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# Molecular Characterization and Panton-Valentine Leucocidin Typing of Community-Acquired Methicillin-Sensitive *Staphylococcus aureus* Clinical Isolates

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**Limited comprehensive molecular typing data exist currently for Panton-Valentine leucocidin (PVL)-positive, methicillin-sensitive *Staphylococcus aureus* (PVL-MSSA) clinical isolates. Characterization of PVL-MSSA isolates by multilocus sequence typing (MLST) and *spa* typing in this study showed a genetic similarity to PVL-positive, methicillin-resistant *S. aureus* (PVL-MRSA) strains, although three novel *spa* types and a novel MLST (ST1518) were detected. Furthermore, the detection of PVL phages and haplotypes in PVL-MSSA identical to those previously found in PVL-MRSA isolates highlights the role these strains may play as precursors of emerging lineages of clinical significance.**

The 1999 CDC report of four pediatric deaths involving the so-called community-acquired, methicillin-resistant *Staphylococcus aureus* (CA-MRSA) isolates and the association of these strains with Panton-Valentine leucocidin (PVL) (8, 18) was followed by the rapid emergence of CA-MRSA, fueling the growing incidence of MRSA worldwide. CA-MRSA strains are striking in their ability to cause infection in young, apparently healthy, immunocompetent hosts, sometimes with severe and fatal outcomes. While the strong epidemiological link between PVL and CA-MRSA is compelling, the precise role of the toxin in virulence and pathogenesis is yet to be elucidated. This bicomponent, pore-forming toxin, encoded by a highly conserved ≈1.9-kb *lukSF-PV* locus consisting of two adjacent, cotranscribed *lukF* and *lukS* genes (26), has 12 major single nucleotide polymorphisms (SNPs), the majority of which are synonymous. A nonsynonymous mutation at position 527, however, serves as the basis of the H and R isoforms (2, 12, 22, 28). Currently, though most research is focused on PVL-positive MRSA (PVL-MRSA), a rising incidence of PVL-positive, methicillin-sensitive *S. aureus* (PVL-MSSA) infections reported recently is the main contributing factor in the increased incidence of PVL-positive strains in some locales, with approximately 60% of total PVL- positive *S. aureus* isolates in England in the past 5 years found to be susceptible to methicillin (6, 16, 29). With clinical and epidemiological characteristics similar to those of CA-MRSA (9, 25), PVL-MSSA may represent a hitherto-unrecognized, overlooked emerging public health threat. Several studies have recently attempted to address this information imbalance (7, 21, 27, 29); however, only two of these reported on PVL gene polymorphisms and their implications (7, 29). In addition to contributing to the limited molecular typing data on PVL-MSSA strains, this study sought to explore PVL gene polymorphisms and phage distribution in this group and how this relates with those previously observed in PVL-MRSA strains. This would aid current understanding of the evolution and emergence of PVL-positive CA-MRSA isolates and help to more accurately assess the current threat posed by these strains.

Nineteen PVL-MSSA clinical isolates recovered by the microbiology laboratory at the Nottingham University Hospitals NHS Trust (NUHT) based on either clinical suspicion or an antibiogram of gentamicin/trimethoprim resistance which had been associated locally with PVL positivity, submitted to the Health Protection Agency's national *Staphyloccus* Reference Unit (SRU) for PVL testing, were analyzed in this study. The isolates were representative of a range of sample types, clinical histories, and patient ages (Table 1) and had no known epidemiological links, with the exception of two clusters; TS6 and TS9 were recovered from different patients on the same hospital ward, while TS18 and TS24 were isolated from different samples of an unrelated patient and TS17 from a relative of this patient. The presence or absence of 13 toxin genes (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, *tst*, *eta*, *etb*, and *etd*) was determined in this population using multiplex PCRs as described previously (1, 20). Sequence-based multilocus sequence typing (MLST) and *spa* typing methods were used in genotyping of isolates. All isolates were *spa* typed using the Ridom GmbH *spa* website, www.spaserver.ridom.de, following sequencing of the variable X region of the *spa* gene as previously described (15), and multilocus sequence types were mapped via the *spa* http://spa.ridom.de/mlst.shtml database. Representative isolates were further characterized by MLST (14) via the *S. aureus* database, http://saureus.mlst.net/. Following amplification and sequencing of two internal fragments of 764 bp and 535 bp in the *lukS* and *lukF* loci, respectively, using the primers LukSF (ATGGTCAAAAAAG ACTATTAGCTG), LukSR (TCAAATTCACTTGTATCTCCTG AG), LukFF (TCAGTAAACGTTGTAGATTATGCACC) and LukFR (nATTTTCATCTTTATAATTATTACCTATC); PVL types were determined based on these sequences. Specific PVL-encoding phage types were detected by the use of nine PCRs

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**TABLE 1** Clinical characteristics of Panton-Valentine leucocidin-positive *Staphylococcus aureus* test isolates

| Clinical characteristic/disease pathology | No. of isolates for patient age group (yrs) | | | | |
|---|---|---|---|---|---|
| | 0–19 | 20–39 | 40–59 | >60 | Total |
| Skin and soft tissue infections | 2 | 5 | 0 | 5 | 12 |
| Bacteremia | 0 | 1 | 0 | 1 | 2 |
| Colonization | 0 | 1 | 1 | 0 | 2 |
| Empyema | 1 | 0 | 0 | 0 | 1 |
| Pneumonia | 0 | 1 | 1 | 0 | 2 |
| Total isolates | 3 | 8 | 2 | 6 | 19 |

(4, 19) which detect six PVL-encoding phages (Table 2), as well as uncharacterized PVL phages classed as either icosahedral or elongated head types.

The 19 PVL-MSSA isolates, comprising both hospital- and community-acquired isolates recovered between November 2008 and May 2009 (Table 2), were predominantly from skin and soft tissue infections, with a single isolate (TS1) recovered from a fatal case of necrotizing pneumonia. A low prevalence of virulence/toxin genes was noted in this population, with *seg* and *sei* genes carried by 89.5% (17/19) of isolates. Only sequence type 722 (ST722) isolates encoded up to 4 of the 13 toxin genes analyzed (Table 2); the *seb* and *tst* genes were not detected in this study group. In a similar trend, 89.5% (17/19) of isolates were trimethoprim resistant, with additional gentamicin resistance observed in 57.9% (11/19) of isolates. The highest resistance (to 4 antibiotics) was observed in a single ST30 isolate (TS12). However, all isolates were susceptible to clindamycin, rifampin, linezolid, vancomycin, fusidic acid, and teicoplanin. Genotyping using the *spa* technique revealed 11 types clustered into 2 *spa* clonal clusters (CCs) (CC005 and CC345/657) and 5 singletons based on the BURP algorithm (StaphType software program; Ridom GmbH, Wurzburg, Germany). Most frequent (47.4%) were t005 and t021, while 7 other types were represented by only a single test isolate. We identified 3 *spa* types (t6642, t6643, and t6769) that have not previously been described. Strain diversity was further noted with the detection of 6 MLST STs grouped by eBURST software analysis into 5 CCs of known MRSA lineages (CC1, CC22, CC30, CC88, and CC152). ST22, which has been specifically associated with gentamicin and trimethoprim resistance (5), occurred most frequently ($n = 9$ [47.4%]). ST1518 (CC152) was identified for the first time ever in this study. This strain, which was isolated from a fatal case of necrotizing pneumonia, is a single-locus variant of ST152 differing by a single mutation in the *glp* allele.

Compared with ΦSLT, the proposed *lukSF-PV* progenitor (30), a total of seven SNPs were noted in the PVL gene sequences of the study strains, five of which occurred in the *lukS* locus (Table 2). Most isolates ($n = 17$; 89.5%) were of the H variant as defined by O'Hara et al. (22), with both H1 and H2 groups present. The nonsynonymous nucleotide 527 A-to-G mutation which defines the R variant occurred only in a single isolate (TS1). In general, the resulting PVL SNP profiles were MLST specific but not exclusively. ST30 isolates exhibited two different PVL SNP profiles (H1 and H2). The outlier H1 profile carried by TS12 was homologous to that of ST22 and ST88 and was found in 63.2% of isolates (12/19). Four known phage types (ΦPVL, Φ108PVL, ΦSa2USA, and ΦSa2mw) were detected in the strains studied. For both ST772 isolates, all phage PCRs were negative, pointing at an unknown phage type. The phage present in TS21 could only be described based on its morphology. A direct relationship was noted between the phage types and clonal lineage in the majority of cases. While all ST22 isolates carried ΦPVL with its corresponding PVL SNP H2 profile, the ST30 isolates showed more variability, with both ΦPVL and Φ108PVL detected within this group. A general lack of correlation of PVL phage type with PVL SNP profile occurred in this group and in the ST1 and ST88 isolates. Carriage of multiple phage types by a single isolate was noted in TS25. Comparing the epidemiologically related isolates, the TS6/9 cluster had identical characteristics, and though TS18 and TS24 from the same patient were identical, TS17 from a relative differed at the *spa* locus (Table 2).

While the molecular epidemiology of CA-MRSA has been explored extensively (10, 11) and interest in CA-MSSA is on the increase, there exists a lack of detailed knowledge on PVL-MSSA, particularly in relation to the genetics of the *lukSF-PV* locus. Represented among the MSSA isolates in this study were major sequence types (ST22, ST88, ST30, and ST1) associated with PVL-MRSA clones (21, 27, 29). Though the majority of PVL-MRSA clones circulating in the United Kingdom belong to either ST8, ST30, or ST80 (13, 17, 23), the predominant group described in this study, ST22, also constitute a significant burden of infection (13). These strains are genetically similar in susceptibility and toxin profile, as well as *spa* type, to the recently described ST22 PVL-MRSA United Kingdom strains, rather than the predominant hospital-acquired MRSA (HA-MRSA) ST22 EMRSA-15 clone (5). This study observed the previously noted geographical and clonal bias in the distribution of the PVL haplotypes, with the R haplotype limited to a few lineages, predominantly in the United States and more recently Australia (22, 29). Due to previous reports of R haplotypes being found in the United Kingdom in significant numbers of MRSA strains (4, 24), the dearth of R haplotypes in this small study population may reflect more on the methicillin susceptibility of the isolates rather than geographical location. No significant functional and structural differences are thought to result from the arginine (R)-to-histidine (H) mutation present in this haplotype (2, 3); however, it is of interest that the only described R haplotype in this study was linked with a fatal case of necrotizing pneumonia in a healthy adult. One evolutionary pathway postulated for the development of CA-MRSA is the acquisition of PVL genes prior to that of the SCC*mec* element. This view of a high genetic similarity between PVL-MSSA and MRSA strains (27) was reinforced in this study, where a number of the PVL-MSSA clinical isolates were found to possess *spa* and toxin gene profiles identical to those of previously described PVL-MRSA clones. The number of MSSA isolates in this study having PVL isoforms identical to those of their previously described MRSA counterparts also lends further credence to this hypothesis (2, 4, 12). This may not be limited to a single evolutionary event. The detection of several PVL phage types observed in the ST30 lineage in this study and described by Boakes and colleagues in two separate studies (4, 5) could perhaps hint at the possibility of multiple phage acquisitions and hence evolutionary events, though this remains to be verified.

In conclusion, due to the gentamicin/trimethoprim resistance bias in isolate selection, this study provides one of the most com-

**TABLE 2** Molecular characterization of 19 PVL-positive *Staphylococcus aureus* clinical isolates[a]

| Strain ID or phage (GenBank accession no.) | Date of isolation[b] (source) | ST (CC) | *spa* type[c] | Antibiogram[d] | Virulence profile | Nucleotide at gene position: *lukS* 33 | *lukS* 105 | *lukS* 181 | *lukS* 216 | *lukS* 345 | *lukS* 470 | *lukS* 527 | *lukS* 663 | *lukF* 1396 | *lukF* 1729 | Sequence isoform | PVL phage typing PCR result[e]: 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | PVL type | PVL isoform |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TS13 | 17/03/09 (CA) | 22 (22) | t005 | Gent, Trim R | *seg*, *sei* | G | T | T | C | C | T | A | G | G | A | H1 | + | + | + | | +* | | | | | ΦPVL | H1 |
| TS14 | 04/03/09 (HA) | 22 (22) | t005 | Gent, Trim R | *seg*, *sei* | G | T | T | C | C | T | A | G | G | A | H1 | + | | + | | +* | | | | | ΦPVL | H1 |
| TS18 | 11/11/08 (CA) | 22 (22) | t005 | Gent, Trim R | *seg*, *sei* | G | T | T | C | C | T | A | G | G | A | H1 | + | | + | | +* | | | | | ΦPVL | H1 |
| TS20 | 03/02/09 (CA) | 22 (22) | t005 | Gent, Trim R | *seg*, *sei* | G | T | T | C | C | T | A | G | G | A | H1 | + | | + | | +* | | | | | ΦPVL | H1 |
| TS24 | 06/11/08 (CA) | 22 (22) | t005 | Gent, Trim R | *seg*, *sei* | G | T | T | C | C | T | A | G | G | A | H1 | + | | + | | +* | | | | | ΦPVL | H1 |
| TS19 | 30/12/08 (CA) | 22 (22) | t310 | Gent, Trim R | *seg*, *sei* | G | T | T | C | C | T | A | G | G | A | H1 | + | | + | | +* | | | | | ΦPVL | H1 |
| TS17 | 04/12/08 (CA) | 22 (22) | t852 | Gent, Trim R | *seg*, *sei* | G | T | T | C | C | T | A | G | G | A | H1 | + | | + | | +* | | | | | ΦPVL | H1 |
| TS6 | 06/05/09 (HA) | 22 (22) | t6642 | Gent, Trim R | *seg*, *sei* | G | T | T | C | C | T | A | G | G | A | H1 | + | | + | | +* | | | | | ΦPVL | H1 |
| TS9 | 07/04/09 (HA) | 22 (22) | t6642 | Gent, Trim R | *seg*, *sei* | G | T | T | C | C | T | A | G | G | A | H1 | + | | + | | +* | | | | | ΦPVL | H1 |
| TS23 | 16/12/08 (CA) | 22 (22) | t6643 | Gent, Trim R | *seg*, *sei* | G | T | T | C | C | T | A | G | G | A | H1 | + | | + | | +* | | | | | ΦPVL | H1 |
| TS1 | 18/03/09 (CA) | 1518 (152) | t1941 | Fully sens | None | G | T | T | C | C | T | G | T | A | G | R | | + | | + | +* | | | | + | ΦSa2USA | R |
| TS5 | 23/04/09 (CA) | 772 (1) | t657 | Trim, Cipro, Ery R | *sea*, *sec*, *seg*, *sei* | G | T | T | C | C | T | A | G | A | A | H2 | | | | | | | | | | | |
| TS8 | 08/04/09 (CA) | 772 (1) | t345 | Trim, Cipro, Ery R | *sea*, *sec*, *seg*, *sei* | G | T | T | C | C | T | A | G | A | A | H2 | | | | | | | | | | | |
| TS25 | 05/12/08 (CA) | 1 (1) | t3342 | Trim R | *sed*, *seg*, *sei* | A | T | T | C | T | T | A | G | A | A | H2 | + | + | | | +* | | + | | | ΦPVL/ΦSa2mw | H1/R |
| TS7 | 04/04/09 (CA) | 30 (30) | t021 | Trim, Doxy R | *sea*, *seg*, *sei* | G | T | T | C | C | A | A | G | A | A | H2 | + | | + | | +* | | | | | ΦPVL | H1 |
| TS12 | 18/03/09 (CA) | 30 (30) | t021 | Gent, Trim, Cipro, Ery R | *seg*, *sei* | G | T | T | C | C | T | A | G | G | A | H1 | + | | + | | +* | | | | | ΦPVL | H1 |
| TS15 | 12/02/09 (CA) | 30 (30) | t021 | Fully sens | *seg*, *sei* | G | T | T | C | C | A | A | G | A | A | H2 | + | | + | | +** | | | | | Φ108PVL | H2 |
| TS16 | 11/02/09 (CA) | 30 (30) | t021 | Trim R | *sea*, *seg*, *sei* | G | T | T | C | C | A | A | G | A | A | H2 | + | | + | | +* | | | | | ΦPVL | H1 |
| TS21 | 11/02/09 (HA) | 88 (88) | t6769 | Trim R | None | G | T | T | C | C | T | A | G | G | A | H1 | | + | | + | +* | | | | | Elongated head | |
| ΦSLT (NC_002661) | | | | | | G | T | T | C | C | T | A | G | A | A | | | + | | + | | | | + | | ΦSLT | H2 |
| Φ108PVL (AB243556.1) | | | | | | G | T | T | C | C | T | A | G | A | A | | + | | + | | +** | | | | | Φ108PVL | H2 |
| ΦPVL (AB009866.2) | | | | | | G | T | T | T | C | T | A | G | G | A | | + | | + | | +* | | | | | ΦPVL | H1 |
| Φ2958PVL (AP009363.1) | | | | | | G | T | T | C | C | T | A | G | G | A | | | + | | + | | + | | | | Φ2958PVL | H1 |
| ΦSa2mw (BA000033) | | | | | | G | T | T | C | C | T | G | T | A | A | | | + | | + | | | + | | | ΦSa2mw | R |
| ΦSa2USA (CP000730.1) | | | | | | G | T | T | C | C | T | G | T | A | G | | | + | | + | | | | | + | ΦSa2USA | R |

[a] Total toxin genes analyzed include *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, *tst*, *eta*, *etb*, and *etd*. ID, identifier; HA (hospital associated), isolated after 48 h of admission; CA, isolated either at the general practice or accident and emergency department or within 48 h of admission.

[b] Day/mo/yr.

[c] The *spa* types underlined were described for the first time in this study.

[d] R, resistant; Gent, gentamicin; trim, trimethoprim; Cipro, ciprofloxacin; Ery, erythromycin; Doxy, doxycycline; sens, sensitive.

[e] "*" and "**" indicate 1,411 bp and 4,340 bp, respectively.

prehensive in-depth analyses of PVL-MSSA ST22 isolates incorporating data on the PVL genes to date. It also highlights the important role PVL-MSSA strains generally play as reservoirs for PVL-MRSA strains due to their direct evolutionary links as seen from the typing results. The data emphasize the need for increased surveillance, which may form the basis for intervention strategies and help curb the emergence and clonal expansion of PVL-MRSA.

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