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Rapid Detection of Panton-Valentine Leukocidin in *Staphylococcus aureus* Cultures by Use of a Lateral Flow Assay Based on Monoclonal Antibodies

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Panton-Valentine leukocidin (PVL) is a virulence factor of *Staphylococcus aureus*, which is associated with skin and soft-tissue infections and necrotizing pneumonia. To develop a rapid phenotypic assay, recombinant PVL F component was used to generate monoclonal antibodies by phage display. These antibodies were spotted on protein microarrays and screened using different *lukF-PV* preparations and detection antibodies. This led to the identification of the optimal antibody combination that was then used to establish a lateral flow assay. This test was used to detect PVL in *S. aureus* cultures. The detection limit of the assay with purified native and recombinant antigens was determined to be around 1 ng/ml. Overnight cultures from various solid and liquid media proved suitable for PVL detection. Six hundred strains and clinical isolates from patients from America, Europe, Australia, Africa, and the Middle East were tested. Isolates were genotyped in parallel by DNA microarray hybridization for confirmation of PVL status and assignment to clonal complexes. The sensitivity, specificity, and positive and negative predictive values of the assay in this trial were 99.7, 98.3, 98.4, and 99.7%, respectively. A total of 302 clinical isolates and reference strains were PVL positive and were assigned to 21 different clonal complexes. In summary, the lateral flow test allows rapid and economical detection of PVL in a routine bacteriology laboratory. As the test utilizes cultures from standard media and does not require sophisticated equipment, it can be easily integrated into a laboratory's workflow and might contribute to timely therapy of PVL-associated infections.

Panton-Valentine leukocidin (PVL) is a phage-borne virulence factor of *Staphylococcus aureus*. It consists of two units (S and F components) encoded by two separate, although colocalized and coexpressed, genes (*lukS-PV* and *lukF-PV*; GenBank accession number BA000033.2; open reading frame [ORF] no. MW1378 and MW1379). Polymers of these molecules form pores in human leukocyte membranes, leading to cell death (1, 2) and cytokine release. Alternatively, low concentrations may induce apoptosis in granulocytes (3).

PVL is structurally, and in terms of sequence similarities, related to other leukocidins, such as *lukE–lukD* and *lukM–lukF-P83* in *S. aureus* and *lukF-int–lukS-int* in *S. intermedius*/*pseudintermedius*, and to the *hlgA–lukF–lukS* gamma-hemolysin/leukocidin locus *hlg*.

PVL is frequently detected in *S. aureus* isolates from skin and soft-tissue infections (SSTI) (4, 5) and is associated with chronic/recurrent infections, such as furunculosis, especially in young and previously healthy adults. PVL-positive *S. aureus* can also cause more severe diseases, such as necrotizing pneumonia. This condition is occasionally a complication of other respiratory tract infections, such as influenza virus, and its fatality rate can be as high as 40% (6). In contrast, PVL is rarely isolated in *S. aureus* from healthy carriers (7–9) or from isolates associated with other types of infections, such as bacteremia (10).

Although PVL was described in the 1930s (11), its existence as a potent leukotoxic toxin produced by some *S. aureus* strains was postulated already in the late 19th century (12). In the 1940s and 1960s, worldwide outbreaks of PVL-positive, methicillin-susceptible *S. aureus* were observed, and by the late 1990s, PVL-positive community-acquired methicillin-resistant *S. aureus* (CA-MRSA) had emerged (13, 14).

Because of its clinical relevance, the detection of *S. aureus* strains which carry the PVL genes warrants aggressive therapy and infection control measures. This includes, in addition to incision and drainage, appropriate antibiotic therapy and the use of mupirocin, in analogy to MRSA eradication protocols. In addition, it is recommended that patients with an acute infection due to PVL-

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TABLE 1 Simplified matrix for the identification of the optimal antibody combination[a]

Labeling antibody	Reactivity for capture antibody and antigen											
	1031			1061			1101			1321		
	Recombinant LukF	Culture supernatant LukF-P83	Culture supernatant ATCC 25923	Recombinant LukF	Culture supernatant LukF-P83	Culture supernatant ATCC 25923	Recombinant LukF	Culture supernatant LukF-P83	Culture supernatant ATCC 25923	Recombinant LukF	Culture supernatant LukF-P83	Culture supernatant ATCC 25923
1031	0	(+)	0	0	0	0	0	+	0	0	(+)	0
1061	0	(+)	0	0	0	0	0	+	0	(+)	++	0
1101	0	+	0	0	(+)	0	0	(+)	(+)	0	++	0
1321	0	+	0	0	(+)	0	(+)	++	0	0	(+)	0
1401	+	++	0	0	+	0	0	+	++	0	+	0
1451	(+)	++	0	0	0	0	++	+++	(+)	+++	+++	0
1631	0	0	0	0	0	0	0	0	0	0	(+)	(+)
1711	0	++	0	0	+	0	(+)	++	0	0	+	0
1771	+	++	0	0	0	0	++	+++	(+)	+++	++++	0
1841	+	+++	0	0	0	0	+++	++++	++	+++	++++	0
1881	0	(+)	(+)	0	0	0	0	(+)	0	0	(+)	0

[a] 0, No reactivity; (+) to +++, weak to strong reactivities, respectively, based on multiple experiments under various conditions.

positive *S. aureus* should not work as health care providers or attend gyms, swimming pools, or saunas (http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1218699411960).

To date, PVL detection is primarily achieved using a molecular method that is essentially limited to reference centers and specialized laboratories with equipment and experience to perform such assays. To facilitate the rapid, nonmolecular detection of PVL by routine clinical microbiology laboratories, we have developed a lateral flow test using monoclonal antibodies. We validated the assay against isolates grown in a variety of different culture media and then evaluated the assay using an international collection of *S. aureus* strains recovered from SSTI.

MATERIALS AND METHODS

Recombinant PVL, F component. The PVL F component gene (*lukF-PV*) was amplified using primers designed to include an EcoRI restriction site and a NotI restriction site at the 5′ and 3′ ends (lukF-PV_fw_5Eco, 5′-CCTGAATTCATGAAAAAAATAGTCAAATC-3′; lukF-PV_rev_5Not, 5′-ATAGCGGCCGCTTAGCTCATAGGATTTT-3′). DNA from the fully sequenced ST1-MRSA-IV reference strain MW2 was used as the template. PCR products were cloned into a commercially available vector (TOPO II; Invitrogen, Karlsruhe, Germany) and sequenced. Resulting sequences were compared to the corresponding GenBank entry (BA000033.2; nucleotide coordinates 1529381 to 153035). Confirmed clones were cut with EcoRI and NotI, and the DNA fragments containing the open reading frame were inserted into the pet28a expression vector (Novagen, Darmstadt, Germany). After ligation, the expression vector was transformed into the *Escherichia coli* strain BL21 (ATCC BAA-1025). Expression of recombinant proteins was achieved in 50 ml lysogeny broth (LB) medium (supplemented with kanamycin) after induction with 50 ml isopropyl β-D-1-thiogalactopyranoside (IPTG; 1 mM). *E. coli* cells were collected by centrifugation and frozen overnight. Expressed recombinant proteins were purified on nickel-nitrilotriacetic acid-agarose (Ni-NTA-agarose) columns (Qiagen, Hilden, Germany) by following the manufacturer's instructions. Aliquots were taken after each step and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the presence of the recombinant protein. Protein concentrations of each sample were determined using the bicinchoninic acid (BCA) protein assay kit (Pierce, Bonn, Germany).

Phage display procedures and initial testing of antibodies. Overexpressed PVL F component was used to generate monoclonal antibodies via phage display. Following immunization of mice, mRNA from B cells was isolated and amplified. Resulting cDNA, specific for the antigen-binding parts of antibodies, was ligated into bacteriophages and then transformed into *E. coli*. Resulting antibodies were purified and characterized initially by enzyme-linked immunosorbent assay (ELISA), and different dilutions were spotted onto microtiter strip-mounted protein microarrays.

Antibody microarrays were used according to previously described protocols (15–18).

For the selection of the optimal combination of capture and labeling antibodies, five different concentrations of each of the 11 selected antibodies were spotted onto protein microarrays. These arrays were tested with recombinant PVL F component, native PVL (in two different concentrations; from the CC30-MSSA strain ATCC 25923), or bovine leukocidin LukM-LukP83 (from a veterinary CC151/705 isolate) as antigens as well as with biotin-labeled preparations of all 11 antibodies as detection antibodies in order to test all possible combinations. Staining then was performed by streptavidin-horseradish peroxidase conjugate and by peroxidase-triggered dye precipitation.

This approach allowed the determination of the optimal combination of capture and detection antibodies (Table 1).

Principles of the lateral flow assay for PVL. The lateral flow assay to detect PVL from primary cultures of *S. aureus* is an immunochromatographic membrane assay that uses the two highly sensitive phage display recombinant monoclonal antibodies selected by the microarray described above. The two selected antibodies against PVL were used to design a lateral flow test where one of the antibodies is used for antigen capture on the test strip while the second is gold labeled and coated in a reaction tube.

The PVL assay device consisted of a test strip, reaction tube, and dropper bottle containing the extraction buffer. The test strip consists of the PVL capture antibody and control protein immobilized onto a membrane support forming two distinct lines. The addition of sample and absorbent pad makes the test strip complete. The control protein is a recombinant antigen not related to *S. aureus*. The reaction tube contains lyophilized, gold-conjugated labeling monoclonal antibodies for both PVL and the control protein.

When performing the test, *S. aureus* isolates or culture supernatants are added to the coated reaction tube. A test strip for the PVL assay is then placed into the reaction tube holding the liquid sample and conjugate. Test results are interpreted after 10 min based on the presence or absence of pink- to purple-colored sample lines. Two bands (PVL line and control line) indicate a valid positive result, and one band (control line) indicates a valid negative result. The absence of a visible control line was interpreted as an invalid test.

Performing the assay. The test was applied to isolates of *S. aureus* from SSTIs (see below) that were also genotyped by microarray hybrid-

TABLE 1 (Continued)

Reactivity for capture antibody and antigen											
1401			1451			1631			1711		
Recombinant LukF	Culture supernatant LukF-P83	Culture supernatant ATCC 25923	Recombinant LukF	Culture supernatant LukF-P83	Culture supernatant ATCC 25923	Recombinant LukF	Culture supernatant LukF-P83	Culture supernatant ATCC 25923	Recombinant LukF	Culture supernatant LukF-P83	Culture supernatant ATCC 25923
(+)	+	0	(+)	++	(+)	0	0	0	0	0	(+)
0	+	0	0	(+)	0	0	0	0	0	+	0
0	(+)	+++	+	++	0	0	0	0	0	+	0
0	(+)	0	++	+++	0	0	0	0	0	0	0
0	(+)	++++	++	+++	+	0	(+)	0	0	(+)	0
+	++	++	0	++	0	0	(+)	0	++	+++	0
0	0	0	0	(+)	0	0	0	0	0	0	0
0	+	0	+++	+++	(+)	0	0	0	0	(+)	0
+	+++	++	(+)	++	(+)	0	(+)	0	++	+++	0
++	+++	+++	0	++	0	0	(+)	0	+++	++++	0
0	(+)	0	0	(+)	(+)	0	(+)	(+)	0	(+)	(+)

ization to determine strain and clonal complex affiliation and their PVL status. Specifically, 280 μl of extraction reagent was added to the coated reaction tube containing lyophilized antibody-Au conjugate. An inoculation loop of *S. aureus* colony material (approximately 10 μl) was harvested, placed into the tube, and thoroughly mixed using the inoculation loop until both cells and conjugate pellet were completely dissolved. When using liquid growth media, 200 μl of buffer and 100 μl of overnight liquid culture were added to the reaction tube and mixed. The test strip was then inserted into the reaction tube. After 10 min of incubation at room temperature, the test strip was withdrawn from the tube and read.

Strains and isolates. A total of 600 *S. aureus* strains and isolates were tested for LukF-PV production, including both methicillin-susceptible *S. aureus* (MSSA) and MRSA strains.

PVL-negative reference strains were Sanger MSSA476 (a sequenced ST1-MSSA-SCC*fus*, i.e., with a staphylococcal casette chromosome element harboring *fusC*; GenBank accession number BX571857.1), Mu50 and N315 (both sequenced ST5-MRSA-II strains; GenBank accession numbers BA000017.4 and BA000018.3), NCTC 8325 (a sequenced ST8-MSSA strain; GenBank accession number CP000253.1), and COL (a sequenced CC8/ST250-MRSA-I isolate; GenBank accession number CP000046.1), as well as West Australian (WA) MRSA-8 (ST75-MRSA-IV 03-17848 [19]) and WA-MRSA-59 (a CC12-MRSA strain with an atypical staphylococcal cassette chromosome *mec* element [SCC*mec*] [20]).

PVL-positive reference strains were MW2-USA400 (a sequenced ST1-MRSA-IV strain; GenBank accession number BA000033.2), USA300-FPR3757 (a sequenced ST8-MRSA-IV strain; GenBank accession number CP000255.1), ATCC 25923 (a historic ST30-MSSA isolate widely used in diagnostic microbiology for quality control purposes [21]), Queensland CA-MRSA (ST93-MRSA-IV 03-16790 [19]), and WA-MRSA-60/Bengal Bay CA-MRSA (ST772-MRSA-V [20]).

In addition, 588 clinical isolates were included that were collected from patients with SSTI.

The clinical isolates originated from Australia (as part of the country-wide Australian Group for Antimicrobial Resistance *Staphylococcus aureus* Surveillance Programs SAP 2008 and SAP 2010 [http://www.agargroup.org/files/FED%20REPORT%20SAP2008%20MRSA%20final.pdf and http://www.agargroup.org/files/FED%20REPORT%20SAP210%20MRSA%20FINAL%20shrink.pdf, respectively]). Further isolates came from diagnostic laboratories in Germany (University Hospital Dresden), Saudi-Arabia (King Fahad Medical City, Riyadh), Spain (Hospital Universitari Germans, Trias i Pujol, Badalona), Sweden (Oerebro University Hospital), Trinidad and Tobago (from various regional hospitals in the country), Uganda (Medical Research Council in Entebbe), and England (including a hospital in the southwest, Bristol, and the national Staphylococcus Reference Unit, HPA, London). The PVL status of all of the isolates from England and of 17 from other countries (eight from Saudi Arabia, seven from Germany, and three from Australia) was known. These isolates were included to maximize representation of a broad range of clonal complexes, but they were excluded from analysis of PVL rates in the different countries of origin.

In addition, 17 isolates were tested for LukF-P83, including 14 *lukM–lukF-P83*-positive isolates from veterinary sources (cattle and goats) belonging to livestock-associated lineages CC133, CC151/705, and CC479. These isolates were from a previous study (22) or were referred from the Friedrich Loeffler Institute, Jena, Germany (courtesy of K. Schlotter [23]). For control purposes, three *lukM–lukF-P83*-negative isolates were included: two CC133 isolates, one from a mute swan (courtesy of D. Gavier-Widén) and one from a human from Dresden University Hospital, and a CC479 isolate from cattle (courtesy of K. Schlotter). No *lukF-P83*-negative CC151/705 isolates were available for testing.

Full datasets for the individual isolates, including results from lateral flow tests, PCRs, and array hybridization profiles, are available in the supplemental material.

Validation of the lateral flow assay using different culture media. Liquid growth media included glucose bouillon (bouillon by Oxoid, Wesel, Germany, with glucose added), brain-heart infusion (Oxoid), 2× TY (tryptone peptone-yeast extract), and Schaedler bouillon with vitamin K3 (bioMérieux, Nürtingen, Germany). A broth comprising yeast extract, Casamino Acids, sodium glycerophosphate, lactate, citric acid, $FeSO_4$, Na_2HPO_4, KH_2PO_4, $MgSO_4$, and $MnSO_4$ that was described by Noda and Kato (24) as being suitable for the production of PVL was also tested.

The following solid media were used: nutrient agar (Oxoid), Mueller-Hinton agar (Oxoid) with and without blood added, Columbia blood (Oxoid), Columbia blood agar with aztreonam and polymixin (CAP), chocolate agar (agar base and sheep blood [Oxoid] plus hemin [Serva] and NAD [Merck]), and commercially available chromogenic MRSA medium (MRSA ID agar; bioMérieux).

Array procedures. For confirmation of PVL status and for assignment to clonal complexes and strains, all isolates were characterized by DNA microarray hybridization (StaphyType; Alere, Jena, Germany).

Following enzymatic lysis using lysostaphin, lysozyme, and RNase, as well as a Qiagen kit buffer and proteinase K, DNA was prepared using commercially available spin columns (Qiagen, Hilden, Germany) or an automated system (Qiagen EZ1) (25).

The following array procedures were carried out in accordance with the manufacturer's instructions; primers, probes and further details have been described previously (26, 27). Briefly, a multiplex primer elongation was performed that amplified and labeled (by incorporation of biotin-16-dUTP) a total of 333 target sequences corresponding to ca. 170 genes. Single-stranded amplification products were hybridized against microar-

TABLE 1 (Continued)

Reactivity for capture antibody and antigen								
1771			1841			1881		
Recombinant LukF	Culture supernatant LukF-P83	Culture supernatant ATCC 25923	Recombinant LukF	Culture supernatant LukF-P83	Culture supernatant ATCC 25923	Recombinant LukF	Culture supernatant LukF-P83	Culture supernatant ATCC 25923
+	++	(+)	+	++	(+)	0	(+)	(+)
0	+	(+)	0	+	(+)	0	(+)	(+)
+	++	0	++	++	++	0	(+)	0
++	+++	(+)	+++	+++	0	0	+	0
++	+++	+	+++	+++	+++	0	++	0
(+)	++	(+)	+	+++	(+)	0	+	0
0	(+)	(+)	0	(+)	(+)	0	(+)	(+)
+++	+++	(+)	+++	++++	(+)	+	++	++
+	++	(+)	+	+++	(+)	0	++	(+)
+	++	0	+	+++	+	0	++	0
0	(+)	(+)	0	(+)	(+)	0	(+)	(+)

rays on which the corresponding probes were spotted. Hybridizations were visualized by adding a streptavidin-horseradish peroxidase conjugate that binds to the biotin tags and by a peroxidase-triggered dye precipitation. The resulting pattern of spots on the array was scanned, analyzed, and compared to a reference database of previously typed strains.

MLST. Multilocus sequence typing (MLST) was performed on selected isolates as previously described by Enright et al. (28). In short, fragments of housekeeping genes *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqiL* were amplified and sequenced using primers and reaction conditions described in reference 28. Resulting sequences were analyzed using the MLST website (http://saureus.mlst.net/) in order to assign a sequence type.

RESULTS

Antibody screening. Based on the screening results summarized in Table 1, a combination of antibody 1401 and antibody 1841 was selected to establish a lateral flow assay capable of detecting PVL (F component) as well as the gene product of *lukF-P83*. The detection limit for the lateral flow test with purified native and recombinant antigen was determined by dilution series to be approximately 1 ng/ml.

Tests of the lateral flow test with different growth media. In the first series of experiments, known strains cultured on different growth media were tested.

Liquid growth media (glucose bouillon, brain-heart infusion, 2× TY, Schaedler broth, and Noda and Kato medium) were tested with PVL-negative Mu50 (ST5-MRSA-II) and NCTC8523 (ST8-MSSA) and known isolates of ST398-MRSA-V and ST8-MSSA, as well as with PVL-positive USA300-FPR3757 (ST8-MRSA-IV, USA300) and isolates of CC30-MSSA and ST93-MRSA-IV (Queensland clone). The PVL-negative ST8-MSSA strain NCTC8325 gave weak but consistent false-positive results in the growth medium described by Noda and Kato (24). This was not observed using genotypically identical clinical isolates of ST8-MSSA. All other results were correct.

Colony material (of PVL-positive ST22-MRSA-IV and of USA300-FPR3757) harvested from plain agar, Mueller-Hinton agar with and without blood, Columbia blood, CAP, and chocolate agar yielded correct positive results. Screening of clinical isolates (see below) was then performed using overnight colonies from Columbia blood agar.

In addition to the aforementioned growth media, a commercially available chromogenic medium for MRSA detection was tested (MRSA ID agar; bioMérieux). The following PVL-positive strains were tested and yielded correct results: CC1-MRSA-IV (MW2 and USA400), CC5-MRSA-IV, ST8-MRSA-IV (USA300-FPR3757), ST22-MRSA-IV, ST30-MRSA-IV (southwest Pacific clone), ST59/ST952-MRSA-V(T) (Taiwan clone), CC80-MRSA-IV (European CA-MRSA clone), CC88-MRSA-IV, and CC152-MRSA-V. PVL-negative strains CC1-MRSA-IV&SCC*fus* (WA-MRSA-1/45), ST22-MRSA-IV (UK-EMRSA-15/Barnim), ST45-MRSA-IV (Berlin EMRSA), ST75-MRSA-IV (WA-MRSA-8), and ST239-MRSA-III (Vienna/Hungarian/Brazilian epidemic strain) and a PVL-negative variant of CC80-MRSA-IV from MRSA ID agar yielded accurate (negative) results.

Detection of LukF-P83. The 14 *lukF-P83*-positive isolates (two CC133, four CC479, and eight CC151) yielded positive results in the lateral flow assay. The three *lukF-P83*-negative isolates (two CC133 and one CC479) were correctly identified as negative.

Screening of clinical isolates using the lateral flow test and the microarray. Compared to the array-based genotyping data, 301 experiments were true positives and 293 were true negatives; there were five false positives (three isolates of CC8, one each of CC15 and CC22) and one false negative (CC30). This corresponds to a sensitivity of 99.7%, a specificity of 98.3%, a positive predictive value (PPV) of 98.4%, and a negative predictive value (NPV) of 99.7%. The six experiments with false results were repeated subsequently and yielded correct results, suggesting operator errors on primary testing.

Overall, 297 test isolates and five reference strains were PVL positive. By array hybridizations, they were assigned to 21 different clonal complexes: CC1 (including ST772), CC5, CC8 (including ST72), CC15, CC22, CC25, CC30, CC45, CC49, CC59, CC80, CC88, CC93, CC96, CC121, CC152, CC188, CC398, ST2479, and ST2482. Table 2 provides further details, such as affiliation with known CA-MRSA strains. The most frequently isolated PVL-positive lineages were CC121 (50 isolates from different regions, all MSSA), CC30 (46 isolates; MSSA and MRSA with SCC*mec* IV elements), CC8 (46 isolates, including MSSA from Trinidad and Tobago as well as USA300 from various regions), and CC93 (42 isolates, MSSA and ST93-MRSA-IV, Queensland CA-MRSA clone, almost exclusively from Australia).

The 287 PVL-negative test isolates and seven reference strains

TABLE 2 Strains tested, their geographic origins, and test results[a]

Clonal complex	Strain	Total no.	No. PVL positive in lateral flow assay	No. PVL negative in lateral flow assay	No. of isolates from:									No. of reference strains
					Australia	Germany	Saudi Arabia	Spain	Sweden	Trinidad and Tobago	Uganda	United Kingdom: London	United Kingdom: Bristol	
CC1	CC1-MSSA	7		7					3	1	2	1		
	CC1-MSSA (PVL$^+$)	6	6		1	1		1	2				1	
	CC1-MSSA-SCC*fus*	8		8	2		1	1				2	1	1
	CC1-MSSA-SCC*fus* (PVL$^+$)	7	7		4							3		
	CC1-MRSA-IV, WA-MRSA-1/57	1		1								1		
	CC1-MRSA-IV (PVL$^+$), USA400	3	3		1								1	1
	CC1-MRSA-IV&SCC*fus*, WA-1/45	1		1			1							
CC1 (ST573/772)	ST573/772-MSSA (PVL$^+$)	2	2		2									
	ST772-MRSA-V (PVL$^+$), Bengal Bay clone/WA-60	4	4									2	1	1
CC5	CC5-MSSA	10		10			1	1	6		2			
	CC5-MSSA (PVL$^+$)	8	8			1			1	5			1	
	CC5-MRSA-II, UK-3/Rhine-Hesse/New York Japan	3		3		1								2
	CC5-MRSA-IV, Pediatric clone	1		1							1			
	CC5-MRSA-IV (*edinA*$^+$), WA-65	1		1	1									
	CC5-MRSA-IV, Pediatric clone (PVL$^+$)	5	5				2					3		
	CC5-MRSA-IVvar, Maltese clone	1		1			1							
	CC5-MRSA-V (PVL$^+$)	1	1									1		
CC6	CC6-MSSA	3		3			1			2				
CC7	CC7-MSSA	9		9		1	1		5	2				
	CC7-MRSA-IV	1		1		1								
CC8	CC8-MSSA	41	3*	38	2	6	1		3	5	21	2		1
	CC8-MSSA (PVL$^+$)	23	23					2	1	18			2	
	CC8-MSSA-SCC*fus*	1		1								1		
	ST250-MRSA-I, Early/Ancestral MRSA	1		1										1
	CC8-MRSA-IV, WA-62	2	2							2				
	ST8-MRSA-IV (PVL$^+$/ACME$^+$), USA300	10	10		2	1				3		3		1
	ST8-MRSA-IV (PVL$^+$/ACME$^-$)	10	10					10						
CC8 (ST72)	ST72-MSSA	13		13	2	1		1		7	2			
	ST72-MSSA (PVL$^+$)	1	1			1								
CC8 (ST239)	ST239-MRSA-III, Vienna/Hungarian/Brazilian clone	13		13			5			6	1	1		
CC9 (ST834)	ST834-MSSA	2		2							2			
	ST834-MRSA-VI	1		1			1							
CC12	CC12-MSSA	8		8					5	1		2		
	CC12-MRSA, WA-59	1		1										1
CC15	CC15-MSSA	29	1*	28	2	1	3	1	18	2	2			
	CC15-MSSA (PVL$^+$)	2	2			1			1					
CC20	CC20-MSSA	3		3					3					
CC22	CC22-MSSA	6	1*	5		3	1		1			1		
	CC22-MSSA (PVL$^+$)	19	19			2	2	5					10	
	CC22-MSSA-SCC*fus* (PVL$^+$)	5	5										5	
	CC22-MRSA-IV, UK-EMRSA-15/Barnim EMRSA	4		4	1	1	1					1		
	CC22-MRSA-IV (PVL$^+$)	5	5			2	1					2		
CC25	CC25-MSSA	10		10			3				6	1		
	CC25-MSSA (PVL$^+$)	2	2		2									
CC30	CC30-MSSA	20		20	1	8	3		7			1		
	CC30-MSSA (PVL$^+$)	35	34	1*	3	4	4	6	4	10	1		2	1

(Continued on following page)

TABLE 2 (Continued)

Clonal complex	Strain	Total no.	No. PVL positive in lateral flow assay	No. PVL negative in lateral flow assay	No. of isolates from:									No. of reference strains
					Australia	Germany	Saudi Arabia	Spain	Sweden	Trinidad and Tobago	Uganda	United Kingdom: London	United Kingdom: Bristol	
	CC30-MRSA-IV (PVL$^+$), Southwest Pacific CA-MRSA clone	11	11		5	1	1					4		
CC30 (ST34)	ST34-MSSA	5		5	1			1	1			2		
CC45	CC45-MSSA	33		33	2	4	1	2	19	2		3		
	CC45-MSSA (PVL$^+$)	2	2		1				1					
	CC45-MRSA-IV, Berlin EMRSA	1		1		1								
CC49	ST49-MSSA (PVL$^+$)	1	1					1						
CC50	CC50-MSSA	3		3					3					
CC59	CC59-MSSA	1		1								1		
	CC59-MSSA (PVL$^+$)	1	1										1	
	CC59-MRSA-V	1		1								1		
	CC59-MRSA-V (PVL$^+$)	1	1									1		
	ST59/ST952-MRSA-V(T) (PVL$^+$), Taiwan CA-MRSA clone	3	3		1							2		
	CC59-MRSA-V&SCC*fus*	2		2						2				
CC75	MSSA, related to ST1223	2		2						2				
	MSSA, related to ST1667	2		2			1			1				
	ST75-MRSA-IV, WA-MRSA-8/79	1		1										1
CC80	CC80-MSSA (PVL$^+$)	5	5				1	1	2		1			
	CC80-MRSA-IV	2		2			2							
	CC80-MRSA-IV (PVL$^+$), European CA-MRSA clone	13	13				11					2		
CC88	CC88-MSSA	2		2	1				1					
	CC88-MSSA (PVL$^+$)	2	2						2					
	CC88-MRSA-IV (PVL$^+$)	2	2				2							
CC93	ST93-MSSA (PVL$^+$)	9	9		9									
	ST93-MRSA-IV (PVL$^+$), Queensland CA-MRSA clone	33	33		30	1						1		1
CC96	CC96/154-MSSA	1		1			1							
	CC96/154-MSSA (PVL$^+$)	1	1			1								
CC97	CC97-MSSA	8		8			1		7					
CC101	CC101-MSSA	3		3		1			1	1				
CC121	CC121-MSSA	16		16				2	8	4	1	1		
	CC121-MSSA (PVL$^+$)	50	50		15	8		4	3		17	2	1	
CC140	CC140-MRSA-IV	2		2							2			
CC152	CC152-MSSA (PVL$^+$)	8	8					2	1	2		1	2	
	CC152-MRSA-V (PVL$^+$)	1	1			1								
CC188	CC188-MSSA	6		6	1			1	2	2				
	CC188-MSSA (PVL$^+$)	2	2		1		1							
CC398	CC398-MSSA	1		1		1								
	CC398-MSSA (PVL$^+$)	1	1			1								
CC398 (ST291/813)	ST291/813-MSSA	1		1			1							
	ST291/813-MSSA (PVL$^+$)	1	1				1							
CC425	ST425-MRSA-XI	1		1								1		
CC509	CC509-MSSA	1		1					1					
CC707	ST707-MSSA	1		1				1						
CC1021	CC1021-MSSA	1		1					1					
CC1290	CC1290/ST2481-MSSA	1		1			1							
ST2479	ST2479-MSSA (PVL$^+$)	1	1					1						
ST2482	ST2482-MSSA (PVL$^+$)	4	4				3		1					
Unidentified	*agr* IV/capsule 5 MSSA	1		1							1			

[a] Strain assignments were based on array hybridization. False results in lateral flow tests are marked with an asterisk.

TABLE 3 Rates of PVL-negative MSSA, PVL-negative MRSA, PVL-positive MSSA, and PVL-positive MRSA by study site[a]

Study site (total no. of isolates)	No. (%) of isolates in each category			
	PVL negative		PVL positive	
	MSSA	MRSA	MSSA	MRSA
Australia ($n = 90$)	14 (15.6)	2 (2.2)	37 (41.1)	37 (41.1)
Germany ($n = 50$)	26 (52.0)	4 (8.0)	17 (34.0)	3 (6.0)
Saudi Arabia ($n = 53$)	21 (39.6)	8 (15.1)	11 (20.8)	13 (24.5)
Spain ($n = 44$)	11 (25.0)	0 (0)	23 (52.3)	10 (22.7)
Sweden ($n = 114$)	95 (83.3)	0 (0)	19 (16.7)	0 (0)
Trinidad and Tobago ($n = 80$)	32 (40.0)	8 (10.0)	35 (43.8)	5 (6.2)
Uganda ($n = 62$)	39 (62.9)	4 (6.5)	19 (30.6)	0 (0)

[a] Three Australian, six German, eight Saudi, and all English isolates, as well as the reference strains, were excluded from this analysis, because their PVL statuses were known already.

were assigned to 31 different clonal complexes: CC1, CC5, CC6, CC7, CC8 (including ST72 and ST239), CC9 (ST834), CC12, CC15, CC20, CC22, CC25, CC30 (including ST34), CC45, CC50, CC59, CC75, CC80, CC88, CC96, CC97, CC101, CC121, CC140, CC188, CC398, CC425, CC509, CC707, CC1021, and CC1290. One isolate could not be allocated to a clonal complex.

Prevalence of PVL-positive *S. aureus* in the different countries. The prevalence of PVL-positive isolates among all SSTI isolates varied widely between the different countries. Rates of PVL positives and negatives for MSSA and MRSA are summarized in Table 3.

The highest rate of PVL positives was observed among the Australian samples, with 82.2% (74 of 90) being PVL positive. Half of the PVL-positive isolates (37 of 74) belonged to CC93, and the majority of them were MRSA (29 of 37 CC93 isolates; 78%), reflecting the burden the so-called Queensland CA-MRSA clone currently causes. The second and third most frequently isolated PVL-positive clones in Australia were CC121-MSSA ($n = 15$) and CC93-MSSA ($n = 8$). Only two isolates of ST8-MRSA-IV (USA300) were identified. PVL-negative *S. aureus* isolates were from multiple CC lineages and included two MRSA clones, ST22-MRSA-IV (UK-EMRSA-15/Barnim EMRSA) and ST5-MRSA-IV (Pediatric clone/WA-MRSA-65).

Among SSTI isolates from Germany, the PVL rate was 40% (20 of 50). The most common strains were CC121-MSSA ($n = 7$) and CC30-MSSA ($n = 4$). One each of ST8-MRSA-IV (USA300) and ST93-MRSA-IV (Queensland CA-MRSA clone) were identified, the latter being associated with travel to Australia. Among the PVL negatives, CC30 and CC8 were the most frequently isolated. Single isolates of PVL-negative CC7-MRSA-IV, CC22-MRSA-IV (UK-EMRSA-15/Barnim EMRSA), ST5/ST225-MRSA-II (UK-EMRSA-3/Rhine-Hesse EMRSA), and CC45-MRSA-IV (Berlin EMRSA) were identified.

Of the isolates from Saudi Arabia, 47.3% (24 of 53) proved to be positive for PVL. Roughly half were MRSA ($n = 13$), with the single most common PVL-positive clone being CC80-MRSA-IV (European CA-MRSA clone; 10 isolates). The most frequently isolated PVL-MSSA clones were CC30-MSSA ($n = 4$) and ST2482-MSSA ($n = 3$). PVL negatives belonged to various clonal complexes (Table 2), and the proportion of MRSA strains was high (8 of 29 PVL negatives), with the most common strain being ST239-MRSA-III (Vienna/Hungarian/Brazilian clone; $n = 4$). Other MRSA strains belonged to a *tst1*-positive variant of CC22-MRSA-IV, a PVL-negative variant of CC80-MRSA-IV, a CC5-MRSA-IV&SCC*fus* strain previously known only from Malta (29), and CC9/ST834-MRSA-VI.

The second highest PVL rate was found in Spain, with 75% (33 of 44) being positive for PVL. Here, the most common clone was an ACME (arginine catabolic mobile element)-negative variant of ST8-MRSA-IV (USA300), to which 10 isolates were assigned. This was followed by CC30-MSSA ($n = 6$) and CC22-MSSA ($n = 5$). The PVL negatives did not include any MRSA strains.

The lowest prevalence for PVL was observed among the Swedish isolates. Only 16.7% (19 of 114) were PVL positive, all MSSA. The most common PVL-positive strains were CC30-MSSA ($n = 4$) and CC121-MSSA ($n = 3$). The most common PVL-negative lineages were CC45 ($n = 19$) and CC15 ($n = 18$). MRSA isolates were not found.

In Trinidad and Tobago, the PVL prevalence was 50% (40 of 80 isolates). The most abundant PVL-positive strain was CC8-MSSA ($n = 18$), which additionally carried enterotoxin genes *sed*, *sej*, *ser*, *sek*, and *seq*. Two CC8-MRSA-IV isolates were identified with the same toxin profile; they lacked ACME and thus resembled WA-MRSA-62. ST8-MRSA-IV (USA300), i.e., carrying the ACME locus and enterotoxin genes *sek* and *seq* only, was identified in three cases. Other frequently isolated PVL-positive strains were CC30-MSSA ($n = 10$) and CC5-MSSA ($n = 5$). PVL-negative isolates included some isolates of unusual strains related to CC75 (ST1223 and ST1667). The PVL-negative MRSA strains were CC59-MRSA-V&SCC*fus* and ST239-MRSA-III (Vienna/Hungarian/Brazilian clone).

In Uganda, 30.6% (19 of 62) were PVL positive, including 17 isolates belonging to CC121-MSSA and single representatives of CC30- and CC80-MSSA. PVL-positive MRSA was not identified. The most common lineage among PVL negatives was CC8 ($n = 22$, plus one isolate each of CC8/ST72 and CC8/ST239). PVL-negative MRSA strains included two ST140-MRSA-IV isolates as well as single isolates of CC5-MRSA-IV (Pediatric clone) and ST239-MRSA-III (Vienna/Hungarian/Brazilian clone).

In contrast to the collections from other countries, the PVL status of the isolates from England were already known, thus the PVL rates cannot be compared to those of the other countries. A variety of different PVL-MRSA strains was identified among the London isolates: CC30-MRSA-IV (Southwest Pacific CA-MRSA clone), CC5-MRSA-IV (Pediatric clone), CC5-MRSA-V, CC80-MRSA-IV (European CA-MRSA clone), ST59/ST952-MRSA-V(T) (Taiwan CA-MRSA clone), ST772-MRSA-V (Bengal Bay CA-MRSA clone/WA-MRSA-60), ST8-MRSA-IV (USA300), and

ST93-MRSA-IV (Queensland CA-MRSA clone). PVL-negative isolates included MRSA strains CC1-MRSA-IV (WA-MRSA-1/57), ST239-MRSA-III (Vienna/Hungarian/Brazilian clone), CC22-MRSA-IV (UK-EMRSA-15/Barnim EMRSA), ST59-MRSA-V, and CC425-MRSA-XI. Another 28 isolates were included from a second center in the southwest of England that were already known to be PVL positive. These were also excluded from the analysis of PVL rates, but their population structure was notable. This group included only two PVL-positive MRSA isolates, ST772-MRSA-V (Bengal Bay CA-MRSA clone/WA-MRSA-60) and CC1-MRSA-IV (USA400). It also included one CC59-MSSA strain that probably was an SCC*mec* deletion mutant of ST59/ST952-MRSA-V(T) (Taiwan CA-MRSA clone). The most common strain in this group was a PVL-positive CC22-MSSA isolate ($n = 10$). Another five PVL-positive CC22 isolates with *spa* type t417 or t1601 carried SCC*fus* elements (*ccrA* and/or *ccrB-1*, Q6GD50, or *fusC*). These isolates originated from patients with an average age of nearly 94 years. This is an unusual finding among PVL positives and suggests a possible association of this clonal complex with care facilities in this region.

DISCUSSION

PVL is a unique virulence marker in *S. aureus*, and it is most commonly associated with clinical symptoms which tend to be either chronic/recurrent or, occasionally, rapidly progressing and life-threatening. A diagnostic test for PVL would be desirable for targeted patient management. The lateral flow assay described in this paper allows the rapid detection of PVL in a routine bacteriological laboratory that is not able to readily perform molecular assays. As it utilizes pure overnight cultures from standard media, such as Columbia blood agar, it can easily be integrated into a routine diagnostic laboratory workflow. Thus, the assay might contribute to timely therapeutic interventions in cases of PVL-associated infections, and it also might help to select isolates for submission for further typing in reference centers.

The amount of PVL released by *S. aureus in vitro* varies widely (30, 31); however, the high concordance between the genotypic and phenotypic assays suggests that *lukS–lukF-PV*-positive strains generally express detectable amounts of PVL using standard culture conditions. In this study, no isolates were identified that harbored PVL genes without producing the toxin *in vitro*. While further media and different formulations still need to be tested, it can be assumed that the probability of false-negative results due to a lack of expression *in vitro* is low. The diversity of PVL- and *lukM–lukF-P83*-positive strains included in this study indicate that possible lineage-specific variations in PVL sequences do not pose an obstacle to PVL detection by the antibodies described here.

Further, the collection of isolates described here provides a snapshot of the molecular epidemiology of *S. aureus* associated with SSTI. Among the PVL-positive methicillin-susceptible *S. aureus* strains, CC121 (50 isolates in total) and CC30 (35 isolates) dominated. PVL-positive CC8-MSSA was abundant in Trinidad and Tobago, although this strain was rare elsewhere. This lends credence to the hypothesis that the USA300 strain emerged in the Caribbean/Latin American region (32).

The study also shows that MRSA strains, PVL positives as well as negatives, pose a serious problem in different parts of the world, being commonly found in SSTI that mostly are community associated. At most study sites, MRSA was isolated frequently, especially in Australia, Saudi Arabia, and Spain. Dominating clones in this study were PVL-positive/ACME-positive ST8-MRSA-IV (USA300), PVL-positive/ACME-negative ST8-MRSA-IV, PVL-positive ST80-MRSA-IV (European CA-MRSA clone), PVL-positive ST93-MRSA-IV (Queensland CA-MRSA clone), and PVL-negative ST239-MRSA-III. In this study, the countries with the lowest MRSA prevalence (or complete absence) were Sweden, which has a very strict policy on MRSA infection control, and Uganda, where the selective pressure on *S. aureus* by the use of antibiotics in health care and veterinary medicine may be more limited than in other countries.

The possibility of combining the lateral flow assay with a chromogenic MRSA screening medium facilitates a quick screening for emerging strains of PVL-positive CA-MRSA. This could be helpful to arrest their dissemination and further expansion. The high percentage of PVL-positive MRSA (ST93-MRSA-IV, Queensland CA-MRSA clone, and ST8-MRSA-IV) among Australian and Spanish isolates, in addition to high rates of PVL-positive MSSA strains, suggests that an expansion of PVL-positive CA-MRSA does not occur at the expense of the established PVL-positive MSSA populations but in addition to it. Besides limiting the efficacy of beta-lactams as a primary therapeutic option, the emergence of PVL-positive CA-MRSA may result in an increased burden of PVL-associated disease. Although the number of isolates in the present study is not sufficient to unambiguously prove such a trend, it warrants further study with respect to the molecular epidemiology of PVL-positive *S. aureus*.

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