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Novel Multiplex PCR Assay for Simultaneous Identification of Community-Associated Methicillin-Resistant *Staphylococcus aureus* Strains USA300 and USA400 and Detection of *mecA* and Panton-Valentine Leukocidin Genes, with Discrimination of *Staphylococcus aureus* from Coagulase-Negative Staphylococci[▽]

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We developed a novel multiplex PCR assay for rapid identification and discrimination of the USA300 and USA400 strains and concomitant detection of Panton-Valentine leukocidin genes, with simultaneous discrimination of methicillin-resistant *Staphylococcus aureus* strains from methicillin-susceptible *S. aureus* strains, *S. aureus* strains from coagulase-negative staphylococci, and staphylococci from other bacteria.

USA300 and USA400 are the predominant community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) strains circulating in North America (8, 12), being implicated in outbreaks of infections associated with significant morbidity and mortality (1, 4, 6). These strains belong to multilocus sequence types (MLST) ST8 and ST1 and staphylococcal protein A (*spa*) types t008 and t128, respectively, and both strains carry Panton-Valentine leukocidin (PVL) genes and the staphylococcal cassette chromosome *mec* (SCC*mec*) type IVa element. However, molecular characterization of these strains can be time consuming and technically laborious. We have designed a multiplex PCR (M-PCR) assay capable of accurately distinguishing USA300 from USA400 strains while simultaneously detecting PVL genes and discriminating MRSA strains from methicillin-susceptible *S. aureus* (MSSA) strains, *S. aureus* strains from coagulase-negative staphylococci (CoNS), and staphylococci from other bacteria.

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Sequence alignment and primer design. Primers for 16S rRNA (Stapy756-F and Staph750-R) (15), thermostable nuclease (*nuc*) (Nuc-1 and Nuc-2) (15), *mecA* (mecA147-F and mecA147-R) (14), and PVL genes *lukS-PV*/*lukF-PV* (Luk-PV-1 and Luk-PV-2) (7) were as previously described. New sets of primers for strains USA300 and USA400 and for prophage ϕSa2usa/ϕSa2mw were designed based on comprehensive analysis and alignment of individual *Staphylococcus* sp. genomes currently available in the GenBank database. Gene targets for each primer pair are as follows: USA300 strain primers arcA-F (5′-GCAGCAGAATCTATTACTGAGCC-3′) and arcA-R (5′-TGCTAACTTTTCTATTGCTTGAGC-3′) target the *arcA* gene on the arginine catabolic mobile element (ACME); USA400 strain primers MW756-F (5′-TGGTTAGCTATGAATGTAGTTGC-3′) and MW756-R (5′-GTCCATCCTCTGTAAATTTTGC-3′) target the gene locus MW0756 on νSa3 of strain MW2; and ϕSa2mw/ϕSa2usa prophage primers phi-int-F4 (5′-CAAATTTTGAAAACTTTACGC-3′) and phi-int-R4 (5′-TCCAGGATTAAAAGAAGCG-3′) target the MW1409 gene locus of the USA400 MW2 strain.

Development of an M-PCR assay for typing MRSA isolates and distinguishing USA300 and USA400 strains. The assay specifically involved targeting the *Staphylococcus* genus-specific 16S rRNA gene sequence (serving to distinguish *Staphylococcus* from other bacteria and acting as an internal PCR control), the *S. aureus*-specific *nuc* gene, the methicillin resistance determinant *mecA*, the PVL genes, the phage marker MW1409, the USA400 genomic island gene locus (MW0756), and the USA300 ACME cassette gene (*arcA*). Amplification in a single M-PCR produced distinct bands corresponding to molecular sizes of 147, 220, 279, 372, 433, 513, and 756 bp for *mecA*, MW1409, *nuc*, MW0756, PVL, *arcA*, and 16S rRNA, respectively (Fig. 1).

Validation of M-PCR assay. The new multiplex PCR assay was validated using 42 representative MRSA/MSSA control strains and 6 ACME-positive ($ACME^+$) or ACME-negative ($ACME^-$) CoNS control strains that had undergone detailed

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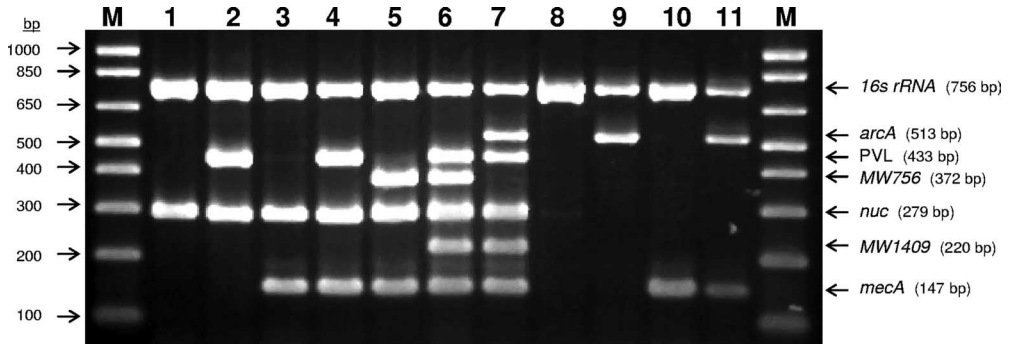

FIG. 1. Novel multiplex PCR assay identifying USA300 and USA400 community-associated MRSA strains, detecting PVL and *mecA* genes, and simultaneously discriminating *S. aureus* from CoNS. The optimized M-PCR was performed as follows: 4.65 μl of template DNA was prepared as previously described (15) in a 25-μl final reaction volume containing 0.30, 0.3, 0.4, 0.32, 0.16, 1.17, and 0.16 μM for each of the 16S rRNA, *arcA*, *lukS-PV*/*lukF-PV*, MW0756, *nuc*, MW1409, and *mecA* primers, respectively, with the thermocycling conditions set at 94°C for 4 min followed by 10 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 45 s and another 25 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 45 s. The PCR amplicons were visualized using a UV light box after electrophoresis on a 2% agarose gel containing 0.5 μg/ml ethidium bromide. Lane 1, strain ATCC 29213 (PVL^- MSSA); lane 2, strain ATCC 49775 (PVL^+ MSSA); lane 3, Canadian epidemic MRSA control strain CMRSA2 (PVL^- non-USA300 and non-USA400 MRSA); lane 4, strain C1538 (PVL^+ non-USA300 and non-USA400 MRSA); lane 5, strain C2901 (PVL^- USA400); lane 6, Canadian epidemic CA-MRSA USA400 control strain CMRSA7 (PVL^+ USA400); lane 7, Canadian epidemic CA-MRSA USA300 control strain CMRSA10 (PVL^+ USA300); lane 8, strain CNS99-PF5 (PVL^- and *arcA*$^-$ MS-CoNS); lane 9, strain CNS99-PF7 (PVL^- but *arcA*$^+$ MS-CoNS); lane 10, strain CNS99-PF6 (PVL^- and *arcA*$^-$ MR-CoNS); lane 11, strain CNS99-PF8 (PVL^- but *arcA*$^+$ MR-CoNS); lanes M, 1 kb Plus DNA ladder (Invitrogen). Refer to Table 1 for details of each strain.

phenotypic and molecular characterization, including analysis of carriage of PVL (7) and other genes, pulsed-field gel electrophoresis (PFGE) fingerprinting (9), SCC*mec* typing (14), *spa* typing (5, 10), and MLST (2) and eBURST (3, 11) analyses (Table 1). The MRSA/MSSA strains differed in their genotypic characteristics and represented 10 major clonal complex groups found in the worldwide MLST collection. The assay was capable of accurately and reproducibly discriminating USA300 from USA400 and other MRSA and MSSA strains while simultaneously detecting PVL genes and ϕSa2mw/ϕSa2usa phages, with a resultant 100% concordance to genotypic features in all these control strains (Table 1). There were seven PVL^+ MRSA and nine PVL^+ MSSA strains, belonging to non-USA300/non-USA400 strains with well-diversified genomic backgrounds, and all were positive for the PVL genes but negative for the ϕSa2mw/ϕSa2usa phage marker, suggesting that PVL genes in these strains may be carried by phages/plasmids other than ϕSa2mw/ϕSa2usa. More interestingly, there were two strains (with non-USA300/non-USA400 PFGE profiles), one a PVL^- MRSA strain (CMRSA5) and one a PVL^+ MSSA strain (SAF516), that were positive for the phage marker. Since this primer pair is a marker for the PVL-bearing phage ϕSa2mw/ϕSa2usa in USA400/USA300, this result suggests that variations of these particular phages can also be present in *S. aureus* strains other than USA300 and USA400 with or without the PVL genes. Further studies are required to better understand this observation.

Applicability and accuracy of M-PCR. To address the applicability and accuracy of the M-PCR assay, we further applied our M-PCR assay to test a total of 1,133 local clinical MRSA isolates randomly selected from our Calgary frozen clinical isolate stock collection for the 18-year period from 1989 to 2006. We were able to accurately identify and classify all strains with available PFGE data, including 54 PVL^+ USA300, 17 PVL^+ USA400, 35 PVL^- USA400, 40 PVL^- CMRSA2, and 34 PVL^- non-USA300/non-USA400/non-CMRSA2 MRSA strains. We were also able to clearly classify the remaining randomly chosen strains, including 514 *S. aureus* and 439 CoNS isolates. Once again, we noted that 10 (1.9%) of the isolates, including 5 PVL^- MRSA and 5 PVL^- MSSA isolates, were positive for the phage gene and yet did not belong to either USA300 or USA400. There was also one MRSA isolate that was positive for the PVL genes but negative for the ϕSa2mw/ϕSa2usa gene. Among 439 CoNS isolates tested, there was a 100% concordance with phenotypic susceptibility to methicillin, with 214 of the isolates being methicillin-susceptible CoNS (MS-CoNS) and 225 being methicillin-resistant CoNS (MR-CoNS). Of the MS-CoNS isolates, 75 (35.0%) of them were positive for *arcA*, while 93 (41.3%) of the MR-CoNS isolates were *arcA* positive. None of the CoNS isolates tested carried PVL, ϕSa2mw/usa phage, or USA400 marker MW0756 gene loci.

Our assay is capable not only of (i) accurately identifying and differentiating USA300 and USA400 strains but also of simultaneously detecting (ii) *mecA* to discriminate MRSA from MSSA and (iii) 16S rRNA and *nuc* to discriminate *S. aureus* from CoNS and of detecting (iv) PVL genes and a ϕSa2mw/usa-specific gene to determine whether the isolates/strains carry PVL genes and whether the PVL genes are carried by the ϕSa2mw/ϕSa2usa phage or other prophages in the population. Our M-PCR assay may also facilitate monitoring of the dynamic exchanges or evolution of genes among strains of MRSA, MSSA, and CoNS. This assay is based on the concept of certain strains carrying unique/specific genes/phages. However, since movement of these genes, phages, and other genetic elements is dynamic, the definition of a strain, as determined by the use of our assay for detection of gene targets uniquely present in individual CA-MRSA strains, may require reconsideration over time.

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TABLE 1. Molecular and genotypic features of *Staphylococcus aureus* and CoNS control strains[a]

Strain type and name	PFGE type (pattern)[b]	*spa* type	Molecular type	Genotype (presence of indicated gene or marker)[c]						
				Staphylococcus sp. specific (16S rRNA)	*S. aureus* specific (*nuc*)	Methicillin resistant (*mecA*)	PVL specific (PVL)	Phage (MW1409)	USA400 (MW0756)	USA300 (*arcA*)
PVL$^+$ USA300										
CMRSA10	USA300-0114 (A)	t008	ST8-MRSA-IVa	+	+	+	+	−	+	
PMRSA-13	USA300-0114 (A)	t008	STnew-MRSA-Iva[d]	+	+	+	+	+	−	+
PMRSA-16	USA300-0114 (A)	t008	ST8-MRSA-IVa	+	+	+	+	+	−	+
PMRSA-46	USA300-0114 (A)	t008	ST8-MRSA-IVa	+	+	+	+	+	−	+
PMRSA-12	USA300 (B)	t008	ST8-MRSA-IVa	+	+	+	+	+	−	+
C21744	USA300 (C)	t008	ST8-MRSA-IVa	+	+	+	+	+	−	+
PVL$^-$ USA400										
C2901	USA400 (B)	t128	ST1-MRSA-IVa	+	+	+	−	−	+	−
C2140	USA400 (A)	t128	ST1-MRSA-IVa	+	+	+	−	−	+	−
PVL$^+$ USA400										
CMRSA7	USA400 (A)	t128	ST1-MRSA-IVa	+	+	+	+	+	+	−
PMRSA-18	USA400 (A)	t128	ST1-MRSA-IVa	+	+	+	+	+	+	−
PMRSA-50	USA400 (A)	t128	ST1-MRSA-IVa	+	+	+	+	+	+	−
C10687	USA400 (A)	t128	ST1-MRSA-IVa	+	+	+	+	+	+	−
C6413	USA400 (C)	t128	ST1-MRSA-IVa	+	+	+	+	+	+	−
PVL$^-$ MRSA										
CMRSA1	USA600	t004	ST45-MRSA-II	+	+	+	−	−	−	−
CMRSA2	Like USA100/800	t002	ST5-MRSA-II	+	+	+	−	−	−	−
C4000	Like USA100/800	t004	ST225-MRSA-II	+	+	+	−	−	−	−
CMRSA3	Like EMRSA1/4/11	t037	ST241-MRSA-III	+	+	+	−	−	−	−
CMRSA6	Like EMRSA1/4/11	t037	ST239-MRSA-III	+	+	+	−	−	−	−
C1777	Like EMRSA1/4/11	t037	ST239-MRSA-III	+	+	+	−	−	−	−
CMRSA4	USA200/EMRSA16	t018	ST36-MRSA-II	+	+	+	−	−	−	−
C23374	USA200/EMRSA 16	t018	ST36-MRSA-II	+	+	+	−	−	−	−
CMRSA5	USA500	t064	ST8-MRSA-IVd	+	+	+	−	+	−	−
CMRSA8	EMRSA 15	t022	ST22-MRSA-IV	+	+	+	−	−	−	−
CMRSA9		t008	ST8-MRSA	+	+	+	−	−	−	−
C74		t1154	ST5-MRSA-IVb	+	+	+	−	−	−	−
PVL$^+$ MRSA										
H435		t311	ST5-MRSA-II	+	+	+	+	−	−	−
PMRSA-34		t437	ST59-MRSA-III	+	+	+	+	−	−	−
MR37	Like USA400	t175	ST1-MRSA-IVa	+	+	+	+	−	−	−
MR138		t044	ST80-MRSA-IVa	+	+	+	+	−	−	−
H434	Like USA1100	t019	ST30-MRSA IVc	+	+	+	+	−	−	−
PMRSA-29	USA1100	t019	ST30-MRSA-IVc	+	+	+	+	−	−	−
C1538	USA1100	t019	ST30-MRSA-IVc	+	+	+	+	−	−	−

PVL$^+$ MSSA										
MS02-W10	USA800	t015	ST5-MSSA	+	+	−	+	−	−	−
SA5		Unnamed[e]	ST121-MSSA	+	+	−	+	−	−	−
SA112		t645	ST121-MSSA	+	+	−	+	−	−	−
SA125		t436	ST25-MSSA	+	+	−	+	−	−	−
SA134		t005	ST22-MSSA	+	+	−	+	−	−	−
SA28		t437	ST59-MSSA	+	+	−	+	−	−	−
MS03-B1		t437	ST59-MSSA	+	+	−	+	−	−	−
SA3		Unnamed[e]	ST30-MSSA	+	+	−	+	−	−	−
H49		t021	ST30-MSSA	+	+	−	+	−	−	−
SAF516		t483	ST30-MSSA	+	+	−	+	+	−	−
PVL$^-$ MS-CoNS										
S. epidermidis (ATCC12228)			ACME$^+$	+	−	−	−	−	−	+
PVL$^-$ MS-CoNS										
S. epidermidis (CNS99-PF5)			ACME$^-$	+	−	−	−	−	−	−
PVL$^-$ MS-CoNS										
S. epidermidis (CNS99-PF7)			ACME$^+$	+	−	−	−	−	−	+
PVL$^-$ MR-CoNS										
S. epidermidis (GISE 12333)			ACME$^+$	+	−	+	−	−	−	+
PVL$^-$ MR-CoNS										
S. epidermidis (CNS99-PF6)			ACME$^-$	+	−	+	−	−	−	−
PVL$^-$ MR-CoNS										
S. epidermidis (CNS99-PF8)			ACME$^+$	+	−	+	−	−	−	+

[a] PFGE, PVL gene typing, SCC*mec* typing, *spa* typing, and MLST were used for strain characterization. The identification of MRSA isolates matching the USA300 and USA400 CA-MRSA strains was based on the similarity of PFGE patterns to those of the USA300 and USA400 control strains and the presence of PVL, SCC*mec* type IVa, *spa* type t008, and MLST type ST8 (for USA300) and of PVL, SCC*mec* type IVa, *spa* type t128, and MLST type ST1 (for USA400).

[b] PFGE patterns A (indistinguishable from the USA300 control strain [CMRSA10]), B (an additional band around 150 kb in size), and C (the band at around 250 bp shifting to around 310 kb) for strain USA300 and patterns A, B, and C for strain SA400 (see reference 13 for details of the patterns) are indicated in brackets.

[c] +, present; −, absent.

[d] New ST (MLST) profile: PMRSA-13 (3-3-1-4-4-4-3).

[e] The *spa* type profiles are unnamed for SA5 (I2H2M) and SA3 (I2H2M).

REFERENCES

1. **CDC.** 2003. Outbreaks of community-associated methicillin-resistant *Staphylococcus aureus* skin infections—Los Angeles County, California, 2002–2003. MMWR Morb. Mortal. Wkly. Rep. **52:**88.
2. **Enright, M. C., N. P. Day, C. E. Davies, S. J. Peacock, and B. G. Spratt.** 2000. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. J. Clin. Microbiol. **38:**1008–1015.
3. **Feil, E. J., B. C. Li, D. M. Aanensen, W. P. Hanage, and B. G. Spratt.** 2004. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J. Bacteriol. **186:**1518–1530.
4. **Francis, J. S., M. C. Doherty, U. Lopatin, C. P. Johnston, G. Sinha, T. Ross, M. Cai, N. N. Hansel, T. Perl, J. R. Ticehurst, K. Carroll, D. L. Thomas, E. Nuermberger, and J. G. Bartlett.** 2005. Severe community-onset pneumonia in healthy adults caused by methicillin-resistant *Staphylococcus aureus* carrying the Panton-Valentine leukocidin genes. Clin. Infect. Dis. **40:**100–107.
5. **Harmsen, D., H. Claus, W. Witte, J. Rothganger, D. Turnwald, and U. Vogel.** 2003. Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for *spa* repeat determination and database management. J. Clin. Microbiol. **41:**5442–5448.
6. **Kazakova, S. V., J. C. Hageman, M. Matava, A. Srinivasan, L. Phelan, B. Garfinkel, T. Boo, S. McAllister, J. Anderson, B. Jensen, D. Dodson, D. Lonsway, L. K. McDougal, M. Arduino, V. J. Fraser, G. Killgore, F. C. Tenover, S. Cody, and D. B. Jernigan.** 2005. A clone of methicillin-resistant *Staphylococcus aureus* among professional football players. N. Engl. J. Med. **352:**468–475.
7. **McClure, J. A., J. M. Conly, V. Lau, S. Elsayed, T. Louie, W. Hutchins, and K. Zhang.** 2006. Novel multiplex PCR assay for detection of the staphylococcal virulence marker Panton-Valentine leukocidin genes and simultaneous discrimination of methicillin-susceptible from -resistant staphylococci. J. Clin. Microbiol. **44:**1141–1144.
8. **McDougal, L. K., C. D. Steward, G. E. Killgore, J. M. Chaitram, S. K. McAllister, and F. C. Tenover.** 2003. Pulsed-field gel electrophoresis typing of oxacillin-resistant *Staphylococcus aureus* isolates from the United States: establishing a national database. J. Clin. Microbiol. **41:**5113–5120.
9. **Mulvey, M. R., L. Chui, J. Ismail, L. Louie, C. Murphy, N. Chang, and M. Alfa.** 2001. Development of a Canadian standardized protocol for subtyping methicillin-resistant *Staphylococcus aureus* using pulsed-field gel electrophoresis. J. Clin. Microbiol. **39:**3481–3485.
10. **Shopsin, B., M. Gomez, S. O. Montgomery, D. H. Smith, M. Waddington, D. E. Dodge, D. A. Bost, M. Riehman, S. Naidich, and B. N. Kreiswirth.** 1999. Evaluation of protein A gene polymorphic region DNA sequencing for typing of *Staphylococcus aureus* strains. J. Clin. Microbiol. **37:**3556–3563.
11. **Spratt, B. G., W. P. Hanage, B. Li, D. M. Aanensen, and E. J. Feil.** 2004. Displaying the relatedness among isolates of bacterial species—the eBURST approach. FEMS Microbiol. Lett. **241:**129–134.
12. **Tenover, F. C., L. K. McDougal, R. V. Goering, G. Killgore, S. J. Projan, J. B. Patel, and P. M. Dunman.** 2006. Characterization of a strain of community-associated methicillin-resistant *Staphylococcus aureus* widely disseminated in the United States. J. Clin. Microbiol. **44:**108–118.
13. **Zhang, K., J. McClure, S. Elsayed, J. Tan, and J. Conly.** 2008. Coexistence of Panton-Valentine leukocidin-positive and -negative community-associated methicillin-resistant *Staphylococcus aureus* USA400 sibling strains in a large Canadian health-care region. J. Infect. Dis. **15:**195–204.
14. **Zhang, K., J. A. McClure, S. Elsayed, T. Louie, and J. M. Conly.** 2005. Novel multiplex PCR assay for characterization and concomitant subtyping of staphylococcal cassette chromosome *mec* types I to V in methicillin-resistant *Staphylococcus aureus*. J. Clin. Microbiol. **43:**5026–5033.
15. **Zhang, K., J. Sparling, B. L. Chow, S. Elsayed, Z. Hussain, D. L. Church, D. B. Gregson, T. Louie, and J. M. Conly.** 2004. New quadriplex PCR assay for detection of methicillin and mupirocin resistance and simultaneous discrimination of *Staphylococcus aureus* from coagulase-negative staphylococci. J. Clin. Microbiol. **42:**4947–4955.