Discriminatory Indices of Typing Methods for Epidemiologic Analysis of Contemporary Staphylococcus aureus Strains

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Abstract: Historically, a number of typing methods have been evaluated for Staphylococcus aureus strain characterization. The emergence of contemporary strains of community-associated S. aureus, and the ensuing epidemic with a predominant strain type (USA300), necessitates re-evaluation of the discriminatory power of these typing methods for discerning molecular epidemiology and transmission dynamics, essential to investigations of hospital and community outbreaks. We compared the discriminatory index of 5 typing methods for contemporary S. aureus strain characterization.

Children presenting to St. Louis Children’s Hospital and community pediatric practices in St. Louis, Missouri (MO), with community-associated S. aureus infections were enrolled. Repetitive sequence-based PCR (repPCR), pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), staphylococcal cassette chromosome A (spa), and staphylococcal cassette chromosome (SCC) mec typing were performed on 200 S. aureus isolates. The discriminatory index of each method was calculated using the standard formula for this metric, where a value of 1 is highly discriminatory and a value of 0 is not discriminatory.

Overall, we identified 26 distinct strain types by repPCR, 17 strain types by PFGE, 30 strain types by MLST, 68 strain types by spa typing, and 5 strain types by SCCmec typing. RepPCR had the highest discriminatory index (D) of all methods (D = 0.88), followed by spa typing (D = 0.87), MLST (D = 0.84), PFGE (D = 0.76), and SCCmec typing (D = 0.68). The method with the highest D among MRSA isolates was repPCR (D = 0.64) followed by spa typing (D = 0.45) and MLST (D = 0.44). The method with the highest D among MSSA isolates was spa typing (D = 0.98), followed by MLST (D = 0.93), repPCR (D = 0.92), and PFGE (D = 0.89). Among isolates designated USA300 by PFGE, repPCR was most discriminatory, with 10 distinct strain types identified (D = 0.63). We identified 45 MRSA isolates which were classified as identical by PFGE, MLST, spa typing, and SCCmec typing (USA300, ST8, t008, SCCmec IV, respectively); within this collection, there were 5 distinct strain types identified by repPCR.

The typing methods yielded comparable discriminatory power for S. aureus characterization overall; when discriminating among USA300 isolates, repPCR retained the highest discriminatory power. This property is advantageous for investigations conducted in the era of contemporary S. aureus infections.

INTRODUCTION

Methicillin-resistant Staphylococcus aureus (MRSA) infections have presented a significant burden in healthcare settings for >50 years, serving as a source of severe morbidity and mortality in compromised hosts and posing substantial financial strain on healthcare institutions. In the late 1990s, novel strains of MRSA emerged, affecting immunocompetent individuals without exposure to healthcare settings and thus were designated community-associated (CA) MRSA. These CA-MRSA strains are clinically and genetically distinct from traditional healthcare-associated (HA) MRSA strains and have caused a clonal epidemic of cutaneous abscesses as well as invasive, life-threatening infections among otherwise healthy individuals.1-8 More recently, this lineage has entered healthcare settings and, in some regions, has become the predominant cause of nosocomial infections.9-11 Worldwide outbreaks of MRSA infections in both community and healthcare settings necessitate optimized strain typing methods in order to elucidate pathogen transmission dynamics.12-14

A number of strain typing methodologies have been developed for investigations of S. aureus molecular epidemiology, each based on a slightly different principle. S. aureus strains most frequently become resistant to methicillin via
acquisition of a staphylococcal cassette chromosome (SCC) mec element carrying mecA. In the United States, HA- and CA-MRSA strains have classically been distinguished based on SCCmec types. SCCmec I, II, and III are most abundant in HA-MRSA strains, whereas SCCmec IV and V are present in most CA-MRSA strains. Pulsed-field gel electrophoresis (PFGE), the now historical “gold standard” for bacterial typing, relies on restriction digestion and subsequent separation of genomic DNA fragments. Minor protocol differences or changes in electrophoresis parameters can result in poor interlaboratory reproducibility. This method is both time-consuming and technically demanding. Whereas MRSA pulotype USA100 represents the traditional strain type causing infections in healthcare settings, USA300 is the predominant MRSA strain type in the community and is also supplanting USA100 in many hospitals. Multilocus sequence typing (MLST) is a sequence-based genotyping method, which compares single nucleotide variants within housekeeping genes to a reference database, providing a sequence type (ST). Staphylococcal protein A (spa) typing analyzes the number and type of point mutations in the repeat region of the spa gene. Repetitive element sequence-based PCR (repPCR) is based on genomic fingerprint patterns to infer relationships among microorganisms using primers that hybridize to intergenic repetitive sequences scattered throughout the genome. Combining repPCR with Diversilab analysis software uses semi-automated objective criteria for assigning strain similarity and provides the ability to simultaneously compare a large number of isolates. A disadvantage of repPCR is that there is no standardized nomenclature at present in use across laboratories for designation of distinct strain types.

Historically, these strain typing methods have been characterized and compared to one another in a variety of settings with classical strains of S. aureus. Given the recent shift in circulating strain types in both community and healthcare settings, particularly the clonal epidemic of USA300 infections, it is essential to re-evaluate the discriminatory power (ie, the capability to identify distinct strains) of these methodologies in the contemporary era to inform approaches used in epidemiologic studies and outbreak investigations. Indeed, in settings with a largely homogeneous population of bacterial strains, genomic sequencing has revealed overestimation of transmission events when methods with inadequate discriminatory power have been used. Thus, the objective of this study was to evaluate the discriminatory index of a variety of strain typing methods in the context of contemporary S. aureus.

**METHODS**

**Microbiology and Molecular Typing**

RepPCR was performed at the Washington University School of Medicine (WUSM, St. Louis, MO) on 1527 infecting isolates obtained as part of a community-associated S. aureus colonization study. The Washington University Human Research Protection Office approved study procedures and written informed consent was obtained from all participants. S. aureus isolates were identified and antibiotic susceptibility testing was performed in accordance with Clinical and Laboratory Standards Institute procedures. DNA was extracted from S. aureus isolates using the BiOstic Bacteremia DNA Isolation Kit (MO BIO Laboratories, Inc, Carlsbad, CA) according to manufacturer’s specifications. RepPCR was performed as previously described using the primer RW3A. PCR products were resolved using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc, Santa Clara, CA), and the resulting banding patterns were compared using the Diversilab System software (bioMérieux, Durham, NC). A similarity index of > 95% was used to group isolates as identical strain types. Each distinct strain type (ie, “reference strain”) was assigned a consecutive number (these repPCR strain types are local designations unique to this study).

As described below, 200 isolates were selected for further analysis. In addition to repPCR, each of these isolates underwent molecular typing by PFGE, spa typing, SCCmec typing, and MLST. SCCmec typing to detect SCCmec types I-V was performed at WUSM via multiplex PCR as described previously. MLST was performed at The McDonnell Genome Institute at WUSM. All S. aureus reagent files used in our in silico typing were downloaded on August 27, 2014 (http://saureus.mlst.net). These data included allele-specific FASTA nucleotide sequences across the 7 representative S. aureus genes (arcC, aroE, glpF, gmk, pta,spa, tpiA, ygiL). For MLST review, all of the strain’s FASTQ files were independently aligned to a single, complete S. aureus reference genome (USA300_TCH1516; GenBank accession no. CP000730.1) using the Burrows-Wheeler Aligner algorithm. Once sample reads were aligned to the reference genome, alignments were collapsed into consensus sequences using various tools (mpileup, bcftools, vcftools, pl vcf2fq) in the samtools package. Once all alleles were assigned a designation for the 7 MLST genes, an ST pattern was defined. Eighteen isolates required manual assignment due to no ST pattern match. For these samples, sequence quality, reference coverage, and read alignments were manually validated using the Tablet genome assembly graphical viewer. For these 18 samples, there were more than adequate levels of high-quality coverage for calling consensus, but the resultant calls included sequence variants (or new ST patterns) not represented in our downloaded MLST identity information, and thus 8 new designations were characterized for the purposes of this study.

PFGE was performed at Emory University School of Medicine (Atlanta, GA) with the Smal restriction enzyme as described previously, now updated using Salmonella enterica serovar Braenderup H9182 as the normalization standard. Gel images were compared by using BioNumerics version 5.01 software (Applied Maths, Austin, TX) and assigned to pulsed-field types at 80% relatedness by use of Dice coefficients and the unweighted-pair group method using average linkages.

Spa typing was performed at Emory University School of Medicine as recommended on DNA prepared using the InstaGene Matrix (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions with an additional 30-min incubation at 65°C followed by 30 min at 37°C with 20 μg/ml of lysostaphin (Sigma-Aldrich, St. Louis, MO) between step 3 and step 4. PCR fragments were sequenced (Beckman Coulter Genomics, Beverly, MA), and sequences were queried and spu type assigned using BioNumerics version 5.01 software (Applied Maths). Although 15 isolates yielded no assigned spa-type designation, based on their complete repeat succession data, we were able to characterize these isolates into 5 novel groups for the purposes of this study.

**Cohort Generation**

It has been established that there is a predominant clone of MRSA circulating in the US at the present time. Therefore, we evaluated S. aureus isolates collected from patients with S. aureus cutaneous and invasive infections presenting to St. Louis Children’s Hospital and community pediatric practices in metropolitan St. Louis, as well as household contacts of these.
patients, from 2008 to 2011. From an initial collection of 1527
S. aureus isolates, a subset of 100 isolates was chosen, selected
to enrich for strain type diversity as determined by repPCR.
These 100 isolates included up to 5 representatives of each of the
less common repPCR strain types, with the remainder of isolates comprising the more common repPCR strain types. To
avoid an inherent bias toward repPCR diversity, we selected
another subset of 100 isolates, chosen from a distinct collection
of 641 isolates amassed during a separate study,21 that had all
been analyzed by MLST. This cohort of 100 isolates was
comprised of all the non-ST8 strains in the collection
(n = 69), with the complement of isolates made up of ST8
strains. The combined cohort of 200 isolates (from 124 patients)
was subjected to all typing methods described above and the
identity of all isolates as S. aureus was confirmed using matrix-
assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS).

**Discriminatory Index**

The discriminatory index (D) of each method was calcu-
lated using the standard formula for this metric:

\[
D = 1 - \frac{1}{N(N - 1)} \sum_{j=1}^{S} n_j(n_j - 1)
\]

where N is the total number of strains in the sample population,
S is the total number of subtypes described, and n_j is the number
of strains belonging to each of the subtypes. A value of 1 is
highly discriminatory and a value of 0 is not discriminatory.27
Confidence intervals (95%) for each D were calculated as previously described.42

**RESULTS**

**Number of Strain Types and Discriminatory Index (D) Per Method**

The first cohort of 100 isolates, generated based on repPCR
diversity, comprised 20 distinct strain types, as determined by
repPCR. We identified 21 distinct strain types by MLST, 16
strain types by PFGE, 43 strain types by spa typing, and 5 strain
types by SCCmec. The methods with the highest D were spa
typing and repPCR (D = 0.89 and D = 0.87, respectively),
followed by MLST (D = 0.79), PFGE (D = 0.75), and SCCmec
(D = 0.56) (Table 1).

In the second cohort of 100 isolates, selected for MLST
diversity, the number of strain types identified by repPCR was 16.
Within this sample set, we identified 21 strain types by MLST, 14
strain types by PFGE, 38 strain types by spa typing, and 5 strain
types by SCCmec. The method with the highest D was MLST
(D = 0.87), followed by repPCR and spa typing (both D = 0.86),
PFGE (D = 0.77), and SCCmec (D = 0.63) (Table 1).

When evaluating the entire collection of 200 S. aureus
isolates, we identified 26 distinct strain types by repPCR, 17
strain types by PFGE, 30 strain types by MLST, 68 strain types
by spa typing, and 5 strain types by SCCmec. The methods with the
highest D were repPCR (D = 0.88) and spa typing
(D = 0.87), followed by MLST (D = 0.84), PFGE (D = 0.76),
and SCCmec (D = 0.60) (Table 1).

**Stratified Analysis by Methicillin Resistance of the Isolates**

Of the 200 S. aureus isolates, 78 (39%) were MRSA and 122
(61%) were methicillin-susceptible S. aureus (MSSA). Among
the MRSA isolates, 9 distinct strain types were identified by
repPCR, 9 by MLST, 5 by PFGE, 2 by SCCmec, and 13 by spa
typing. The method with the highest D among MRSA isolates was
repPCR (D = 0.64) followed by spa typing (D = 0.45) and MLST
(D = 0.44). Among the MSSA isolates, 24 distinct strain types
were identified by repPCR, 25 by MLST, 14 by PFGE, 4 by
SCCmec, and 61 by spa typing. The method with the highest D
among MSSA isolates was spa typing (D = 0.98), followed by
MLST (D = 0.93), repPCR (D = 0.92), and PFGE (D = 0.89)
(Table 1). We identified 45 MRSA isolates which were classified
as identical by PFGE, MLST, spa typing, and SCCmec typing
(USA300, ST8, t008, SCCmec IV, respectively); within this
collection, there were 5 distinct strain types identified by repPCR.

**Analysis of USA300 Subset**

Of the 200 S. aureus isolates, 92 (46%) were designated
USA300 by PFGE. Within these USA300 isolates, 10 distinct
strain types were identified by repPCR, 13 by MLST, and 18 by
spa typing. The method with the highest D among the USA300
isolates was repPCR (D = 0.63) followed by spa typing
(D = 0.44) and MLST (D = 0.42) (Table 1).

The 108 non-USA300 S. aureus isolates represented 16
other PFGE strain types. Of the non-USA300 isolates, 24
distinct strain types were identified by repPCR, 26 by MLST,
and 55 by spa typing. The method with the highest D among
the non-USA300 isolates was spa typing (D = 0.98), followed by
MLST (D = 0.93) and repPCR (D = 0.91) (Table 1).

**Most Frequent Strain Types per Method**

Within the collection of 200 S. aureus isolates, the most
colonial strain type identified by PFGE was USA300 (46%), by
MLST was ST8 (37%), by SCCmec was IV (44%), and by spa
was t008 (35%). The 2 most common strain types identified by
repPCR (repPCR type 16 and repPCR type 17) each made up
21% of the collection (Table 2).

**DISCUSSION**

Staphylococcus aureus molecular typing is essential for
epidemiologic studies and outbreak investigations. The optimal
typing method may vary depending on the context, including
the predominant strain types present in the population, relative
clonality of the strains within a collection, and whether the desired
resolution is on a local or more global level. Although accurate
strain typing and robust discriminatory power is essential, there is
at present no consensus in the field regarding a single best
typing method. Several studies have conducted epidemiologic investi-
gations discriminating strain types based on a variety of
phenotypic and genotypic factors, including methicillin resistance.
Bocchini and colleagues investigated the similarity of S. aureus
isolates recovered from recurrent CA S. aureus infections in 700
otherwise healthy patients presenting to Texas Children’s Hos-
pital. Classifying strains based only on methicillin resistance,
this group reported that 90% of recurrences within the first 12
months after initial infection arose from the same strain,
compared with 79% of recurrences observed > 12 months after

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*Comparisons of S. aureus Typing Methods*
<table>
<thead>
<tr>
<th>Method</th>
<th>Total N = 200</th>
<th>Selecting for repPCR diversity N = 100</th>
<th>Selecting for MLST diversity N = 100</th>
<th>MRSA isolates only N = 78</th>
<th>MSSA isolates only N = 122</th>
<th>USA300 isolates only N = 92</th>
<th>Non-USA300 isolates only N = 108</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of unique strain types</td>
<td>D (95% CI)</td>
<td>No. of unique strain types</td>
<td>D (95% CI)</td>
<td>No. of unique strain types</td>
<td>D (95% CI)</td>
<td>No. of unique strain types</td>
</tr>
<tr>
<td>Methicillin resistance</td>
<td>2</td>
<td>0.48 (0.45, 0.51)</td>
<td>2</td>
<td>0.47 (0.42, 0.52)</td>
<td>2</td>
<td>0.49 (0.45, 0.52)</td>
<td>–</td>
</tr>
<tr>
<td>SCCmec</td>
<td>5</td>
<td>0.60 (0.56, 0.63)</td>
<td>5</td>
<td>0.56 (0.52, 0.60)</td>
<td>5</td>
<td>0.63 (0.58, 0.69)</td>
<td>2</td>
</tr>
<tr>
<td>repPCR</td>
<td>26</td>
<td>0.88 (0.86, 0.91)</td>
<td>20</td>
<td>0.87 (0.82, 0.92)</td>
<td>16</td>
<td>0.86 (0.81, 0.90)</td>
<td>9</td>
</tr>
<tr>
<td>MLST</td>
<td>30</td>
<td>0.84 (0.79, 0.88)</td>
<td>21</td>
<td>0.79 (0.72, 0.86)</td>
<td>21</td>
<td>0.87 (0.82, 0.91)</td>
<td>9</td>
</tr>
<tr>
<td>PFGE</td>
<td>17</td>
<td>0.76 (0.70, 0.82)</td>
<td>16</td>
<td>0.75 (0.67, 0.83)</td>
<td>14</td>
<td>0.77 (0.69, 0.84)</td>
<td>5</td>
</tr>
<tr>
<td>spa^1</td>
<td>68</td>
<td>0.87 (0.83, 0.92)</td>
<td>43</td>
<td>0.89 (0.83, 0.94)</td>
<td>38</td>
<td>0.86 (0.79, 0.92)</td>
<td>13</td>
</tr>
</tbody>
</table>

CI = confidence interval, D = discriminatory index, MLST = multilocus sequence typing, MRSA = methicillin-resistant S. aureus, MSSA = methicillin-susceptible S. aureus, PFGE = pulsed-field gel electrophoresis, repPCR = repetitive sequence-based PCR, SCCmec = staphylococcal cassette chromosome mec, spa = staphylococcal protein A.

^18 isolates that do not match a known MLST (9% of the 200 strains overall) have been categorized into 8 novel types A-H based on sequences.

^15 isolates that do not match a known spa type (8% of the 200 strains overall) have been categorized into 9 novel types 1–9 based on sequences.

14 MSSA isolates were SCCmec type I, 12 III, and 14 IV; the remaining 92 isolates were negative for SCCmec.

^1 isolate each of SCCmec type I (MSSA), II (MRSA), and III (MSSA); 18 isolates were negative for SCCmec (all MSSA); the remaining isolates were SCCmec type IV (69 MRSA, 2 MSSA).
TABLE 2. Most Frequent Strain Types per Method, N = 200

<table>
<thead>
<tr>
<th>Methicillin resistance (%)</th>
<th>SCCmec (%)</th>
<th>repPCR* (%)</th>
<th>MLST (%)</th>
<th>PFGE (%)</th>
<th>spa (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSSA (61)</td>
<td>IV (44)</td>
<td>RT 16 (21)</td>
<td>ST 8 (37)</td>
<td>USA 300 (46)</td>
<td>t008 (35)</td>
</tr>
<tr>
<td>MRSA (39)</td>
<td>III (6)</td>
<td>RT 17 (21)</td>
<td>ST 5 (9)</td>
<td>USA 200 (11)</td>
<td>t002 (5)</td>
</tr>
<tr>
<td></td>
<td>NEG (46)</td>
<td>RT 3 (13)</td>
<td>ST 30 (9)</td>
<td>USA 800 (9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>RT 20 (10)</td>
<td>ST 72 (6)</td>
<td>USA 600 (8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>RT 4 (5)</td>
<td>ST 45 (5)</td>
<td>USA 900 (5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>RT 24 (5)</td>
<td>ST 15 (5)</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>ST 59 (5)</td>
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</tr>
</tbody>
</table>

NOTE: strain types accounting for < 5% of sample not shown. MLST = multilocus sequence typing, MRSA = methicillin-resistant Staphylococcus aureus, MSSA = methicillin-susceptible Staphylococcus aureus, NEG = negative, PFGE = pulsed-field gel electrophoresis, repPCR = repetitive sequence-based PCR, RT = repPCR type, SCCmec = staphylococcal cassette chromosome mec, spa = staphylococcal protein A.

*repPCR strain types are local designations unique to this study.

Among the 24 USA100 isolates, 79% were ST5, 88% were spa type t002, and 96% carried SCCmec II. The investigators then evaluated the specificity, sensitivity, and positive and negative predictive values of these other typing methods (spa type t008, presence of the PVL, arcs, and opp3 genes, MLST ST8, and SCCmec type IV) to predict the USA300 pulsotype. The optimal combination of methods by receiver operator characteristic analysis was the presence of the arcs and PVL genes (area under the curve 0.98, 95% confidence interval 0.95 – 1.0).53

Similar to several published studies, in the present study of community-associated S. aureus, considering the entire cohort of 200 isolates, all molecular typing methods (repPCR, spa typing, PFGE, and MLST) yielded comparable discriminatory power.51,54 RepPCR performed superiorly when evaluating a homogenous population of isolates, such as might be studied in an outbreak setting or in discerning transmission dynamics. Specifically, within a cluster of 45 MRSA isolates classified as 1 identical strain type by a combination of typing methods (USA300, ST8, t008, and SCCmec IV), repPCR offered further discrimination among these isolates, discerning 5 distinct strain types. Strikingly, within our population of MSSA isolates, spa typing yielded the highest discriminatory power (D = 0.98). However, the findings in our study of contemporary S. aureus strains recovered from otherwise healthy children are in contrast to several other studies, which have found superior discriminatory power of PFGE compared to repPCR.21,44,55–57 The discrepancies between these studies could be attributable to methodological variation (eg, primers used and automated kits), study populations (hospitalized patients vs individuals in the community), geographic and temporal differences, and predominant circulating strain types.

Whole genome sequencing (WGS) is emerging as the ultimate strain typing tool.58 Using WGS, Köser and colleagues conducted a case-control study of MRSA isolates recovered from patients in the neonatal intensive care unit in the United Kingdom associated with an outbreak situation. Of 14 isolates sequenced (7 from cases associated with the outbreak and 7 isolates unaffiliated with the outbreak), 10 isolates had identical sequence types by MLST (of note, these were consistent with the most common MRSA clone recovered from hospitals in the UK). Within this group of 10 isolates deemed identical by MLST, phylogenetic analysis generated by WGS grouped the 7 outbreak-associated isolates as identical and differentiated these
isolates from the 3 strains not associated with the outbreak. Additionally, WGS illuminated a separate transmission event among the nonoutbreak isolates that had not been previously detected. Similarly, Price and colleagues conducted an evaluation of *S. aureus* transmission and acquisition among ICU patients in the UK. Isolates recovered from surveillance cultures from these patients were subjected to spa typing, epidemiologic evaluation (ie, determination of overlapping patient time in the ICU), and WGS. On the basis of WGS, 3 transmission events detected by the combination of spa typing and epidemiologic evaluation were discounted; WGS also detected additional acquisition and transmission events that were missed by conventional criteria. Although WGS provides ultimate discriminatory power among genotyping methods, a major challenge with WGS is determining the definition of a “strain type,” taking into consideration to which “gold standard” strain all isolates should be compared, what degree of genetic variation defines a distinct strain type, and what number of single nucleotide variants are expected due to evolution during microbial DNA replication. At present, given the specialized equipment, expense, time, and technical expertise required to conduct sequencing and analysis, WGS is not yet practical for most routine clinical settings.

The strengths of the present study are the large number of isolates evaluated and the comprehensive comparison of multiple typing methods with formal calculation of discriminatory index. This study also has several limitations. First, the MRSA isolates were all recovered in 1 geographic region (metropolitan St. Louis, MO). Additionally, the selection of isolates for the first cohort on the basis of diversity as determined by repPCR may have biased our results toward repPCR having a superior discriminatory index; however, within the second cohort of isolates, chosen based on MLST diversity, repPCR produced an almost identical discriminatory index to that of the first cohort of isolates, minimizing the likelihood of this bias.

In conclusion, in our study comparing molecular typing methods for *S. aureus* characterization in the contemporary era, whereas all methods yielded comparable results overall, repPCR demonstrated the highest discriminatory power within the USA300 subset. When planning and implementing epidemiologic studies and outbreak investigations, the discriminatory index of typing methods is an important consideration, particularly in the context of a predominant circulating clone.

REFERENCES


