

Pathogen Profiling: Rapid Molecular Characterization of *Staphylococcus aureus* by PCR/Electrospray Ionization-Mass Spectrometry and Correlation with Phenotype[∇]

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There are few diagnostic methods that readily distinguish among community-acquired methicillin (meticillin)-resistant *Staphylococcus aureus* strains, now frequently transmitted within hospitals. We describe a rapid and high-throughput method for bacterial profiling of staphylococcal isolates. The method couples PCR to electrospray ionization-mass spectrometry (ESI-MS) and is performed on a platform suitable for use in a diagnostic laboratory. This profiling technology produces a high-resolution genetic signature indicative of the presence of specific genetic elements that represent distinctive phenotypic features. The PCR/ESI-MS signature accurately identified genotypic determinants consistent with phenotypic traits in well-characterized reference and clinical isolates of *S. aureus*. Molecular identification of the antibiotic resistance genes correlated strongly with phenotypic *in vitro* resistance. The identification of toxin genes correlated with independent PCR analyses for the toxin genes. Finally, isolates were correctly classified into genotypic groups that correlated with genetic clonal complexes, repetitive-element-based PCR patterns, or pulsed-field gel electrophoresis types. The high-throughput PCR/ESI-MS assay should improve clinical management of staphylococcal infections.

Invasive infections caused by methicillin (meticillin)-resistant *Staphylococcus aureus* (MRSA) are among the most common complications of health care in the United States. Klevens et al. estimate that 94,360 invasive MRSA infections occur in the United States each year, with associated deaths in 18,650 cases (26). Infections are subcategorized as health care-associated MRSA (HA-MRSA) infections or as community-associated MRSA (CA-MRSA) infections; the latter occur in otherwise healthy people who have not experienced a hospital stay within the past 12 months (24, 35). CA-MRSA infections have largely been attributable to a few strains, designated pulsed-field types (PFTs) USA300 and USA400 (26, 29). Once introduced into a health care environment, CA-MRSA strains can be readily transmitted, blending with or replacing entrenched HA-MRSA strains (25, 35).

Microbial genotyping analysis allows investigation into the clonality of an outbreak and risk factors associated with infection (5). Methods such as pulsed-field gel electrophoresis (PFGE) (39), repetitive-element-based PCR (rep-PCR) (42), and multilocus sequence typing (MLST) (10, 11) are used for microbial genotyping but are costly and labor-intensive and

do not enable specific characterization of acquired genetic elements encoding virulence factors or toxins or of genes that mediate antibiotic resistance (40). A rapid technique for determining the MRSA strain genotype and its broader complement of genetic elements would enable a more comprehensive understanding of transmission dynamics and could lead to more effective actionable decisions related to bed management, prioritization of infection control resources, and treatment.

Here, we describe the use of a rapid and high-throughput method to simultaneously genotype and characterize *S. aureus* specimens with respect to acquired genes encoding virulence factors, toxins, and antibiotic resistance determinants. The method is based on PCR coupled to electrospray ionization-mass spectrometry (ESI-MS) (8, 22, 37).

The PCR/ESI-MS assay uses several novel strategies. First, broad-range primers, targeting sites that are highly conserved in all members of a microbe family, are used to amplify PCR products from groupings of microbes rather than single species. These primers are coupled with species- or strain-specific primers for the identification of specific pathogens or antibiotic targets. Second, PCR conditions are, by design, permissive and thus tolerant of mismatches, so that even sequences from novel strains can be amplified. Third, inosine and other “wild-card” nucleotides are used in primers to facilitate PCR analysis of mispaired sequences. Fourth, because MS simply measures the mass-to-charge ratio (m/z), the sequence of the amplicon need not be known in order to detect it. The technology offers

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TABLE 1. Primer pairs used in this study

Primer pair code	Target(s)	GenBank accession no.	Amplicon sequence coordinates	Primer sequence
BCT2249	<i>tufB</i>	NC_002758	615038–616222	5'-TGAACGTGGTCAAATCAAAGTTGGTGAAGA-3' 5'-TGTACCAGCTTCAGCGTAGTCTAATAA-3'
BCT2095	PVL genes, <i>lukE-lukD</i>	NC_003923	1529595–1531285	5'-TGAGCTGCATCAACTGTATTGGATAG-3' 5'-TGGAAAACCTCATGAAATTTAAAGTGAAAGGA-3'
BCT879	<i>mecA</i>	Y14051	4507–4581	5'-TCAGGTACTGCTATCCACCCTCAA-3' 5'-TGGATAGACGTCATATGAAGGTGTGCT-3'
BCT2056	<i>mecRI</i>	NC_003923	41798–41609	5'-TTTACACATATCGTGAGCAATGAACTGA-3' 5'-TTGTGATATGGAGGTGTAGAAGGTGTTA-3'
BCT3016	<i>mupA</i>	X75439	2482–2573	5'-TAGATAATTGGGCTCTTTCTCGCTTAAAC-3' 5'-TAATCTGGCTGCGGAAGTGAAAT-3'
BCT2256	<i>nuc</i>	NC_002758.2	894288–894974	5'-TACAAAGGTCAACCAATGACATTCAGACTA-3' 5'-TAAATGCACTTGTCTTCAGGCCATAT-3'
BCT2081	<i>ermA</i>	NC_002952	55890–56621	5'-TAGCTATCTTATCGTTGAGAAGGGATTTGC-3' 5'-TGAGCATTTTTATATCCATCTCCACCAT-3'
BCT2086	<i>ermC</i>	NC_005908	2004–2738	5'-TCTGAACATGATAATATCTTTGAAATCGGCTC-3' 5'-TCCGTAGTTTTGCATAATTTATGGTCTATTTCAA-3'

advantages over routine single-target and multiplex PCR in that it is a full bioinformatics sequence analysis system.

After amplification, MS is used to rapidly determine the precise mass-to-charge ratio for the amplified nucleic acid fragments present, and the A, C, T, and G contents (i.e., the base composition) of each amplicon are determined using proprietary software that creates a signature to allow organism identification and genotyping. This novel MS technology enables the rapid, sensitive, cost-effective, and simultaneous detection of a wide range of typical pathogenic organisms.

We used the PCR/ESI-MS assay to analyze a well-characterized set of *S. aureus* strains from the CDC and geographically distinct clinical isolates from Maryland and Arizona. The PCR/ESI-MS technology effectively combines genotyping and characterization on a single high-throughput platform suitable for surveillance, infection control interventions, and patient management.

MATERIALS AND METHODS

Bacterial isolates. A total of 317 *S. aureus* isolates were studied. Thirty were selected to represent the diversity of isolates in the CDC database, including strains historically responsible for HA and community-acquired disease (29, 40). Two hundred forty clinical isolates of *S. aureus* from Johns Hopkins Hospital (JHH) were analyzed. Isolate sources were blood samples ($n = 31$), wound/skin specimens ($n = 43$), nasal samples obtained for surveillance purposes ($n = 57$), respiratory specimens not obtained for surveillance purposes ($n = 78$), urine samples ($n = 10$), samples from sterile body sites ($n = 10$), and specimens collected from medical devices such as catheter tips ($n = 11$). *S. aureus* isolates from the University of Arizona collection included 47 clinical isolates obtained from wound specimens, abscesses, drainage fluids, sputum samples, cardiovascular catheter tip specimens, and tissues. Twenty-nine coagulase-negative staphylococcal isolates, isolated at the University of Arizona, were evaluated as specificity controls, along with a *Staphylococcus schleiferi* isolate from the CDC.

Genotyping of all isolates was performed previously by PCR/ESI-MS and by a variety of reference methods, such as PFGE, MLST, and rep-PCR, as described elsewhere (17). In addition to genotype characterization and assignment to clonal complexes, as described by Hall et al. (17), further characterization of the same bacterial isolates was performed as described herein.

Primer pairs for PCR/ESI-MS. General methods for PCR/ESI-MS using the commercially available Ibis T5000 instrument have been described previously (9, 19). Genus-wide detection of *Staphylococcus* spp. was achieved with a primer pair targeting part of the region of *tufB* encoding domain II of EF-Tu (20). Primers were designed using an alignment of *tufB* genes extracted from multiple, diverse bacteria and were selected for the ability to specifically prime DNA from *Staphy-*

lococcus spp. and provide a PCR product with a base composition that distinguishes *S. aureus* from other *Staphylococcus* spp.

Further confirmation of the presence of *S. aureus* was provided by a primer pair targeting the *S. aureus*-specific thermostable nuclease gene *nuc* (4). An amplicon from the primer pair BCT2256, which targets the *nuc* gene encoding the thermonuclease enzyme, indicates the presence of *S. aureus*. The *nuc* gene is believed to be unique to *S. aureus*; it has not been found in other *Staphylococcus* spp. (12). Thus, an amplicon from the *tufB* primer pair with the expected *S. aureus* base composition and the presence of an amplicon from the *nuc* gene will unambiguously identify an isolate as *S. aureus*. Primer sequences are listed in Table 1.

Two primer pair sets were used to detect high-level methicillin resistance (Table 1). One pair (BCT879) directly targets the open reading frame of the *mecA* gene (genomic reference accession no. Y14051.1) (43), and the second pair (BCT2056) targets a region spanning the 5' end of the *mecRI* regulatory gene (23) and the intergenic region just upstream of the *mecRI* gene (genomic reference accession no. NC_003923.1) (1). Amplicons with the expected base compositions from these two primer pairs suggest the presence of an intact methicillin resistance gene and an associated regulatory system.

Other antibiotic targets were also included. The presence of *ermA* and *ermC* genes was detected by primer pairs targeting the open reading frames of these genes (primer pairs BCT2081 and BCT2086, respectively). In addition, high-level resistance to mupirocin was detected by a primer pair (BCT3016) targeting the *mupA* gene (reference accession no. X75439.1) (21). While the clinical relevance of high- and low-level mupirocin resistance is not completely known (2), resistance is on the rise in parts of the world (33, 38) and may hamper efforts to use mupirocin in hospitals that employ it to decolonize patients. Therefore, the *mup* targets were included, as there are interpretive criteria (7) and the ability to assess resistance trends is of interest to investigators.

Regions of two leukocidin toxin-producing operons were amplified by a single pair of primers, BCT2095, designed according to the sequence with accession number NC_003923.1. The genes encoding Pantone-Valentine leukocidin (PVL) were detected by a primer pair targeting a region of the PVL operon that includes the junction of the 3' end of *lukS-PV* and the 5' end of *lukF-PV* (28) (Fig. 1A). The same primer pair simultaneously detects and differentiates (by virtue of base composition) the homologous leukotoxin genes *lukE* and *lukD* (16). Thus, analysis of an isolate has four potential outcomes: PVL positive, *lukE-lukD* positive, positive for both, or positive for neither. Three of these outcomes are illustrated in Fig. 1B. No independent confirmation of *lukD* status was performed.

Antimicrobial resistance detection. At JHH, all isolates were tested for susceptibility to oxacillin, erythromycin, clindamycin, vancomycin, and trimethoprim-sulfamethoxazole by the agar dilution method per CLSI guidelines (6). Clindamycin susceptibility was confirmed using the D-test (6). Methicillin resistance detection and other in vitro antimicrobial susceptibility testing of *S. aureus* isolates at the University of Arizona were performed according to disk diffusion testing standards established by the CLSI (6).

Susceptibility to mupirocin was evaluated with the mupirocin Etest according to the instructions of the manufacturer (AB Biodisk, Solna, Sweden). Mupirocin-resistant strains were grouped into two distinct categories: those with low-level or

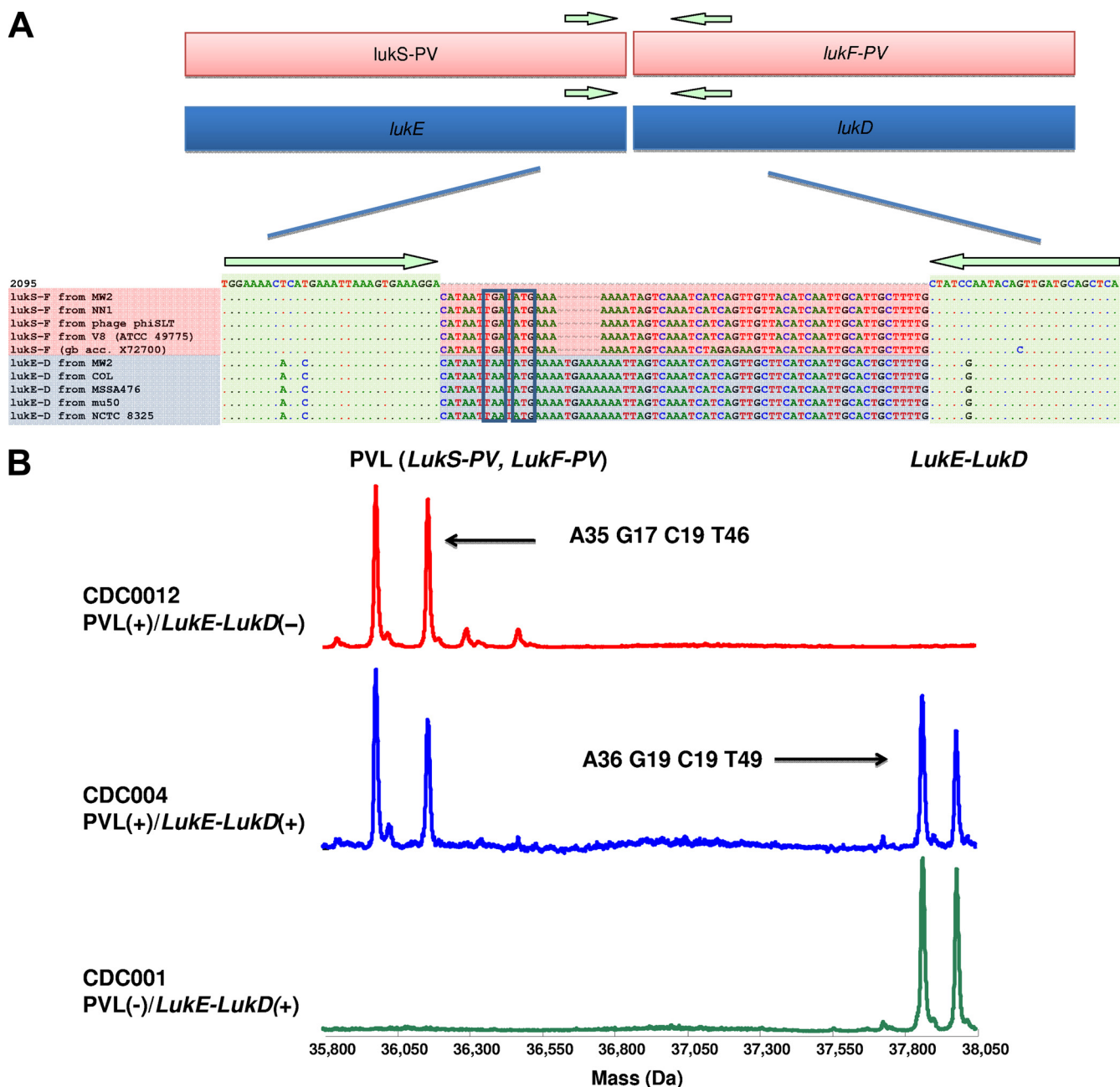


FIG. 1. Detection and identification of genes encoding leukocidin toxins. (A) Alignment of leukocidin toxin-encoding genes. The *lukS-PV* and *lukF-PV* genes (orange), encoding the PVL toxin, and the *lukE* and *lukD* genes (blue) have structural and sequence homology that enables detection of both operons with the same primer pair. The translational termination and initiation codons are boxed. A 6-nucleotide insertion (encoding two additional amino acids) in the *lukE-lukD* operon provides a length variation that, coupled with base composition variation, distinguishes the operons in PCR/ESI-MS. gb acc., GenBank accession number. (B) Spectra from representative isolates from the CDC showing the combinations of leukocidin genes found in different isolates. Forward and reverse PCR product strand mass measurements are shown in the spectra. The base composition shown is for the forward strand.

intermediate resistance, for which MICs were 4 to 256 µg/ml, and those with high-level resistance (Mup^r strains), for which MICs were ≥512 µg/ml.

PCR detection of genetic targets. The presence of genes encoding toxic shock syndrome toxin 1 and PVL was assessed by real-time PCR in a duplex reaction using primers, probes, and concentrations described previously (28, 31). *S. aureus* strains ATCC 51650 and ATCC 49775 were utilized as positive controls for toxic shock syndrome toxin 1 and PVL genes, respectively, and as negative controls for the respective alternate gene targets.

Genotyping. SCCmec typing of isolates from the CDC was performed according to the method of Zhang et al. (44). JHH isolates were genotyped by PFGE using a

modification of the protocol of McDougal et al. (29). Patterns were interpreted using the criteria of Tenover et al. (39). Patterns were compared to those for the USA100 to USA800 control strains obtained from the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA), and isolates with three or fewer band differences from the USA clones were presumed to be the same strain as the clone. Arizona isolates were analyzed by rep-PCR using a Diversilab system according to the instructions of the manufacturer (bioMérieux, Marcy l’Etoile, France).

Human subjects. Procedures were performed in accordance with ethical standards as reviewed by the University of Arizona and the Johns Hopkins Institutional Human Subjects Protection Committees (13).

RESULTS

The PCR/ESI-MS method enables identification and characterization of *Staphylococcus* isolates at several levels. At the genus level, primer pair BCT2249 (Table 1) hybridizes with a region of the *tufB* gene (encoding EF-Tu) and amplifies DNA isolated from all known species within the *Staphylococcus* genus but not from other genera of bacteria (Fig. 2A). The base composition of the amplicon distinguishes *S. aureus* from all other species of staphylococci. Representative spectra from *S. aureus* isolates and several coagulase-negative *Staphylococcus* species are shown in Fig. 2B. In ESI-MS analysis of PCR amplicons, the forward and reverse strands of the DNA are separated, producing two distinct peaks in the mass spectrum. Almost all unique base compositions are distinguishable by MS, even those separated by single nucleotide substitutions. Thus, this primer pair is capable of detecting all species of staphylococci for which sequence data exist based on bioinformatics analyses of sequence databases and unambiguously distinguishes *S. aureus* from other species. Analyses of amplicons from more than 1,000 confirmed *S. aureus* isolates (317 in this study) to date have shown identical base compositions (43 A, 28 G, 19 C, and 35 T residues). The ability of this primer pair to unambiguously identify strains of non-*S. aureus* staphylococci to the species level has not yet been determined experimentally; however, in specificity testing, 29 coagulase-negative *Staphylococcus* isolates were correctly categorized in the coagulase-negative *Staphylococcus* grouping and showed no cross-reactions suggesting *S. aureus* identification patterns.

The *nuc* primer pair was shown to have 100% sensitivity and specificity for all isolates in this study. Additional primer pairs were used to determine the presence or absence of other genes important in the characterization of *S. aureus*.

Using the primers shown in Table 1, we employed PCR/ESI-MS to characterize 30 *S. aureus* isolates from the CDC that had been genotyped previously by PFGE, MLST (23 isolates), and multilocus PCR/ESI-MS and characterized by real-time PCR (17). These isolates represent diverse genotypes corresponding to each of the major clonal complexes. The results of PCR/ESI-MS characterization are shown in Table 2. As expected, the *tufB*- and *nuc*-targeting primer pairs generated amplicons consistent with *S. aureus* for all isolates except CDC0031, a blind unknown isolate that was *nuc* negative and gave a PCR/ESI-MS *tufB* signature inconsistent with *S. aureus* but consistent with its correct identification, *S. schleiferi*.

All isolates were either positive for both *mecA* and *mecR1* genes or negative for both. PCR/ESI-MS results were consistent with previous characterization at the CDC by broth microdilution and *mecA* PCR. The presence or absence of *ermA* and *ermC* as detected by PCR/ESI-MS was concordant with in vitro resistance to erythromycin and clindamycin as determined at the CDC. PCR/ESI-MS detection of *mupA* in CDC006 was consistent with broth microdilution and PCR results; all other isolates were phenotypically mupirocin susceptible and negative for *mupA* by PCR/ESI-MS. The *lukE* and *lukD* genes of this sample set were not characterized independently, so the PCR/ESI-MS results for these genes could not be corroborated.

There was no correlation between the presence or absence of PVL genes or any of the antibiotic resistance genes and PCR/ESI-MS types or clonal complexes, suggesting that the

acquisition of these genes happened on a more recent time scale than the evolution of the housekeeping genes used in MLST analysis. However, the presence or absence of *lukE-lukD* was highly correlated with both PCR/ESI-MS types and clonal complexes (Table 2). All isolates belonging to clonal complex 8 (CC8) and CC5 were *lukE-lukD* positive, and all isolates of CC30, CC45, and CC59 were *lukE-lukD* negative, suggesting a stable relationship between the housekeeping genes used in MLST and the *lukE-lukD* operon.

Two hundred forty isolates of *S. aureus* obtained from JHH were characterized by PCR/ESI-MS. These isolates, previously genotyped by PFGE at JHH, were grouped by PCR/ESI-MS into 23 unique genotypes (Fig. 3A), primarily from six major clonal complexes, with the largest numbers of isolates from CC8 (82 of 240; 34%) and CC5 (70 of 240; 29%). Upon analysis of these same isolates for the presence of the *lukE-lukD* operon, PVL genes, *mecA*, *ermA*, *ermC*, and *mupA*, the number of unique PCR/ESI-MS genotypes increased from 23 to 59. A visualization of the occurrence of these genes in conjunction with the PCR/ESI-MS genotype and clonal complex assignment is shown in a radial plot in Fig. 3A. The presence of the *lukE-lukD* operon, PVL genes, the *mec* gene, and either *ermA* or *ermC* is depicted by the stippled segments, and the absence of these genes is shown using the background color for the clonal complex. Thirty-four of the 240 isolates (14%) were PVL positive, and the majority of these (29 isolates) were of PCR/ESI-MS type 1 (corresponding to CC8, which contains USA300 isolates). All but three of these were also *mecA* positive. Overall, 95 of the 240 isolates (40%) were *mecA* positive, 69 (29%) were *ermA* positive, 44 (18%) were *ermC* positive, and only 4 (<2%) were *mupA* positive. Only three of the *erm*-positive isolates were both *ermA* and *ermC* positive. The distribution of the antibiotic resistance genes was less correlated with PCR/ESI-MS types and clonal complexes than was that of the leukotoxin operons (see the two outer rings of Fig. 3A). The results obtained from PCR/ESI-MS compared favorably with the results of phenotypic methods to detect resistance to oxacillin, erythromycin, and mupirocin and with PCR and PFGE typing data (Table 3). Sensitivities and specificities ranged from 90 to 100%, except for erythromycin resistance. The presence of either *ermA* or *ermC* was highly predictive of erythromycin resistance detected by culture (specificity, 98%). The absence of an *ermA* or *ermC* gene did not ensure erythromycin susceptibility (70%), however, implying resistance by other mechanisms, such as *msrA*.

S. aureus isolates obtained from the University of Arizona were also characterized by PCR/ESI-MS, and the data compared to results from conventional culture methods for species identification and antimicrobial resistance as shown in Fig. 3B. PCR/ESI-MS spectra from the *tufB* and *nuc* gene-targeting primers showed the characteristic spectral signatures for *S. aureus*, with no variation in base composition from the canonical signatures. The 47 *S. aureus* isolates comprised six PCR/ESI-MS types and three clonal complexes, predominantly CC5 and CC8. All but one of the isolates contained the *lukE-lukD* operon. Isolates belonging to CC5 (e.g., USA100) were PVL negative, and all but one of the isolates belonging to CC8 (e.g., USA300 and USA500) were PVL positive. The *mecA* gene was present in 42 of 47 isolates (89%), the *ermA* gene was present in 36 (77%), and the *ermC* gene was present in 4 isolates (9%);

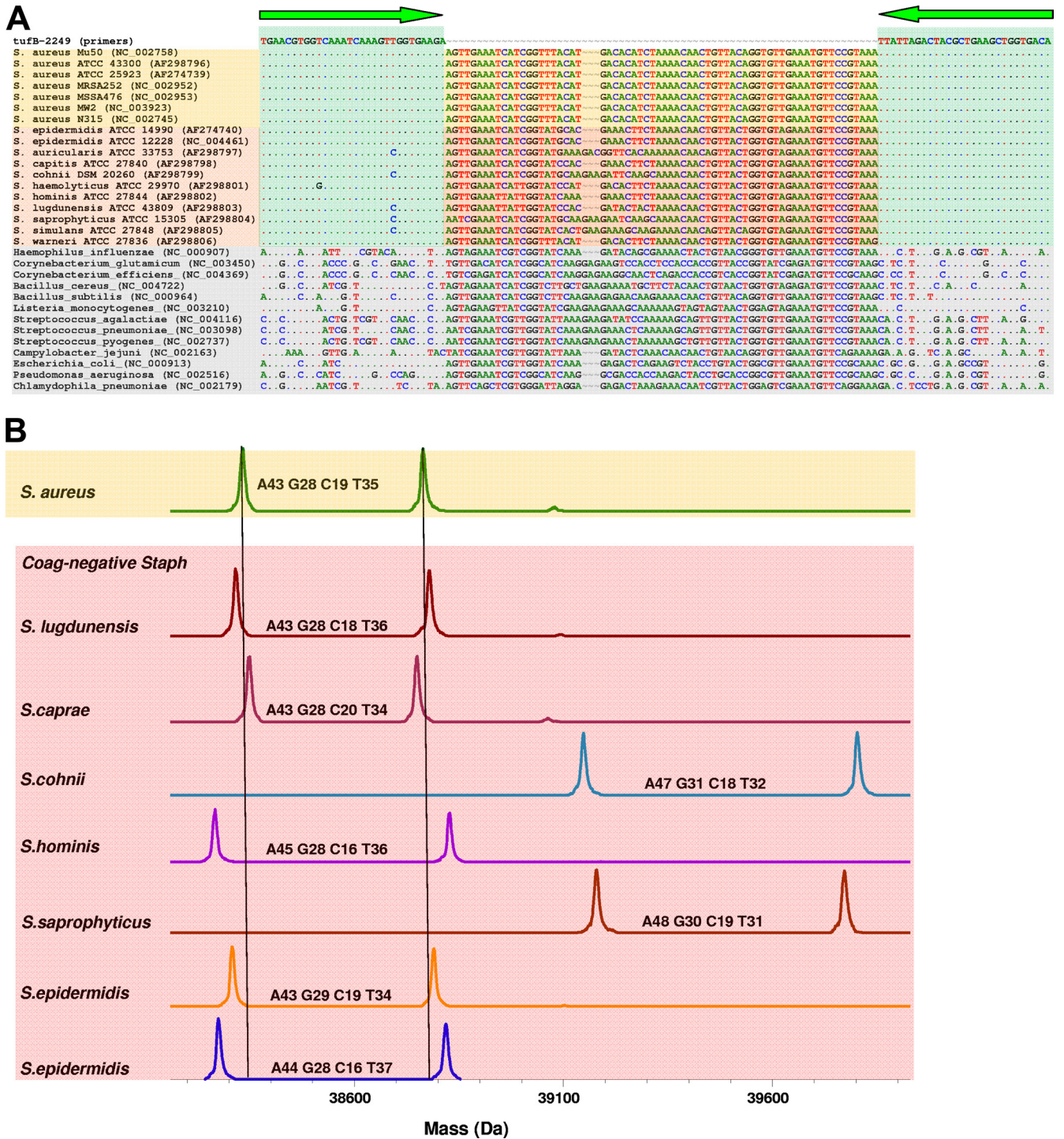


FIG. 2. Detection and identification of multiple *Staphylococcus* spp. by base composition analysis with a genus-level primer pair targeting the *tufB* gene. (A) Alignment showing the target and amplified region for the *Staphylococcus*-specific *tufB* primer pair BCT2249. The primer binding sites in the species with sequences amplified by the primers are shaded in light green. Yellow shading highlights the names of and amplified regions from the multiple *S. aureus* strains tested. Orange shading indicates coagulase-negative *Staphylococcus* species with sequences amplified by the primer pair and distinguished from *S. aureus* sequences by base composition. Unamplified sequences of organisms representing a diverse collection of non-*Staphylococcus* bacterial species, including related members of the *Bacillales* (*Bacillus* and *Listeria* spp.) and gram-positive and gram-negative bacteria, are shaded in grey. (B) Differentiation of *Staphylococcus* spp. using a base composition signature from the *tufB* gene. Forward and reverse PCR product strand mass measurements are shown in the spectra. The base composition labels represent only the forward strands. DNA was amplified from representative coagulase-negative *Staphylococcus* (coag-negative Staph) spp., and species were identified based upon matches to calculated base compositions from known organisms.

TABLE 2. PCR/ESI-MS signatures of CDC panel of *S. aureus* isolates^a

Clonal complex ^a	PCR/ESI-MS type	Sample identification no.	USA PFT(s) ^b	Result for leukotoxin (primer pair):		Result for species identifier (primer pair):			Result for antibiotic resistance gene(s) (primer pair):				
				LukE-LukD (BCT2095)	PVL (BCT2095)	<i>tdiB</i> (BCT2249)	<i>nuc</i> (BCT2256)	<i>mecA</i> (BCT1879)	<i>mecR1</i> (BCT2056)	<i>ermA</i> (BCT2081)	<i>ermC</i> (BCT2086)	<i>mupA</i> (BCT2313)	
CC8	1	CDC0010	Archaic	+	-	<i>S. aureus</i>	+	+	+	+	-	-	-
		CDC0026	EMRSA10	+	-	<i>S. aureus</i>	+	+	+	+	+	+	-
		CDC0030	Iberian	+	-	<i>S. aureus</i>	+	+	+	+	+	+	-
		CDC0015	USA300	+	+	<i>S. aureus</i>	+	-	-	-	-	-	-
		CDC0014	USA300	+	+	<i>S. aureus</i>	+	+	+	+	+	+	-
		CDC004	USA300	+	+	<i>S. aureus</i>	+	+	+	+	+	+	-
		CDC0019	USA500	+	-	<i>S. aureus</i>	+	+	+	+	+	+	-
		CDC008	USA300/USA500	+	-	<i>S. aureus</i>	+	+	+	+	+	+	-
		CDC0023	Brazilian	+	-	<i>S. aureus</i>	+	+	+	+	+	+	-
		CDC0025	EMRSA9	+	-	<i>S. aureus</i>	+	+	+	+	+	+	-
CC5	2	CDC001	USA100	+	-	<i>S. aureus</i>	+	+	+	+	+	-	
		CDC0022	USA100	+	-	<i>S. aureus</i>	+	+	+	+	+	-	
		CDC006	USA100	+	-	<i>S. aureus</i>	+	+	+	+	+	+	
		CDCVRS1	USA100	+	-	<i>S. aureus</i>	+	+	+	+	+	-	
		CDCVRS2	ND	+	-	<i>S. aureus</i>	+	+	+	+	+	+	
		CDC007	USA800	+	-	<i>S. aureus</i>	+	+	+	+	+	-	
		CDC0027	USA100	+	-	<i>S. aureus</i>	+	+	+	+	+	-	
CC30	3	CDC0011	USA200	-	-	<i>S. aureus</i>	+	-	-	-	-	-	
		CDC0021	USA200	-	-	<i>S. aureus</i>	+	+	+	+	+	-	
		CDC0012	USA100	-	+	<i>S. aureus</i>	+	+	+	+	-	-	
CC45	6	CDC002	USA600	-	-	<i>S. aureus</i>	+	+	+	+	+	-	
		CDC0028	USA600	-	-	<i>S. aureus</i>	+	+	+	+	+	-	
CC59	5	CDC005	USA1000	-	-	<i>S. aureus</i>	+	+	+	+	+	-	
		CDC0018	USA1000	-	+	<i>S. aureus</i>	+	+	+	+	+	-	
CC1	11	CDC0029	USA400	+	+	<i>S. aureus</i>	+	+	+	+	-	-	
CC15	12	CDC0020	USA900	+	-	<i>S. aureus</i>	+	-	-	-	+	-	
CC80	8	CDC0013	ST80	+	+	<i>S. aureus</i>	+	+	+	+	-	-	
CC22	13	CDC0024	EMRSA15	-	-	<i>S. aureus</i>	+	+	+	+	-	-	
None	9	CDC0016	USA1200	+	-	<i>S. aureus</i>	+	-	-	-	-	-	
None	7	CDC003	USA700	+	-	<i>S. aureus</i>	+	+	+	+	-	-	
NA ^c	NA	CDC0031	NA	-	-	<i>S. schleiferi</i>	-	-	-	-	-	-	

^a Clonal complex as defined by Enright et al. (10, 11).^b USA PFT as defined by McDougal et al. (29).^c NA, not applicable.

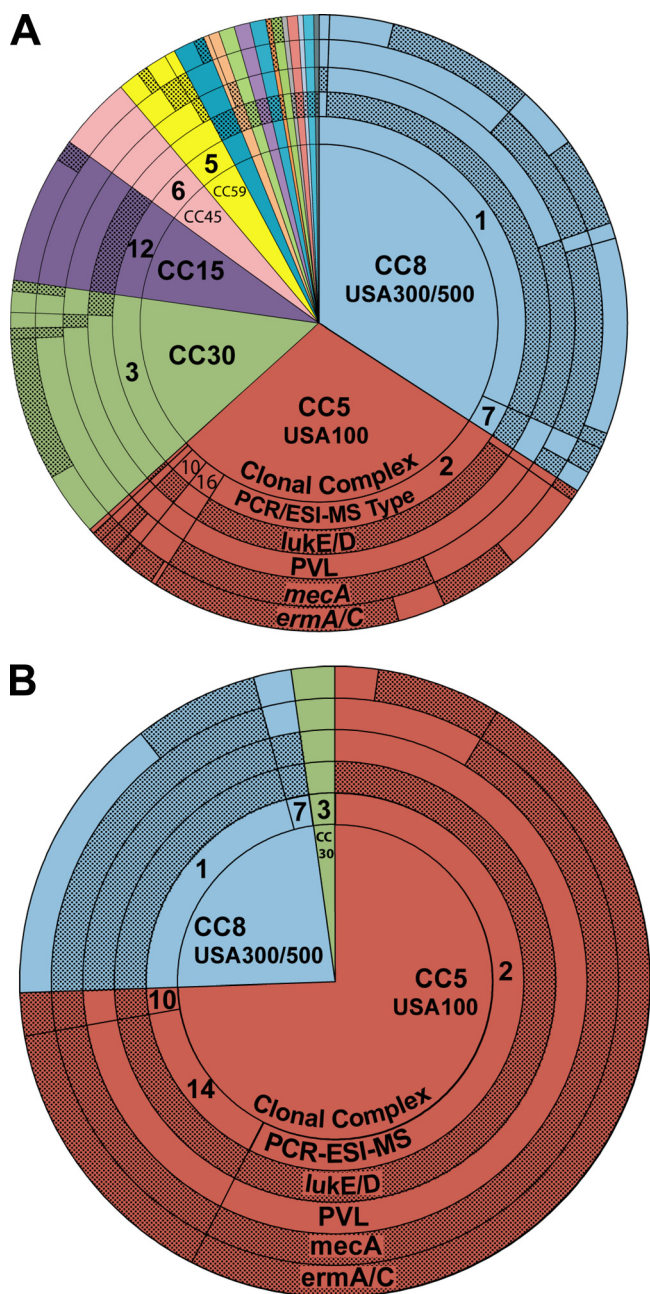


FIG. 3. PCR/ESI-MS analysis of *S. aureus* isolates obtained from geographically distinct hospital collections. (A) Radial plot of results from genotyping and characterization of 240 *S. aureus* isolates from JHH. The area of each wedge corresponds to the number of isolates with the given genotypic signature. The innermost circle corresponds to clonal complex groupings based on PCR/ESI-MS genotypes, showing a predominance of CC8 and CC5, and these groupings are subdivided in the next circular band. Sections corresponding to different clonal complexes are uniquely colored. The presence or absence of the *lukE-lukD* operon is depicted in the third band from the center, with the presence of the genes indicated by stippling within the segment; the absence of the genes is shown using the background color for the clonal complex. The same patterns are used in outer bands for PVL genes, the *mec* gene, and either the *ermA* or *ermC* gene, as described above. (B) Radial plot of results for 47 isolates from the University of Arizona, with circles, wedges, and coding symbolizing data as described above.

3 of the 4 were both *ermA* and *ermC* positive. The presence of the *mecA* gene correlated with in vitro cefoxitin resistance for all 47 isolates. Of the 37 isolates that contained either an *ermA* or *ermC* gene, all but 2 were erythromycin resistant; however, as was observed with the CDC and JHH isolates, the absence of *ermA* or *ermC* did not ensure erythromycin susceptibility. Of the 10 *ermA*- and *ermC*-negative isolates, only 2 were susceptible to erythromycin. Of the 47 isolates, only 1 had the *mupA* gene; this isolate was confirmed to be mupirocin resistant in vitro. The other isolates were mupirocin susceptible.

DISCUSSION

S. aureus isolates demonstrate a wide variety of virulence properties and antibiotic resistance profiles, which make clinical and prognostic assessments of infections caused by HA-MRSA and those caused by CA strains of MRSA and methicillin-susceptible *S. aureus* difficult. Here, we describe a rapid and high-throughput method of analysis, pathogen profiling via PCR/ESI-MS, which is suitable for use in clinical research and surveillance and may have future merit in a clinical laboratory setting. The method identifies key genetic elements that distinguish *S. aureus* from coagulase-negative *Staphylococcus* species and detects the presence of the *mecA*, *ermA* and *ermC*, and *mupA* genes, known to impart antibiotic resistance to methicillin, erythromycin, and mupirocin, respectively. In addition, the assay provides high-resolution genotypic signatures for *Staphylococcus* isolates and detects the presence and absence of the *lukE-lukD* operon and the PVL operon. One limitation of the present study is that the *lukD* reactions were not confirmed via PCR for *lukD*; although the role of the *lukD* toxin is uncertain, further confirmation of this genetic target is warranted.

The PCR/ESI-MS method will enable testing to move beyond single-target molecular methods. It allows near-real-time antimicrobial susceptibility testing for several antibiotics, whereas current methods target methicillin alone (12, 34). This study assessed the accuracy of testing of four antibiotic resistance targets. There was a high degree of correlation between the presence of the *mecA* gene in bacterial isolates and resistance to cefoxitin and associated oxacillin and methicillin resistance; discordant results from PCR/ESI-MS and traditional phenotypic susceptibility testing for some samples were observed. The exact cause of these discrepancies is unknown; future studies will need to explore the reasons for these different outcomes.

Likewise, a high degree of correlation between the presence of the *mupA* gene and mupirocin resistance was observed, although no conclusions about the assay's performance can be drawn due to the limited number of Mup^r strains found at the collaborating sites. Testing with a sample set of Mup^r strains will be necessary. In contrast, while the presence of an *ermA* or *ermC* gene correlated with erythromycin resistance, the absence of these genes did not predict erythromycin susceptibility. This finding is not surprising, as other mechanisms for erythromycin resistance have been described previously (36). Two isolates contained an *ermA* or *ermC* gene but were erythromycin susceptible; the cause of this phenomenon is unknown but may be a mutation or other genetic changes that rendered the isolates susceptible to erythromycin. Results are promising for many of the targets used in the PCR/ESI-MS assays; however, since this sample set was derived from banked isolates,

TABLE 3. Sensitivity and specificity of PCR/ESI-MS with respect to phenotypes for Johns Hopkins isolates

Determinant or factor	PCR/ESI-MS result	No. of isolates with agar dilution susceptibility result:		No. of isolates with real-time PCR result:		No. of isolates with Etest result:		No. of isolates with agar dilution susceptibility result:		Sensitivity (%)	Specificity (%)
		Oxacillin resistant	Oxacillin susceptible	PVL positive	PVL negative	Mup ^r	Mup ^s	Erythromycin resistant	Erythromycin susceptible		
<i>mecA</i> ^a	Positive	89	6							94	96
	Negative	6	139								
PVL	Positive			89	6					90	97
	Negative			3	139						
<i>mupA</i>	Positive					4	0			100	100
	Negative					0	236				
<i>erm</i> ^b	Positive							102	2	70	98
	Negative							44	79		

^a *mecA* as defined by PCR/ESI-MS versus oxacillin agar dilution methods.

^b *erm* as defined by PCR/ESI-MS for *ermA* and *ermC* versus erythromycin agar dilution methods.

further testing of fresh isolates will need to be performed to assess the performance for specimens that have not been banked and frozen.

The PCR/ESI-MS configuration will require improvements before it can be reliably used for the assessment of erythromycin resistance, and the panel could be improved by the inclusion of analysis for genetic markers for other antibiotics, such as clindamycin, daptomycin, and linezolid. Nevertheless, the method as described here allows rapid molecular testing for genes that result in resistance to a number of antibiotics, and it should be useful for the diagnosis and treatment of MRSA infections. For example, while CA-MRSA is known to historically exhibit resistance to macrolides and oxacillin and susceptibility to ciprofloxacin, clindamycin, tetracycline, trimethoprim-sulfamethoxazole, and vancomycin, these susceptibility patterns are changing rapidly and cannot be used to determine appropriate empirical therapy. As a consequence, the ability to correctly predict antibiotic resistance may help to correctly target the therapeutic choices and limit inappropriate antibiotic therapy. Clearly, this strategy would require knowledge of the prevalent molecular basis for antibiotic resistance.

The additional health risks associated with CA strains and the pathogenesis and epidemiology of these strains merit further investigation, yet few commercial methods currently provide rapid options for staphylococcal toxin testing. For example, the PVL genes are markers of CA strains (40, 41) and are associated with increased virulence (3, 15, 27). Although not proven to be responsible for all the severe morbidity associated with CA strains, the presence of PVL genes is associated with greater risks to certain patient populations and identification of these genes may prove to be valuable. The use of experimental interventions such as the administration of intravenous immunoglobulin containing antibodies to PVL (14, 18) and combination antimicrobial therapy to quell the surge of toxins (30) has been reported to combat severe infections with PVL-positive strains. In such cases, rapid detection of the PVL operon may be useful. The *lukE-lukD* leukocidin operon (16, 32) has been less well studied but is associated with diarrhea-causing strains of *S. aureus* (16); therefore, further investigation of the *lukE-lukD* leukocidin toxin is warranted. The PCR/ESI-MS assay could easily be adapted to include other toxin gene targets to facilitate research to further characterize the epidemiology of the array of known toxins.

In summary, our PCR/ESI-MS approach allowed us to determine the genotypes of *Staphylococcus* spp. and to detect the presence or absence of genetic elements encoding potential virulence factors and antibiotic resistance elements. For the samples from the CDC and geographically disparate health care sites, the PCR/ESI-MS method correctly identified *S. aureus* isolates and distinguished them from coagulase-negative isolates. This methodology is suitable for surveillance and research purposes and, in the future, may support hospitals and state public health labs alike in epidemiologic tracking of HA- and CA-MRSA. The T5000 instrument platform can perform high-throughput testing for staphylococcal genotyping and characterization at a rate of approximately 84 samples per day. Plans for a clinical laboratory PCR/ESI-MS system with higher throughput and a variety of different test panels are under way; however, the present system is available for research use only at this time. Implementation of this rapid, high-throughput approach is a unique tool that can facilitate a broader understanding of staphylococcal epidemiology, transmission dynamics, virulence factors, and drug resistance patterns.

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