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Panton-Valentine Leukocidin (PVL)-Positive Health Care-Associated Methicillin-Resistant *Staphylococcus aureus* Isolates Are Associated with Skin and Soft Tissue Infections and Colonized Mainly by Infective PVL-Encoding Bacteriophages

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The emergence of Panton-Valentine leukocidin (PVL)-positive methicillin-resistant *Staphylococcus aureus* (MRSA) is a public health concern worldwide. PVL is associated with community-associated MRSA and is linked to skin and soft tissue infections (SSTIs). However, PVL genes have also been detected in health care-associated (HA) MRSA isolates. The diseases associated with PVL-positive HA-MRSA isolates and the distributions of PVL-encoding bacteriophages in HA-MRSA have not been determined. In this study, a total of 259 HA-MRSA strains isolated between 2009 and 2012 in China from inpatients with SSTIs, pneumonia, and bacteremia were selected for molecular typing, including staphylococcal cassette chromosome *mec* typing, multilocus sequence typing, and staphylococcal protein A gene typing. The PVL genes and PVL bacteriophages in the MRSA isolates were characterized by PCR. Among the tested MRSA isolates, 28.6% (74/259) were PVL positive. The high prevalence of PVL-carrying HA-MRSA was observed to be associated with SSTIs but not with pneumonia or bacteremia. The PVL-positive HA-MRSA isolates were colonized mainly by infective PVL phages, namely, Φ7247PVL, ΦSLT, and ΦSa2958. The distribution of PVL-carrying bacteriophages differed geographically. Our study highlights the potential risk of the emergence of multidrug-resistant HA-MRSA strains with increased virulence.

Staphylococcus aureus, particularly methicillin-resistant *S. aureus* (MRSA), is a major human pathogen. MRSA can cause skin and soft tissue infections (SSTIs), as well as pneumonia, bacteremia, and sepsis (1). The majority of MRSA infections are caused by only a small number of clones. For example, the major clones in the United Kingdom are epidemic MRSA 15 (EMRSA-15) (clonal complex 22 [CC22] and sequence type 22 [ST22]) and EMRSA-16 (CC30 and ST36), whereas in Japan, Hungary, and the United States, the predominant clones are ST5-staphylococcal cassette chromosome *mec* element (SCC*mec*) II, ST239-SCC*mec* III, and ST8-SCC*mec* IV, respectively (1, 2). In China, the major clones are ST239-SCC*mec* III and ST5-SCC*mec* II, while ST59 is the third most common ST (3).

MRSA infections can be caused by either health care-associated (HA) MRSA or community-associated (CA) MRSA. However, CA-MRSA is phenotypically and genotypically different from HA-MRSA (4). CA-MRSA is believed to mainly cause SSTIs in healthy persons without risk factors for MRSA acquisition, and it rarely causes necrotizing pneumonia (5, 6). CA-MRSA typically harbors SCC*mec* type IV (21 to 24 kb) or V (28 kb) and is less resistant to non-β-lactam antibiotics. HA-MRSA persists in hospitals, causes cutaneous and invasive infections, and usually contains larger SCC*mec*, such as types I (34 kb), II (53 kb), or III (67 kb); thus, the resistance of HA-MRSA isolates is not restricted to β-lactam antibiotics, and these pathogens are often resistant to multiple types of drugs (1).

Panton-Valentine leukocidin (PVL), a bicomponent pore-forming cytotoxin assembled by LukS-PV and LukF-PV, has been demonstrated to have a significant function in the pathogenesis of MRSA by targeting polymorphonuclear cells, monocytes, and macrophages (7). Epidemiological studies have revealed that the PVL genes are carried mainly by CA-MRSA (8). However, PVL genes carried by HA-MRSA strains have also been described (9, 10). In China, an analysis conducted between 2005 and 2006 of 702 MRSA isolates from 18 teaching hospitals in 14 cities showed that the carriage rate of PVL genes in MRSA strains was 2.3%, and all PVL-positive isolates were considered to be HA-MRSA based on patient medical records (11). In a teaching hospital in Wenzhou, 11.9% of the HA-MRSA isolates were PVL positive (12). The risk of the prevalent PVL-positive HA-MRSA strains is a se-

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TABLE 1 PCR-based scheme for typing PVL phages

PVL phage	Primer name	Target genes/loci	Size (bp) of PCR product	Reference
Φ108PVL	intF-2/108-aR	*int*, *ant*	4,340	15
ΦSa2958	intF-2/2958-aR	*int*, JP004	2,238	15
ΦSLT	intF-2/SLT-aR	*int*, *ssb*	8,770	15
ΦSa2MW	intF-2/MW2-aR	*int*, *cro*	4,065	15
ΦSa2usa	Sa2USA_F/Sa2USA_R	*phiSLT ORF484-like*	680	14
Φ7247PVL	intF-2/repR	*int*, *rep*	2,965	16

rious concern that can result in the emergence of multidrug-resistant HA-MRSA isolates with increased virulence.

The diffusion of PVL genes to different MRSA lineages is mediated by PVL bacteriophages (also called phages). Seven PVL phages (Φ108PVL, ΦPVL, ΦSLT, ΦSa2958, ΦSa2MW, ΦSa2usa, and Φ7247PVL) have been described in detail (13–18). ΦPVL and Φ108PVL were observed to be defective phages because they carry truncated genes related to tail formation (13, 18). ΦSLT and ΦSa2958 have intact infectious ability, and the infective Φ7247PVL phage can also be induced (15–17). Moreover, the integrase gene is conserved among the PVL-carrying phages, the attachment sites of which are present in various MRSA lineages. Therefore, the spread of PVL phages to different HA-MRSA lineages is expected. HA-MRSA infections can be further divided into hospital-onset or community-onset infections (19). However, whether the distribution of PVL varies among hospital-onset or community-onset infections is unknown.

This study aimed to (i) conduct comprehensive molecular analyses of PVL-positive MRSA isolates recovered from six hospitals in five cities in China, (ii) investigate the frequency of PVL-positive HA-MRSA in diverse clinical syndromes, which include not only SSTIs but also pneumonia and bacteremia, and (iii) characterize the PVL phages among hospital-onset and community-onset HA-MRSA isolates.

MATERIALS AND METHODS

Study population and strain collection. We retrospectively studied hospitalized patients treated between January 2009 and March 2012. The patients were diagnosed with an SSTI, pneumonia, or bacteremia in six hospitals in China, namely, Urumqi General Hospital (Urumqi, Xinjiang Province), Nanfang Hospital and Guangzhou General Hospital (Guangzhou, Guangdong Province), 411 Hospital (Shanghai), Liao Ning North Hospital (Shenyang, Liaoning Province), and Xinqiao Hospital (Chongqing). Staphylococcal SSTIs were defined based on published recommendations (20). The diagnosis of MRSA pneumonia required the presence of respiratory symptoms and signs, such as cough, tachypnea, and rales, consistent findings on a chest X-ray examination or computed tomography (CT) scan, and the positive isolation of MRSA from a sputum specimen and/or blood culture (21). Bacteremic infections of MRSA were defined by patients with positive blood culture results and patients who met the following criteria: exclusion of endocarditis, SSTI, and pneumonia, no implanted prostheses, and follow-up blood cultures conducted on specimens obtained 2 to 4 days after the initial set that did not grow MRSA, as described previously (22).

A total of 259 single-patient MRSA isolates were recovered from respiratory tract secretions, blood, drainage, pus, and wound samples of patients with pneumonia, an SSTI, or bacteremia. Among the selected isolates, 166 strains (including two blood-cultured strains) were recovered from pneumonia patients ($n = 166$) with respiratory symptoms and signs and consistent findings on chest X-rays (70.5% cases) and a CT scan (29.5% cases), and the pneumonia cases showing alveolar infiltrates on chest X-rays may have cavitated. Sixty-six isolates were recovered from patients diagnosed with staphylococcal SSTIs ($n = 66$), including five positive blood culture cases with wound infections. Twenty-seven isolates were recovered from patients with bacteremia ($n = 27$). All the isolates were characterized as staphylococcal strains using a MicroScan WalkAway-40 automatic bacteria analyzer (Dade Behring, USA) and subsequently confirmed to be MRSA isolates through the detection of their *mecA* and *femB* genes using multiplex PCR, as described in previous studies (23, 24).

Definition of HA-MRSA. We used the criteria for HA-MRSA proposed by the Centers for Disease Control and Prevention of the United States (19). HA infections were further classified as either community onset (cases with a health care risk factor but with a culture obtained ≤48 h after hospital admission; the health care risk factors included the presence of an invasive device at the time of admission, history of MRSA infection or colonization, history of surgery, hospitalization, and/or dialysis, or residence in a long-term care facility in the 12 months preceding the culture date) or hospital onset (cases with a culture obtained >48 h after admission, regardless of whether they also had other health care risk factors), as proposed by Klevens et al. (19).

Molecular typing. Genomic DNA was extracted using a TIANamp bacterial DNA kit (Tiangen, Beijing, China). Molecular typing methods, including SCC*mec* typing, multilocus sequence typing (MLST), and staphylococcal protein A (*spa*) gene typing, were applied to the MRSA strains of interest, as described in a previous study (3).

PVL gene detection and characterization of PVL-carrying phages. A 433-bp nucleotide fragment located in the *lukS-PV* and *lukF-PV* operons was amplified by PCR using the primers and conditions described by Lina et al. (5). The integrase gene of the PVL-encoding phages belonging to the Sa2 group was detected using PCR (25).

An eight-PCR assay was developed by Ma et al. (15) to detect five PVL phages. PCR1 and PCR2 detect icosahedral- or elongated-head phages. PCR3 and PCR4 link the morphologies of the phages to PVL genes. PCR5 to PCR8 detect the five PVL-carrying prophages (Φ108PVL, ΦPVL, ΦSLT, ΦSa2958, and ΦSa2MW). However, most of the PCR1-positive strains were also PCR2 positive. Thus, PCR1 and PCR2 are not discriminative. The fragments amplified by PCR3 and PCR4 are >9,000 nucleotides, which can lead to amplification failure. The identification of the third PVL phage type, such as Φ7247PVL, compromised the utility of PCR1 to PCR4. Thus, we used only PCR5 to PCR8 to detect PVL-encoding phages. As noted by Otter et al. (26), the 1,411-bp fragment is not suitable for inferring the presence of ΦPVL. Two additional sets of PCRs were developed by Boakes et al. (14) and Zhang et al. (16) to detect ΦSa2usa and Φ7247PVL. Thus, six PVL phages (Φ108PVL, ΦSLT, ΦSa2958, ΦSa2MW, ΦSa2usa, and Φ7247PVL) were detected using the available PCR primers. The PCR-based scheme for typing of PVL phages is summarized in Table 1.

Statistical analysis. The SPSS statistical software was used to analyze the data. A χ^2 or Fisher's exact test was used to compare categorical variables. All analyses were two-tailed, and a P value of <0.05 was considered statistically significant.

RESULTS

Population characteristics and PVL-positive HA-MRSA-associated diseases. The comparison of the baseline population charac-

TABLE 2 Comparison of baseline population characteristics of patients with HA-MRSA SSTIs, pneumonia, and bacteremia carrying the PVL genes with those of patients with MRSA infections not carrying the PVL genes[a]

Patient characteristic	PVL positive ($n = 74$)	PVL negative ($n = 185$)	P value
Age (mean ± SD) (yr)	46.1 ± 21.3	53.8 ± 20.3	0.007
Age ≥ 70 yr	11/74 (14.9)	37/185 (20.0)	0.337
Female sex	23/74 (31.1)	47/185 (25.4)	0.353
SSTIs	27/74 (36.5)	39/185 (21.1)	0.010
Hospital onset	18/50 (36.0)	32/50 (64.0)	0.242
Community onset	9/16 (56.3)	7/16 (43.7)	
Pneumonia	35/74 (47.3)	131/185 (70.8)	<0.001
Hospital onset	30/154 (19.5)	124/154 (79.5)	0.132
Community onset	5/12 (41.7)	7/12 (58.3)	
Bacteremia	12/74 (16.2)	15/185 (8.1)	0.071
Hospital onset	7/21 (33.3)	14/21 (66.7)	0.060
Community onset	5/6 (83.3)	1/6 (16.7)	

[a] Unless otherwise specified, the data represent the no./total no. (%).

teristics of patients with HA-MRSA SSTIs ($n = 66$), pneumonia ($n = 166$), and bacteremia ($n = 27$) carrying the PVL genes with those of patients with MRSA infections not carrying the PVL genes is shown in Table 2. The patients with PVL-positive HA-MRSA were younger than their PVL-negative counterparts ($P = 0.007$). No significant differences were observed in older people (≥70 years old) or for patient gender ($P = 0.337$ and 0.353, respectively). Among the 259 HA-MRSA isolates, 28.6% (74/259) were PVL positive, as detected by PCR using a pair of primers spanning the *lukS-PV* and *lukF-PV* genes. This PVL carriage rate among HA-MRSA isolates (28.6%) was higher than the rates reported several years ago by Liu et al. (11) and Yu et al. (12).

With regard to the significance of the increasing PVL carriage among HA-MRSA isolates in China, the diseases caused by HA-MRSA carrying the PVL genes and those caused by HA-MRSA not carrying the PVL genes were compared. The rate of PVL-positive HA-MRSA-caused SSTIs (36.5% [27/74]) was higher than that caused by PVL-negative HA-MRSA (21.1% [39/185]) ($P = 0.010$). However, the rate of PVL-positive HA-MRSA-caused pneumonia (47.3% [35/74]) was lower than that caused by PVL-negative HA-MRSA (70.8% [131/185]) ($P < 0.001$). No significant difference was observed in PVL-positive HA-MRSA-caused bacteremia and PVL-negative HA-MRSA-caused bacteremia ($P = 0.071$) because of the small number of bacteremia cases studied (Table 2). These results indicate that PVL-positive HA-MRSA strains are associated with SSTIs, whereas PVL-negative HA-MRSA strains are associated with pneumonia. We also compared the PVL-positive-to-PVL-negative HA-MRSA ratios among hospital-onset and community-onset infections for each disease (SSTIs, pneumonia, or bacteremia). However, no significant difference was observed (Table 2).

MRSA molecular types and PVL gene carriage. The 259 HA-MRSA isolates were subjected to molecular typing to investigate if they carried the PVL genes. The most frequent SCC*mec* type associated with PVL among all isolates was SCC*mec* IV (60.9% [14/23]), followed by SCC*mec* V (50.0% [4/8]), SCC*mec* I (37.5% [3/8]), SCC*mec* III (26.6% [41/154]), and SCC*mec* II (17.7% [11/62]). Of the nontypeable SCC*mec* isolates, 25% (1/4) carried PVL. The MLST results show that the predominant STs of HA-MRSA that carried PVL were ST59 (83.3% [15/18]), ST239 (27.7% [38/137]), ST45 (20.0% [3/15]), and ST5 (16.7% [12/72]). Several minor STs (ST88, ST30, ST267, ST188, and ST217) of the MRSA isolates were also found to carry PVL. Four major *spa* types, namely, t030 (28.6% [20/70]), t037 (31.8% [14/44]), t002 (15.2% [7/46]), t437 (80.0% [12/15]), and several minor *spa* types (t021, t138, t441, t570, t632, t852, t1081, t2460, t7281, t7637, and t7825) were also identified to be PVL positive (Table 3).

Distribution of PVL phages. An MRSA strain typically has more than one prophage integrated in its genome. The integrase gene of a phage is used for its categorization. Seven major groups of prophages have been described, and all known PVL-encoding prophages are in the same group (25). Using the phage group-characterizing primers developed previously, we confirmed that all the 74 PVL-positive HA-MRSA isolates had the same integrase gene, which belongs to the Sa2 integrase group.

The spread and transfer of PVL phages to HA-MRSA hosts may result in increasing PVL gene carriage in HA-MRSA isolates. To verify this condition, we characterized the PVL phages in these PVL-positive MRSA isolates using PVL phage-specific PCR primers described in previous studies (14–16, 18). The PVL phages in 59 MRSA isolates were characterized, whereas the remaining 15 isolates were not determined, indicating the existence of new types of PVL-encoding phages. Four species of PVL phages were characterized, of which the most prevalent was Φ7247PVL (32.2% [19/59]), followed by Φ108PVL (25.4% [15/59]), ΦSLT (23.7% [14/59]), and ΦSa2958 (18.7% [11/59]).

When we analyzed the distribution of PVL phages and the molecular typing results, we did not observe a specific association between the PVL phages and different STs (Table 3). However, the major hospital-onset HA-MRSA clones ST239 and ST5 were colonized mainly by infective PVL phages, namely, Φ7247PVL, ΦSLT, and ΦSa2958. The major community-onset HA-MRSA clone ST59 is colonized by infective and defective PVL phages, namely, Φ7247PVL and Φ108PVL.

Furthermore, we observed that the distribution of the PVL phages differed geographically. Φ108PVL was the most predominant PVL-encoding prophage among the HA-MRSA isolates recovered from Chongqing, whereas Φ7247PVL dominated in Guangzhou, ΦSLT dominated in Shanghai, and ΦSa2958 dominated in Shenyang and Urumqi (Table 4).

TABLE 3 Distribution of PVL-encoding phages in HA-MRSA strains of various genotypes

Clonal complex	Sequence type (no.)	*spa* type(s) (no.)	*SCCmec* type(s) (no.)	PVL phage(s) (no.)
CC239	ST239 (38)	t030 (20), t037 (14), t437 (1), t632 (3)	I (1), II (1), III (36)	Φ7247PVL (11), ΦSa2958 (7), Φ108PVL (6), ΦSLT (5), unknown (9)
CC59	ST59 (15)	t437 (11), t441 (2), t138 (1), t7281 (1)	I (2), III (1), IV (10), V (2)	Φ108PVL (7), Φ7247PVL (6), ΦSa2958 (1), unknown (1)
CC5	ST5 (12)	t002 (7), t2460 (3), t570 (2)	II (9), III (2), IV (1)	ΦSLT (5), ΦSa2958 (3), Φ108PVL (1), Φ7247PVL (1), unknown (2)
CC45	ST45 (3)	t1081 (2), t7825 (1)	III (1), IV (1), V (1)	ΦSLT (1), Φ7247PVL (1), unknown (1)
CC5	ST267 (1)	NT (1)[a]	III (1)	ΦSLT (1)
CC88	ST88 (1)	t7637 (1)	NT (1)	ΦSLT (1)
CC1	ST188 (1)	t189 (1)	V (1)	Unknown (1)
CC22	ST217 (1)	t852 (1)	IV (1)	Φ108PVL (1)
CC30	ST30 (1)	t021 (1)	IV (1)	Unknown (1)
Others	New ST (1)	t2460 (1)	II (1)	ΦSLT (1)

[a] NT, nontypeable.

DISCUSSION

The emerging prevalence of PVL-positive MRSA is a major health concern. PVL genes are seldom detected in HA-MRSA isolates recovered before 2000 and are usually considered to be unambiguous markers of CA-MRSA (4, 8). However, the PVL marker for CA-MRSA was disputed in recent years, not only because the PVL-positive rates in CA-MRSA recovered from certain regions were <10% (27), but also because HA-MRSA was also confirmed to carry the PVL genes. Mariem et al. (9) reported that 21 of 41 (51%) HA-MRSA strains were positive for PVL in Tunisian hospitals, and another study reported that 5.7% of the HA-MRSA isolates were PVL positive in Asian countries (10). The increasing prevalence of PVL-encoding HA-MRSA is a serious concern that may worsen MRSA infections.

Clinically, CA-MRSA with PVL are typically associated with pyogenic SSTIs and life-threatening diseases, most notably necrotizing pneumonia (5, 6). Although the function of PVL as a virulence determinant was questioned in mouse models (28), the increasing carriage of PVL genes in HA-MRSA was hypothesized to affect clinical MRSA infections. To test this hypothesis, the diseases caused by HA-MRSA that carried PVL and those caused by HA-MRSA without PVL were compared. We observed that PVL-positive HA-MRSA was associated with SSTIs but not with pneumonia and bacteremia.

In our study, the overall PVL-positive rate was 28.6% (74/259), which was higher than that reported in two previous studies in which the PVL-positive HA-MRSA rates were 2.3% and 11.9%, respectively (11, 12). The staphylococcal PVL genes are carried by PVL phages that target *S. aureus*. Three groups of structurally distinct PVL phages have been described to date (16). The increasing carriage of PVL genes in a certain MRSA lineage may result from the spread and transfer of PVL-encoding phages. Using the available PVL phage-specific primers (14–16, 18), we characterized 59 PVL-encoding phages belonging to various phage groups, whereas the remaining phages in 15 PVL-positive MRSA isolates were not determined, which would indicate the existence of new PVL-encoding phages that have yet to be characterized, such as the recently characterized PVL phages ΦIND772 and Φ7401 (9, 29). We also determined that hospital-onset HA-MRSA isolates were colonized mainly by PVL phages with high infectivity, such as Φ7247PVL, ΦSLT, and ΦSa2958, which might explain the increasing prevalence of PVL in HA-MRSA isolates.

In a previous study describing the PVL gene sequence variation and PVL phages among *S. aureus* strains collected from children in mainland China, the authors observed that ST59 was the most frequent PVL-positive MRSA clone (30). A national surveillance study of MRSA in China also revealed that ST59 is a major PVL-positive ST (31). In the current study, we revealed a high percentage of PVL-positive ST59 isolates associated with community-onset HA-MRSA infections. These data collectively indicate that PVL-positive ST59 clones are spreading into hospitals. In contrast, several groups have reported PVL-positive ST239 and ST5 MRSA isolates (11, 12, 24, 32, 33). ST239 and ST5 are the major HA-MRSA clones prevalent in mainland China. Φ7247PVL is

TABLE 4 Distribution of different clones carrying PVL-encoding phages by city

City (total no. of clones)	Clone names (no.)
Chongqing (12)	ST59-t437-Φ108PVL (5), ST239-t037-Φ108PVL (2), ST239-t030-Φ108PVL (1), ST59-t138-Φ108PVL (1), ST239-t037-ΦSLT (2), ST239-t030-unknown (1)
Guangzhou (34)	ST239-t037-Φ7247PVL (7), ST239-t030-Φ7247PVL (4), ST59-t437-Φ7247PVL (4), ST59-t441-Φ7247PVL (2), ST5-t002-Φ7247PVL (1), ST45-t1081-Φ7247PVL (1), ST45-t1081-ΦSLT (1), ST5-t002-ΦSa2958 (1), ST59-t437-ΦSa2958 (1), ST239-t030-ΦSa2958 (1), ST59-t7281-Φ108PVL (1), ST239-t037-Φ108PVL (1), ST239-t030-Φ108PVL (1), ST59-t437-Φ108PVL (1), ST217-t852-Φ108PVL (1), ST45-t7825-unknown (1), ST30-t021-unknown (1), ST59-t437-unknown (1), ST239-t437-unknown (1), ST239-t037- unknown (1), ST239-t030-unknown (1)
Shanghai (14)	ST5-t002-ΦSLT (3), ST5-t2460-ΦSLT (2), ST239-t632-ΦSLT (3), ST88-t7637-ΦSLT (1), ST267-NT-ΦSLT (1), New ST-t2460-SLT (1), ST5-t2460-Φ108PVL (1), ST5-t002-unknown (1), ST239-t037-unknown (1)
Shenyang (3)	ST5-t570-ΦSa2958 (2), ST5-t002- unknown (1)
Urumqi (11)	ST239-t030-ΦSa2958 (6), ST239-t030-unknown (4), ST188-t189-unknown (1)

mainly distributed in the ST59 lineage in Taiwan and is infective (16). In this study, we also observed that Φ7247PVL is colonized in ST239 and ST5. The high prevalence of PVL-positive ST59 clones may maintain the PVL phage reservoir for HA-MRSA. The distribution of the characterized 59 PVL phages differed geographically. This situation was similar to that in other areas. In the United States, ΦSa2usa and ΦSa2MW are the main PVL phages, whereas in Japan, Φ108PVL and ΦSa2958 are the main PVL phages (15).

In conclusion, the PVL-carrying HA-MRSA isolates were observed to be associated with patients diagnosed with SSTIs but not with patients with pneumonia or bacteremia. The hospital-onset HA-MRSA strains in this study were observed to be inhabited mainly by the infective PVL-encoding phages. These data may partly explain the increasing prevalence of PVL-encoding HA-MRSA.

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