



# Typing of Panton-Valentine Leukocidin-Encoding Phages and *lukSF-PV* Gene Sequence Variation in *Staphylococcus aureus* from China

Huanqiang Zhao<sup>1†</sup>, Fupin Hu<sup>2†</sup>, Shu Jin<sup>3</sup>, Xiaogang Xu<sup>2</sup>, Yuhan Zou<sup>3</sup>, Baixing Ding<sup>2</sup>, Chunyan He<sup>1</sup>, Fang Gong<sup>4</sup> and Qingzhong Liu<sup>1\*</sup>

<sup>1</sup> Department of Clinical Laboratory, Shanghai General Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai, China, <sup>2</sup> Institute of Antibiotics, Huashan Hospital, Fudan University, Shanghai, China, <sup>3</sup> Experimental Research Center, Shanghai People's Hospital of Putuo District, Shanghai, China, <sup>4</sup> Department of Clinical Laboratory, The Third Hospital Affiliated to Nantong University, Wuxi, China

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### \*Correspondence:

Qingzhong Liu  
jiaodamedicine@foxmail.com

<sup>†</sup>These authors have contributed  
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Panton-Valentine leukocidin (PVL, encoded by *lukSF-PV* genes), a bi-component and pore-forming toxin, is carried by different staphylococcal bacteriophages. The prevalence of PVL in *Staphylococcus aureus* has been reported around the globe. However, the data on PVL-encoding phage types, *lukSF-PV* gene variation and chromosomal phage insertion sites for PVL-positive *S. aureus* are limited, especially in China. In order to obtain a more complete understanding of the molecular epidemiology of PVL-positive *S. aureus*, an integrated and modified PCR-based scheme was applied to detect the PVL-encoding phage types. Phage insertion locus and the *lukSF-PV* variant were determined by PCR and sequencing. Meanwhile, the genetic background was characterized by staphylococcal cassette chromosome *mec* (SCC*mec*) typing, staphylococcal protein A (*spa*) gene polymorphisms typing, pulsed-field gel electrophoresis (PFGE) typing, accessory gene regulator (*agr*) locus typing and multilocus sequence typing (MLST). Seventy eight (78/1175, 6.6%) isolates possessed the *lukSF-PV* genes and 59.0% (46/78) of PVL-positive strains belonged to CC59 lineage. Eight known different PVL-encoding phage types were detected, and  $\Phi$ 7247PVL/ $\Phi$ ST5967PVL ( $n = 13$ ) and  $\Phi$ PVL ( $n = 12$ ) were the most prevalent among them. While 25 (25/78, 32.1%) isolates, belonging to ST30, and ST59 clones, were unable to be typed by the modified PCR-based scheme. Single nucleotide polymorphisms (SNPs) were identified at five locations in the *lukSF-PV* genes, two of which were non-synonymous. Maximum-likelihood tree analysis of attachment sites sequences detected six SNP profiles for *attR* and eight for *attL*, respectively. In conclusion, the PVL-positive *S. aureus* mainly harbored  $\Phi$ 7247PVL/ $\Phi$ ST5967PVL and  $\Phi$ PVL in the regions studied. *lukSF-PV* gene sequences, PVL-encoding phages, and phage insertion locus generally varied with lineages. Moreover, PVL-positive clones that have emerged worldwide likely carry distinct phages.

**Keywords:** *Staphylococcus aureus*, Panton-Valentine leukocidin, sequence variation, phage typing, genetic background

## INTRODUCTION

*Staphylococcus aureus* causes a spectrum of diseases from minor skin and soft tissue infections (SSTIs) to life-threatening conditions due to its potential to produce many toxins and efficiency at overcoming antibiotics (David and Daum, 2010; Uhlemann et al., 2014). Panton-Valentine leukocidin (PVL), found in some strains of *S. aureus*, plays a key role in the leukocytolysis and tissue necrosis (Shallcross et al., 2013). The genes (*lukSF-PV*) encoding PVL are composed of two co-transcribed open reading frames (*lukS-PV* and *lukF-PV*), and located on lysogenized bacteriophages integrated into *S. aureus* chromosome (Boyle-Vavra and Daum, 2007; Shallcross et al., 2013).

To date, all known *S. aureus* phages belong to the order *Caudovirales*, which can be separated into three major families (*Podoviridae*, *Siphoviridae*, and *Myoviridae*) depending on the tail morphology (Xia and Wolz, 2014). At least 10 PVL phages have been described and sequenced, and all of them belong to the *Siphoviridae* family characterized by double-stranded DNA and a long non-contractile tail (Zhang et al., 2011; Xia and Wolz, 2014). As the mobile genetic elements, phages can be mobilized and transferred among *S. aureus* strains. Hence, investigating the typing of PVL-encoding phages among *S. aureus* may help to better understand the evolution of these pathogens. The PCR-based scheme targeting a small part of the phage genome is a cursory means of predicting phages types inexpensively. However, there is no unified pattern for this scheme. To identify as many PVL-encoding phages as possible, this article summarized a strategy by integrating and modifying the expanded PCR-based scheme described previously (Ma et al., 2008; Boakes et al., 2011; Chen et al., 2013; Sanchini et al., 2014). Subsequently, by the usage of this strategy, fifteen-reaction PCR assay was carried out to detect 10 of the PVL-encoding phages ( $\Phi$ PVL,  $\Phi$ 108PVL,  $\Phi$ tp310-1,  $\Phi$ Sa2958,  $\Phi$ Sa2mw,  $\Phi$ SLT,  $\Phi$ Sa2USA,  $\Phi$ TCH60,  $\Phi$ 7247PVL/  $\Phi$ ST5967PVL, and  $\Phi$ Sa119) in *S. aureus* from China.

At least 22 single-nucleotide polymorphisms (SNPs) have been identified in the *lukSF-PV* genes (Wolter et al., 2007; O'Hara et al., 2008; Boakes et al., 2011; Li et al., 2012; Chen et al., 2013; Sanchini et al., 2014). And several PVL protein isoforms carrying non-synonymous mutations have recently been revealed (O'Hara et al., 2008; Li et al., 2012), which may implicate functional significance. As we all know, phages lysogenize into the bacterial chromosome through the integrative pathway. Boakes et al. (2011) analyzed the sequence diversity at the insertion site for the different PVL-encoding phages and found two lineage-specific insertion sites within the *S. aureus* chromosome: Ins1 and Ins2. Of note, site-specific attachment sequences (*attL* and *attR*) are also conserved across lineages of PVL-carrying strains although some variations were found (Boakes et al., 2011; Chen et al., 2013).

Due to the absence of a uniform detection method, complete data on Chinese epidemiology of PVL-encoding phages are limited (Li et al., 2012; Hu et al., 2015). In addition, no details yet about chromosomal PVL-encoding phage insertion sites for *S. aureus* in China are reported.

In the present study, we aimed to obtain a more complete description of the molecular epidemiology of PVL-positive *S. aureus* from China by detecting PVL-encoding phage types, analyzing PVL variant alleles and the chromosomal phage insertion junctions, and determining the genetic background. Moreover, the relationships among them were also elucidated.

## MATERIALS AND METHODS

### Bacterial Isolates

During January 2010 to May 2015, a total of 1175 consecutive, non-duplicate clinical *S. aureus* isolates were collected from seven hospitals in China, namely, Shanghai General Hospital, Shanghai sixth People's Hospital, Tongren Hospital, Ruijin hospital, Shanghai People's Hospital of Putuo District, Zhejiang Xiaoshan Hospital, and The Central Hospital of Lishui City, Zhejiang province (Figure 1). All the isolates, including 924 methicillin-resistant *S. aureus* (MRSA) strains and 251 methicillin-susceptible *S. aureus* (MSSA) strains, were identified by VITEK Systems (BioMérieux, Marcy l'Étoile, France), the susceptibility of cefoxitin (30  $\mu$ g, Oxoid, Basingstoke, UK), and the presence of *mecA* and *mecC* genes (Bignardi et al., 1996; Ganesan et al., 2013; Clinical Laboratory Standards Institute, 2014). The presence of *lukSF-PV* genes was determined by PCR according to previously published method (Lina et al., 1999).

### Single-Nucleotide Polymorphisms of the *lukSF-PV* Genes

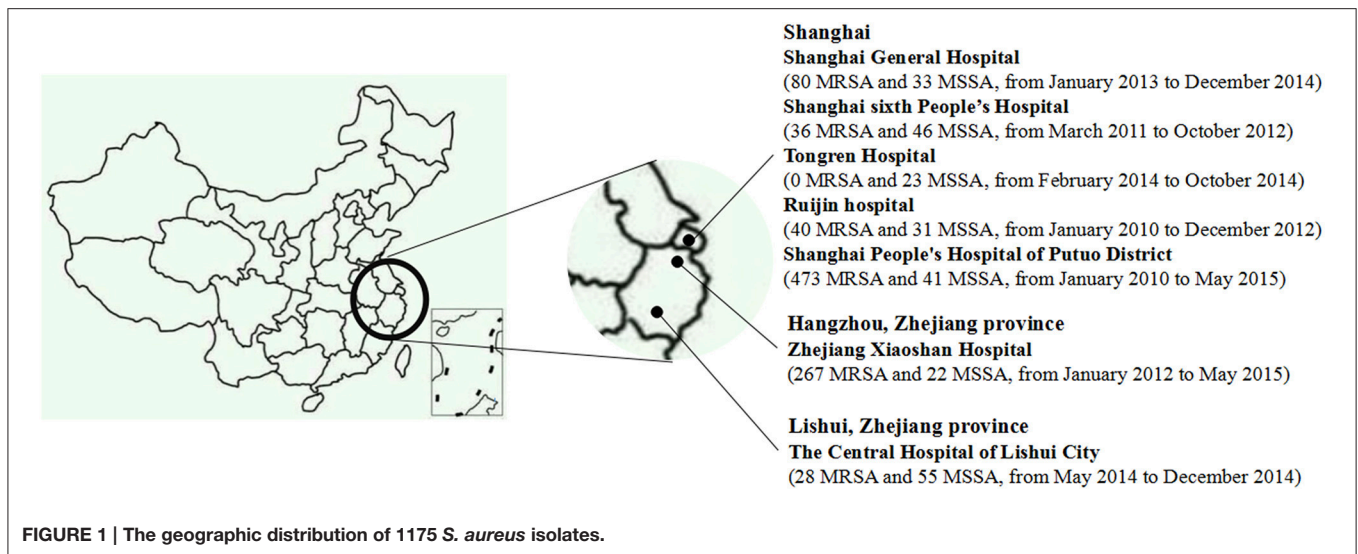
All isolates were cultured on blood agar and incubated overnight at 37°C. Genomic DNA was extracted by TIANamp Bacterial DNA Kit (TIANGEN BIOTECH Co., Ltd., Beijing, China). Three primer pairs were designed to amplify three fragments (654, 718, and 680 nucleotides in length, respectively) of *lukSF-PV* genes as described by Boakes et al. (2011). All the products of PCR were sequenced in both directions by Shanghai Sangon Biotech.

### Characterization of PVL-Positive Isolates

PVL-positive *S. aureus* were characterized by staphylococcal cassette chromosome *mec* (*SCCmec*) typing (Zhang et al., 2005), staphylococcal protein A (*spa*) gene polymorphisms typing (Koreen et al., 2004), pulsed-field gel electrophoresis (PFGE) typing (Mulvey et al., 2001), accessory gene regulator (*agr*) locus typing (Lina et al., 2003) and multilocus sequence typing (MLST; Enright et al., 2000). The *spa* type for each isolate was obtained based on a website (<http://www.ridom.de/spaserver/>). Sequence types (STs) were determined by allelic profile according to the MLST database (<http://saureus.mlst.net/>). And clustering of related STs into clonal complexes (CCs) was analyzed using eBURST (<http://www.mlst.net>).

### PVL-Encoding Phage Typing

Integrating the expanded PCR-based scheme described by previous studies (Ma et al., 2008; Boakes et al., 2011; Chen et al., 2013; Sanchini et al., 2014) with a little modification,



three sets of PCRs including 15 PCR reactions (Set 1: PCR-1, -4 -7, and -8, Set 2: PCR-2, -5, and -9 to -13, and Set 3: PCR-3, -6, -14, and -15), were carried out to detect 10 of the PVL-encoding phages ( $\Phi$ PVL,  $\Phi$ 108PVL,  $\Phi$ tp310-1,  $\Phi$ Sa2958,  $\Phi$ Sa2mw,  $\Phi$ SLT,  $\Phi$ Sa2USA,  $\Phi$ TCH60,  $\Phi$ 7247PVL/ $\Phi$ ST5967PVL, and  $\Phi$ Sa119) (Table 1). PCR-1 to -3 (step-1) targeting genes encoding icosahedral or elongated head shape allowed classification of elongated-head group, icosahedral-head group I and icosahedral-head group II phages; PCR-4 to -6 (step-2) were used to link these morphologically specific tail genes to the *lukSF-PV* genes with primer pairs commonly conserved among each of the groups; PCR-7 to -15 (step-3) were designed to detect 10 specific PVL-encoding phages. All the PVL-positive isolates were detected by PCR-1 to -3 (step-1). Only when a positive result was obtained from step-1, were step-2, and step-3 performed. Phages which were positive by icosahedral/elongated head classification assays (PCR-1 to -3) and characterization assays (PCR-7 to -15) but negative for linkage assays (PCR-4 and -6), were defined as “phage-like” depending on the existence of known individual phage type (Chen et al., 2013). The workflow and the detailed PCR-based scheme for PVL-encoding phage typing were summarized in Figure 2 and Table 1, respectively.

In case of the fragments amplified >3000 nucleotides, the PCRs were carried out using PrimeSTAR<sup>®</sup> Max DNA Polymerase (TaKaRa, Dalian, China) adhering to the protocols recommended by manufacturer.

### Phage Insertion Locus Sequencing

PCR designed to detect the proximal and distal junctions of PVL-encoding phage insertion sites were performed by the method described previously (Boakes et al., 2011). Sequences were aligned using CLUSTA L X 2.0. Phylip3.6 was used to construct a maximum-likelihood phylogenetic tree with 500 bootstrap replicates for the junction region sequences.

## RESULTS

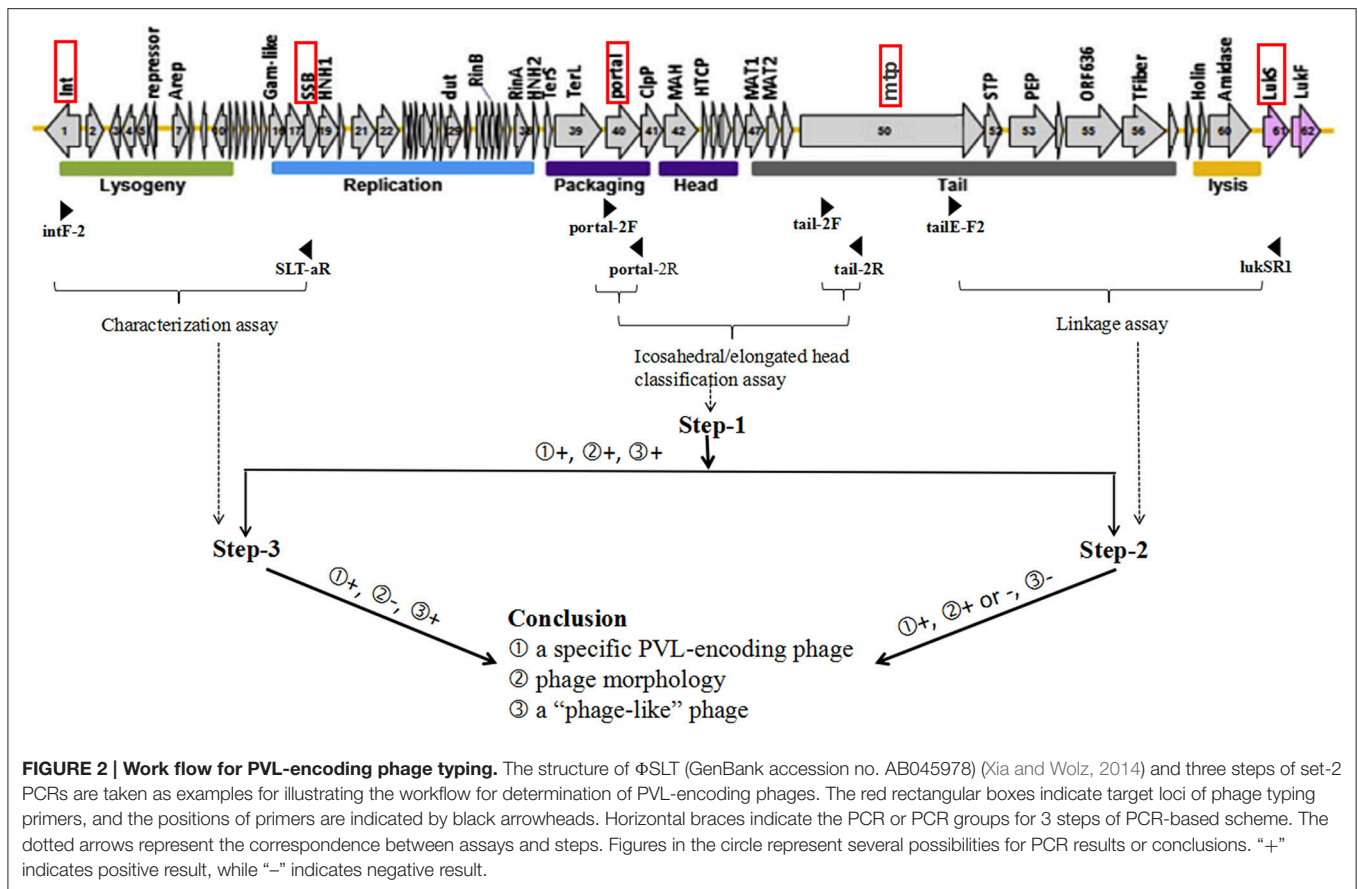
### Frequency and Characterization of *S. aureus* Harboring *lukSF-PV* Genes

Of 1175 *S. aureus* isolates, 78 (6.6%) isolates were *lukSF-PV* genes positive, including 62 MRSA and 16 MSSA. Among the 78 *lukSF-PV* genes positive strains, 66 (55 MRSA and 11 MSSA) were *agr1*, 9 (6 MRSA and 3 MSSA) were *agr3*, and 2 (MSSA) were *agr4*. One isolate was unable to be classified in any of the established *agr* groups, which may be an *agr*-negative variant associated with the extensive use of antibiotics (Paulander et al., 2013). Of course, point mutation occurring in primer binding sequence may be another reason. MLST results showed 15 ST types were produced in isolates studied, namely ST1 (CC1), ST9 (CC9), ST22 (CC22), ST25 (CC25), ST30 (CC30), ST59 (CC59), ST88 (CC88), ST188 (CC1), ST149 (CC5), ST217 (CC22), ST338 (CC59), ST398 (CC398), ST1301 (CC121), ST160 (CC121), and ST172 (singleton). ST59 (64.5%, 40/62) was the most frequent ST in MRSA isolates, while ST398 (25%, 4/16) was the most prevalent type in MSSA strains. Two STs identified in this study have not found matching profiles in the MLST database, and subsequently were designated ST160 and ST172 after we uploaded the data to the website (<http://pubmlst.org/saureus/>). By sequence analysis of PCR products of the *spa* gene, 23 *spa* types were yielded in 78 PVL-positive isolates. The most common *spa* type identified was t437 (48.7%, 38/78). Two *spa* types determined in this study were not found in Ridom SpaServer, which were subsequently designated t15796 and t15797 after our submission. It is notable that 94.7% (36/38) of t437 isolates were associated with CC59. In addition, most of t034 strains (75%, 3/4) related to CC398. PFGE typing showed that 78 PVL-positive isolates were divided into 17 patterns. Most of isolates were clustered into PFGE type A (subtypes A1 to A7), type B (subtypes B1 to B3), and type C (subtypes C1 to C7) together accounting for 44.9% (35/78). In 62 PVL-positive MRSA isolates, 57 could be typed (SCC*mec* I-V) by SCC*mec* typing method, and 5 (8.1%) were non-typeable. The SCC*mec* type most commonly found

**TABLE 1 | PCR-based scheme for PVL-phage typing.**

Primer use	Step 1				Step 2				Step 3								
	Primer name	Target locus	Size of PCR product (bp)	References	Primer use	Primer name	Target locus	Size of PCR product (bp)	References	PVL phage(s)	Primer use	Primer name	Target locus	Size of PCR product (bp)	GenBank accession no.	References	PCR's order number
Set 1 Icosahedral-head group I	portal-1F	<i>por</i>	569	Ma et al., 2008	To link icosahedral-head group I specific tail genes to the PVL genes.	tail-ico-F	<i>mtp</i>	8570	Ma et al., 2008	ΦPVL	To Classify individual PVL phages by detecting the gene lineage between the integrase gene and the genes located downstream of the gene.	inIF-2/PVL-aR	<i>int1/JP030</i>	1411	AB009866	Ma et al., 2008	PCR-7
	portal-1R	<i>por</i>		Ma et al., 2008				10,497		Φ108PVL		inIF-2/108-aR	<i>int1/ant</i>	4340	AB243556	Ma et al., 2008	PCR-8
	tail-1F	<i>mtp</i>	489	Ma et al., 2008		lukSR1	<i>lukS-PV</i>	8574	Ma et al., 2008	Φtp310-1*		inIF-2/PVL-aR / 108-aR*	<i>int1/JP030 / ant</i>	1411/4579*	EF462197	Ma et al., 2008; Chen et al., 2013	PCR-7, -8
	tail-1R	<i>mtp</i>		Ma et al., 2008													
Set 2 Elongated-head group	portal-2F	<i>por</i>	656	Ma et al., 2008	To link elongated-head group specific tail genes to the PVL genes.	tailE-F2	<i>mtp</i>	9483	Otter et al., 2010	ΦSaz2958	To Classify individual PVL phages by detecting the gene lineage between the integrase gene and the genes located downstream of the gene.	inIF-2/2958-aR	<i>int1/JP004</i>	2238	AP009363	Ma et al., 2008	PCR-9
	portal-2R	<i>por</i>		Ma et al., 2008				9484		ΦSaz2mw		inIF-2/MM2-aR	<i>int1/cro</i>	4065	BA000033	Ma et al., 2008	PCR-10
	tail-2F	<i>mtp</i>	468	Ma et al., 2008		lukSR1	<i>lukS-PV</i>	9486	Ma et al., 2008	ΦSLT		inIF-2/SLT-aR	<i>int1/ssb</i>	8770	AB045978	Ma et al., 2008	PCR-11
	tail-2R	<i>mtp</i>		Ma et al., 2008				9484		ΦSaz2USA		Saz2USA-F / Saz2USA-R2	<i>phSLT / ORF484-like / lukS-PV</i>	679	CP000255	Boakes et al., 2011; Sanchini et al., 2014	PCR-12
Set 3 Icosahedral-head group II	portal-3F	<i>por</i>	535	Sanchini et al., 2014	To link icosahedral-head group II specific tail genes to the PVL genes.	TAL-5	<i>mtp</i>	9164	Sanchini et al., 2014	Φ7247PVL / ΦST5967PVL	To Classify individual PVL phages by detecting the gene lineage between the integrase gene and the genes located downstream of the gene.	inIF-2/repR	<i>int1/rep</i>	2965	AP011956 / AP011955	Ma et al., 2008; Chen et al., 2013; Sanchini et al., 2014	PCR-14
	portal-3R	<i>por</i>		Sanchini et al., 2014				10,728		ΦSai119		inIF-2/SA119Ant1	<i>int1/ant</i>	4918	KJ596420	Ma et al., 2008; Sanchini et al., 2014	PCR-15
	tail-3F	<i>mtp</i>	842	Sanchini et al., 2014		lukSR1	<i>lukS-PV</i>										
	tail-3R	<i>mtp</i>															

\*Φtp310-1 harbors an icosahedral head and can be identified by the presence of PCR products specific both for ΦPVL and Φ108PVL (positive both for PCR-7 and -8).



was type III (53.2%, 33/62), followed by type IVa (32.3%, 20/62), type II (3.2%, 2/62), type IVb, and type V (1.6%, 1/62 each). The detailed molecular characterization of PVL-positive isolates by SCCmec, agr, spa, PFGE, and MLST typing was shown in Table 2.

### Typing of PVL-Encoding Phages

Applying the expanded PCR-based scheme described above, it was possible to identify 10 of known phages carrying *lukSF-PV* genes. Our results showed that 67.9% (53/78, 42 MRSA and 11 MSSA) of PVL-positive isolates could be divided into 8 phage types [ $\Phi$ PVL ( $n = 12$ , including one  $\Phi$ PVL-like),  $\Phi$ 108PVL-like ( $n = 1$ ),  $\Phi$ tp310-1 ( $n = 4$ ),  $\Phi$ Sa2958 ( $n = 7$ ),  $\Phi$ Sa2USA ( $n = 5$ ),  $\Phi$ TCH60 ( $n = 5$ , including one  $\Phi$ TCH60-like),  $\Phi$ 7247PVL/ $\Phi$ ST5967PVL ( $n = 13$ ), and  $\Phi$ Sa119 ( $n = 6$ , including one  $\Phi$ Sa119-like)], which were almost equally split among elongated-head group ( $n = 17$ ), icosahedral-head group I ( $n = 17$ ), and II ( $n = 19$ ). Phage typeability was 68.8% in MSSA (11 out of 16) and 67.7% in MRSA (42 out of 62). Unfortunately, 25 isolates (32.1%) could not be typed by the present scheme. Four MRSA isolates were considered to be "phage-like," named  $\Phi$ PVL-like,  $\Phi$ 108PVL-like,  $\Phi$ TCH60-like and  $\Phi$ Sa119-like, according to the definition from materials and methods. The details of PVL-encoding phages types were shown in Table 2.

### Variation of *lukSF-PV* Genes

The amplification products of the *lukSF-PV* genes from 78 isolates were sequenced, and nucleotide variations were seen at five sites (position 527 and 663 located in the *lukS* locus and position 1022, 1396, 1729 located in the *lukF* locus) using the *lukSF-PV* genes of  $\Phi$ SLT as a reference. Of 78 PVL-positive isolates, 71 (91.0%, 71/78) were of H variant as defined by O'Hara et al. (2008), which can be further separated into H1 (Genbank Accession no. EF571669) (23.1%, 18/78), H2 (Genbank Accession no. EF571668) (66.7%, 52/78), and H3 (Genbank Accession no. EF571713) (1.3%, 1/78) groups differed at nucleotide position 1396 and 663 (Table 2). The rest of seven isolates were identified as R variant displaying non-synonymous nucleotide 527 A to G mutation, and further divided into R1 (Genbank Accession no. EF571829) (5.1%, 4/78), and R2 (Genbank Accession no. EF571830) (3.8%, 3/78) groups according to the distinction of nucleotide site 1729 (Table 2).

### DNA Sequencing of Phage/Chromosome Junctions

To investigate the chromosomal insertion site of phage in the present study, we performed PCR based on the known phage/chromosome junctions in the other strains (Boakes et al., 2011; Li et al., 2011). Seventy two (92.3%) isolates showed

**TABLE 2 | Characteristics and PVL-phage typing of 78 PVL-positive *S. aureus* isolates.**

Isolate ID	location	ST	CC	SCCmec	spa type	agr type	PFGE	attL cluster	attR cluster	Phage morphology	PVL-phage type	Isoform of PVL
<b>MRSA (n = 62)</b>												
7	Zhejinag	ST1	CC1	V	t114	I	K	I	I	Icosahedral II	Φ7247PVL/ ΦST5967PVL	H2
LS2183	Zhejinag	ST59	CC59	NT	t437	I	B1	I	I	Icosahedral II	NT	H1
161	Zhejinag	ST188	CC1	NT	t189	I	M	NS	NS	Icosahedral II	NT	H2
SH12	Shanghai	ST88	CC88	NT	t12147	III	A1	IV	III	Icosahedral I	ΦPVL	H2
244	Zhejinag	ST88	CC88	NT	t7637	III	C7	IV	III	Elongated	ΦTCH60	H2
104	Zhejinag	ST88	CC88	NT	t5269	III	N	IV	III	Elongated	ΦTCH60*	R2
148	Zhejinag	ST9	CC9	IVb	t309	I	O	VI	II	Icosahedral I	ΦPVL	H2
203	Zhejinag	ST1	CC1	IVa	t127	III	H3	I	NS	Icosahedral I	Φ108PVL*	H1
SH9	Shanghai	ST59	CC59	IVa	t441	I	I1	I	I	Icosahedral I	ΦPVL	H1
209	Zhejinag	ST59	CC59	IVa	t437	I	E3	II	I	Elongated	ΦSa2958	H1
LS373	Zhejinag	ST59	CC59	IVa	t1751	I	B3	I	I	Icosahedral II	NT	H2
60	Zhejinag	ST59	CC59	IVa	t437	I	D3	NS	I	Icosahedral II	NT	H2
34	Zhejinag	ST59	CC59	IVa	t437	I	J	I	I	Icosahedral II	NT	H2
65	Zhejinag	ST59	CC59	IVa	t437	I	D1	I	I	Icosahedral II	NT	H2
79	Zhejinag	ST59	CC59	IVa	t437	I	F2	I	I	Icosahedral II	ΦSa119	H2
186	Zhejinag	ST59	CC59	IVa	t437	I	I1	I	I	Icosahedral II	ΦSa119	H2
238	Zhejinag	ST59	CC59	IVa	t437	I	C3	II	I	Icosahedral II	ΦSa119*	H2
212	Zhejinag	ST59	CC59	IVa	t437	I	E1	II	I	Elongated	ΦSa2958	H2
226	Zhejinag	ST59	CC59	IVa	t437	I	C3	II	I	Elongated	ΦSa2958	H2
246	Zhejinag	ST338	CC59	IVa	t437	I	C5	II	III	Elongated	ΦTCH60	H2
202	Zhejinag	ST88	CC88	IVa	t7637	III	H1	IV	III	Elongated	ΦTCH60	H2
213	Zhejinag	ST59	CC59	IVa	t1451	I	H1	NS	NS	Icosahedral II	NT	H2
LS1939	Zhejinag	ST59	CC59	Iva	t437	I	B3	I	I	Icosahedral II	NT	H2
SH6	Shanghai	ST59	CC59	IVa	t441	I	A3	I	I	Icosahedral I	ΦPVL	H2
40	Zhejinag	ST59	CC59	IVa	t437	I	J	II	I	Icosahedral II	ΦSa119	R1
218	Zhejinag	ST59	CC59	IVa	t437	NT	E2	II	NS	Icosahedral II	NT	R2
206	Zhejinag	ST59	CC59	IVa	t437	I	H2	II	I	Icosahedral I	ΦPVL	R2
LS2032	Zhejinag	ST59	CC59	III	t437	I	A2	I	I	Icosahedral II	NT	H1
130	Zhejinag	ST59	CC59	III	t441	I	F2	I	I	Icosahedral II	ΦSa119	H1
242	Zhejinag	ST338	CC59	III	t441	I	C2	I	I	Icosahedral II	Φ7247PVL/ ΦST5967PVL	H1
256	Zhejinag	ST59	CC59	III	t5983	I	D2	I	I	Icosahedral II	Φ7247PVL/ ΦST5967PVL	H1
211	Zhejinag	ST22	CC22	III	t5983	I	G1	VI	II	Icosahedral I	Φtp310-1	H1
LS1268	Zhejinag	ST59	CC59	III	t437	I	B3	I	I	Icosahedral II	NT	H1
233	Zhejinag	ST30	CC30	III	t1749	III	L	VII	V	Icosahedral II	NT	H2
219	Zhejinag	ST338	CC59	III	t437	I	C1	I	I	Icosahedral II	NT	H2
82	Zhejinag	ST59	CC59	III	t437	I	F1	I	I	Icosahedral II	NT	H2
253	Zhejinag	ST59	CC59	III	t437	I	D1	I	I	Icosahedral II	NT	H2
8	Zhejinag	ST59	CC59	III	t437	I	D1	I	NS	Icosahedral II	NT	H2
204	Zhejinag	ST59	CC59	III	t437	I	E3	I	I	Icosahedral II	NT	H2
210	Zhejinag	ST59	CC59	III	t437	I	E4	I	I	Icosahedral II	NT	H2
214	Zhejinag	ST22	CC22	III	t5983	I	G1	IV	II	Icosahedral I	ΦPVL	H2
221	Zhejinag	ST338	CC59	III	t437	I	C1	I	I	Icosahedral I	ΦPVL	H2
69	Zhejinag	ST217	CC22	III	t309	I	G3	III	II	Icosahedral I	ΦPVL*	H2
195	Zhejinag	ST59	CC59	III	t437	I	P	I	I	Icosahedral II	ΦSa119	H2
51	Zhejinag	ST59	CC59	III	t441	I	D2	I	I	Elongated	ΦSa2958	H2

(Continued)

**TABLE 2 | Continued**

Isolate ID	location	ST	CC	SCCmec	spa type	agr type	PFGE	attL cluster	attR cluster	Phage morphology	PVL-phage type	Isoform of PVL
220	Zhejiang	ST338	CC59	III	t437	I	C1	I	I	Icosahedral II	Φ7247PVL/ ΦST5967PVL	H2
74	Zhejiang	ST59	CC59	III	t437	I	F1	I	I	Icosahedral II	Φ7247PVL/ ΦST5967PVL	H2
170	Zhejiang	ST59	CC59	III	t437	I	F2	I	I	Icosahedral II	Φ7247PVL/ ΦST5967PVL	H2
254	Zhejiang	ST59	CC59	III	t437	I	D1	I	I	Icosahedral II	Φ7247PVL/ ΦST5967PVL	H2
237	Zhejiang	ST59	CC59	III	t437	I	C4	I	I	Icosahedral II	Φ7247PVL/ ΦST5967PVL	H2
236	Zhejiang	ST59	CC59	III	t437	I	C4	I	I	Icosahedral II	Φ7247PVL/ ΦST5967PVL	H2
108	Zhejiang	ST59	CC59	III	t437	I	F2	I	I	Icosahedral II	Φ7247PVL/ ΦST5967PVL	H2
145	Zhejiang	ST59	CC59	III	t437	I	D2	I	I	Icosahedral II	Φ7247PVL/ ΦST5967PVL	H2
217	Zhejiang	ST59	CC59	III	t437	I	E1	I	I	Icosahedral II	Φ7247PVL/ ΦST5967PVL	H2
16	Zhejiang	ST59	CC59	III	t437	I	D3	I	I	Icosahedral II	Φ7247PVL/ ΦST5967PVL	H2
205	Zhejiang	ST22	CC22	III	t5983	I	G1	I	II	Icosahedral I	Φtp310-1	H2
239	Zhejiang	ST22	CC22	III	t5983	I	G2	I	II	Icosahedral I	Φtp310-1	H2
RJ117	Shanghai	ST338	CC59	III	t437	I	A4	I	I	Icosahedral II	NT	H2
LS2137	Zhejiang	ST59	CC59	III	t2755	I	A6	I	I	Icosahedral II	NT	H2
3	Zhejiang	ST59	CC59	III	t034	I	Q	III	III	Elongated	ΦSa2USA	H2
222	Zhejiang	ST149	CC5	II	t437	I	C6	I	I	Elongated	ΦSa2USA	H2
255	Zhejiang	ST149	CC5	II	t437	I	K	II	III	Icosahedral I	Φtp310-1	H2
<b>MSSA (n = 16)</b>												
LS126	Zhejiang	ST30	CC30	-	t318	I	B2	I	I	Icosahedral II	NT	H1
SH14	Shanghai	ST398	CC398	-	t034	I	A5	III	III	Elongated	NT	H1
LS1985	Zhejiang	ST22	CC22	-	t309	I	B1	IV	II	Icosahedral I	ΦPVL	H1
LS2074	Zhejiang	ST25	CC25	-	t227	I	B1	NS	IV	Icosahedral I	ΦPVL	H1
SH26	Shanghai	ST25	CC25	-	t078	I	A2	NS	NS	Elongated	ΦSa2958	H1
SH135	Shanghai	ST1301	CC121	-	t12145	IV	A1	V	VI	Icosahedral I	NT	H1
LS1940	Zhejiang	ST30	CC30	-	t318	III	A7	I	VI	Icosahedral II	NT	H1
LS2078	Zhejiang	ST172	singleton	-	t078	I	B1	NS	VI	Icosahedral I	ΦPVL	H1
LS1004	Zhejiang	ST160	CC121	-	t15797	IV	B3	V	VI	Icosahedral I	NT	H2
LS1966	Zhejiang	ST88	CC88	-	t15796	III	B2	IV	III	Icosahedral I	ΦPVL	H2
SH13	Shanghai	ST88	CC88	-	t2310	III	A3	IV	III	Elongated	ΦTCH60	H2
SH25	Shanghai	ST217	CC22	-	t309	I	A4	VIII	II	Elongated	ΦSa2958	H2
SH19	Shanghai	ST217	CC22	-	t309	I	A4	VIII	II	Elongated	ΦSa2958	H3
SJ1775	Shanghai	ST398	CC398	-	t034	I	I2	III	III	Elongated	ΦSa2USA	R1
SH3	Shanghai	ST398	CC398	-	t1255	I	A3	III	NS	Elongated	ΦSa2USA	R1
LS1911	Zhejiang	ST398	CC398	-	t034	I	B3	III	III	Elongated	ΦSa2USA	R1

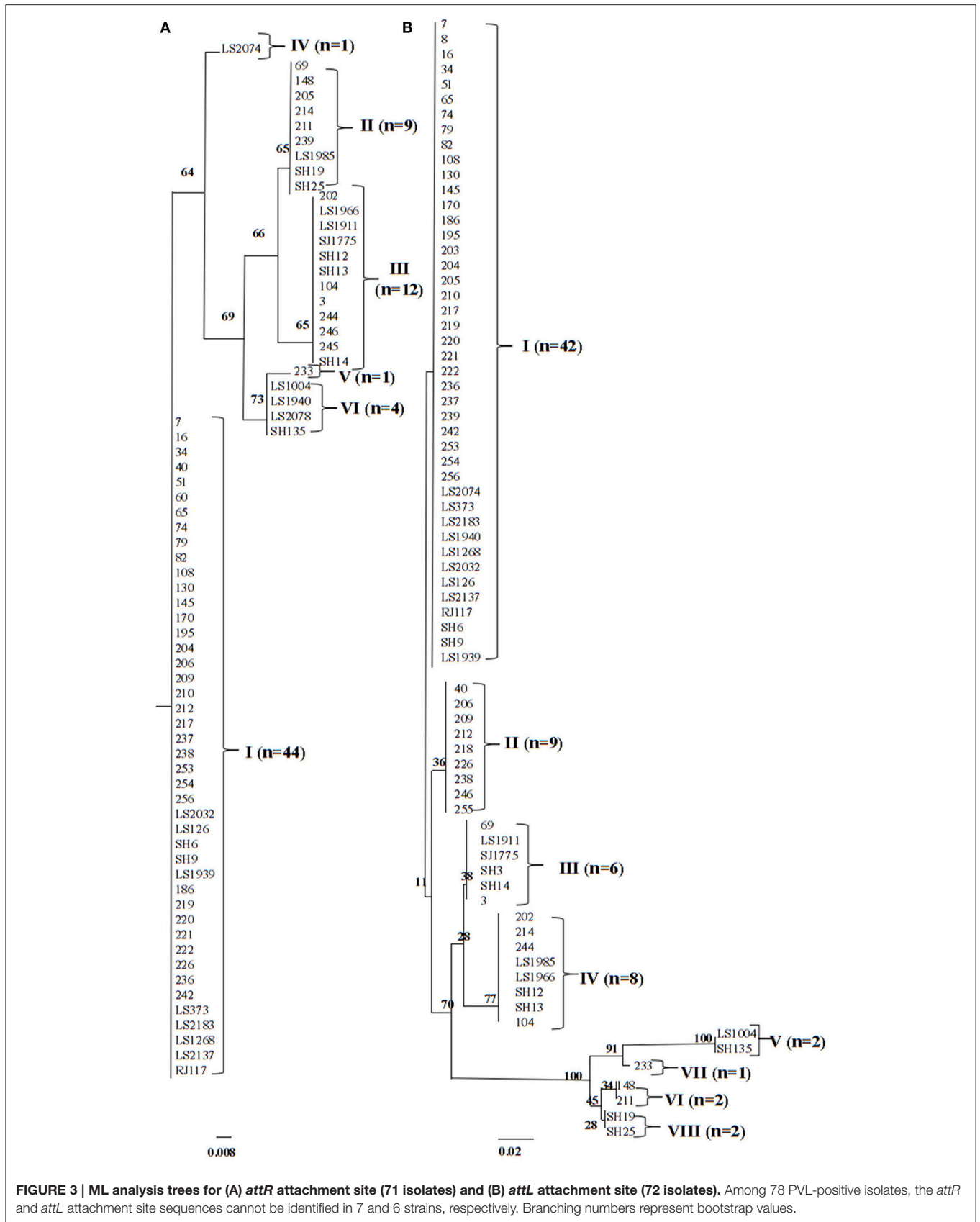
\* Phages-like, positive by icosahedral/elongated head classification assays (PCR-1 to -3) and characterization assays (PCR-7 to -15) but negative for linkage assays (PCR-4 and -6).  
 -, no SCCmec elements was detected (MSSA).

NT, non-typeable.

NS, could not be sequenced.

positive amplification for *attL* sequences, and 71 (91.0%) for *attR* sequences. This indicated a relatively conserved PVL-encoding phage integration site regardless of difference among genetic features of strains, PVL-encoding phage types, and *lukSF-PV* genes variants. Maximum-likelihood phylogenetic

tree analysis of the *attR* and *attL* attachment sites showed four clusters (I, II, III, and VI) and two singletons (IV and V) for *attR* and seven clusters (I, II, III, IV, V, VI, and VIII) and one singleton (VII) for *attL* (Figure 3, Table 2).



**FIGURE 3 | ML analysis trees for (A) attR attachment site (71 isolates) and (B) attL attachment site (72 isolates).** Among 78 PVL-positive isolates, the attR and attL attachment site sequences cannot be identified in 7 and 6 strains, respectively. Branching numbers represent bootstrap values.



## DISCUSSION

PVL-positive *S. aureus*, strongly associated with SSTI and necrotizing pneumonia, has gained great attention in recent years (David and Daum, 2010; Shallcross et al., 2013). In China, the PVL positive rate ranged from 5.5 to 28.6% in HA-MRSA isolates (Yu et al., 2008; Fan et al., 2009; Li et al., 2012; Xiao et al., 2013; Hu et al., 2015). The major clone with *lukSF-PV* genes was ST59 in MRSA, while ST398, and ST88 in MSSA according to the previous studies (Yu et al., 2008; Fan et al., 2009; Li et al., 2012; Xiao et al., 2013; Hu et al., 2015). In the current study, we also revealed a high percentage of PVL-positive ST59 clones associated with MRSA and ST398 with MSSA strains.

A previously published eight-reaction PCR assay was performed to identify five ( $\Phi$ Sa2958,  $\Phi$ Sa2mw,  $\Phi$ PVL,  $\Phi$ 108PVL, and  $\Phi$ SLT) of the PVL-encoding phages in *S. aureus* (Ma et al., 2008). Subsequently, this scheme was improved to include the detection of  $\Phi$ Sa2USA (Boakes et al., 2011),  $\Phi$ TCH60,  $\Phi$ tp310-1, and  $\Phi$ 7247PVL (Chen et al., 2013). Recently, Sanchini et al. (2014) divided PVL-encoding phages into three groups including the elongated-head group, icosahedral-head group I, and icosahedral-head group II, and incorporated  $\Phi$ Sa119 and  $\Phi$ ST5967PVL belonging to icosahedral-head group II into the scheme. However, this PCR-based scheme could not discriminate  $\Phi$ ST5967PVL from  $\Phi$ 7247PVL (Chen et al., 2013; Sanchini et al., 2014), because the genomes of both phages showed more than 99% identity (Zhang et al., 2011). Therefore, these two phages were marked as " $\Phi$ 7247PVL/ $\Phi$ 5967PVL" in this study.

Applying the PCR-based strategy, the distribution of PVL-encoding phages was investigated all over the world. Two morphologically distinct phages ( $\Phi$ PVL and  $\Phi$ Sa2958) were found to be predominant among Japanese PVL-positive MRSA (Ma et al., 2008), and  $\Phi$ Sa2USA was the most prevalent phage in Italy (Sanchini et al., 2014), while in United Kingdom, the most common phage types were  $\Phi$ Sa2USA and  $\Phi$ Sa2mw (Otter et al., 2010; Boakes et al., 2011). In this study, the results revealed that  $\Phi$ 7247PVL/ $\Phi$ ST5967PVL, and  $\Phi$ PVL were the major PVL phage types in the isolates studied. Notably, all the untypeable isolates were positive for PCR-3 and belonged to ST30 and ST59 strains, indicating that both the clones may harbor novel unknown icosahedral-head group II phage type(s) in China. In line with the previous observations (Ma et al., 2008; Otter et al., 2010; Boakes et al., 2011; Chen et al., 2013; Sanchini et al., 2014), lineage-specificity of PVL-encoding phages were noted in this study. For instance, 92.3% of  $\Phi$ 7247PVL/ $\Phi$ ST5967PVL-carrying isolates belonged to CC59 lineage, harboring cluster I *attR*, and *attL*, and 60% of  $\Phi$ Sa2USA-hosting isolates pertained to ST398. However, the  $\Phi$ PVL-carrying isolates showed more variability of genetic characterization, with CC22, CC25, CC59, CC88, and CC9 detected within this group.  $\Phi$ Sa119, recently identified in CC5 lineage by Sanchini et al. (2014), was detected in CC59 lineage.  $\Phi$ Sa2958, associated with MRSA belonging to CC30 (Ma et al., 2008), was related to CC22 and CC59 in the present study.

These findings are similar with those reported in China previously (Li et al., 2012; Hu et al., 2015). However,  $\Phi$ 108PVL, which was the predominant phage type in CA-MRSA from

children in mainland China (Li et al., 2012), and  $\Phi$ SLT, which was prevalent in 74 health care-associated PVL-positive MRSA strains (Hu et al., 2015), are not common in the present study. The difference of regions across China and sources of isolates may account for the dissimilarity of the predominant PVL-encoding phages among these studies.

The correlation between the presence of PVL in *S. aureus* infections and the clinical outcome has been controversial because of some conflicting data from epidemiological study or *in vivo* animal models (Hermos et al., 2010; Otto, 2011). Corresponding protein models of two *lukSF-PV* sequence variants, named R and H variant, may indicate certain functional significance (Wolter et al., 2007; John and Lindsay, 2008; O'Hara et al., 2008). In our study, the H- and R-PVL isoforms could be identified in three morphological phage groups. Of note, the R1 isoform was mainly associated with  $\Phi$ Sa2USA, similar to the previous study (Chen et al., 2013). Of the five SNPs in the present study, two were non-synonymous, including a previously described arginine to histidine replacement at amino acid residue 176 (nucleotide 527) and a valine to isoleucine replacement at amino acid 340 (nucleotide 1022). The latter amino acid change presented in only one sequence (LS2074) and decreased the predicted molecular size of LukF from 36962Da to 36948Da, but did not influence the theoretical isoelectric point (9.1). The nucleotide sequence of the *lukSF-PV* genes has been deposited in GenBank under the Accession no. KX443594. Further work is required to confirm whether this non-synonymous replacement results in clinical significance.

Another interesting finding was that all the *attR* cluster I-harboring isolates possessed the same *agr* type (*agr* I) and were mostly observed within CC59 lineage. With the exception of one strain (strain 148), all of the *attR* sequences of cluster II strains were identified in CC22 lineage. The CC88 isolates were completely associated with *attR* cluster III and *attL* cluster IV. The main variations in *attL* were located in the phage binding sites (P1 and P2) and those in *attR* were located in the phage binding site P3 and chromosome binding sites B2. These indicated that the diversity of junction sequences was mainly due to differences in the phage DNA, which was different from the previous report that the main variations in *attR* were located in the 29-bp chromosome binding sites (B2) (Chen et al., 2013).

There were two limitations in this study. First, the identification of PVL-encoding phages was on the basis of PCR assays targeting only a part of the phage genome. Since phages tend to have recombination events, positivity by PCR should be supposed to be a family of PVL-encoding phages with variable genomic portions, instead of being taken as a specific phage (Sanchini et al., 2014; Xia and Wolz, 2014). Second, the proportion of phage-untypable PVL-positive isolates is a little high (32.1%), although the rate is within the range of 16.4–77.6% reported previously (Ma et al., 2008; Boakes et al., 2011; Li et al., 2012; Hu et al., 2015) by the PCR-based scheme. This could be because some unknown or newly published PVL-encoding phages such as  $\Phi$ 7401PVL (Mariem et al., 2013) have not being included in the scheme. Therefore, unknown

PVL-encoding phages remain to be discovered and new primer pairs are expected to be designed to further expand this scheme. Whatever, next-generation sequencing may provide us with a way to achieve perfect epidemiological picture of diversity of PVL-encoding phages, which seems to represent a trend in the future.

## CONCLUSION

This study characterized PVL-encoding phages, the chromosomal phage insertion sites, the polymorphism of *lukSF-PV* genes, and the genetic background of PVL-positive *S. aureus* clinical isolates from China, and found the existence of some correlation among them. Our findings may contribute to the understanding of the epidemiology and evolution of PVL-positive *S. aureus*, and add the evidence that PVL-positive strains disseminating worldwide likely carry distinct PVL phages.

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## AUTHOR CONTRIBUTIONS

HZ, FH performed the experiments; QL designed and conceived the study; HZ, QL, CH analyzed the clinical data and wrote this manuscript; QL, SJ, XX, YZ, BD, and FG collected the clinical samples. All authors read and approved the final manuscript.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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