Characterization of Community-acquired Methicillin-resistant Staphylococcus aureus by Pulsed-field Gel Electrophoresis, Multilocus Sequence Typing, and Staphylococcal Protein A Sequencing: Establishing a Strain Typing Database

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Characterization of Community-Acquired Methicillin-Resistant *Staphylococcus aureus*
by Pulsed-Field Gel Electrophoresis, Multilocus Sequence Typing, and Staphylococcal
Protein A Sequencing: Establishing a Strain Typing Database

by

Jill Carolyne Roberts

A dissertation submitted in partial fulfillment
of the requirements for the degree of
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Department of Global Health
College of Public Health
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Dedication

I would like to dedicate this dissertation to my mother Carolyne Roberts. Without your support I would never have realized and greatly exceeding my educational goals. Your faith in my ability to accomplish any goal, encouragement to strive for independence, strength of character, and resilience have contributed immensely to my success. A daughter cannot wish for a better role model and for that you will always have my deepest respect and love.
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Characterization of Community-Acquired Methicillin-Resistant \textit{Staphylococcus aureus} by Pulsed-Field Gel Electrophoresis, Multilocus Sequence Typing, and Staphylococcal Protein A Sequencing: Establishing a Strain Typing Database

Jill Carolyne Roberts

\textbf{Abstract}

\textit{Staphylococcus aureus} has long been recognized as a leading cause of nosocomial infection. However, several recent publications have demonstrated this pathogen as the cause of community-acquired severe wound infections and necrotizing pneumonia in otherwise healthy individuals. These highly virulent endemic clones have been reported in several locations in the United States and Canada. The rapid spread of the organism, the ability of certain clones to cause serious infection, and the antibiotic resistance of the endemic clones, illustrates the importance of infection control measures. In this study we examined three \textit{S. aureus} typing techniques; pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), and Staphylococcal protein A (\textit{spa}) sequencing for subspeciation of community-acquired methicillin-resistant \textit{S. aureus} (CA-MRSA). It is hypothesized that PFGE will result in a higher level of discrimination among the strains, while MLST and \textit{spa} typing will result in highly portable data that lacks the discriminatory power of PFGE. Thirty CA-MRSA isolates that were obtained from Florida and Washington State were characterized by molecular typing methods. Whole genome restriction analysis was performed by PFGE using the \textit{SmaI} enzyme. Sequence-
based typing analyses, MLST and spa typing, were performed by polymerase chain reaction (PCR) followed by sequencing. PFGE data was analyzed using the BioNumerics® software package and sequence-based data was analyzed using DNAstar®. MLST Alleles were assigned using the online MLST database (www.mlst.net) and spa types were assigned using the Ridom SpaServer (www.ridom.de/spaserver). Molecular characterization of the 30 isolates resulted in 21 pulsotypes, four MLST sequence types (STs), and six spa types. Combining data from both MLST and spa typing resulted in only seven strain categories, many of which grouped isolates that are not epidemiologically linked. These data demonstrate that techniques such as MLST and spa typing are not well suited for tracking isolates with limited evolutionary diversity such as the CA-MRSA epidemic clones.
Introduction

Impact of *Staphylococcus aureus*

*Staphylococcus aureus* is a Gram positive, nonmotile cocci that is one of the leading causes of nosocomial infection. Approximately 2% of all patients admitted to the hospital will develop *S. aureus* infection (66), which is a major public health concern due to the associated morbidity, mortality, and cost of care. Infection with *S. aureus* results in twice the length of stay, deaths, and medical costs as compared to patients without infection who are receiving the same treatment (94). The average cost of hospital stay in a study of patients in New York City hospitals was $13,263 without complications versus $32,100 for patients with hospital-acquired *S. aureus* infection (94). Furthermore, the economic impact is increasing due to the spread of antibiotic resistance, particularly methicillin resistance, among *S. aureus* isolates. A 2004 report from the National Nosocomial Infections Surveillance (NNIS) System, a network of healthcare agencies which report nosocomial infection surveillance data, demonstrated that nearly 60% of all *S. aureus* isolates in US hospitals are methicillin resistant (11). The cost of treating infections caused by methicillin-resistant *S. aureus* (MRSA) is approximately $2,500 more than treating susceptible *S. aureus* due to the increased length of stay and more expensive antibiotic treatment (94).

Among the many types of disease caused by *S. aureus* are localized skin infections including carbuncles, furuncles, impetigo, and sties, and one disseminated skin...
condition known as scalded skin syndrome. More serious conditions caused by *S. aureus* include septicemia, wound infection, post-surgical infection, pneumonia, osteomyelitis, toxic shock syndrome, and endocarditis (96). NNIS surveillance data from 1990 to 1996 demonstrated that *S. aureus* was the most common cause of nosocomial pneumonia and surgical site infection, and the second leading cause of nosocomial bloodstream infections (10). Furthermore, *S. aureus* is one of the leading causes of food-borne illness (63). Most cases of food-borne illness result from the toxin produced by the organism and therefore there is little surveillance data to elucidate the true contribution of *S. aureus* to the estimated 76 million cases of food-borne illness that occur annually in the United States (63). However, in at least one documented case, MRSA organism was spread from a food handler to restaurant patrons via the gastrointestinal route (52).

*S. aureus* is found in the anterior nares of both adults and children with approximately one-third of the population colonized during any given time (19, 67, 85). Among the carriers, between three and 10% of individuals will be colonized with MRSA (19, 46, 67, 85). The rates of MRSA carriage are highest among individuals with human immunodeficiency virus (HIV), intravenous drug users, patients with open abscesses, and those persons who were recently hospitalized (85). However, the number of children colonized with MRSA, who have no potential risk factors for MRSA carriage, has increased significantly in the past four years (19, 46). The emergence of these community-acquired MRSA (CA-MRSA) strains is of particular concern because colonization typically precedes infection, with greater than 80% of infecting isolates originating from the nose (18, 19). Asymptomatic carriers of *S. aureus* can be a source of
spread of the organism in both the community and hospital setting. However, not all *S. aureus* strains are equal in their ability to cause serious disease.

**Staphylococcus aureus Pathogenesis**

Complete genome sequencing has been performed on seven *S. aureus* strains, including four MRSA isolates. Comparison of these genomes indicates that *S. aureus* has a stable core of vertically transmitted genes, present in all seven genomes, but also frequently acquires genes by horizontal transfer (66). Approximately 25% of the *S. aureus* genome consists of genes that are horizontally transferred by mobile elements including bacteriophages, chromosomal cassettes, plasmids, and transposons (66). Many of these genes encode virulence and resistance determinants (44, 66). Further information on these determinants has been elucidated by comparison of the seven *S. aureus* genomes to a related but less pathogenic species, *Staphylococcus epidermidis* (66). These studies suggest that the pathogenesis of *S. aureus* is largely due to the presence of MSCRAMMS (microbial surface components recognizing adhesive matrix molecules), capsule production, antibiotic resistance genes, and toxins (66, 96).

MSCRAMMS are bacterial proteins present in a number of pathogenic organisms that aid in binding to human tissues and artificial surfaces. MSCRAMMS have been identified in *S. aureus* that bind to fibronectin, fibrinogen, and collagen components of the host cell wall (35). Most strains produce two fibronectin binding proteins, FnBPA and FnBPB which are involved in host cell attachment and binding to plasma clots and to artificial surfaces (35). Fibrinogen binding is mediated by two proteins, ClfA and ClfB, that are not only involved in the same attachment processes as the fibronectin-binding proteins, but also seem to have a role in endocarditis (35). Collagen binding, mediated by
the Cna protein, is not a characteristic of most isolates. However, Cna is necessary for binding to collagen and therefore has an important role in septic arthritis caused by *S. aureus* (35).

The classical experiments by Griffith illustrating the genetic transformation principle using *Streptococcus pneumoniae* also demonstrated the role of the bacterial capsule in pathogenesis. These experiments demonstrated that unencapsulated non-virulent *S. pneumoniae* isolates could “acquire” the ability to form capsule if mixed with encapsulated strains and these strains could then cause pneumonia in a mouse model (68). Although considered a seminal contribution to the field of genetics, this experiment also highlighted the role of the capsule in *S. pneumoniae* virulence. Although often overlooked as a virulence factor, it is of particular note that all of the organisms responsible for septic meningitis, *Neisseria meningitidis*, *S. pneumoniae*, and *Haemophilus influenzae* produce polysaccharide capsules (96). The *S. aureus* capsule has several roles in pathogenesis including host cell binding via MSCRAMMS as described above, biofilm formation, and immune system avoidance. Although the MSCRAMMS likely play a role in biofilm formation, a number of capsular genes are also involved (35, 96). The type of capsule present on a particular strain, determining the serotype of that isolate, has been related to virulence (118). Finally, the staphylococcal protein A component of the *S. aureus* capsule has been shown to bind to antibodies without leading to lysis of the cell (96). This antibody coat may allow the organism to escape the immune response (96).

One of the most well studied areas of *S. aureus* pathogenesis is its array of antibiotic resistance genes. The organism has acquired resistance by mutation of genes
on the chromosome as well as transfer of resistance determinants from other organisms by conjugation, transformation, and transduction (44, 51, 62). Although resistance to many antibiotics is common in *S. aureus*, methicillin resistance is of particular concern due to its public health impact in terms of mortality, morbidity, and cost. MRSA was reported in European hospitals in the early 1960s immediately following the introduction of methicillin to clinical practice (13). Resistance occurs in these isolates due to the production of a penicillin-binding protein, PBP 2a, that has a low affinity for binding β-lactam antibiotics (13). The PBP 2a protein thus performs the cell growth functions of the high-affinity proteins while simultaneously providing the cell resistance to an entire class of antibiotics (13). Phylogenetic studies have demonstrated that genetic exchange has resulted in *S. aureus* acquiring methicillin resistance at least five times since the first isolates were described in 1960, (20, 27, 28, 31, 32) and MRSA isolates have since spread worldwide (1, 5, 15, 25, 37, 56, 58, 87, 102, 110, 122).

*S. aureus* isolates differ in their ability to cause disease due in part to the expression of toxin genes present in their genome. Certain toxins such as Panton-Valentine Leukocidin (PVL) are known to cause necrotizing pneumonia and wound infections (36, 39, 50, 65, 117), while others such as Staphylococcal enterotoxin A and B are related to food poisoning (55, 57, 63, 69, 96, 99, 104). Many of the *S. aureus* toxin genes reside in four Staphylococcal pathogenicity islands (SaP1 – SaP4) along with other virulence factors while some of the genes are phage or plasmid encoded (66). A summary of staphylococcal toxin genes and their function is shown in Table 1.
## TABLE 1. Summary of *S. aureus* toxin genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Location and Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>seb</td>
<td>SaP1 – superantigen – food poisoning</td>
<td>Klotz et al. (2003)</td>
</tr>
<tr>
<td>sec1,2,3</td>
<td>SaP2 – 3 related superantigens – food poisoning</td>
<td>Klotz et al. (2003)</td>
</tr>
<tr>
<td>see</td>
<td>Phage encoded – superantigen – food poisoning</td>
<td>Klotz et al. (2003)</td>
</tr>
<tr>
<td>sej</td>
<td>Phage encoded – superantigen – unknown function</td>
<td>Seergev et al. (2004)</td>
</tr>
<tr>
<td>sel</td>
<td>SaP2 – superantigen – food poisoning</td>
<td>Seergev et al. (2004)</td>
</tr>
<tr>
<td>seo</td>
<td>SaP3 – superantigen – unknown function</td>
<td>Klotz et al. (2003)</td>
</tr>
<tr>
<td>sep</td>
<td>Phage encoded – superantigen – food poisoning</td>
<td>Kuroda et al. (2001)</td>
</tr>
<tr>
<td>seq</td>
<td>SaP1 – superantigen – unknown function</td>
<td>Seergev at al. (2004)</td>
</tr>
<tr>
<td>seu</td>
<td>Superantigen – unknown function</td>
<td>Klotz et al. (2004)</td>
</tr>
<tr>
<td>TSST</td>
<td>SaP1 – toxic shock syndrome toxin</td>
<td>Salyers et al. (2002)</td>
</tr>
<tr>
<td>eta</td>
<td>Phage encoded – exfoliative toxin, scalded skin syndrome</td>
<td>Salyers et al. (2002)</td>
</tr>
<tr>
<td>etb</td>
<td>Phage encoded – exfoliative toxin, scalded skin syndrome</td>
<td>Salyers et al. (2002)</td>
</tr>
<tr>
<td>hla</td>
<td>Gamma hemolysin component – red blood cell lysis</td>
<td>Kuroda et al. (2001)</td>
</tr>
<tr>
<td>hlb</td>
<td>Gamma hemolysin component – red blood cell lysis</td>
<td>Kuroda et al. (2001)</td>
</tr>
<tr>
<td>hid</td>
<td>Alpha hemolysin – red blood cell lysis and tissue necrosis</td>
<td>Kuroda et al. (2001)</td>
</tr>
<tr>
<td>lukS</td>
<td>Phage encoded – PVL – pneumonia, etc.</td>
<td>Gillet et al. (2002)</td>
</tr>
<tr>
<td>lukF</td>
<td>Phage encoded – PVL – pneumonia, etc.</td>
<td>Gillet et al. (2002)</td>
</tr>
</tbody>
</table>

### Role of *S. aureus* in Bioterrorism

The deliberate contamination of food products with microbial agents including organisms and/or their toxins is a distinct possibility due to the complexity of the food service industry (63). Food is particularly vulnerable during the processing, preparation, and delivery stages (63). *Staphylococcal enterotoxin B* (SEB), considered a select agent, can be used to contaminate food or low-volume water supplies, or alternatively it can be administered as an aerosol (63, 93). Contamination of food supplies with SEB is a low-tech means of incapacitating a large number of people by simultaneously inducing an
outbreak of vomiting and diarrhea. In addition to these symptoms, typical *S. aureus* food-borne intoxication includes fever, chills, headache, non-productive cough, and shortness of breath (93). In most cases, the gastrointestinal symptoms clear quickly and full recovery is likely, but the cough is long-lasting and persons are usually incapacitated for up to two weeks (93). *S. aureus* food poisoning could therefore be a useful tactic to a terrorist who wishes to do economic damage to a particular company or to induce panic in a particular population.

A more deadly scenario occurs when SEB is aerosolized. Studies have shown that SEB acts as a superantigen that activates T-cells leading to the release of massive amounts of cytokines within the respiratory mucosa which can lead to death by severe pulmonary edema (69). Although this route of exposure is unlikely due to the technical issues involved in mass production and aerosolizing SEB, just a few hundred cases would overwhelm the capacities of most local medical institutions to provide ventilation support (69, 93).

Although most research has focused on the detection of SEB in food products both for bioterrorism detection (55) and food-borne illness detection (99), the other *S. aureus* toxins should also be considered as possible bioweapons. The majority of food-borne illness outbreaks are due to staphylococcal enterotoxin A (SEA) and newly described enterotoxins elicit a gastrointestinal syndrome in mouse models (57). Furthermore, *S. aureus* isolates capable of causing serious disease contain various combinations of toxin genes, and many isolates do not contain *seb* genes. Therefore detection methods for *S. aureus* intoxication should include a wide variety of toxins (99).
Community-acquired Methicillin-Resistant *S. aureus* (CA-MRSA)

MRSA has been circulating in hospitals since the early 1960s but reports of infection in the community were relatively rare. However, in the early 1990s strains of highly virulent CA-MRSA were reported in Western Australia (112). In recent years the incidence of CA-MRSA has been increasing with outbreaks occurring in the United States (5, 7, 12, 14, 37, 54, 70, 71, 79, 81, 112), Canada (77), and overseas (2, 25, 36, 39, 110). CA-MRSA strains differ from hospital-acquired MRSA (HA-MRSA) in their antibiotic resistance profiles, their virulence determinants, and their ability to cause disease in patients without traditional risk factors (24, 43, 119).

Methicillin resistance in *S. aureus* is encoded by the *mecA* gene located in the staphylococcal cassette chromosome *mec* (SCC*mec*) region. This region also contains the regulatory and recombinase genes. Several different *SCCmec* complexes have been described that contain insertions and deletions in the regulatory and recombinase genes (3, 64). CA-MRSA strains frequently carry variants of the *SCCmec* IV element, the smallest of the known *SCCmec* elements (2, 3, 64, 116). This finding has lead to a number of hypotheses concerning the spread of the CA-MRSA isolates. While one group believes that CA-MRSA isolates evolved from HA-MRSA (2), another introduces the differences in *SCCmec* complexes as evidence that CA-MRSA and HA-MRSA are in fact not related (116). Interestingly, the same studies report different results when comparing growth rates of CA-MRSA and HA-MRSA strains. In two studies CA-MRSA isolates displayed a faster doubling time than the HA-MRSA isolates (116, 123) and the authors postulate that this occurs due to the small size of the *SCCmec* complex. However, another study found no difference in growth rates between the two types of isolates (2).
Regardless of the evolutionary background of the isolates, it seems clear that MRSA is replacing methicillin-sensitive *S. aureus* (MSSA) in the community.

Many CA-MRSA strains carry the genes for encoding PVL, a toxin involved in tissue necrosis (117). Binding of PVL to neutrophils induces an immune cascade that includes secretion of degradative enzymes and generation of superoxide ions which promotes necrosis of tissue (123). Several studies have demonstrated a link between PVL-producing *S. aureus* isolates and primary skin infections and necrotizing pneumonia (7, 12, 14, 25, 36, 37, 39, 50, 54, 61, 65, 71, 77), although other virulence factors many also play a role (95). Pneumonia caused by PVL-positive CA-MRSA isolates has a high mortality rate as shown in a study of 23 patients in which 14 patients (61%) died from the disease (65). Wound infections caused by similar isolates are not usually fatal but are often accompanied by serious complications such as the need for reconstructive surgery to reverse tissue damage and prolonged stays in the intensive care unit (71).

Pulsed-field gel electrophoresis (PFGE) of 957 MRSA isolates resulted in the identification of eight major lineages of *S. aureus* strains known as USA 100 – USA 800 (70). While most were hospital-associated strains, USA 300 and USA 400 were associated with community-acquired infections (70). Recent studies have indicated that these two clones are spreading rapidly and they are now referred to as epidemic clones. USA 300 epidemic clone has been reported as a cause of serious wound infections in football players in Missouri and Connecticut among otherwise healthy, young individuals (5, 54). USA 300 is also responsible for the sharp increase in necrotizing wound infections reported in California (71) and may be responsible for another outbreak in California reported in the same time frame with similar epidemiology (75). Alarmingly,
studies in Texas have also demonstrated the emergence of an epidemic clone among pediatric isolates in Houston and Dallas (14, 72, 81). Although these studies did not provide conclusive evidence that the responsible clone is USA 300 in the form of pulsed-field patterns, the antibiotic resistance data, epidemiological data, and the multilocus sequence type (MLST) 8, are all consistent with USA 300 and not USA 400 (14, 72, 81). The USA 400 epidemic CA-MRSA clone, which has a distinct PFGE pattern and multilocus sequence type, has been reported as the cause of wound infections in New York and Canada (7, 77). Finally, a retrospective study of patient charts identified CA-MRSA isolates in Georgia, Minnesota, and Maryland (37). However, this study provided only antibiotic resistance data and the epidemic clones can not be differentiated without genomic subspeciation.

**Techniques for subtyping *S. aureus***

Bacterial subtyping is useful for a number of epidemiological and infection control practices including tracking the spread of strains of interest, determining isolates involved in outbreaks, monitoring trends in endemic isolates, and source tracking. Earlier techniques for subtyping *S. aureus* were phenotypic and biochemical in nature but many of these have been replaced with genomic techniques that offer higher discriminatory power. The “Gold Standard” for typing *S. aureus* isolates is PFGE. It has been shown to be more discriminatory than phenotypic techniques such as bacteriophage typing (4, 70), that is based on the susceptibility of certain strains for bacteriophage lysis detected by plaque assay, and antibiogram typing, based on antibiotic susceptibility patterns (48, 74). Phage typing was used by the Centers for Disease
Control and Prevention (CDC) for many years to type *S. aureus* isolates as it had higher discriminatory power than other techniques available at the time, despite the fact that nearly 20% of isolates were non-typeable by this method (4). The CDC has since replaced phage typing with pulsed-field typing for a number of organisms including *S. aureus* (4). Likewise, PFGE has replaced many biochemical typing methods such as biotyping, which uses a panel of biochemical reactions to identify strains, capsular typing, based on serotyping using monoclonal antibodies, and zymotyping, based on the electrophoretic mobility patterns of esterases (97).

Early molecular techniques used for *S. aureus* genotyping include plasmid profile analysis, whole genome restriction enzyme analysis (REA), and ribotyping. Plasmid profile analysis is limited both by the number of isolates that lacked plasmids, and that plasmids are mobile and can therefore be lost (48). REA is limited by the large number of fragments generated in the digest that results in overlapping fragments not separated by agarose gel electrophoresis (111). Ribotyping, based on the use of probes to detect the ribosomal rRNA genes, has had mixed results in its use for typing *S. aureus*. While the technique has been shown to be useful in a hospital outbreak of MSSA (82), it lacks the discriminatory power to differentiate MRSA strains (21, 82, 89, 111). Many PCR-based techniques have also been used for *S. aureus* typing (103). Multiplex PCR allows the rapid identification of MRSA versus MSSA isolates, but cannot identify strains (84). Hypervariable region PCR (HVR-PCR) can classify MSSA but cannot discriminate epidemic and sporadic MRSA (47, 98) and arbitrarily primed PCR (RAPD), has been useful in typing isolates from cattle (86, 111), but lacks the discriminatory power of PFGE for MRSA strains. Some techniques that have excellent discriminatory power
include GeneChips and whole genome sequencing, but their use is restricted due to high cost, lengthy analysis, and required technical expertise (26, 51, 62, 66).

Methicillin resistance in *S. aureus* is believed to have been acquired at least 5 times since the 1960s and has spread throughout hospitals worldwide (27). In contrast, community-acquired MRSA have only appeared in the last decade, a relative short time period on an evolutionary scale. As such, CA-MRSA lacks the genetic diversity that is often seen in HA-MRSA (34). Due to the fact that MRSA strains, and particularly CA-MRSA strains were derived from relatively few clones, a typing technique must be used that has sufficient discriminatory power to differentiate the strains into epidemiologically related and non-related groups (107, 111).

Pulsed-field Gel Electrophoresis for Subtyping Methicillin-Resistant *S. aureus*

The first step in PFGE involves embedding the microorganisms of interest in an agarose block to prevent shearing of the DNA during electrophoresis. Immobilization allows the organisms to be lysed in situ followed by macrorestriction of the DNA using the restriction enzyme of choice. The *Sma*I enzyme is used for *S. aureus* PFGE as it results in 10-20 fragments, depending on the isolate, which range in size from 10 to 700 kb which can be easily separated on a PFGE gel (42). The agarose “plugs” containing the restricted DNA are then loaded on the gel and an electrical current is applied. The direction of the current (switch) is alternated during the electrophoresis to allow large fragments of DNA to align themselves with the current (108, 111). The run time, voltage, and switch times are experimentally determined based on the size of the fragments generated by macrorestriction. The resulting fingerprint patterns can be
visualized using ethidium bromide and analyzed either by sight and application of the Tenover criteria for strain typing (108), or by computer using BioNumerics®, GelCompar® or other software packages. To date no *S. aureus* isolate has ever been reported that is non-typeable by PFGE and epidemiological data suggests that unique DNA fingerprint patterns are consistent with strain designations.

PFGE has been used for a wide variety of applications in healthcare institutions, research laboratories, public health laboratories, and other government laboratories. The SENTRY program uses both PFGE and ribotyping to track multidrug-resistant bacteria in healthcare organizations (23). PFGE can be used to track highly virulent, epidemic clones such as USA 300 and USA 400 (12, 70, 119). DNA fingerprint patterns can be used to determine if particular strains are related to disease outcome (12, 70, 114, 119). In particular, the patterns may predict if strains are PVL positive and which SCC*mec* cassette they are carrying (121). PFGE may also be used to compare methicillin-sensitive to methicillin-resistant strains (8). One of the largest programs utilizing this technique is the PulseNet program at the CDC and worldwide.

PulseNet is a network of public health laboratories that submit isolates involved in suspected food-borne illness outbreaks for molecular typing and comparison to other isolates in the database (105). This technique has been used successfully to identify outbreaks, to determine the number of isolates involved, and to track the source of the outbreak (38, 90, 105). PulseNet participants must use a standardized protocol and pass rigorous quality control training before they may submit samples to the database. Currently there exists no PulseNet database for *S. aureus* and no standardized protocol has been developed for *S. aureus* PFGE. A recent publication suggests that CDC is
developing a database of *S. aureus* PFGE fingerprints aimed at tracking the CA-MRSA epidemic clones (70), however it is unclear if this database will be a part of PulseNet and therefore available for worldwide sample submission. MRSA fingerprint databases do exist in Europe, Canada, and Australia and efforts to standardize protocols have been successful. A protocol developed in Canada has shortened the *S. aureus* PFGE procedure from five days to 48 hours and laboratories using the procedure reported excellent reproducibility and interlaboratory comparison (76). Similarly, a harmonized European protocol was tested by 10 laboratories in eight countries and demonstrated sufficient interlaboratory reproducibility to track epidemic MRSA strains in Europe (78).

Although PFGE has been used for a number of applications, there are as many disadvantages as advantages to using the technique (Table 2) and other molecular techniques are under investigation.

**TABLE 2. Advantages and disadvantages of pulsed-field gel electrophoresis**

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Highly discriminatory</td>
<td>• May be too discriminatory</td>
</tr>
<tr>
<td>• Reproducible</td>
<td>• Time-consuming</td>
</tr>
<tr>
<td>• All strains can be typed</td>
<td>• Expensive</td>
</tr>
<tr>
<td>• Patterns are stable over time</td>
<td>• Technically demanding</td>
</tr>
<tr>
<td>• BioNumerics software allows comparison between laboratories</td>
<td>• Interlaboratory comparison may be difficult as software is subjective</td>
</tr>
<tr>
<td>• Demonstrated use in epidemiological studies</td>
<td>• Need to store marker isolate</td>
</tr>
<tr>
<td>• Not necessary to standardize DNA plug preparation procedure, type of agarose used, or plug digestion.</td>
<td>• Must standardize cell concentrations, lysis conditions, PFGE equipment, electrophoresis conditions, run times, and analytical methods</td>
</tr>
<tr>
<td>• Genomic Information not needed.</td>
<td>• Need control strain on every gel</td>
</tr>
</tbody>
</table>
Multilocus Sequence Typing of *S. aureus*

Genome-based sequencing techniques have been explored as an alternative to gel-based techniques such as PFGE to circumvent problems associated with cost, time, technical expertise required, and interlaboratory comparison of resulting data. One of the more recent techniques used for molecular typing of *S. aureus* is multilocus sequence typing (MLST). MLST was designed based on the principles of multilocus enzyme electrophoresis (MLEE) in which the electrophoretic mobilities of housekeeping enzymes of isolates of interest are compared (29). In MLST, the gel analysis is bypassed by comparing nucleotides from housekeeping genes, chosen because they likely represent the stable core genome as opposed to virulence genes (31). The number of housekeeping genes that must be sequenced depends on the organism typed and the genes used for the analysis. Organisms such as *Enterococcus faecalis* can be typed using just two genes while typing of *S. aureus* requires seven housekeeping genes (29, 80). A standardized protocol has been established for *S. aureus* MLST which requires amplification of the genes of interest, followed by sequencing of approximately 450 base pair fragments (http://saureus.mlst.net). This fragment size was selected because the entire length of the fragment can easily be sequenced in either direction with just one reaction (113). Each gene sequence is assigned an allele and the sequences of all seven genes make up an allelic pattern. One of the major advantages of using MLST is that the gene sequences once resolved can be entered into the database and alleles assigned by comparison with sequences already entered. The same principle allows allelic patterns to be assigned as sequence types (STs) if a match of all seven alleles exists in the database. Therefore any
isolate that is typed can easily be compared to all previously typed isolates, allowing worldwide tracking of STs of interest (28).

MLST was primarily designed for global epidemiology and the online database includes a phylogenic program, eBURST, that subdivides isolates that share high genetic similarity into clonal clusters (CCs) using allelic profile data (33). Isolates within each clonal cluster share 100% genetic identity at six of seven loci with at least one other member of the group (32). The founding genotype is identified parsimoniously as the genotype that differs from the highest number of other genotypes in the CC at only one locus and usually corresponds to a strain that is present in the population in high numbers due to increased fitness or random genetic drift (32, 33). The eBURST program generates radial diagrams that can be used to address basic questions about the evolutionary and population biology of bacterial species (28). *S. aureus* CCs are usually simple with a single founder surrounded by isolates with allelic profiles that differ from that of the founder in only one housekeeping gene, known as single locus variants (SLVs) (32). This is especially true of MRSA because MLST assesses variations that accumulate slowly in the population and MRSA isolates have not had much time to accumulate variations in their housekeeping genes to distinguish them from their MSSA ancestors (28, 31).

The evolution of *S. aureus* and the acquisition of methicillin-resistance have been examined by a large number of MLST studies. Comparison of isolates from carriers were identical to those with invasive disease suggesting that *S. aureus* disease-causing isolates do not differ genetically from carrier strains, in contrast to other studies that suggest disease is caused by hypervirulent clones (31, 120). However, MLST
demonstrated that some STs are commonly found in high-risk patients, such as intravenous drug users, and these STs correlated with the disease presentation (73). In depth analysis of the nature of mutations within alleles between members of a clonal group, demonstrated that *S. aureus* clonal diversification occurs 15-fold more frequently by point mutation than by recombination (31). Similar fine-scale analyses indicate that methicillin-resistance in *S. aureus* may have been horizontally acquired at least 20 times and that these strains exist in five major lineages of *S. aureus* (17, 27, 113). MLST demonstrated that the early MRSA isolates were highly related to MSSA isolates (28). Furthermore, modern MRSA lineages are unrelated by eBURST analysis to the early MRSA isolates further demonstrating that *mecA* has been acquired by horizontal transfer (92). The horizontal acquisition of *mecA* into MSSA isolates has been corroborated by two separate studies that demonstrated that the first MRSA evolved from an epidemic MSSA isolate in Europe (20, 92). A number of recent studies have attempted to use MLST to demonstrate the relationship between sporadic isolates, those occurring in one or a few patients, HA-MRSA, and CA-MRSA (2, 16). As previously mentioned, these studies contradicted one another concerning the evolution of these epidemic clones from hospital-acquired strains (2, 16). Three recent studies highlight the use of MLST in global epidemiology to track epidemic isolates; the identification of two major genotypes of MRSA in Asia with distinct geographic distributions (58), the introduction of epidemic ST239 in Saudi Arabia and Romania (15), and the first detection of an epidemic MRSA clone in Spain (87).

MLST using housekeeping genes has demonstrated use in defining clonal complexes (40) and in global epidemiological applications but it is time consuming and
is not well suited for monitoring localized outbreaks. The adaptation of the MLST protocol for use on the Light Cycler platform eliminates the need for electrophoresis and may diminish the processing time (6). Similarly, the use of DNA array technology allows scanning of all genes of interest simultaneously which although time saving is at this time cost prohibitive (115). Another variation of the technique is multilocus restriction fragment typing (MLRFT) in which housekeeping genes are amplified, digested, and visualized on an agarose gel. This technique avoids the costs involved in sequencing but does not result in the genomic data necessary for the use of MLST in global studies. While financial and technical factors may result in limited use of MLST for public health applications, further studies are needed to examine the possible use of virulence genes, in addition to housekeeping genes, to increase the discriminatory power for monitoring local outbreaks (17).

**Staphylococcal Protein A Gene Sequencing (spa Typing)**

MLST has shown promise in studies of global epidemiology but the technique is time consuming due to the amplification and sequencing of seven genes for each isolate tested. Therefore recent efforts have aimed at the use of single markers such as clumping factor B (clfB) typing, coagulase (coa) typing, and staphylococcal protein A (spa) gene typing for *S. aureus* subtyping (59, 60, 101). The three techniques are similar in that each requires amplification and sequencing of repeat regions. These studies have demonstrated that *spa* typing has the highest discriminatory power of the three genes, but it may also be used in combination with the other techniques for even higher resolution (59, 60, 101).
The *spa* gene contains a polymorphic 24-base pair variable-number tandem repeat (VNTR) region, with unknown biological function, immediately upstream of the region coding the C-terminal cell wall attachment sequence (100). The diversity of the repeat regions within *spa* is likely a result of deletion and duplication of the repetitive units and by point mutation (100). Amplification and sequencing of *spa* is simplified by the existence of well-conserved regions flanking the repeats (83). Repeats are easily located within a *spa* sequence by identifying the conserved regions. The 24-base pair repeats can then be compared with known repeats using the online database (www.ridom.de/spaserver). The number of repeats and their order can then be used to determine the *spa* type using the spaserver. Although the online server is currently free, an advanced database program, RidomStaphType®, is available that simplifies the technique by reading chromatograms directly from the sequencer and assigning *spa* types (41). There is some disagreement on the acceptable length of *spa* repeats with one repeat reported as 21 base pairs long (53), but the database currently contains 96 reported repeats, most of which are 24 base pairs long. Similarly, one study reported three of 41 isolates were non-typeable by *spa* sequencing (1) while another successfully typed thousands of isolates (59). Repeat combinations ranging from 1 repeat per isolate to 23 repeats in one isolate are represented in the online database resulting in the designation of 1165 *spa* types from 21 countries to date (www.ridom.de/spaserver). The assignment of simple numeric codes, use of just one gene for amplification and sequencing, standardization of the protocols among laboratories, and the ease of data storage illustrates some of the advantages of using simple PCR-based approaches such as *spa* typing (100, 106). However, studies using this technique have found that while strains
with different spa types can generally be considered not related, strains with the same spa type are not necessarily epidemiologically related (106).

Several recent studies have correlated spa type 44 with CA-MRSA isolates that are PVL positive recovered from infections in Denmark (30), Belgium (22), and Germany, France, and Switzerland (121). In a German hospital, spa typing was successfully used to track epidemic isolates and to disprove that person-to-person transmission of MRSA was occurring within the facility which allowed better use of infection control resources (41). A comparison of HA-MRSA with HA-MSSA and CA-MSSA in Portugal using spa typing found that the three major MRSA clones were not related to any of the MSSA isolates tested (1). This data suggests that MRSA in Portugal did not result from acquisition of SCCmec by MSSA isolates in Portugal but likely was imported from abroad (1). A study of isolates in Hungary demonstrated that MRSA isolates recently introduced into the hospital environment varied little in spa types but MRSA clones circulating for longer periods of time and spread among several hospitals showed a higher degree of variability (83). This study further suggests that the spa gene may play a role in attachment and pathogenesis as an explanation for why certain epidemic clones, with stable spa types, are highly successful and have been circulating for decades (83). The comparison of CA-MRSA isolates to HA-MRSA isolates suggested once again that CA-MRSA is the result of recent acquisition of SCCmec into a MSSA background because all CA-MRSA isolates were related by spa typing but none were related to the HA-MRSA isolates (34). Some studies suggest that spa is relatively stable and can therefore be used to characterize both macro- and microevolution in the primarily clonal population structure of S. aureus (59, 100). However, a recent study of
*S. aureus* isolates recovered from persistently infected cystic fibrosis patients found that one genetic change occurs in *spa* every 70 months (53). While some of the problems encountered with the use of *spa* gene typing, including low discriminatory power, may be overcome by combining the techniques such as *coa* typing and hypervariable region typing (74, 103), the expense of sequencing will continue to restrict the use of PCR/sequence based typing methods.
Objectives

Recent studies have demonstrated the emergence of highly virulent community-acquired isolates of \textit{S. aureus} that are capable of causing serious disease in otherwise healthy populations. These infections are of concern due to increased morbidity, mortality, and cost of care. Surveillance programs should be implemented by public health, hospital, and research laboratories to determine the extent of CA-MRSA dissemination, to better understand the epidemiology behind the spread of the organism, to propose infection control measures, and to provide adequate treatment. Currently there is no ongoing surveillance of \textit{S. aureus} infections in the United States and the objective of this project was to determine what typing methods would be suitable for monitoring \textit{S. aureus}. The suspected outcome is that no one typing method will be suitable for each \textit{S. aureus} typing study and that the method should be selected based on the desired outcome. The following specific aims were designed to test this hypothesis:

1. To establish a database of \textit{S. aureus} isolates and categorize the isolates by PFGE.
2. To type a subset of the \textit{S. aureus} isolates using MLST.
3. To type the same subset of \textit{S. aureus} isolates using \textit{spa} Typing.
4. To compare and contrast the usefulness of the 3 typing methods for public health applications.
Materials and Methods

*S. aureus* Isolates

A total of 403 isolates were used in this study, including community-associated methicillin-resistant *S. aureus* (CA-MRSA) organisms isolated from wound, nose, sputum, blood, and other sources. CA-MRSA isolates were requested from two hospitals in Washington, 1 hospital in Florida, and Quest Diagnostics, a regional testing laboratory in Florida. All clinical isolates were collected from patients treated on an outpatient basis, within 48 hours of consultation. Control strains USA 100 – USA 800 were obtained from the Network on Antimicrobial Resistance in *S. aureus* (NARSA) (Table 3). The PFGE standardization control strain, NCTC 8324 (NRS77) was also obtained from NARSA. Several additional strains were obtained from the American Type Tissue Collection (ATCC) (Manassas, VA) (Table 3). The identification of all isolates as methicillin-resistant *S. aureus* was based on Gram stain reaction and cell morphology, catalase reaction, Microdase disk oxidase test (REMEL Inc., Lenexa, KS), slide and tube coagulase testing in rabbit plasma with EDTA (REMEL Inc.) and the API STAPH® identification system (bioMerieux, Inc., Hazelwood, MO) supplemented with lysostaphin testing (REMEL Inc.). Profiles were analyzed using the API STAPH® Codebook, Reference # 20590, 14th Edition. Methicillin-resistance was determined on Mueller Hinton agar by the direct colony method of standardized disk diffusion susceptibility testing against 1 μg oxacillin and 30 μg cefoxitin disks incubated at 35°C
and 30°C, respectively (REMEL Inc., Technical Insert No. 33000) (49). Zones of inhibition ≤10mm around the oxacillin disk and ≤19 mm at cefoxitin are indicative of oxacillin and methicillin resistance (9). The presence of the meca gene product was confirmed using the Penicillin Binding Protein (PBP2') Latex Agglutination Test (Oxoid Limited, Hampshire, England). A list of all strains used in the study appears in Appendix A.

<table>
<thead>
<tr>
<th>CBD Designation</th>
<th>Identifier</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBD0007</td>
<td>ATCC 14458A</td>
</tr>
<tr>
<td>CBD0019</td>
<td>ATCC 51740</td>
</tr>
<tr>
<td>CBD0034</td>
<td>ATCC 29213</td>
</tr>
<tr>
<td>CBD0036</td>
<td>ATCC 14458B</td>
</tr>
<tr>
<td>CBD 0044</td>
<td>ATCC 25923</td>
</tr>
<tr>
<td>CBD0544</td>
<td>ATCC 6538</td>
</tr>
<tr>
<td>CBD0545</td>
<td>ATCC 12600</td>
</tr>
<tr>
<td>CBD0623</td>
<td>NRS77 (NCTC 8325)</td>
</tr>
<tr>
<td>CBD0653</td>
<td>ATCC 33593</td>
</tr>
<tr>
<td>CBD0654</td>
<td>NRS 100 (COL)</td>
</tr>
<tr>
<td>CBD0655</td>
<td>NRS 271A</td>
</tr>
<tr>
<td>CBD0656</td>
<td>NRS 271B</td>
</tr>
<tr>
<td>CBD0797</td>
<td>NRS 70 (N315)</td>
</tr>
<tr>
<td>CBD0798</td>
<td>NRS 123</td>
</tr>
<tr>
<td>CBD0835</td>
<td>ATCC 49775A</td>
</tr>
<tr>
<td>CBD0836</td>
<td>ATCC 49775B</td>
</tr>
<tr>
<td>CBD1064</td>
<td>NRS382 (USA 100)</td>
</tr>
<tr>
<td>CBD1065</td>
<td>NRS383 (USA 200)</td>
</tr>
<tr>
<td>CBD1066</td>
<td>NRS384 (USA 300)</td>
</tr>
<tr>
<td>CBD1067</td>
<td>NRS123 (USA 400)</td>
</tr>
<tr>
<td>CBD1068</td>
<td>NRS385 (USA 500)</td>
</tr>
<tr>
<td>CBD1069</td>
<td>NRS22 (USA 600)</td>
</tr>
<tr>
<td>CBD1070</td>
<td>NRS386 (USA 700)</td>
</tr>
<tr>
<td>CBD1071</td>
<td>NRS387 (USA 800)</td>
</tr>
</tbody>
</table>

**TABLE 3. Control strains**

Pulsed-field Gel Electrophoresis

A single isolated colony of *S. aureus* was aseptically transferred from a BBL™ Trypicase Soy Agar Plate (Becton Dickinson, Sparks, MD) to 5 ml BBL™ Trypticase™ Soy Broth (Becton Dickinson, Sparks, MD). The broth culture was incubated 16 hours in
a 37°C water bath with gentle agitation. Cells were pelleted for 10 min at 4°C, 5200 g and resuspended in 1.5 ml of PIV solution (10 mM Tris-HCl, 1 M NaCl). Plugs were prepared by adding 300 μl of cells to 300 μl of InCert agarose (Cambrex Bioscience, Rockland, ME), pipeting the mixture into reusable plug molds (Bio-Rad, Hercules, CA), and allowing them to cool for 10 min at 4°C. The plugs were then incubated for 5 hr at 37°C in 7 ml of EC-Lysis solution (6 mM Tris-HCl (pH 7.6), 1 M NaCl, 100 mM EDTA (pH 7.5), 0.5% Brij-58, 0.2% Deoxycholate, 0.5% sarkosyl, 20 μg/ml RNase, 1 mg/ml lysozyme). Lysostaphin (no. L7386, Sigma, St. Louis, MO) was added to the EC-Lysis buffer at a concentration of 3 U/ml. EC-lysis solution was replaced with ESP solution (0.5 M EDTA (pH 9.0), 1% sarkosyl, 50 μg/ml proteinase K) and the plugs were incubated overnight at 50°C. Plugs were then washed three times (45 min each wash) with TE (10 mM Tris-HCl (pH 7.5)), 1 mM EDTA (pH 7.5) and stored at 4°C until needed.

Plugs were digested in SmaI overnight at room temperature (or overnight at 37°C for EagI and SacII digestions). Plugs were then melted at 69°C for 10 min, and loaded into the wells of a 1.6% Seakem Gold (Cambrex BioScience, Rockland, ME) gel prepared by adding 3.2 g of agarose to 200 ml of 0.5X TBE (Tris – Boric Acid – EDTA). SmaI digested NCTC 8325 was used as a marker for all gels. PFGE was performed using a DR-II CHEF Mapper (Bio-Rad, Hercules, CA) using the following parameters: 200V, 14°C, 5.3 s initial switch, 34.9 s final switch, 0.5X TBE running buffer, and 20 to 22 hrs run time. The run time varied based on which of two available DR-II CHEF Mapper gel boxes was utilized. Following electrophoresis, gels were stained for 20 min in 200 ml of water containing 1 μg/ml ethidium bromide and destained for 20 min in water (repeat
Gels were then visualized using the Bio-Rad Gel Doc System utilizing the Quantity One version 4.6 software (Bio-Rad, Hercules, CA). Data was analyzed using BioNumerics® (Applied Math, Sint-Martens, Belgium) Version 3.0. Dendrograms were derived from the unweighted pair group method using arithmetic averages (UPGMA) and based on Dice coefficients. Band position tolerance and optimization were set at 1.00%.

**Virtual Digestion to Identify Secondary Enzymes for *S. aureus* PFGE**

A list of 262 commercially available enzymes was compiled using catalog lists from New England Biolabs (Beverly, MA), Fisher Scientific (Pittsburgh, PA) Roche Applied Science (Indianapolis, IN), and Invitrogen (Carlsbad, CA). A list of all enzymes tested appears in Table 7. Restriction digestion of the *S. aureus* MW2 genome was performed for all 262 enzymes online at The Institute for Genomic Research (TIGR) website using the Restriction Digest Tool (www.tigr.org/tigr-scripts/CMR2/restrict_display.pl). Using a subset of the enzymes, the genomes of other available *S. aureus* genomes including, *Staphylococcus aureus* Michigan VRSA and *Staphylococcus aureus* MU50 were digested. This resulted in the same size fragments for the *S. aureus* MW2 genome thus it was determined that virtual digestion of the MW2 genome was sufficient. Only those enzymes that resulted in 10 to 50 fragments were further considered for *S. aureus* PFGE.

**DNA Isolation for MLST and *spa* Typing**

Genomic DNA was isolated from all *S. aureus* strains using the MagNA Pure LC DNA Isolation Kit III (Bacteria, Fungi) (Roche Diagnostics, Indianapolis, IN) protocol as
follows. Cells were grown for 16 hours at 37°C in 4 ml BBL™ Trypticase™ Soy Broth (Becton Dickinson, Sparks, MD). Pellets were obtained by centrifugation of 1 ml of the overnight cell culture at 8,000 g for 10 min. After discarding the supernatant, the samples were lysed by adding 130 μl of Bacterial Lysis Buffer, followed by mixing, then adding 20 μl of Proteinase K provided in the kit and incubation for 10 min at 65°C. Organisms were then inactivated by boiling samples at 95°C for 10 min. Magnetic glass particles, which bind to the DNA, were added to the samples immediately before loaded onto the MagNA Pure LC Instrument (Roche Diagnostics, Indianapolis, IN). Samples were loading following onscreen instructions and the instrument performed a series of washes that removed unbound substances. The DNA was then eluted and it was experimentally determined through a series of dilutions that 1:20 dilutions of MagNA Pure genomic DNA sample was sufficient as template for both MLST and spa typing polymerase chain reactions.

**PCR for Multilocus Sequence Typing**

MLST of 30 *S. aureus* isolates was performed as per the standardized protocol (http://saureus.mlst.net/misc/info.asp). Internal fragments of the following seven housekeeping genes; *arc* (carbamate kinase), *aro* (shikimate dehydrogenase), *glp* (glycerol kinase), *gmk* (guanylate kinase), *pta* (phosphate acetyltransferase), *tpi* (triosephosphate isomerase), *yqi* (acetyl coenzyme A acetyltransferase) were amplified by PCR. PCR master mixes were prepared for each amplification using reagents from the Takara PCR Amplification Kit (Fisher Scientific, Pittsburg, PA) including 6μl MgCl₂, 5 μl 10x PCR buffer, and 4 μl dNTPs. Each reaction included 1 μl each forward and reverse primers (1
μg/μl), 0.25 μl (1.2U) Takara Taq polymerase (Fisher Scientific, Pittsburg, PA), and 30 μl water for each reaction. Approximately 3μl of 1:20 MagNA Pure isolated genomic DNA was added as template. The PCR protocol included an initial 5 min denaturation at 95°C, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55° C for 1 min, extension at 72°C for 1 min, and a final extension step of 72°C for 5 min. The annealing temperature was raised to 65°C for PCR amplification of the \textit{gmk}, \textit{pta}, and/or \textit{yqi} gene for some isolates to obtain a single band. A list of all primers used for MLST PCR appears in Table 4.

**PCR for \textit{spa} Typing**

Amplification of the \textit{spa} gene was performed for 30 \textit{S. aureus} isolates using the same master mix, DNA concentration, and primer concentration as reported above for MLST. The primers used for \textit{spa} are listed in Table 4. Isolates CBD0614 and CBD0633 required the use of \textit{spaF2} (\textit{spa} Forward 2) primer, all other isolates were amplified with \textit{spaF1}. The PCR protocol included an initial 10 min denaturation at 95°C, followed by 30 cycles of 30 seconds denaturation at 95°C, annealing at 60°C for 30 sec, extension at 72°C for 45 sec, and a final extension step of 72°C for 10 min (83).
TABLE 4. MLST and spa typing primers – primer sequences obtained from www.mlst.net and www.ridom.de/spaserver/.

<table>
<thead>
<tr>
<th>Primer Designation</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>arc Forward</td>
<td>TTG ATT CAC CAG CGC GTA TTG TC</td>
</tr>
<tr>
<td>arc Reverse</td>
<td>AGG TAT CTG CTT CAA TCA GCG</td>
</tr>
<tr>
<td>aro Forward</td>
<td>ATC GGA AAT CCT ATT TCA CAT TC</td>
</tr>
<tr>
<td>aro Reverse</td>
<td>GGT GTT GTA TTA ATA ACG ATA TC</td>
</tr>
<tr>
<td>glp Forward</td>
<td>CTA GGA ACT GCA ATC TTA ATC C</td>
</tr>
<tr>
<td>glp Reverse</td>
<td>TGG TAA AAT CGC ATG TCC AAT TC</td>
</tr>
<tr>
<td>gmk Forward</td>
<td>ATC GGT TTA TCG GGA CCA TC</td>
</tr>
<tr>
<td>gmk Reverse</td>
<td>TCA TTA ACT ACA ACG TAA TCG TA</td>
</tr>
<tr>
<td>pta Forward</td>
<td>GTT AAA ATC GTA TTA CCT GAA GG</td>
</tr>
<tr>
<td>pta Reverse</td>
<td>GAC CCT TTT GTT GAA AAG CTT AA</td>
</tr>
<tr>
<td>tpi Forward</td>
<td>TCG TTC ATT CTG AAC GTC GTG AA</td>
</tr>
<tr>
<td>tpi Reverse</td>
<td>TTT GCA CCT TCT AAC AAT TGT AC</td>
</tr>
<tr>
<td>yqi Forward</td>
<td>CAG CAT ACA GGA CAC CTA TTG GC</td>
</tr>
<tr>
<td>yqi Reverse</td>
<td>CGT TGA GGA ATC GAT ACT GGA AC</td>
</tr>
<tr>
<td>spaF1 (Forward 1)</td>
<td>GAC GAT CCT TCG GTG AGC</td>
</tr>
<tr>
<td>spaF2 (Forward 2)</td>
<td>CAG CAG TAG TGC CGT TTG C</td>
</tr>
<tr>
<td>spa Reverse</td>
<td>GAA CAA CGT AAC GGC TTC ATC C</td>
</tr>
</tbody>
</table>

**Wizard® PCR Clean-up Kit for MLST and spa Typing**

PCR products were purified (removal of amplification primers and primer dimers) for sequencing using the Wizard® PCR Preps DNA Purification System (Promega, Madison, WI) as follows. 100µl of Direct PCR Purification Buffer was added to the PCR product followed by mixing. The DNA was then added to 1 ml of resin and vortexed briefly 3 times over a 1 min interval to bind the DNA to the resin. The DNA/resin mixture was then transferred to a 3 ml syringe with a Wizard® Minicolumn attached. The syringe was then used to push the resin/DNA slurry into the minicolumn. A wash step was performed by adding 2 ml 80% isopropanol to the syringe and pushing it through the minicolumn. The minicolumn was then transferred to a 1.5 ml microcentrifuge tube followed by centrifugation at 10,000 g for 2 min. The minicolumn was then transferred to a second 1.5 ml microcentrifuge tube. To elute the DNA, 30 µl of 65°C water was
added to the column and allowed to stand 1 min. Centrifugation for 1 min eluted the DNA which was then stored at -20°C until needed.

**Preparation of DNA Sequencing Reactions for MLST and spa Typing**

All sequencing reactions were performed on a CEQ 8000 Sequencer (Beckman Coulter, Fullerton, CA) using the CEQ Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter, Fullerton, CA) as follows. Sequencing reactions were performed in 0.2 ml tubes containing 8.0 μL DTCS Quick Start Master Mix, 2.0 μL primer (25 pmol/μL), 2 μL template, and 8.0 μL of water. The recommended thermal cycling program was 30 cycles of 96°C for 20 sec, 50°C for 20 sec, and 60°C for 4 min. Ethanol precipitation of all templates was then performed as follows. The templates were added to sterile 0.5 ml microfuge tubes and 4 μL of stop solution (equal volumes of 1.5 M NaOAc (pH 5.2) and 50 mM EDTA (pH 8.0) and 1 μL of glycogen (20 mg/ml) was added. Following a brief mix, 60 μL of cold 95% ethanol was added to each template and mixed. The samples were then centrifuged at the maximum speed for 15 min at 4°C. The supernatants were then removed without disturbing the pellets. The pellets were then washed with 200 μL of cold 70% ethanol and centrifuged at maximum speed for 10 min at 4°C. The wash step was then repeated and the pellets were dried in a concentrator for 20 min. Dried pellets were resuspended in 40 μL of sample loading solution and transferred to a 96-well sequencing plate (Beckman-Coulter, Fullerton, CA). A drop of mineral oil was added to each sample before loading onto the CEQ 8000 and running the LFR (Long Fragment Run) sequencing program. All sequences were aligned using the
SeqMan II component of the Lasergene Expert Sequence Analysis Software package (DNASTAR, Madison, WI). MLST alleles and sequence types were assigned using online MLST database (http://saureus.mlst.net). All spa typing alleles and spa types were assigned using the online database (http://spa.ridom.de/spatypes.shtml).
Results

*S. aureus* BioNumerics® Database

PulseNet has demonstrated that PFGE is useful for tracking trends in the spread of microorganisms of interest. A large collection of *S. aureus* isolates including clinical isolates from Florida and Washington was used to develop a database of DNA fingerprint patterns that could be used to investigate the trends of *S. aureus* infection in these two states. Macroculture followed by pulsed-field gel electrophoresis was performed for every *S. aureus* isolate in the Center for Biological Defense (CBD) collection. The *S. aureus* PFGE size standard, *SmaI* digested NRS77 (NCTC 8325), was used for normalization. This standard is required by the software to allow comparison across the length of the agarose gels and to allow gel to gel comparison. The standard was run in lanes 1, 6, 11 and 15 on gels prepared with a small comb (15 wells), and in lanes 1, 5, and 10 on gels prepared with a larger comb (10 wells) (Figure 1). PFGE was performed on a total of 403 isolates and all isolates were typeable by this technique. Images from a total of 44 gels were imported into the BioNumerics® database and a UPGMA dendrogram based on Dice coefficients was generated.

A total of 88 isolates matched the USA 100 pulsotype and 136 isolates matched the USA 300 pulsotype. The remaining 161 isolates (does not include non-epidemic control strains) demonstrated pulsotypes consistent with sporadic strains as the patterns were either unique to that strain or were present in a low number of isolates. A
A dendrogram was generated using BioNumerics® to represent all of the strains included in the study (Figure 2). In this figure, USA 100 and USA 300 epidemic clone control strains represent 88 and 136 isolates respectively (Figure 2). Data for each individual strain is included in Appendix A.

Figure 1. Pulsed-field gel electrophoresis of *Staphylococcus aureus*. *S. aureus* isolates were run on 1.0% agarose gels following macrorestriction with *Sma*I enzyme. The standard marker (NCTC 8325) was run in lanes 1, 5, and 10 to allow normalization across the gel as well as gel to gel comparison. Images were uploaded to the BioNumerics® software package for dendrogram creation.
Figure 2. Dendrogram of sporadic isolates and epidemic controls. Data was analyzed using BioNumerics® (Applied Math, Sint-Martens, Belgium) Version 3.0. Dendrograms were derived from the unweighted pair group method using arithmetic averages (UPGMA) and based on Dice coefficients. Band position tolerance and optimization were set at 1.00%.
Figure 2. (Continued) Dendrogram of sporadic isolates and epidemic controls.
Figure 2. (Continued) Dendrogram of sporadic isolates and epidemic controls.
Figure 2. (Continued) Dendrogram of sporadic isolates and epidemic controls.
Analysis of *S. aureus* Epidemic Clone USA 300 in *S. aureus* PFGE Database.

A number of studies have demonstrated the epidemiology of this strain and its link to wound infections and necrotizing pneumonia (12, 37, 71). The dendrogram generated by BioNumerics® analysis indicated that a large number of isolates possessed pulsotypes consistent with the USA 300 epidemic clone control strain. This analysis demonstrated the first documented USA 300 isolates in both Florida and Washington State. Excluding those isolates from unknown sources, non-clinical sources, unknown clinical background, and/or non-MRSA, the database contains 332 CA-MRSA clinical isolates. A total of 136 CA-MRSA isolates were found to possess pulsotypes consistent with the USA 300 clone including 119 isolates from Florida and 17 isolates from Washington. Although the clinical background of these isolates was limited, they were grouped according to their source, i.e. wound, sputum, blood, nose, and other. Isolates that were reported as abscess, wound, furunculosis, and cellulitis were grouped as wound infections, while those listed as nasal, nose, and nares were grouped as nose infections. Clinical sources listed as other included urine, unspecified body fluid, eye, feces, and cervix. The clinical source for each individual isolate is listed in Appendix 1. Wound infections accounted for the majority of the 332 clinical isolates in the database, 194 (58%) of the isolates. A total of 121 of these wound isolates (63%) demonstrated the USA 300 pulsotype (Table 5). These data demonstrated both that the majority of isolates in the database are from wounds and that the majority of the wound isolates are USA 300 pulsotype. Although USA 300 was present in isolates from the other clinical sources, it constituted a minority of the isolates (Table 5).
TABLE 5. Summary of results for all isolates tested

<table>
<thead>
<tr>
<th>Source of Isolates</th>
<th>Total no. of isolates from source (% of total)</th>
<th>Total no. of isolates</th>
<th>No. (%) of isolates:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>USA 300 positive</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FL</td>
</tr>
<tr>
<td>Wound</td>
<td>194 (58)</td>
<td>106 (55)</td>
<td>15 (8)</td>
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<tr>
<td>Sputum</td>
<td>16 (5)</td>
<td>1 (6)</td>
<td>0</td>
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<tr>
<td>Blood</td>
<td>21 (6)</td>
<td>1 (5)</td>
<td>1 (5)</td>
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<tr>
<td>Nose</td>
<td>87 (26)</td>
<td>10 (11)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Other</td>
<td>14 (4)</td>
<td>1 (7)</td>
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</tr>
<tr>
<td>Total</td>
<td>332 (100)</td>
<td>119 (36)</td>
<td>17 (5)</td>
</tr>
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</table>

**Analysis of *S. aureus* Epidemic Clone USA 100 in *S. aureus* PFGE Database.**

There is a paucity of data concerning the epidemiology of the USA 100 epidemic clone but it is believed to be primary an HA-MRSA (70). Analysis of the *S. aureus* BioNumerics® database demonstrated that 88 of the isolates have pulsotypes consistent with USA 100 (no more than 1 band difference). Using the same exclusion criteria as the USA 300 analysis, the database containing CA-MRSA isolates includes 82 USA 100 isolates from Florida and 6 USA 100 isolates from Washington State. USA 100 isolates constituted the majority of the nose (47%) and blood (58%) isolates and was rarely present in wound, sputum, and isolates from other clinical sources (Table 6). The clinical source for each individual isolate is listed in Appendix 1. Few blood isolates were present in the database (21 total), limiting the conclusions that can be made concerning the etiology of blood-borne infections caused by this clone. However, *S. aureus* was
identified in 87 nose isolates confirming the nose as the second most common isolation site behind wound isolates (Table 6). Due to the limited clinical information provided for the isolates in this study, it is unclear whether these nose isolates were a cause of infection or if they are present as colonizing strains.

<table>
<thead>
<tr>
<th>Source of Isolates</th>
<th>No. (%) of isolates:</th>
<th>Total no. of isolates from source (% of total)</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>USA 100 positive</td>
<td>USA 100 negative</td>
</tr>
<tr>
<td></td>
<td>FL</td>
<td>WA</td>
</tr>
<tr>
<td>Wound</td>
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<td>1 (1)</td>
</tr>
<tr>
<td>Sputum</td>
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<td>3 (19)</td>
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<tr>
<td>Blood</td>
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<td>2 (10)</td>
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<td>Nose</td>
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<td>0</td>
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<tr>
<td>Other</td>
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<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>82 (25)</td>
<td>6 (2)</td>
</tr>
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</table>

Use of Restriction Digestion of the MW2 Genome to Identify Secondary Enzymes for S. aureus PFGE.

Studies with organisms other than S. aureus have demonstrated that differences in strains are often not detected by the use of just one restriction enzyme for PFGE (88). Virtual digestion of the S. aureus enzyme was performed in an attempt to find a secondary enzyme for S. aureus PFGE. A total of 262 commercially available restriction enzymes were tested for their ability to virtually digest the MW2 S. aureus genome into 10 to 50 fragments. Restriction digestion was performed using the TIGR Restriction Digest Tool (www.tigr.org/tigr-scripts/CMR2/restrict_display.pl) and the S. aureus
MW2, Michigan VRSA, and MU50 genomes. After testing 25 enzymes it was determined that the same number of restriction fragments resulted regardless of the \textit{S. aureus} genome selected and further digestions used only the \textit{S. aureus} MW2 genome. The majority of the enzymes, 242, resulted in >50 fragments which cannot be resolved on a conventional pulsed-field gel. Eleven enzymes either failed to cut or had recognition sites in the \textit{S. aureus} MW2 genome that would result in too few bands for epidemiological studies. Two enzymes were excluded based on expense as their use would exceed $1,000 per gel. Two additional enzymes were excluded because the resulting digestion pattern would contain bands that are too close together to be resolved by PFGE. This technique also identified $XmaI$ and $XmaCI$, both of which are isoschizomers of $SmaI$. Because their use would result in the same pattern as $SmaI$ and since $SmaI$ is less expensive and in common use, these two enzymes were also excluded. Results for each enzyme tested are shown in Table 7. Virtual digestion and application of the criteria mentioned above resulted in the identification of two enzymes, in addition to $SmaI$, \textit{Eag}I and \textit{Sac}II, which could potentially be used for \textit{S. aureus} PFGE.
<table>
<thead>
<tr>
<th>Enzyme</th>
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<th>Fragments</th>
<th>Enzyme</th>
<th>Fragments</th>
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<td>BspH I</td>
<td>1073</td>
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<td>BciV I</td>
<td>319</td>
<td>BspM I</td>
<td>535</td>
</tr>
<tr>
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<td>BspLU11 I</td>
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Table 7. Virtual digestion of *S. aureus* MW2 genome. A total of 262 enzymes were used to virtually digest the *S. aureus* genome. The resulting fragment numbers are listed in the table. Isolates with digestion results of 10 to 50 fragments (bold) were considered for further analysis.
<table>
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<tr>
<th>Enzyme</th>
<th>Fragments</th>
<th>Enzyme</th>
<th>Fragments</th>
<th>Enzyme</th>
<th>Fragments</th>
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<td>Mvn I</td>
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**Table 7 (Continued).** Virtual digestion of *S. aureus* MW2 genome. A total of 262 enzymes were used to virtually digest the *S. aureus* genome. The resulting fragment numbers are listed in the table. Isolates with digestion results of 10 to 50 fragments (bold) were considered for further analysis.
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Table 7 (Continued). Virtual digestion of S. aureus MW2 genome. A total of 262 enzymes were used to virtually digest the S. aureus genome. The resulting fragment numbers are listed in the table. Isolates with digestion results of 10 to 50 fragments (bold) were considered for further analysis.
Analysis of the PFGE Patterns of 12 USA 300 Epidemic Clone Isolates Using SmaI, EagI, and SacII.

Twelve CA-MRSA isolates that have identical SmaI pulsotypes consistent with the USA 300 epidemic clone were used to test the ability of the two additional enzymes to discriminate identical isolates (Figure 3). Restriction digestion of these isolates with EagI resulted in approximately the same number of fragments as digestion with SmaI, although the pulsotype generated was distinct from that of SmaI (Figure 4). Phylogenetic analysis of the EagI PFGE using the BioNumerics® software package determined that the 12 isolates were identical. Likewise, digestion with SacII resulted in a unique pulsotype, but with approximately the same number of bands as the other two enzymes (Figure 5). As shown in Figure 5, digestion with SacII also confirmed that the 12 CA-MRSA USA 300 isolates are identical. Thus, S. aureus PFGE was successfully performed with the three enzymes predicted by virtual digestion and BioNumerics® analysis of the three pulsed-field gels was in agreement that the 12 isolates are 100% identical.
Figure 3. USA 300 isolates demonstrating 100% identity by SmaI PFGE. Isolates possessing pulsotypes identical to the USA 300 epidemic clone were used to demonstrate the use of SmaI for digestion for PFGE. The original clinical source information is present in the figure. Quest represents isolates obtained from Quest Diagnostics in Tampa, Florida.
Figure 4. USA 300 isolates demonstrating 100% identity by *Eagl* PFGE. The same isolates present in Figure 3 were used to demonstrate the use of *Eagl* for digestion of *S. aureus* for PFGE. The original clinical source information is present in the figure. Quest represents isolates obtained from Quest Diagnostics and TGH isolates obtained from Tampa General Hospital both in Tampa, Florida.
Figure 5. USA 300 isolates demonstrating 100% identity by SacII PFGE. The same isolates present in Figure 3 were used to demonstrate the use of SacII for digestion of S. aureus for PFGE. The original clinical source information is present in the figure. Quest represents isolates obtained from Quest Diagnostics and TGH isolates obtained from Tampa General Hospital both in Tampa, Florida.
Analysis of the PFGE Patterns of Seven Pairs of Identical Isolates using \textit{SmaI}, \textit{EagI}, and \textit{SacII}.

Seven pairs of CA-MRSA isolates that were 100\% identical by \textit{SmaI} PFGE (Figure 6) were chosen to test the ability of \textit{EagI} and \textit{SacII} to further discriminate the pairs. Digestion with both enzymes again resulted in approximately the same number of resolvable fragments as \textit{SmaI} as predicted by virtual digestion. However, digestion with \textit{EagI} and subsequent BioNumerics® analysis demonstrated one to two band differences between isolates within three of the seven pairs (Figures 7). Similarly, digestion with \textit{SacII} followed by BioNumerics analysis also detected one to two band differences between isolates in the same three pairs as identified using \textit{EagI} (Figure 7 and 8). Thus, \textit{EagI} and \textit{SacII} were able to discriminate between isolates that were identical by \textit{SmaI} (Figures 6-8).
Figure 6. Seven pairs of identical isolates by Smal PFGE. Smal digestion was used to demonstrate seven pairs of identical isolates. The original source of the isolates is shown. The NRS100 control strain, obtained from NARSA, is included in this set of isolates.
Figure 7. Seven pairs of isolates further distinguished by Eag\textit{l} PFGE. \textit{Eag}\textit{l} digestion was used to demonstrate that the isolates digested by \textit{Sma}\textit{l}, shown in Figure 6, could be further distinguished by \textit{Eag}\textit{l} digestion. The original source of the isolates is shown. The NRS100 control strain, obtained from NARSA, is included in this set of isolates.
Figure 8. **Seven pairs of isolates further distinguished by SacII PFGE.** SacII digestion was used to demonstrate that the isolates digested by SmaI, shown in Figure 6, could be further distinguished by SacII digestion. The original source of the isolates is shown. The NRS100 control strain, obtained from NARSA, is included in this set of isolates.
Selection of CA-MRSA Isolates for Multilocus Sequence Typing.

In order to explore the use of genomic typing methods for CA-MRSA subspeciation, 30 isolates that were previously typed by PFGE were chosen for MLST. The isolates were selected to represent both Florida and Washington State (Figure 9). CA-MRSA isolated from a variety of clinical sources including wounds, urine, blood, sputum, and nose were included to represent the variety of disease caused by *S. aureus* (Figure 9). Furthermore, isolates possessing several pulsotypes as determined using the PFGE database were included (Figure 9). Isolates with identical pulsotypes were also included among the 30 CA-MRSA as controls as shown in Figure 9.

PCR Amplification of Housekeeping Genes for MLST

Following isolation of genomic DNA, internal fragments of the following seven housekeeping genes; *arc, aro, glp, gmk, pta, tpi*, and *yqi* were amplified by PCR using the primers listed in Table 4. PCR was performed according to the standardized protocol for *S. aureus* (saureus.mlst.net) for most isolates. However, adjustment of the annealing temperature, increasing from 60°C to 65°C, was necessary for PCR amplification of the *gmk, pta, and/or yqi* genes for some isolates to obtain a single band for sequencing. The PCR primers, primer dimers, and enzymes were removed from the amplification reactions prior to sequencing using the Wizard PCR Preps® kit and 1 μl of the resulting product was run on a gel to confirm a single band was present (Figure 10).
Figure 9. BioNumerics® Analysis. A UPGMA derived dendrogram illustrates the pulsotypes of the 30 CA-MRSA isolates used for MLST.
Figure 10. PCR amplification of the internal fragment of the *gmk* gene for MLST. PCR reactions were purified using the Wizard PCR Preps® kit to remove primer dimers and amplification primers. The result was a single band of 429 base pairs corresponding to the internal fragment of the *gmk* gene. The marker used for all agarose gels was Promega Benchtop 1 Kb Ladder.
DNA Sequencing, Alignment, and Assigning of Alleles and Sequence Types for CA-MRSA MLST.

A minimum of four forward and reverse sequencing reactions were performed for each PCR product analyzed, comprising 28 sequencing reactions for each of the 30 isolates examined. The resulting DNA sequences were uploaded to the SeqMan® program for alignment. Each gene was analyzed separately for each isolate using both forward and reverse sequencing reactions to remove ambiguity in base calling. The consensus sequence data from the alignments was then entered into the MLST database for comparison to known housekeeping gene sequences (alleles). The online allele tool allowed consensus sequences to be cut and paste into the database and compared to known sequences. Alleles were then assigned for the seven gene sequences from all isolates used in this study as each sequence matched a known allele present in the database (Table 8). The resulting allelic pattern could then be entered into the database to assign a sequence type. For example, the following pattern, \(arcC\) – allele 3, \(aroE\) – allele 3, \(glpF\) – allele 1, \(gmK\) – allele 1, \(pta\) – allele 4, \(tpi\) – allele 4, and \(yqi\) – allele 3 corresponds to ST 8. All isolates used in this study possess known STs as indicated in Table 8. As illustrated by Table 8 and Figure 11, there is little variation of the alleles present among the CA-MRSA isolates used for this study. Although a large number of alleles have been described for each of the seven housekeeping genes (Table 9), our isolates failed to show much variation. Consequently, despite the presence of 734 known STs in the \(S.\ aureus\) database, our isolates were comprised of only four sequence types (Table 8).
Figure 11. Clustal W (1.82) multiple sequence alignment of arc housekeeping gene fragment. Alignment of the sequences of the arc genes from three S. aureus isolates demonstrates 100% identity resulting in the assignment of the same allele at this loci for these isolates.
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Table 8. Multilocus sequence typing alleles and sequence types for 30 *S. aureus* isolates. Thirty *S. aureus* isolates were chosen for MLST which represent strains from both Florida and Washington State and clinical sources including nose, sputum, urine, blood, and wound isolates. The alleles and ST types were assigned using the online *S. aureus* database and sequences obtained from the housekeeping genes of the 30 isolates.
### TABLE 9. Comparison of *S. aureus* MLST alleles

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**Comparison of PFGE Data and MLST Data for 30 CA-MRSA Isolates.**

A dendrogram was prepared for the 30 *S. aureus* isolates typed by MLST using the *S. aureus* PFGE database. The isolates were grouped into 21 pulsotypes by PFGE, as compared to four STs, as shown in Figure 12. Isolates did not cluster based on either the state in which they were isolated or on their disease manifestations. Isolates with the same pulsotype always had the same MLST sequence type, however isolates with the same sequence type were frequently further differentiated by PFGE (Figure 12). For example, CBD 0665 and CBD 0677 were both assigned to ST 8 by MLST, but the PFGE patterns differ by >7 bands. The largest cluster of isolates was a match to the USA 300 epidemic clone (control not shown), which is also ST 8 by MLST (Figure 12). Database searches for the four resulting STs determined that they are found worldwide (Table 10).
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<th>Geographical Location (s) of ST</th>
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<td>EMRSA 3 European endemic strain, isolated from blood, pus, etc in hospital. Also frequently isolated from carriers in numerous countries.</td>
</tr>
<tr>
<td>8</td>
<td>UK, Canada, Netherlands, Scotland, Ireland, Australia, US (NY,MD) France, Germany, Denmark, Sweden, Greece, Switzerland, Belgium</td>
<td>EMRSA 2,6,7,12,13,14 European endemic strains, isolated from blood, pus, etc in hospital. Also frequently isolated from carriers in numerous countries</td>
</tr>
<tr>
<td>45</td>
<td>Canada, Belgium, Germany, Sweden, Finland, Netherlands, UK, US (NY), Australia, Switzerland</td>
<td>European endemic strains, isolated from blood, pus, etc in hospital. Also frequently isolated from carriers in numerous countries</td>
</tr>
<tr>
<td>105</td>
<td>US (FL), Switzerland</td>
<td>Only 3 strains added to date, 1 isolated from urine, two from unknown clinical source. All isolates from hospital</td>
</tr>
</tbody>
</table>
Figure 12. **BioNumerics® analysis and MLST data.** Dendrograms were derived from the unweighted pair group method using arithmetic averages and based on Dice coefficients. Band position tolerance and optimization were set at 1.0%. The sequence types for MLST as assigned using the online database (saureus.mlst.net) appear in the final column.
PCR Amplification of *spa* Region of CA-MRSA

The 30 CA-MRSA isolates that were characterized by PFGE and MLST were further characterized by *spa* typing. PCR was performed for the CA-MRSA using the genomic template isolated for MLST and the primers listed in Table 4. Amplification for *spa* typing required the use of two different forward primers, spaF1 or spaF2 (Table 4). PCR amplification of all isolates was initially performed using the spaF1 primer. These initial results indicated that CBD0614 produced a product that was much smaller in size than the rest of the isolates tested. This isolate was amplified again using the spaF2 primer which resulted in the same small sized fragment suggesting that this isolate possesses a low number of *spa* repeats (Figure 13). The initial amplification of CBD0633 using the spaF1 primer failed but was successful using the spaF2 primer as shown in Figure 13. Amplification of the *spa* region resulted in the same size fragments for all isolates except CBD0614 (Figure 13), CBD0633 (Figure 13), CBD0691 (Figure 14), and CBD0885 (Figure 14). The PCR primers, primer dimers, and enzymes were removed from the amplification reactions prior to sequencing using the Wizard PCR Preps® kit and 1 μl of the resulting product was run on a gel to confirm a single band was present (not shown).
**Figure 13. Amplification of spa region.** Polymerase chain reaction was performed on 30 *S. aureus* isolates to amplify the spa region for molecular typing. In the 15 isolates pictured, the amplified region ranged in size due to the number of 24 base pair spa repeats in each isolate. CBD0614 possesses only two spa repeats, and CBD0633 possesses only nine spa repeats, accounting for the small size of the products pictured, while the remaining isolates all contain 10 spa repeats. The marker used on this gel was Promega Benchtop Ladder. The last lane pictured was the no DNA control.
Figure 14. Amplification of _spa_ region. Polymerase chain reaction was performed on 30 _S. aureus_ isolates to amplify the _spa_ region for molecular typing. In the 15 isolates pictured, the amplified region ranged in size due to the number of 24 base pair _spa_ repeats in each isolate. CBD0691 possesses only seven, and CBD0885 only six _spa_ repeats, accounting for the small size of the products pictured. The remaining isolates all contain 10 _spa_ repeats. The marker used on this gel was Promega Benchtop Ladder.
DNA Sequencing, Alignment, and Assigning of Repeats and *spa* Types for CA-MRSA *spa* Typing.

A minimum of four forward and four reverse sequencing reactions were performed for each PCR product analyzed, comprising 8 sequencing reactions for each of the 30 isolates examined. The resulting DNA sequences were uploaded to the SeqMan® program for alignment. The use of eight sequences for each isolate removed ambiguity in base calling. The repeat regions were easily identified in the aligned sequences by visual examination of the consensus sequence (Figure 15). The majority of the isolates in the study were found to contain 10 repeats (Table 11). Exceptions included CBD0614 (2 repeats), CBD0633 (9 repeats), CBD0691 (7 repeats) and CBD0855 (6 repeats) as shown in Table 11. Each repeat region was then entered into the *spa* database for comparison to known *spa* repeats worldwide. The online repeat finder allowed individual repeats to be cut and paste into the database and repeats assigned. All isolates used in this study contained repeats of 24 base pairs that had previously been identified and were therefore able to be assigned numbers using the online tool (Table 11). The pattern of the repeats was then entered into the database for comparison against known *spa* types and *spa* types were assigned when possible. For example, CBD0479 contained 10 repeats with the following pattern: R11-19-12-21-17-34-24-34-22-25, a pattern previously identified, and assigned to *spa* type 8 (Table 11). A total of six *spa* types were identified in the study, including *spa* type 2, *spa* type 8, *spa* type 586, and three isolates with unknown *spa* types that were not identical to one another. Information from the worldwide database on the known *spa* types is presented in Table 12.
Figure 15. Clustal W (1.82) multiple sequence alignment for spa typing. Alignment of the sequences of the spa genes from three S. aureus isolates demonstrates 100% identity resulting in the assignment of the same repeats and the same spa type for these three isolates.
TABLE 11. Summary of *spa* repeats and *spa* type

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>State</th>
<th>Repeat Pattern</th>
<th><em>spa</em> Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBD0467</td>
<td>Blood</td>
<td>WA</td>
<td>R26-23-17-34-17-20-17-12-17-16</td>
<td>2</td>
</tr>
<tr>
<td>CBD0468</td>
<td>Wound</td>
<td>WA</td>
<td>R11-19-12-21-17-34-24-34-22-25</td>
<td>8</td>
</tr>
<tr>
<td>CBD0471</td>
<td>Urine</td>
<td>WA</td>
<td>R26-23-17-34-17-20-17-12-17-16</td>
<td>2</td>
</tr>
<tr>
<td>CBD0472</td>
<td>Blood</td>
<td>WA</td>
<td>R26-23-17-34-17-20-17-12-17-16</td>
<td>2</td>
</tr>
<tr>
<td>CBD0479</td>
<td>Wound</td>
<td>WA</td>
<td>R11-19-12-21-17-34-24-34-22-25</td>
<td>8</td>
</tr>
<tr>
<td>CBD0482</td>
<td>Wound</td>
<td>WA</td>
<td>R26-23-17-34-17-20-17-12-17-16</td>
<td>2</td>
</tr>
<tr>
<td>CBD0541</td>
<td>Wound</td>
<td>WA</td>
<td>R11-19-12-21-17-34-24-34-22-25</td>
<td>8</td>
</tr>
<tr>
<td>CBD0542</td>
<td>Blood</td>
<td>WA</td>
<td>R26-23-17-34-17-20-17-12-17-16</td>
<td>2</td>
</tr>
<tr>
<td>CBD0611</td>
<td>Wound</td>
<td>FL</td>
<td>R11-19-12-21-17-34-24-34-22-25</td>
<td>8</td>
</tr>
<tr>
<td>CBD0613</td>
<td>Nose</td>
<td>FL</td>
<td>R26-23-17-34-17-20-17-12-17-16</td>
<td>2</td>
</tr>
<tr>
<td>CBD0614</td>
<td>Wound</td>
<td>FL</td>
<td>R26-16</td>
<td>586</td>
</tr>
<tr>
<td>CBD0618</td>
<td>Nose</td>
<td>FL</td>
<td>R26-23-17-34-17-20-17-12-17-16</td>
<td>2</td>
</tr>
<tr>
<td>CBD0633</td>
<td>Nose</td>
<td>FL</td>
<td>R26-23-17-34-17-12-17-17-16</td>
<td>Unknown</td>
</tr>
<tr>
<td>CBD0637</td>
<td>Nose</td>
<td>FL</td>
<td>R26-23-17-34-17-20-17-12-17-16</td>
<td>2</td>
</tr>
<tr>
<td>CBD0644</td>
<td>Nose</td>
<td>FL</td>
<td>R26-23-17-34-17-20-17-12-17-16</td>
<td>2</td>
</tr>
<tr>
<td>CBD0652</td>
<td>Nose</td>
<td>FL</td>
<td>R26-23-17-34-17-20-17-12-17-16</td>
<td>2</td>
</tr>
<tr>
<td>CBD0665</td>
<td>Nose</td>
<td>FL</td>
<td>R11-19-12-21-17-34-24-34-22-25</td>
<td>8</td>
</tr>
<tr>
<td>CBD0674</td>
<td>Nose</td>
<td>FL</td>
<td>R11-19-12-21-17-34-24-34-22-25</td>
<td>8</td>
</tr>
<tr>
<td>CBD0677</td>
<td>Nose</td>
<td>FL</td>
<td>R11-19-12-21-17-34-24-34-22-25</td>
<td>8</td>
</tr>
<tr>
<td>CBD0685</td>
<td>Wound</td>
<td>FL</td>
<td>R11-19-12-21-17-34-24-34-22-25</td>
<td>8</td>
</tr>
<tr>
<td>CBD0691</td>
<td>Nose</td>
<td>FL</td>
<td>R8-16-2-43-34-17-34</td>
<td>Unknown</td>
</tr>
<tr>
<td>CBD0692</td>
<td>Nose</td>
<td>FL</td>
<td>R11-19-12-21-17-34-24-34-22-25</td>
<td>8</td>
</tr>
<tr>
<td>CBD0704</td>
<td>Sputum</td>
<td>FL</td>
<td>R11-19-12-21-17-34-24-34-22-25</td>
<td>8</td>
</tr>
<tr>
<td>CBD0717</td>
<td>Nose</td>
<td>FL</td>
<td>R26-23-17-34-17-20-17-12-17-16</td>
<td>2</td>
</tr>
<tr>
<td>CBD0727</td>
<td>Blood</td>
<td>FL</td>
<td>R26-23-17-34-17-20-17-12-17-16</td>
<td>2</td>
</tr>
<tr>
<td>CBD0728</td>
<td>Blood</td>
<td>FL</td>
<td>R26-23-17-34-17-20-17-12-17-16</td>
<td>2</td>
</tr>
<tr>
<td>CBD0734</td>
<td>Nose</td>
<td>FL</td>
<td>R26-23-17-34-17-20-17-12-17-16</td>
<td>2</td>
</tr>
<tr>
<td>CBD0736</td>
<td>Nose</td>
<td>FL</td>
<td>R26-23-17-34-17-20-17-12-17-16</td>
<td>2</td>
</tr>
<tr>
<td>CBD0737</td>
<td>Wound</td>
<td>FL</td>
<td>R11-19-12-21-17-34-24-34-22-25</td>
<td>8</td>
</tr>
<tr>
<td>CBD0885</td>
<td>Urine</td>
<td>FL</td>
<td>R11-34-24-34-22-25</td>
<td>Unknown</td>
</tr>
</tbody>
</table>
### TABLE 12. Summary of spa Type Information

<table>
<thead>
<tr>
<th>ST</th>
<th>Geographical Location (s) of ST</th>
<th>Additional Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>France, Germany, Norway, Sweden, Denmark, Hungary, Austria, Italy, Belgium</td>
<td>EMRSA-3, New York clone, Japan clone, Pediatric, Match to isolates with USA100 and USA800 pulsotypes. Matches isolates with MLST types 5, 231, 113</td>
</tr>
<tr>
<td>8</td>
<td>Germany, France, Sweden, Norway, Denmark, Netherlands, Austria, Italy, Belgium</td>
<td>Northern German MRSA (subclone), Matched isolates with USA 300 pulsotype, Archaic/Iberian. Matches isolates with MLST type 8.</td>
</tr>
<tr>
<td>586</td>
<td>Germany</td>
<td>Only 2 isolates in Germany, no further information available.</td>
</tr>
</tbody>
</table>

### Comparison of PFGE, MLST, and spa Typing Data for 30 CA-MRSA Isolates.

Sequence types and spa types were added to the dendrogram prepared for the 30 S. aureus isolates using the S. aureus PFGE database (Figure 16). The isolates were grouped into four sequence types by MLST and six spa types (Figure 16). As previously noted in the worldwide database (Table 12) isolates with patterns consistent with USA 300 controls were spa type 8, and isolates consistent with USA 100 controls were spa type 2 (control data not shown). The 30 CA-MRSA isolates were assigned to four sequence types by MLST and six spa types by staphylococcal protein A typing. However, these subtypes were further differentiated into 21 pulsotypes by PFGE as demonstrated in Figure 17.
Figure 16. **BioNumerics® analysis including PFGE, MLST, and spa data.** Dendrograms were derived from the unweighted pair group method using arithmetic averages and based on Dice coefficients. Band position tolerance and optimization were set at 1.0%. The sequence types for MLST appear in the second to last column and the *spa* types appear in the final column.
* CBD0471, CBD0727, CBD0728 have identical pulsotypes
** CBD0472, CBD0482, CBD0736 have identical pulsotypes
*** CBD0468, CBD0541, CBD0611, CBD0674, CBD0685, CBD0727 are USA300 pulsotype

**Figure 17.** Summary of PFGE pulsotypes, spa types, and Sequence types for 30 CA-MRSA isolates. Typing the 30 CA-MRSA resulted in 21 PFGE pulsotypes, 6 spa types, and 4 MLST sequence types as illustrated.
Discussion

*Staphylococcus aureus* causes a wide variety of diseases both within the hospital environment and in the community. *S. aureus* is one of the most commonly diagnosed bacterial infections which causes diseases ranging from self-limiting food poisoning to life-threatening septicemia and pneumonia. Control and treatment options for this organism are complicated by its remarkable ability to adapt to its environment especially through the acquisition of antibiotic resistance determinants. The most alarming trend in recent years was the acquisition of methicillin resistance that rendered the use of all β-lactam antibiotics useless for *S. aureus* infections. More recently, MRSA strains have been identified in persons in the community who lack the traditional risk factors for *S. aureus* infection. The CA-MRSA infections are of particular public health concern because they result in serious diseases including necrotizing fasciitis and necrotizing pneumonia. The high morbidity, mortality, and cost of care for strains such as these highlights the need for public health, hospital, and other laboratories to accurately identify these microorganisms. These factors illustrate the need for a proactive approach to surveillance, identification of hypervirulent strains, and infection control measures to prevent spread of the organism and to provide the most effective treatment options to patients.

In this project *S. aureus* isolates that were collected from Florida and Washington states and from a variety of clinical diseases were used to establish a large database of
DNA fingerprint patterns. Only community-acquired, methicillin-resistant isolates were included in this study. The definition of community-acquired *S. aureus* includes isolates collected within 72 hours of hospitalization, non-multi-drug resistant isolates, and isolates collected from persons with no prior history of hospitalization (64). The present study used slightly more stringent characteristics in that CA-MRSA is defined as isolates collected in an outpatient setting, or isolates collected no more than 48 hours after hospitalization, and only one isolate per patient was used in the analyses. The DNA fingerprint patterns resulting from PFGE were analyzed using the BioNumerics® software package to create dendrograms used for phylogenetic comparison of the isolates. A total of 403 isolates were analyzed and compared to a number of control strains (Appendix 1). Although a large number of the isolates included in the study demonstrated sporadic pulsotypes (Figure 2), the dendrogram contained two pulsotypes consistent with the control strains USA 300 and USA 100. Identifying these repeat pulsotypes among the isolates was the first step in surveillance for hypervirulent clones and it established the presence of the epidemic CA-MRSA isolate USA 300 in both Florida and Washington states, geographic regions in which neither strain had previously been identified (91).

A total of 136 of the CA-MRSA isolates analyzed possessed patterns consistent with USA 300 a strain that is known to carry the PVL toxin genes and to cause serious wound infection. Consistent with other reports, the majority of these organisms were isolated from wound cultures, and most of the wound cultures included in this study contained the USA 300 strain. This finding has important implications for public health measures to control the spread of this isolate. First, this strain accounts for the greatest
majority of all of the isolates included in this study, a fact that is probably related to its epidemic potential as described in numerous other studies (12, 24, 70, 72, 110). Second, the strain is known to carry the PVL toxin genes which likely contribute it its ability to cause serious diseases including necrotizing fasciitis and pneumonia in an otherwise healthy person with none of the usual risk factors related to *S. aureus* infection (12, 37, 71). Finally, this strain, while resistant to methicillin and therefore all of the β-lactam antibiotics, is not generally resistant to antimicrobials such as trimethoprim-sulfamethoxazole (Bactrim®), and therefore the usual recommended treatment for MRSA, vancomycin, can be avoided. The use of vancomycin for the treatment of MRSA should be discouraged as it is believed to contribute to the evolution of VRSA (vancomycin-resistant *S. aureus*) (45). However, vancomycin is commonly used to treat MRSA as most cases are believed to be multi-drug resistant and therefore untreatable by any other antimicrobial therapy. The knowledge that this strain is circulating within a community can be used along with other clinical factors to guide the treatment plan for patients.

The presence of the USA 100 strain among the CA-MRSA, accounting for 88 of the isolates included in this study, was unexpected as this isolate was previously reported as a hospital-acquired strain (70). This strain was identified primarily in isolates from the nose and blood (Table 6). It is interesting to note that USA 100 strain does not carry the PVL genes and has not been associated with any particular disease etiology in contrast to USA 300. Therefore, it is possible that USA 100 is present in a large number of isolates collected from the nose because it is primarily involved in colonization rather than infection. The presence of USA 100 in blood isolates is not easily interpreted due to
the low number of blood isolates included in this study. There are two major public health implications of the presence of this strain in the community. First, it appears that a classically hospital-acquired isolate has moved into the community (70). Traditionally CA-MRSA included only those isolates harboring the SCC\textit{mec} IV element. In contrast, USA 100 carries the SCC\textit{mec} type II resistance element. Second, studies have shown that MRSA carriers are most likely to develop staphylococcal disease caused by the same isolate that they harbor (18, 19, 46). The spread of isolates within the community, and from the hospital to the community, are both scenarios of public health concern. One possible mechanism for the spread of epidemic clones from the hospital environment to the community may be transmission by asymptomatic carriers. A study of CA-MRSA epidemiology suggested that surveillance for \textit{S. aureus} isolates should include periodic nasal colonization studies, a method which could also be used to track the USA 100 isolates (119). Monitoring the rates of MRSA nasal carriage among otherwise healthy individuals followed by appropriate treatment to eliminate strains, even in persons who do not display symptoms of disease, may be warranted to stop the spread of epidemic strains.

The identification of the two epidemic strains has illustrated the usefulness of PFGE in surveillance studies and it is to data considered the “Gold Standard” for typing of \textit{S. aureus} isolates. However, an unanswered concern regarding \textit{S. aureus} PFGE is that the use of just one restriction enzyme does not explore the potential discriminatory power of the technique. Studies using organisms such as \textit{Salmonella} have demonstrated that the use of multiple enzymes often unmasked relationships that were not seen using single digest macrorestriction (88). The present study demonstrated through the use of virtual
digestion that secondary enzymes could be identified for *S. aureus* PFGE (Table 7) but that the discriminatory power was unchanged by the use of more than one enzyme (Figures 3-8). Therefore *Sma*I digestion is sufficient for *S. aureus* PFGE but unfortunately results in data that at present cannot easily be shared between laboratories.

Compilation of data on the spread of CA-MRSA in the United States currently requires a review of the pertinent literature as no nationwide database for *S. aureus* exists. The CDC currently compiles DNA fingerprint data on a number of organisms to track food-borne outbreaks in a program referred to as PulseNet. In a 2003 publication the CDC reported in house efforts at establishing a national database for MRSA (70). However, since that publication there has been no standardization of the *S. aureus* PFGE protocol, no guidelines for certifying laboratories to submit isolates, and conflicting information concerning the pulsotypes which are considered epidemic clones (70, 109). A standardized protocol will be required for *S. aureus* researchers to compare data and control strains will be necessary for each group to determine if epidemic isolates are circulating in their communities. Furthermore, the criteria for certifying laboratories to submit data to PulseNet is extensive and the data flow is unidirectional. One of the major concerns of PulseNet participants, as presented at the 2005 PulseNet Update Meeting in Atlanta, was that data is sent to CDC and no analysis is returned to the submitting group. Without a detailed report on the isolates present in the community the chain from identification to treatment is broken and the user gets very little out of participating in PulseNet.

As a result of requesting CA-MRSA isolates from various clinical entities this study essentially monitored those communities for the presence of *S. aureus* isolates of
interest during the period the isolates were collected. Using the database created from PFGE data two epidemic *S. aureus* isolates were identified that could potentially have considerable public health impact. Ideally this data could now be shared with clinicians working in the effected area. However, the study was limited to distinct geographical areas, primarily Central Florida, and Washington State, and little is known how these isolates compare to those present in the rest of the United States or worldwide. Such information would be of great value as data comparison would result in better understanding of how the isolates are spread which in turn can direct public health efforts to prevent their spread. While the United States has not yet established a network for tracking *S. aureus* isolates, other countries such as Canada, Australia, and several European countries routinely track *S. aureus* isolates using PCR-based techniques (2, 25, 77, 87).

The use of gel-based techniques for molecular typing has a considerable number of advantages and disadvantages as listed in Table 2. PFGE is considered the “Gold Standard” for *S. aureus* typing due in large part to the following advantages listed in the table; high discriminatory power, all isolates are typeable, and results are highly reproducible. Regardless of these advantages, the disadvantage of low portability of data and the time-consuming nature of performing PFGE makes the technique less than desirable for some public health applications. As a consequence, efforts are underway to identify a technique that is fast, has high discriminatory power, and results in highly portable data. Any technique that results in numerical or sequence data is going to be highly portable while those involving analysis of gels are no more applicable than PFGE. Therefore the many available techniques that involve restriction digest to generate
fingerprints such as REA, riboprinting, and MLEE were not considered for this study. The PCR and sequence-based techniques such as multilocus sequence typing that directly compares sequence data for the presence of single nucleotide changes, and spa typing in which tandem repeats in a particular genomic region are analyzed and compared, were considered the best options for this study. These two typing techniques have been extensively used in characterizing *S. aureus* isolates overseas but neither has been applied to the study of CA-MRSA isolates in the United States. Thirty isolates from the PFGE database which represent both Florida and Washington, a number of disease etiologies, and a variety of pulsotypes as determined by PFGE, were analyzed by both MLST and spa typing (Figure 9). The goal of this analysis was to determine if these PCR-based techniques would be useful in identifying the hypervirulent clones among the CA-MRSA isolates thereby providing an alternative to PFGE typing.

Multilocus sequence typing was performed on the 30 isolates according to the well-established protocol and resulted in allele assignment and sequence types for all isolates tested (Table 8). The genes used for the MLST were housekeeping genes that are present in all isolates with has two advantages for the technique. First, these genes must be present in all *S. aureus* isolates so therefore all isolates should be typeable by MLST. Second, variation in the housekeeping genes is slow and genetic relationships between clones can therefore be detected. Unfortunately, there is little variation in the housekeeping genes among CA-MRSA isolates as demonstrated in Table 8. Sequence results from one of the regions, internal fragment of the *glp* gene, demonstrated the same result for all isolates, except one (CBD0691), indicated that nearly all of the isolates have the same allele at this locus. Alleles with no variation such as this are of no value to the
technique and omission of this gene would have resulted in the same overall result. The lack of variation among the isolates resulted in only 4 STs identified among the 30 CA-MRSA isolates. Furthermore, isolates with significantly different PFGE patterns, as well as different phenotypes (data not shown), such as CBD0885 and CBD0467, possessed the same allelic pattern and were therefore assigned to the same sequence type.

The MLST approach was designed to measure the evolutionary differences between \textit{S. aureus} isolates. Variations in housekeeping genes accumulate very slowly and MLST is therefore suitable to measure changes among specific clones. MLST has been used successfully to create models for the evolution of MRSA from MSSA ancestors and these studies have concluded that MRSA has arisen from at least 5 different lineages (27-29, 31-33). The significance of this is that isolates identified in any location can be compared using the eBURST algorithm to determine how MRSA was introduced into a specific geographic location. Studies such as these using MLST have demonstrated that MRSA evolved from MSSA strains already present within a country in some cases such as Denmark, Germany, and France. Understanding the long-term epidemiology of the spread of MRSA isolates worldwide will likely contribute to efforts to control the spread of other \textit{S. aureus} strains of interest. For example, other studies have demonstrated the spread of antibiotic resistance genes, other than \textit{mecA}, and virulence determinants, such as toxin genes, within particular clonal complexes as defined by MLST (113). Each of these studies involved the evolution of a set of genetically related isolates, often described as a clone. However, highly related isolates such as the 30 CA-MRSA isolates used in this study may belong to the same clonal complex and the same clone, but these clones may contain isolates with considerably
different pathogenic potential. Isolates belonging to the same clone as defined by MLST in this study were associated with different disease etiologies, possessed different virulence factors, and varied in their resistance patterns (data not shown), all of which contribute to the public health measures necessary for their control and the available treatment options. Therefore it is often necessary to identify \textit{S. aureus} at the strain level rather than the level of a particular clone, especially when treatment options are considered.

The relatively stable “core” genome of the \textit{S. aureus} isolates renders the MLST housekeeping gene protocol of little use for typing highly related strains such as MRSA and particularly CA-MRSA which has only been identified in the United States in the last 6 years. Modification of the existing MLST protocol to eliminate genes of little use such as \textit{glp} and replacing them with hypervariable genes such as surface antigen genes may increase the discriminatory power of the technique. A mix of both conserved and hypervariable genes may be required for adequate discriminatory power that represents the true relationship at the level of the strain and still corresponds to clinical significance. Conversely, it is entirely possible that the use of too many genes may result in closely related stains appearing different due to the increased discriminatory power. Experimentation with different combinations of conserved and hypervariable genes will be required to create a more discriminatory MLST protocol for \textit{S. aureus}. However, regardless of the genes used another major disadvantage of the MLST approach is that it is comparable to PFGE in the amount of time required for the analysis. The required PCR amplification and sequencing of seven genomic regions was not only time-consuming but significant ambiguity existed in the assignment of alleles for each region.
Although multiple sequence reactions were prepared in both the forward and reverse directions, the sequence-calling software often gave numerous results for the same nucleotide leading to repeat sequencing. Furthermore, the large number of sequences required for the assignment of an ST type for a single isolate is costly when compared to PFGE.

The major advantage of the technique was that multilocus sequence typing resulted in data that was highly portable and was easily compared to MLST results obtained for strains worldwide using the online database. The four STs identified among the 30 CA-MRSA isolates were present in the database indicating that they had been identified in other studies. For each of the four STs, the database contained information concerning the geographic locations in which isolates with the same ST have been identified. Significantly, the database also contains information on the background of the isolates deposited including such information as clinical site of infection, carrier status, matches to known epidemic clones, and PFGE patterns (Table 10). However, the database is limited to mostly European countries as MLST typing in the rest of the world is rarely performed. A more comprehensive database would be desirable for continued use of this technique, but a similar typing technique that is not as time-consuming or as costly, but results in higher discriminatory power would be more applicable.

Several recent studies have demonstrated the applicability of *spa* typing for typing *S. aureus* isolates of interest (1, 41, 59, 83, 103, 106). This technique is attractive in that it requires PCR amplification of only the variable region of the *spa* gene followed by sequencing as opposed to the PCR amplification of seven regions required for MLST. Consequently, the amount of time required and costs involved in *spa* typing are
considerably less than MLST. The 30 CA-MRSA isolates that were typed by MLST were characterized by spa typing in the hopes that this technique would be highly discriminatory, rapid, and inexpensive. The spa typing technique proved to be more rapid than either MLST or PFGE in that results could be obtained in 48 hours as opposed to five days required for the other two techniques. Sequencing of the relatively short spa repeat regions resulted in sequence data that was unambiguous and much more quickly analyzed in comparison to MLST data. However, as with MLST, the discriminatory power of spa typing for the CA-MRSA isolates was considerably less than that of PFGE with only 6 distinct spa types present among the isolates as compared to 21 distinct pulsotypes (Table 11). Furthermore, although all of the repeats identified in this study were present in the spa database, the pattern of three of the isolates was unknown and these could therefore not be assigned to spa types. As demonstrated with the MLST data, spa typing data could be used to search the worldwide database for information regarding the spread of particular spa types, once again including geographical location, clinical data, MLST typing data, and pulsotypes (Table 12). Technically, because spa typing was able to further differentiate subtypes within sequence types defined by MLST, it is the more discriminatory of the two techniques but insomuch as analysis of CA-MRSA isolates is concerned, it does not approach the discriminatory power of PFGE.

The variability of the spa gene between MSSA and MRSA isolates appears to be considerably higher than that among CA-MRSA isolates and spa typing has been used successfully to differentiate epidemic and sporadic isolates during nosocomial outbreaks (103). At least two European hospitals have reportedly replaced PFGE with spa typing for analyzing S. aureus isolates present in their facilities (1, 2, 41). Similar to MLST
studies, *spa* typing was used to determine that MRSA strains present in Portugal are not descended from long-established MSSA strains present within the country but were likely imported from abroad (1). Another study of nosocomial outbreaks determined that in each case where *spa* types were different between isolates, other typing techniques confirmed that the isolates were in fact not related (106). The same study also suggested, and has been demonstrated in the present study, that isolates with the same *spa* type are not necessarily epidemiologically related (106). The use of *spa* typing for tracking *S. aureus* isolates for some applications such as nosocomial spread of isolates and its considerable advantages in terms of time and portability of data would be useful as an initial screen to rule out the necessity of more discriminatory technique such as PFGE.

The presence of *S. aureus* isolates with increased virulence, antibiotic resistance determinants, and high transmissibility is a major public health concern. Active surveillance and application of the appropriate infection control measures will be required to control the spread of these strains. In the present study molecular methods for typing *S. aureus* isolates, the first step in surveillance, were compared for their ability to type a set of closely related CA-MRSA isolates. No one particular method excelled at typing these isolates in terms of discriminatory power, length of analysis, cost, and portability of data. These data suggest that the technique used for typing should be selected based on the purpose of the typing study. Cost and availability of the required equipment will of course also determine what typing methods can be used to type *S. aureus*. All of the techniques discussed herein require highly specialized and costly equipment including PFGE gel boxes, thermocyclers, and nucleic acid sequencers. Many laboratories may not have access to any of this equipment and may be required to send
samples to other laboratories for testing. However, an ever-increasing number of hospital and public health laboratories do have access to this equipment and an application of each of the techniques to particular studies follows.

Infection control in a hospital environment is critical to prevent acquisition of nosocomial infection. Outbreaks of nosocomial infection caused by *S. aureus* in the hospital environment are common and can be caused by either MSSA or MRSA. Infection control measures must be implemented to control the spread as quickly as possible but what measures are used depends on the extent of the spread. For example, if the surgical and ICU wards are both reporting *S. aureus* outbreaks, it is imperative that the infection control practitioners know if the same strain is responsible for the outbreaks in both locations. A quick way to determine if the strains are different is to use *spa* typing. Strains with different *spa* types are epidemiologically unrelated and therefore infection control measures unique to each ward should be initiated. Conversely, those strains with identical *spa* types may be related, and this could then be confirmed using PFGE. In this case, the outbreak is likely from a common source and efforts should be directed at identifying infected personnel working in both wards or shared equipment. Following this same scenario, *spa* typing could then be used to rule out infection from healthcare workers who may be colonized with *S. aureus*. In the event of a matching *spa* type, PFGE could then be used to confirm the outbreak strain originated with the healthcare worker. Infection control measures can then be aimed at treating the healthcare worker as well as providing infection control prevention education. Another use of *spa* typing is to rule out recurrent infection in carriers as opposed to successive infection with a different strain. An initial screening by *spa* typing can aid in determining
if post-surgical infections are most likely to occur due to the patient’s own flora or
nosocomially-acquired infection as hospital-acquired strains are significantly different in
spa types from community-acquired S. aureus. Finally, in an outbreak situation where
PFGE confirmed that USA 300 is present in the community, strains from a patient with a
wound infection who is otherwise healthy can be analyzed by spa typing to determine if
the strain belongs to ST 8 consistent with all USA 300 isolates. This patient could then
be considered part of the outbreak and treated with the appropriate antibiotics. Although
most of the studies that are listed above as examples of spa typing applications could also
be performed using MLST, spa typing for these applications has considerable advantages
in terms of time and cost.

Large-scale epidemiological studies are often more concerned with the origins of
the strains present in a geographic location during a particular time frame. MLST studies
have been used to track the origins of MRSA in many countries, and have in particular
demonstrated that MRSA originated when MSSA strains previously identified in the
country developed methicillin-resistance by acquisition of one of the SCCmec mobile
elements. These studies have provided a considerable amount of data concerning the
worldwide spread of antibiotic-resistant strains. Further applications of this technique
will be to track the worldwide spread of the Panton-Valentine Leukocidin toxin genes,
other newly described toxin genes, and other virulence factors. MLST analysis of strains
that are present in a particular community over a long time period will demonstrate which
clonal complexes are most successful in that community. Analysis of the strains present
within that clonal complex can then be used to determine what factors likely determine
the evolutionary advantage that allows those strains to be successful. Although spa
typing can be used for similar studies, the lack of an algorithm to define the precise evolutionary relationships between spa types, as is present for sequence types in the eBURST analysis program, limits the use of spa typing for long-term evolutionary studies.

As demonstrated with the CA-MRSA isolates, often it is only the most discriminatory of the techniques that is required for a particular study. Only PFGE was able to identify the presence of the USA 300 isolates in Florida and Washington State. The presence of this highly virulent, highly transmissible pathogen in the community has significant public health consequences. The treatment of these patients can be guided by the knowledge that this strain carries the methicillin-resistance SCCmec IV element and is known to be susceptible to other antibiotics, negating the need to perform antibiotic susceptibility testing, and hopefully preventing the use of vancomycin to treat the infection. It is well known that this isolate is easily spread among athletes, prisoners, and others sharing common facilities which can guide infection control measures within these facilities. Often hospitals are contaminated with particular isolates for long periods of time and the source of the isolates are never discovered. Detailed information regarding the susceptibilities of these nosocomial inhabitants is often well known and nosocomial infections within that facility are treated accordingly. PFGE testing of hospital strains can determine what isolates are present and therefore more likely to cause nosocomial infection and have in the past detected changes from one dominant strain to another, leading to changes in the appropriate therapy. Periodic nasal colonization studies in the community can be used to determine what strains are present among carriers. These strains are important as most S. aureus infections are caused by the person’s normal flora.
As demonstrated in the present study, PFGE identified the USA 100 epidemic clone among community-acquired *S. aureus* isolates. The public health significance of this finding is that a traditionally hospital-acquired MRSA is present in large numbers in the community. This data can be used to create a model for the spread of this isolate and to guide public health efforts to prevent further spread. As mentioned above, the use of *spa* typing can rule out infections with identical strains in many cases, but PFGE must be used to confirm that the strains are in fact identical. Although PFGE provides considerable advantages in terms of discriminatory power, global studies involving the technique will have to wait for the establishment of PFGE fingerprinting databases and standardized protocols.

Surveillance programs for the spread of *S. aureus* isolates should be implemented by laboratories at the local, state, federal, and worldwide levels to guide infection control practices, to determine effective treatment therapies, to describe the epidemiology of the spread of *S. aureus*, and to study the evolution of the strains. No one typing method is available to address all of these areas. Those typing methods such as PFGE that detect rapid variation are well-suited to outbreak investigations, while the typing methods that categorize isolates by relatively stable genetic loci such as MLST and *spa* typing are more suited to long-term epidemiological and evolutionary studies.

Future studies should include improvements to each of the techniques. A worldwide database of DNA fingerprint patterns obtained using a standardized PFGE protocol is necessary to address the issue of portability of data. Furthermore, newer PFGE protocols for *S. aureus* are under development to shorten the time period required for analysis. Modification of the MLST protocol to include both variable and highly-
conserved genes is necessary to increase the discriminatory power of the technique. The development of guidelines to determine the genetic relationships between spa types is necessary for this technique to be useful for evolutionary studies. Finally, efforts should continue to test existing typing techniques and to develop newer techniques that provide reliable, portable, typing data rapidly and at low cost.
References


Evaluation of protein A gene polymorphic region DNA sequencing for typing of


120. **Wertheim, H. F., W. B. Leeuwen, S. Snijders, M. C. Vos, A. Voss, C. M. Vandenbroucke-Grauls, J. A. Kluytmans, H. A. Verbrugh, and A. Belkum.**


Appendices
Appendix A. List of all *Staphylococcus aureus* isolates in BioNumerics® database

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About the Author

Jill Roberts received a Bachelor of Science degree in Microbiology from Indiana University in 1996. She received a Master of Public Health degree in Tropical Infectious Diseases from the University of South Florida in 1998. She received a Master of Science degree from the University of Texas in Microbiology and Molecular Genetics in 2003. She entered the Ph.D. program in Global Communicable Diseases at the University of South Florida, College of Public Health in August of 2003.