columbia blood agar and cultured at 35°C overnight.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of *S. aureus* isolates was performed with a commercial VITEK Compact II system. Antibiotics susceptibility profiles were determined according to Clinical Laboratory Standards Institute Performance Standards of Antimicrobial Susceptible Testing (CLSI document M100 27th edition).

MLST and SCCmec genotype

MLST analysis of *S. aureus* isolates was performed according to previously described procedures.[12] In the MLST database (https://pubmlst.org/saureus/), the sequences of seven housekeeping genes (*arc*C, *aro*E, *glp*F, *gmk, pta, tpi*, and *yqi*) were compared to existing sequences by the assignment of allelic numbers. The SCC*mec* genotype was also identified as previously described.[13]

Phylogenetic analysis

ST22 clinical isolates were compared with two representatives of epidemic ST22 *S. aureus* strains, H-EMRSA-15 (NZ_CP007659.1:538355-539911) and HO 5096 0412 (HE681097.1:474448-476002), to construct phylogenetic tree for 16S rRNA gene using MEGA X software.[14] 16S rRNA sequencing of clinical *S. aureus* isolates was carried out by a commercial company (Beijing Ruibiotech) using a primer pair, 27F 5-AGAGTTTGATCCTGGCTCAG and 1492R 5-TACGGCTACCTTGTTACGACTT.

Detection of virulence genes

All *S. aureus* isolates were screened for toxic shock syndrome toxin 1 gene (*tst-1*), Panton–Valentine leukocidin genes (*lukS/lukF*), three types of Staphylococcal exfoliative toxin (ET) genes (*etA, etB, etD*), epidermal cell differentiation inhibitor genes (*edinA* and *edinB*), and intracellular adhesion molecule genes (*icaA* and *icaD)* by PCR with appropriate primers as previously described.[15–22]

Biofilm formation assay

Biofilm formation contributes to the pathogenesis of *S. aureus*. Quantification of biofilms was performed using a modified Microtiter plate method.[23] Briefly, the bacterial isolates were cultured in TSB and incubated at 37ºC overnight. The cultures were diluted with fresh TSB to 10^6 CFU/mL. Two hundred microliters of the diluted solution was added to sterile 96-well plates and incubated at 37ºC for 24 hrs. The negative control wells contained TSB alone. After 24-hr incubation, broth was removed and the wells were gently washed three times with phosphate-buffered saline. After the wells were dried at room temperature, they were stained with 0.1% crystal violet. Then, the crystal violet bound to the biofilms in the wells was dissolved by 30% acetic acid. The optical density (OD) was measured at 570 nm by BioTek Synergy H1 reader. Each assay was performed in triplicate wells and

was repeated three times. The cutoff of OD (ODc) was defined as three standard deviations above the mean OD of the negative control. The ability of biofilm formation was classified as follows: OD≤ODc, negative; ODc<OD≤2xODc, weak; 2xODc<OD≤4xODc, moderate; and 4xODc<OD, strong.

Statistical analysis

The correlation between the antibiotic resistance and biofilm formation was evaluated by the Pearson Chi-Square test. *P*-values less than 0.05 were regarded as significant. The statistic software used in our study was MedCalc 15.2.2 (Seoul, Korea).

Results

Staphylococcus aureus isolates

One hundred fifteen purulent SSTIs outpatients were enrolled in this study from April 2015 to April 2017. *S. aureus* was the most common pathogen, accounting for 38.3% (44/115). A total of 44 non-duplicate *S. aureus* isolates were recovered from abscess.

Molecular characteristics of CA-*S. aureus*

Of 44 CA *S. aureus* strains, MSSA accounted for 79.6% (n=35), and MRSA for 20.4% (n=9). All CA-MRSA harbored SCC*mec* Ⅳ or Ⅴ. Eleven distinct sequence types (STs) were identified among the 44 isolates (Table 1), among which the most prevalent ST22 accounted for 40.9% (n=18). Furthermore, the predominant ST among MSSA was ST22 (17/35, 48.6%), followed by ST398 (5/35, 14.3%), ST188 (4/35, 11.4%), ST121 (2/35, 5.7%), and ST5 (2/35, 5.7%) respectively. Meanwhile, the most prevalent ST within MRSA was the clonal complex 59 (CC59) including ST59 (5/9, 55.6%) and ST338 (2/9, 22.2%). Other clones were isolated sporadically.

Phylogenetic tree

Neighbor-Joining tree for 16S rRNA gene of predominant ST22 *S. aureus* showed two clades (Figure 1). Five out of 18 (27.8%) ST22 isolates exhibit the same evolutionary origins as the epidemic ST22 EMRSA-15 (HO 5096 0412 and H-EMRSA-15) in Europe.

Virulence genes

The prevalence of PVL positive *S. aureus* was 50% (22/44), and there was no significant difference (p=0.45) between MRSA (66.7%, 6/9) and MSSA (45.7%, 16/35). 83.3% (15/18) of ST22 was detected positive for *lukS/lukF*. All ST398, ST188, ST121, and ST5 isolates did not carry the *lukS/lukF* genes. Forty-one isolates, except two ST121 and one ST398, carried the *tst-1* gene accounting for 93.2% (38/41). Only ST121 isolates carried *et*A and *edin*A, accounting for 4.5%. However, none were found positive for *et*B, *et*D, and *edin*B. All of the isolates possessed two genes i*ca*A and i*ca*D coding for critical intracellular adhesion molecules.

Biofilm formation

The results showed that 74.3% (26/35) of isolates were weak biofilm producers, and 25.7% (9/35) MSSA isolates were moderate biofilm producers, while 77.8% (7/9) MRSA were moderate biofilm producers (Table 2). All CC59 isolates, including ST59 and ST338, exhibit moderate biofilm formation ability, irrespective of methicillin susceptibility.

Comparison of susceptibility profiles of different biofilm producers

Antibiotics sensitivity was different between weak and moderate biofilm producers among MSSA subgroup. Within the MSSA subgroup, the sensitivity to benzylpenicillin, gentamycin, ciprofloxacin, levofloxacin, and erythromycin of weak biofilm-producing isolates were higher than that of moderate biofilm producers; however, the differences were not statistically significant. The only significant difference of antibiotic sensitivity was observed in sulfamethoxazole/trimethoprim (92.3% vs 55.6%, p=0.03) (Table 3). Although MRSA subgroup consisted of only nine isolates, the majority of them are moderate biofilm producers. All MRSA and MSSA isolates were sensitive to vancomycin, rifampicin, linezolid, moxifloxacin, and tigecycline.

Discussion

In the present research, we determined the clonal distribution, virulence factors, and biofilm formation ability of *S. aureus* clinical isolates from purulent SSTIs. Several findings were reported. Firstly, an endemic PVL^+ ST22 clone similar to EMRSA-15 appeared in this region. Secondly, CA-MRSA accounted for 20.4% of SSTIs. The local circulating MRSA clone belongs to CC59, and all CC59 isolates have stronger biofilm formation ability compared to ST22. Finally, the majority of isolates harbored *tst-1* and *lukS/lukF* genes.

Table 1 MLST typing, biofilm formation, and virulence factors genes of 44 *S. aureus* isolations from SSTI patients

No.	Sex	Age (years)	Phenotype	SCCmec Type	MLST	Biofilm	*lukS/lukF*	*tst-1*	*etA*	*edinA*	*icaA*	*icaD*
1	F	51	MRSA	Ⅳ	338	Moderate	P	P	N	N	P	P
2	F	4	MRSA	Ⅴ	338	Moderate	P	P	N	N	P	P
3	M	25	MRSA	Ⅴ	59	Moderate	P	P	N	N	P	P
4	F	19	MRSA	Ⅴ	59	Moderate	P	P	N	N	P	P
5	F	30	MRSA	Ⅴ	59	Moderate	P	P	N	N	P	P
6	F	29	MRSA	Ⅳ	59	Moderate	N	P	N	N	P	P
7	F	2	MRSA	Ⅴ	59	Moderate	N	P	N	N	P	P
8	M	11	MSSA		59	Moderate	P	P	N	N	P	P
9	F	39	MRSA	Ⅳ	7	Weak	N	P	N	N	P	P
10	F	50	MSSA		7	Weak	N	P	N	N	P	P
11	M	3	MRSA	Ⅳ	22	Weak	P	P	N	N	P	P
12	M	34	MSSA		22	Weak	P	P	N	N	P	P
13	F	22	MSSA		22	Weak	P	P	N	N	P	P
14	F	29	MSSA		22	Weak	P	P	N	N	P	P
15	M	37	MSSA		22	Weak	P	P	N	N	P	P
16	F	34	MSSA		22	Weak	P	P	N	N	P	P
17	F	41	MSSA		22	Weak	P	P	N	N	P	P
18	M	26	MSSA		22	Weak	P	P	N	N	P	P
19	M	2	MSSA		22	Weak	N	P	N	N	P	P
20	M	11	MSSA		22	Weak	P	P	N	N	P	P
21	M	4	MSSA		22	Weak	N	P	N	N	P	P
22	F	46	MSSA		22	Moderate	P	P	N	N	P	P
23	M	9	MSSA		22	Weak	P	P	N	N	P	P
24	F	34	MSSA		22	Weak	N	P	N	N	P	P
25	F	29	MSSA		22	Weak	P	P	N	N	P	P
26	M	8	MSSA		22	Weak	P	P	N	N	P	P
27	M	22	MSSA		22	Weak	P	P	N	N	P	P
28	F	73	MSSA		22	Weak	P	P	N	N	P	P
29	M	33	MSSA		398	Weak	N	P	N	N	P	P
30	M	20	MSSA		398	Weak	N	N	N	N	P	P
31	M	23	MSSA		398	Moderate	N	P	N	N	P	P
32	M	29	MSSA		398	Moderate	N	P	N	N	P	P
33	M	59	MSSA		398	Weak	N	P	N	N	P	P
34	M	61	MSSA		188	Moderate	N	P	N	N	P	P
35	M	35	MSSA		188	Weak	N	P	N	N	P	P
36	M	27	MSSA		188	Weak	N	P	N	N	P	P

(*Continued*)

Table 1 (Continued).

No.	Sex	Age (years)	Phenotype	SCCmec Type	MLST	Biofilm	*lukS/lukF*	*tst-1*	*etA*	*edinA*	*icaA*	*icaD*
37	M	9	MSSA		188	Weak	N	P	N	N	P	P
38	F	3	MSSA		121	Weak	N	N	P	P	P	P
39	M	28	MSSA		121	Weak	N	N	P	P	P	P
40	F	28	MSSA		5	Moderate	N	P	N	N	P	P
41	F	85	MSSA		5	Weak	N	P	N	N	P	P
42	M	24	MSSA		88	Moderate	P	P	N	N	P	P
43	F	26	MSSA		6	Moderate	N	P	N	N	P	P
44	M	28	MSSA		1	Moderate	N	P	N	N	P	P

Abbreviations: *lukS/lukF*, Panton–Valentine leukocidin genes; *icaA and D*, Intracellular adhesion molecules A and D; *tst-1*, Toxic shock syndrome toxin-1; *etA*, Exfoliative toxin A; *edinA*, Epidermal cell differentiation inhibitor A.

It is worth noting that we screened a dominant clone PVL$^+$ ST22 CA-MSSA circulating in this Beijing community, whereas previous study[6] suggested the PVL$^+$ ST398 was the most dominant clone among CA-MSSA with SSTIs around Beijing. It is likely that ST22 has replaced ST398 as the dominant lineage in Beijing. This trend deserves attention from the physicians for the following reasons. Firstly, ST22 clone has a strong ability to spread. ST22-IVh (EMRSA-15 clone) is a pandemic lineage originated from Europe, which spread rapidly through hospitals following introduction into Singapore, replacing the endemic ST239 population.[24,25] Recent studies reported several outbreaks of ST22 CA-MSSA or CA-MRSA.[11,26,27] In our study, the phylogeny analysis indicated local PVL$^+$ ST22 CA-MSSA is similar to epidemic EMRSA-15, which could serve as a reservoir for CA-MRSA. Secondly, ST22 clone is highly virulent. DeLencastre et al,[28] document that ST22-IV effectively invade cells in vitro and is highly pathogenic in vivo compared with ST228-I. In addition, a long-term familial infection cluster was caused by a novel PVL-positive ST22 CA-MRSA in Japan.[29]

The spread of CA-MRSA has become a serious problem worldwide. Our data showed the prevalence of CA-MRSA was 20.4%, which is much higher than previously reported ~4% in China.[6] The prevalence of CA-MRSA was found dramatically increasing in a majority of regions.[2,5,6,30] The trend would pose a serious challenge to local infection control.

As far as the clonal distribution of CA-MRSA is concerned, five CA-MRSA clones, ST1, ST30, ST80, ST59, and ST8, account for the vast majority of CA-MRSA infections worldwide.[31] Different clonal lineages spread in specific regions, for instance, ST8 in Germany[32] and ST59 in Taiwan.[33] Several surveillance reports suggested that the predominant clone would shift across regions. A multicenter study in China between 2009 and 2011 revealed that the most prevalent sequence type was ST121 (19/51, 37.3%) among CA-MRSA with SSTIs,[2] however, ST59 was found to be the most prevalent ST in China since 2012.[34–36] We also found the circulating clone CC59 accounted for 77.8% CA-MRSA. Indeed, ST59 CA-MRSA has become a persistent pandemic clone causing invasive infections around China now.[36]

PVL is a key virulence factor of *S. aureus*, mainly associated with necrotic lesions in the skin or mucosa.[18] The prevalence of *lukS/lukF* ranged from over 20% to

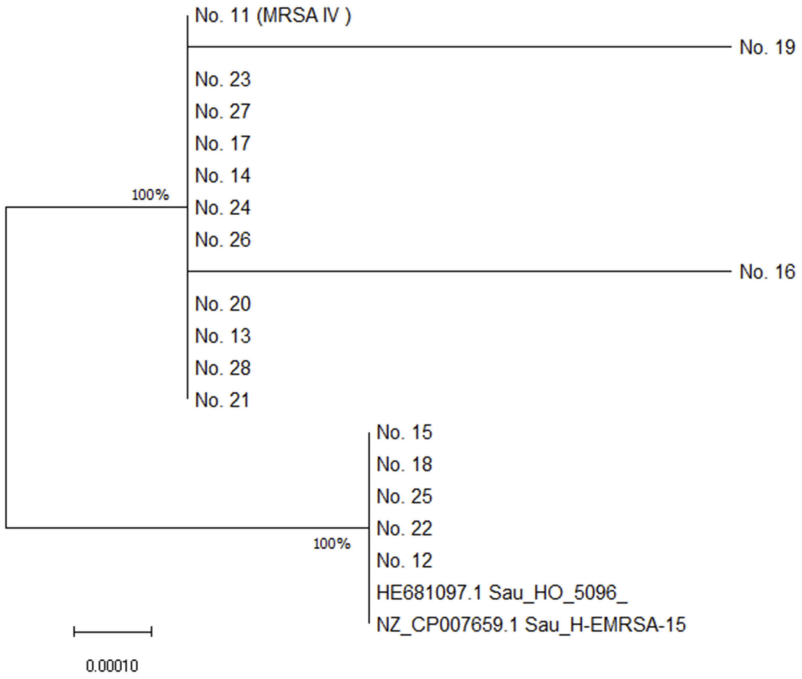

Figure 1 Neighbor-Joining tree of ST22 *S. aureus* isolates. The tree was rooted by the representative reference genomes of ST22 EMRSA-15 (HO 5096 0412 and H-EMRSA-15). The optimal tree with the sum of branch length =0.00212286 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA X.

Table 2 Biofilm formation ability of MSSA and MRSA

Biofilm formation	MSSA		MRSA	
	Number	Percent	Number	Percent
Moderate	9	25.7	7	77.8
Weak	26	74.3	2	22.2

Table 3 The sensitivities of antibiotics between weak biofilm and moderate biofilm producers among MSSA subgroup

Antibiotics	Weak biofilm (%) (N=26)	Moderate biofilm (%) (N=9)	*p*
Benzylpenicillin	15.4	11.1	0.62
Clindamycin	46.2	22.3	0.19
Erythromycin	42.3	33.3	0.47
Sulfamethoxazole/ trimethoprim	92.3	55.6	0.03*
Gentamycin	92.3	66.7	0.1
Ciprofloxacin	92.3	77.8	0.27
Levofloxacin	96.15	77.8	0.16
Rifampicin	100	100	1
Linezolid	100	100	1
Moxifloxacin	100	100	1
Vancomycin	100	100	1
Tetracycline	100	100	1
Tigecycline	100	100	1

approximately 80% of *S. aureus* isolates.[6,30,34,37,38] We detected 50% of *S. aureus* harboring *lukS/lukF*. Particularly, 83.3% of ST22 isolates were PVL positive. Chen[39] reported that ST22 tends to harbor a *lukS/lukF* gene compared to other ST types in China. Surprisingly, Gu[5] detected no *lukS/lukF* gene among CA-MRSA with SSTIs in Jiangsu Province, China. So far, it is unknown whether PVL^+ CA-MRSA clones arose through acquisition of the mec element from strains with a PVL^+ MSSA or conversely, through acquisition of PVL phage by strains with a methicillin resistance background. Overall, we are concerned whether the PVL^+ ST22 CA-MSSA clone would arise to become the dominant CA-MRSA clone around the city. Further studies are needed to uncover the possible evolution of the dominant PVL^+ ST22 CA-MSSA over time in China.

TSST-1-producing *S. aureus* are more likely to cause complicated infections. In this study, we found the *tst-1* gene was detected among 93.2% CA-*S.aureus* isolates. The overall prevalence of *tst-1* carrying *S. aureus* in Iran was 21.3%, ranging from 0% to 68%.[40] Moreover, a recent survey in China[41] reported that only 3.9% MRSA strains harbored the *tst-1* gene. Concerning the *tst-1* gene, the prevalence is much higher in our study, so we presume

high virulence isolates circulate around this community in Beijing.

ETs are the sole virulence factors responsible for SSSS, a disease mostly affecting neonates and children under 5 years of age. In Italy, *etA* positive ST5 clone *S. aureus* had ever caused an outbreak of skin infections in neonates.[42] We found two ST121 clone strains contain both the *etA* gene and the *edinA* gene associated with epidermal hyperplasia. Yet, the *etA* gene positive *S. aureus* strains were rarely reported in China.[34] Continuous monitoring ETs positive *S. aureus* in this community may be of interest.

Biofilm formation is another primary virulence factor of *S. aureus*.[43] Different strains possess different adhesins and differ in their ability to produce biofilms. In this study, the majority of ST22 clones had poor biofilm formation ability, whereas all CC59 strains can produce robust biofilms. CA-MRSA isolates have stronger biofilm formation ability than CA-MSSA, 77.8% vs 25.7%. Therefore, the biofilm formation ability is likely related to the genetic background or the clonal types. In fact, it is necessary to validate the hypothesis with a larger size of samples. The intracellular adhesion molecules are the main mechanism of biofilm formation. All of the isolates harbored the *ica*A and i*ca*D genes in our study, which implies poor biofilm producers of ST22 would have potential to become strong biofilm producers. The bacterial biofilm communities are surrounded by exopolysaccharides matrix.[44] Within this environment, bacteria develop polymicrobial interactions[45] and increase antibiotics resistance.[46] And some researchers reported a variable biofilm-producing ability of different MRSA isolates.[47] According to our analysis, the moderate biofilm producers had a significantly lower sensitivity to Sulfamethoxazole/Trimethoprim. In addition moderate biofilm producers also exhibited lower, although not significantly, sensitivities against several other antibiotics including benzylpenicillin, gentamycin, ciprofloxacin, levofloxacin, and erythromycin. Furthermore, there were no differences in the sensitivities to many other antibiotics including rifampicin, linezolid, moxifloxacin, vancomycin, tetracycline, and tigecycline between different biofilm producers.

Conclusion

In summary, we documented a prevalent community epidemic PVL^+ ST22 CA-MSSA which had previously described as a sporadic clone in Beijing, China.[6] The changing epidemiology of CA-*S. aureus* necessitates further surveillance to inform mechanisms of evolution, empiric treatment guidelines, and prevention strategies.

Ethics approval

The ethics committee of Beijing Tsinghua Changgung Hospital approved the study procedure. The reference number of the approval is 19167-0-01.

Abbreviations

CA-*S. aureus*, community-associated *Staphylococcus aureus*; SSTIs, skin and soft tissue infections; CA-MSSA, community-associated methicillin-susceptible *S.aureus*; CA-MRSA, community-associated methicillin-resistant *S. aureus*; MLST, multilocus sequence typing; PVL, Panton-Valentine Leukocidin; i*ca*, intracellular adhesion molecules; *tst-1*, toxic shock syndrome toxin-1; *et*A, exfoliative toxin A; *edin*A, epidermal cell differentiation inhibitor A; ST, sequence types; CC, clonal complex; TSS, toxic shock syndrome; SSSS, Staphylococcal scalded skin syndrome.

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Disclosure

The authors report no conflicts of interest in this work.

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