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DEVELOPMENT OF A NOVEL MULTIPLEX PCR METHOD FOR DETECTING THE
MAJOR CLONAL COMPLEXES OF HEALTHCARE- AND COMMUNITY-ASSOCIATED
METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA)

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an increasingly common cause of healthcare- and community-associated infections. MRSA is capable of causing many different types of infections due to the large number of virulence factors it produces. The recent emergence of healthcare-associated MRSA (HA-MRSA) strains with resistance to the best available antibiotic treatments and community-associated (CA-MRSA) strains capable of highly virulent infections has made the detection and characterization of these strains even more important. Several molecular methods have been used for either the detection of MRSA strains or the characterization and identification of MRSA clones. However, a need exists for a method that provides a link between the detection and clonal characterization of isolates. To fill this void, a novel clonal complex multiplex PCR (CC M-PCR) was developed in the present study. The CC M-PCR targets virulence genes specific to the major clonal complexes (CCs) of HA-MRSA and CA-MRSA in the United States. The CC M-PCR was used to characterize MRSA isolates from 67 presumptive MRSA positive nasal swabs taken upon admission to the ICUs at The Penn State Milton S. Hershey Medical Center. A majority (41/67) of the isolates were correctly identified as CC 5 strains, the primary cause of HA-MRSA in the northeastern U.S. The remaining isolates represented CCs 1, 8, 30, 45, 59, 133, and 5 isolates were identified as not being *S. aureus*. A reliable means for the rapid detection and characterization of MRSA strains is now critical due to CA-MRSA strains causing an increasing number of infections in healthcare settings. The CC M-PCR method developed in the present study can serve as a rapid screening method to detect which clonal complex a MRSA isolate belongs to before it is subsequently subtyped to the strain level using more sophisticated, but more time-consuming molecular methods.

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METHICILIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

Staphylococci are Gram-positive, catalase-positive, facultative anaerobes with a low DNA G+C content (23, 83). The *Staphylococci* were first identified in 1882 by Alexander Ogston, as micrococci in the “grouped form” (75). The *Staphylococci* are nonmotile, do not form spores, and exhibit limited capsule formation. The genus *Staphylococcus* contains 36 recognized species, including the important human pathogen *S. aureus* (38).

S. aureus forms round “golden” colonies and frequently produces a zone of β -hemolysis when grown on sheep blood agar (8). *S. aureus* is primarily distinguished from other *Staphylococci* by its ability to produce coagulase (65). A very small minority of *S. aureus* strains do not produce coagulase, while some strains of *S. schleiferi* test positive for coagulase production leading to some misidentification by this method (99). The production of DNase and the fermentation of mannitol in 7.5% NaCl are also used to distinguish *S. aureus* from other *Staphylococci* (58). The high salt tolerance of *S. aureus* allows it to exist commensally on human skin and in the nasal cavities of nearly 30% of the population (37, 104).

MRSA Disease

From the initial discovery of *S. aureus* until the introduction of penicillin as an antibiotic in the early 1940s, *S. aureus* infections had nearly an 80% mortality rate. As early as 1942, the first penicillin-resistant strain of *S. aureus* was observed. By 1960, approximately 80% of all *S. aureus* strains isolated from infected patients were penicillin-resistant. In 1959, methicillin was introduced as “penicillinase-resistant” penicillin for use against penicillin-resistant strains of *S. aureus* (17). Two years later, the first methicillin-resistant *Staphylococcus aureus* (MRSA) strains were observed in hospitals (50). By 1992, 35.9% of healthcare-associated *S. aureus*

infections in the National Nosocomial Infections Surveillance System were caused by MRSA, and by 2003 the percentage had increased to 64.4% (53).

As a whole, MRSA infections kill approximately 19,000 hospitalized Americans per year, which is equivalent to the numbers killed by AIDS, TB, and viral hepatitis combined in the United States. MRSA causes a wide variety of diseases including skin/soft tissue infections, bacteremia, endocarditis, pneumonia, and toxic shock syndrome (5). The skin/soft tissue infections caused by MRSA range from the more common, less severe carbuncles and furuncles to the less common, more severe scalded-skin syndrome and necrotizing fasciitis (30, 68, 78). MRSA bacteremia is increasingly observed as a persistent infection and sometimes leads to endocarditis. Both persistent bacteremia and endocarditis are very severe infections that have a high mortality rate, ranging from 17-35% in patients with medical devices (5). MRSA is the cause of 10-20% of pneumonia cases observed during hospitalization, and has been implicated primarily as an opportunistic infection following influenza-like illness (80). MRSA toxic shock syndrome is caused by a superantigenic toxin carried in some MRSA strains, which leads to uncontrolled release of inflammatory cytokines and chemokines and often death (59).

SCC*mec* and the Evolution of Methicillin-Resistance

Resistance to methicillin and all other β -lactam antibiotics in MRSA is due to the acquisition of the *mecA* gene, which encodes the 78-kDa penicillin-binding protein 2' (PBP2'). As shown in Figure 1.1, the transcription of *mecA* is repressed by the DNA-binding MecI, which also represses the transcription of itself and the β -lactam-sensing signal-transducer MecR1. In the presence of a β -lactam, MecR1 cleaves itself and MecI, allowing the production of PBP2'. PBP2' is a modified version of the PBPs found in all *S. aureus* strains. The native PBPs in the

cell wall bind to β -lactam antibiotics, and cell wall synthesis is disrupted, killing the bacterium. In the presence of PBP2', β -lactam antibiotics cannot bind to the native PBPs and cell wall synthesis is unaffected (3).

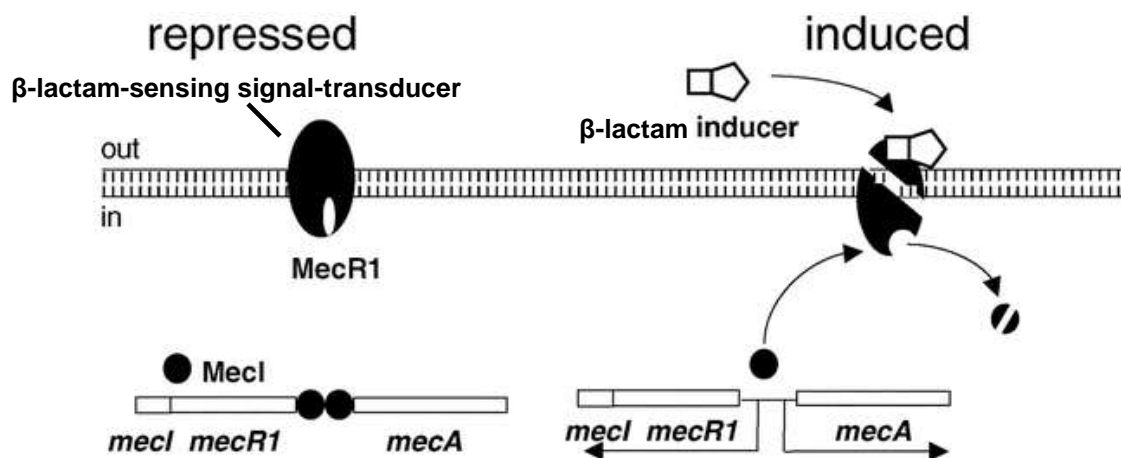


FIG 1.1. In the absence of a β -lactam inducer, transcription of *mecA*, *mecI* and *mecR1* is repressed by the repressor MecI. In the presence of a β -lactam inducer, MecR1 cleaves itself and MecI allowing the transcription of *mecA*, *mecI* and *mecR1* and the production of the penicillin-binding protein 2' from the *mecA* gene product. Adapted from Berger-Bachi et al. (3).

The *mecA* gene cluster is carried on the Staphylococcal Cassette Chromosome *mec* (SCC*mec*) element. Each SCC*mec* element also contains at least one cassette chromosome recombinase (*ccr*) gene which is responsible for the integration and excision of the SCC*mec* element from the chromosome (52). There are eight major types of SCC*mec* elements (types I to VIII) that have been distinguished, varying in size from 20.9 to 66.9 kilobases (9, 17). The additional gene content and length of the noncoding “junkyard regions” of each SCC*mec* type account for the size differences between elements. SCC*mec* types I, IV, V, VI, and VII provide resistance only to β -lactams, while SCC*mec* types II and III provide resistance to additional

drugs due to antimicrobial resistance genes carried on integrated plasmids or the Tn554 transposon (17). SCC*mec* type II typically contains the pUB110 plasmid and Tn554, while SCC*mec* type III is a composite element of SCC*mec* type III and SCC*mercury* that typically contains the plasmids pI258 and pT181 and two copies of Tn554 (12). pUB110 carries resistance to aminoglycosides, kanamycin, tobramycin and bleomycin (17). Tn554 provides inducible resistance to macrolides, lincosamides, streptogramin and resistance to cadmium. pT181 encodes tetracycline resistance, and pI258, which is part of SCC*mercury*, carries resistance to penicillin, mercury, and other heavy metals giving the cassette its name (12). Some structural variants of SCC*mec* exist which differ from the consensus sequences by not containing a copy of a plasmid or transposon or containing different ‘junkyard regions’ (17).

The first MRSA strain was isolated in 1960 in a hospital in Colindale, United Kingdom and contained SCC*mec* type I, and thus was given the name COL (9). The Archaic clone, of which COL is a member, spread through Europe and the United States until the 1970s (13). Over the following ten years, the Archaic clone disappeared from European hospitals (9). SCC*mec* type I continued to persist in the Italian and Rome clones into the 1990s and persists to the present in the Iberian clone (17, 64). In 1962, the first SCC*mec* type II strain was observed in Japan. The SCC*mec* type II strain disseminated around the world and was designated the New York/Japan clone (17). The New York/Japan clone is the predominant healthcare-associated MRSA (HA-MRSA) strain in the northeastern United States, with healthcare facilities defined as hospitals, nursing homes, and outpatient clinics (79). Some strains harboring SCC*mec* type II have acquired partial or complete resistance to vancomycin, the antibiotic of choice for MRSA infections (94, 96). SCC*mec* type III was detected in New Zealand in 1985, and has persisted

more successfully than *SCCmec* type I (9). Until the 1990s, all MRSA strains that were observed only caused healthcare-associated infections.

In the 1980s, the first community-associated MRSA (CA-MRSA) cases were observed in Michigan (82). The first isolates of CA-MRSA known to carry *SCCmec* type IV were observed in indigenous populations in Western Australia. These strains were distinguished from other Australian MRSA strains by their susceptibility to antibiotics other than β -lactams (9). Between 1997 and 1999, the first well-documented cases of CA-MRSA with *SCCmec* type IV in the United States were observed. Like the Australian CA-MRSA strains, the CA-MRSA strains in the United States were susceptible to antibiotics other than β -lactams. However, the strains were very virulent, killing all four of the infected children (49). The MRSA strain that emerged to cause the U.S. outbreak was the USA400 clone, designated as such because of the banding pattern produced by the strain following pulsed-field gel electrophoresis (PFGE) (86). Between 1999 and 2001, a second CA-MRSA clone, USA300, surfaced and displaced USA400 as the primary cause of CA-MRSA infections in the United States (9, 93). Since 2001, MRSA strains harboring *SCCmec* type V primarily have been observed in Australia, *SCCmec* type VI strains have been observed in Portugal, and *SCCmec* type VII strains have been observed in Taiwan (46, 77, 91). While the presence of newer *SCCmec* types is limited geographically, the prevalence of the USA300 clone in the United States grew significantly from 2001 to 2004 (92).

Emergence of USA300

Initially, USA300 was restricted to being a source of CA-MRSA infections. However, USA300 is now replacing HA-MRSA strains in healthcare settings. In a Houston, TX medical center it was reported that 60% of the nosocomial MRSA cases were caused by the USA300 clone (35). In emergency departments in eleven American cities, 76% of *S. aureus* isolates were identified as MRSA, and 97% of those were USA300 (70). Mathematical modeling suggests that USA300 will become the dominant MRSA clone in healthcare facilities resulting in an increase in the number of severe infections caused by USA300 in an environment of susceptible hosts (14). Horizontal gene transfer between MRSA strains causes additional concern about the invasion of USA300 into hospitals where it can co-exist with HA-MRSA strains. If horizontal gene transfer were to occur between USA300 and HA-MRSA, the increased virulence of USA300 compared to other prevalent strains and the vancomycin intermediate resistance of some HA-MRSA strains could lead to a novel, highly virulent, multi-drug-resistant MRSA strain (18, 62, 96). Thus, it is not surprising that a USA300 strain with vancomycin intermediate resistance has been observed (40). The prospect of facing an infection which is both quickly fatal and resistant to the last reliable treatment is not a good one.

MRSA Virulence Factors

Because of their multifunctional and redundant nature, the factors that allow MRSA to be pathogenic and have increased virulence are not completely understood. The surface structures of *S. aureus* mediate the attachment of MRSA to host tissues, as well as serve other roles in virulence. Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), include clumping factors and binding proteins for fibrinogen, collagen, and

bone sialoprotein mediate the attachment of *S. aureus* to multiple host tissues (28, 95). The MSCRAMM Staphylococcal protein A (SpA) is a superantigen, and it binds to the Fc region of immunoglobulins inhibiting recognition of the foreign cell (42). Two other surface proteins with similar function, the staphylococcal superantigen-like protein 10 (SSL10) and the *Staphylococcus aureus* binder of IgG (Sbi), also bind the Fc region of IgG, underscoring the redundancy of virulence genes produced by *S. aureus* (42, 48). Many *S. aureus* strains are able to form biofilms and capsules on host surfaces, which decrease their recognition and phagocytosis by the host immune system. MRSA infections on prosthetic devices often involve the formation of biofilms making the clearance of the bacteria very difficult (36). In addition to the antiphagocytic effect of the *S. aureus* capsule, it exists in a zwitterionic form that is implicated in abscess formation in the host (97). *S. aureus* also secretes several enzymes and toxins that act as virulence factors.

Many of the virulence genes in *S. aureus* are carried in staphylococcal pathogenicity islands (SaPIs) located within the chromosome, while others are part of the core genome. SaPIs are mobile genetic elements, each of which carries a unique set of virulence genes. At least twenty different SaPIs are known, and each *S. aureus* strain carries at least one SaPI with most strains carrying a few (74). The expression of virulence genes in both the core genome and the SaPIs is thought to be regulated by a group of two-component regulatory systems including the *agr* two-component system and the SarA protein family. The accessory gene regulator *agr* is thought to be the most important regulator of virulence based on growth phase (73). However, these results were only observed *in vitro*, and the exact role of *agr in vivo* is not completely understood (25).

S. aureus strains are capable of producing a wide range of toxins with diverse and overlapping functions. At least ten different enterotoxin genes can be expressed by *S. aureus* strains (20). The enterotoxins are typically associated with staphylococcal food poisoning, which does not require the presence of the organism (15). *S. aureus* is also capable of producing exfoliative toxins and toxic shock syndrome toxin-1, which are responsible for the severe scalded skin syndrome and toxic shock syndrome, respectively (68, 74). Several enzymes are produced that are involved in tissue invasion including lipases, serine and cysteine proteases, and phospholipase C (36).

The immunoglobulin binding of SpA, SSL10, and Sbi is not the only mechanism *S. aureus* uses to evade the host immune response. In addition to binding IgG, Sbi forms a complex with the proteins C3b and factor H, which are involved in the complement pathways inhibiting that response (42). SSL10 is part of a larger cluster of SSL genes, whose functions are mostly unknown (48). However, SSL7 has been shown to form a complex with IgA and complement protein C5 to inhibit both of these responses (60). The iron-regulated surface determinant protein IsdH and Sbi are both capable of degrading the important opsonization factor C3b (101). The staphylococcal complement inhibitor SCIN and its homologues the extracellular fibrinogen-binding protein Efb and the extracellular complement-binding protein Ecb all inhibit the complement response by blocking the action of the convertases responsible for continuation of the complement cascade (51). The chemotaxis inhibitory protein of *S. aureus* CHIPS inhibits the complement response by blocking the receptor for the anaphylatoxin C5a (57). While most enzymes and toxins synthesized by *S. aureus* are present in multiple clones, USA300 has several hypothesized virulence factors which are expressed differently than in many other MRSA clones.

USA300 Virulence

USA300 strains typically contain the Panton-Valentine leukocidin (PVL), the arginine catabolic mobile element (ACME), and the phenol-soluble modulins (PSMs) (19). PVL is a toxin that forms pores in leukocytes, and was implicated as the primary virulence factor responsible for the spread of USA300 and USA400 (100). However, multiple models have shown that normally expressed PVL does not affect USA300 virulence in skin/soft tissue infections, bacteremia, or pneumonia (7, 56, 102). ACME is physically linked to the SCC mec type IV element in USA300 and contains two gene clusters thought to be involved in L-arginine catabolism and inhibition of nitric oxide production (19). The PSMs are found in all *S. aureus* strains, but they are produced at very low levels in clones other than USA300. The leukocidin activity of PSMs is much more potent than that of PVL. Additionally, PSMs are regulated by the RNAIII regulatory molecule, which is not found in many HA-MRSA strains (103).

Molecular Epidemiologic Methods

Molecular epidemiologic subtyping methods provide an important tool for investigating the population genetics of MRSA. Subtyping methods allow the surveillance of MRSA to determine which strains are responsible for disease in an area and to identify the independent risk factors that are associated with those strains. They are also used to confirm MRSA outbreaks, as well as monitor the endemic level of MRSA in an area. For the purpose of molecular subtyping, a clone is defined as a group of isolates descended from a common ancestor as part of a direct chain of replication and transmission. Thus, if a large population of MRSA isolates is characterized by a molecular subtyping method, then inferences can be made about the population structure of MRSA (90).

A molecular epidemiologic method with a high efficacy and efficiency is ideal for characterizing microbial isolates. A highly efficient method is rapid, low cost, and easy to use, traits that are often necessary during an epidemiologic investigation. The efficacy of a typing method evaluates the ability of that method to repeatedly and accurately predict the correct identity of each strain. Efficacy criteria include the typability of the method, which shows the proportion of strains assigned a type by the method, and reproducibility, which is the ability of the typing method to assign the same type to a strain in independent assays. Discriminatory power (D) and epidemiologic concordance (E) are the two most important criteria for determining the efficacy of a molecular epidemiologic method. D is the average probability that the method will assign two randomly sampled unrelated strains to different types. E is the probability that epidemiologically related strains thought to be from single-clone outbreaks are classified in the same clone. Ideally both D and E should be as close to 1.0 as possible, though in practice $D > 0.95$ is sufficient for a molecular epidemiologic typing method to be deemed trustworthy (90).

SCC*mec* Typing

The most basic method of molecular epidemiologic subtyping in MRSA is SCC*mec* typing. Several methods have been developed for SCC*mec* typing using sequences unique to each SCC*mec* element as genetic markers. Ito et al. (45) developed the first SCC*mec* typing method targeting the *mec* complex and the *ccr* genes with multiple PCRs to determine their structure. More recently, multiplex PCR (M-PCR) methods were developed by Boye et al. (6) and Zhang et al. (105), which allowed detection of SCC*mec* types I-V and the subtypes of type IV. These assays identify single genetic markers on the SCC*mec* element, which lowered their

discriminatory power, but increased their speed and ease of use. Prior to the development of those methods, Oliveira et al. (76) had developed an M-PCR which detected *SCCmec* types I-IV and used multiple genetic markers for each *SCCmec* element. This method was updated to include *SCCmec* types V and VI and provided higher discriminatory power than those methods which only used a single genetic marker. This new method detected newer *SCCmec* types not detected by the older methods (67). While the other M-PCR methods are still in use, it is likely that the updated Oliveira et al. method will become the most used method for *SCCmec* detection. However, detection of *SCCmec* is not enough to investigate the evolutionary patterns of *SCCmec*.

Changtrakool et al. (12) proposed a novel uniform method for the typing of *SCCmec*, which can be used to type the entire *SCCmec* element and allow evolutionary studies. The naming system for this method consists of a number followed by a capital letter and additional numbers, for example *SCCmec* type IVb is type 2B2.1. The first number is the *ccr* gene present on the *SCCmec* element, which are given their numbers based on the chronological order of their discovery. The letter stands for the *mec* complex type of the element, and the numbers that follow the letter characterize the 'junkyard regions' of the *SCCmec* element, which also are numbered chronologically (12). A scheme using five M-PCRs was developed using this naming method was developed, but it was not feasible for regular use because of the large number of reactions needed for typing (17, 54). *SCCmec* typing is a useful first step in the molecular epidemiologic investigation of MRSA, but it does not provide the level of epidemiologic concordance necessary to track MRSA epidemics.

Pulsed-Field Gel Electrophoresis

Pulsed-Field Gel Electrophoresis (PFGE) was the first molecular epidemiologic method developed for subtyping MRSA and is used by Centers for Disease Control and Prevention (CDC) as the 'gold standard' for molecular subtyping of pathogens, including MRSA. The first step in PFGE is the digestion of MRSA chromosomal DNA with the restriction enzyme *SmaI*. Agarose gel electrophoresis in an alternating voltage gradient is then used to separate the different-sized DNA fragments. A computer program that uses Dice comparison and Unweighted Pair Group Matching Analysis (UPGMA) is then used to analyze the banding patterns of the DNA fragments. The protocols used to classify Pulsed-Field Types (PFTs) have problems with reproducibility and nomenclature (17). The PFT-based nomenclature used in the United States developed by McDougal et al. (66) in 2003 originally identified 8 clonal lineages of MRSA and named them USA100 through USA800. Since 2003, an additional 4 lineages have been designated USA900 through USA1200 (17, 33). An international standard nomenclature does not truly exist for PFTs, but the McDougal et al. naming scheme has become more widely used with the proliferation of USA300 (17). PFGE is a highly discriminatory method, but it does not allow analysis of the chromosomal DNA sequence, making determination of MRSA evolutionary patterns problematic.

Multilocus Sequence Typing

Multilocus sequence typing (MLST) utilizes DNA sequences in housekeeping genes to investigate the evolution of MRSA. Sequences of ~500 bp fragments from seven housekeeping genes *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqi* are used for MLST analysis of MRSA isolates. Unique sequences of each gene fragment are assigned as distinct alleles. The allelic profile of all

7 genes is then used to assign a Sequence Type (ST) to each strain (21). STs which contain 5 of 7 identical alleles are assigned to the same clonal complex (CC) by the Based Upon Related Sequence Types (BURST) algorithm. Within a CC, STs with the fewest number of nucleotide differences in a single locus, single locus variants (SLVs), are determined to be most closely related by BURST. CCs are named after the putative ancestral ST for that CC which contains the most SLVs. BURST analysis of SLVs and double locus variants allowed determination of evolutionary events that led to the formation of newer STs (22).

MLST is very useful for determining the evolutionary history and population structure of MRSA, but it does have several drawbacks. The ability to relate isolates based on mutations in well-conserved housekeeping genes provides a useful long-term evolutionary picture of MRSA. However, the method lacks short-term epidemiologic concordance since MRSA epidemics are typically caused by shifts in virulence not mutations in housekeeping genes. An example of this problem is that two strains could belong to the same ST yet have radically different virulence profiles and habitats, as is the case for the ST8 strains USA300 and USA500 (66). USA300 causes widespread CA-MRSA infections, while USA500 causes more limited HA-MRSA infections. In addition to problems with characterizing short-term evolution, MLST based on housekeeping genes is comparatively expensive and time consuming compared to other molecular epidemiologic methods. Seven gene fragments must be sequenced and analyzed for every isolate investigated, which requires a significant amount of reagents and time (17). A possible solution to this is a microarray based determination of STs, which was developed by Van Leeuwen et al. (98). The method probes for known Single Nucleotide Polymorphisms (SNPs) within the MLST loci to determine the ST (98). However, the discovery of new STs would lead to the microarray needing to be constantly updated (17).

***spa* Typing**

Typing of the *spa* gene which encodes staphylococcal protein A has become an increasingly common molecular epidemiologic subtyping method for characterizing MRSA. Region X of *spa* consists of polymorphic repeats of approximately 24 bp, which are sequenced to classify MRSA isolates. The repeats contain large amounts of deletions, duplications and point mutations creating great diversity at a single locus. The series of repeats from an isolate is designated the *spa* type for that isolate. The series of repeats is easily detected using the StaphType software, which uses raw sequence data in the form of sequence chromatograms as an input (41). Two naming systems were originally developed for *spa* types, causing difficulty in interpreting published data (17). Since then, the use of ‘st’ followed by three numbers based on when the type was discovered has become the predominant naming system used for the SpaServer database maintained by the SeqNet.org Initiative to provide access to the repeats and combinations of repeats used in *spa* typing (41). *spa* typing is considered a more versatile method than either PFGE or MLST. The discriminatory power of *spa* typing is higher than that of MLST as multiple *spa* types fall within each ST, but some *spa* types are also found in multiple STs within the same MLST CC. The high discriminatory power and ease of *spa* typing makes it ideal for short-term tracking of MRSA epidemics similar to the use of PFGE. Since *spa* typing analyzes the genetic sequence, it can also be used to conduct long-term MRSA evolutionary studies, providing versatility that PFGE and MLST lack (17, 89). The Based Upon Repeat Pattern (BURP) algorithm can be used to generate *spa*-clonal complexes (*spa*-CCs) relating *spa* types in a similar way to the relation of STs into CCs by MLST. Clonal relationships determined by *spa* typing, PFGE and MLST have been shown to have good concordance, giving each of these methods equal validity (89). However, typing of a single locus, as in *spa* typing, can lead to

problems with discriminatory power due to recombination events between strains of different CCs. Some important MLST CCs cannot be distinguished by *spa* typing, such as CC1 and CC80 and CC8 and CC45 (87). Thus, the use of *spa* typing warrants the concurrent typing of another locus, such as *clfB* or *SCCmec*, which have been combined with *spa* typing to create double-locus typing schemes (17, 55).

Development of Novel Molecular Epidemiologic Methods

As the name would suggest, multi-virulence-locus sequence typing (MVLST) is very similar in design to MLST, the only difference being the target is now virulence genes instead of housekeeping genes. A MVLST scheme was originally developed for *Listeria monocytogenes* with the aim of providing a greater degree of discrimination between strains and a greater epidemiologic concordance than PFGE and MLST methods available at that time for molecular epidemiologic subtyping of *L. monocytogenes* (107). MVLST of *L. monocytogenes* originally targeted 3 virulence genes and 3 virulence-associated genes and was able to differentiate outbreak clones from epidemic clones (11). The successful use of the method in *L. monocytogenes* led to the hypothesis that an MVLST scheme might also be developed for the molecular subtyping of MRSA. The MVLST scheme in the present study targeted *essC*, *etaA*, *htrA*, *alt*, *geh* and *sdrC*. Preliminary results demonstrated a higher discriminatory power for MVLST than MLST, likely also corresponding to a higher epidemiologic concordance for MVLST than MLST. Therefore, the goal of this thesis research project was to develop a M-PCR based on virulence genes to detect the major CCs of HA-MRSA and CA-MRSA, which is discussed in detail in the following chapter.

NOVEL MULTIPLEX PCR METHOD

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) emerged as a healthcare-associated pathogen in 1961 two years after the introduction of methicillin (50). Healthcare-associated MRSA (HA-MRSA) causes bacteremia, pneumonia, and skin/soft-tissue infections, and also increased morbidity, length of hospitalization and associated costs (61, 69, 71, 72, 81). The emergence of community-associated MRSA (CA-MRSA) was first reported in the 1980s (82) and has greatly increased the incidence of MRSA skin/soft-tissue infections (31, 70) and severe necrotizing pneumonia (29, 34). While MRSA is associated with many diseases, asymptomatic colonization of the anterior nares is found in approximately 1.5% of the human population (37). Although both HA-MRSA and CA-MRSA are capable of asymptomatic colonization, they are epidemiologically and clonally distinct pathogens (22, 39). Recently, CA-MRSA strains have become an increasingly common cause of healthcare-associated infections (84), blurring the distinction between HA-MRSA and CA-MRSA.

The evolution and epidemiology of MRSA have been studied using various molecular subtyping methods. Typing of the Staphylococcal Cassette Chromosome *mec* (SCC*mec*) element, which carries the *mecA* gene, can be used to differentiate many HA-MRSA and CA-MRSA strains via multiplex polymerase chain reaction (M-PCR) (6, 10, 47, 63, 76, 105). HA-MRSA strains typically carry SCC*mec* types I, II or III, which provide resistance to various antimicrobials in addition to methicillin. In contrast, CA-MRSA primarily contains the smaller SCC*mec* type IV element that provides resistance to only methicillin (45, 63). The value of SCC*mec* typing is limited by the characterization of only one genetic element and this results in

incomplete differentiation of HA-MRSA and CA-MRSA strains. While *SCCmec* typing is the most common M-PCR for molecular subtyping MRSA strains, other M-PCR methods and various molecular subtyping methods are also in use. M-PCRs have been developed for the identification of CA-MRSA strains (4, 27, 88, 106) and the virulence gene profiling of MRSA strains (27, 43). M-PCR methods for the characterization of CA-MRSA strains provide little information about HA-MRSA strains, and virulence gene profiling is not typically used for molecular subtyping. However, a carefully selected combination of virulence genes based on known clonal relationships might reveal the major clonal complexes (CCs) of MRSA (11).

The clonal relationships of MRSA have been identified using pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) (1). MLST determines the evolutionary relationships of MRSA isolates through the sequencing of 7 housekeeping gene fragments, allowing the isolates to be grouped into CCs (21, 24). PFGE is a highly discriminatory method that distinguishes the USA300 and USA500 pulsed-field types (PFTs), which are epidemiologically distinct clones that belong to the same MLST sequence type (ST) (66). However, both MLST and PFGE are expensive and require a minimum of a full day to produce results (21, 66). Therefore, the goal of the present study was to develop a novel M-PCR for the rapid and accurate identification and differentiation of the major CCs of HA-MRSA and CA-MRSA.

Materials and Methods

Bacterial strains. Reference strains were obtained from the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) and the Centers for Disease Control and Prevention (CDC). Presumptive MRSA isolates were also obtained from nasal swabs of patients taken upon admission of patients to intensive care units at the Pennsylvania State University Milton S. Hershey Medical Center (HMC) and screened as MRSA positive by identification of the *orfX* integration site. Descriptions of the isolates/reference strains used in the present study are shown in Table 2.1. MRSA positive swabs were subsequently transported to the Foodborne Pathogen Laboratory of Dr. Stephen Knabel in the Food Science Department at Penn State University, University Park, PA, where they were streaked on Columbia agar (Columbia Blood Agar Base [Sigma, St. Louis, MO]) containing 4 mg/l methicillin (MRSA Selective Supplement [Fluka, St. Louis, MO]) and grown at 35 °C overnight. Single, isolated presumptive MRSA colonies were subsequently inoculated into tryptic soy broth (Difco, Sparks, MD) and grown overnight at 35 °C. Chromosomal DNA was then isolated using the UltraClean Microbial DNA Isolation Kit (MoBio Laboratories, Solana Beach, CA).

TABLE 2.1. Number of MRSA isolates or reference strains obtained from three different sources in the present study, and a description of the isolates or reference strains.

Source	No. of Isolates/Strains	Description
NARSA	11 strains	Reference strains COL, N315, MSSA476, USA100, USA200, USA300, USA500, USA600, USA700, USA800, USA1000 and USA1100.
CDC	5 strains	Reference strains SCC <i>mec</i> type I and III, USA100, USA300 and USA400.
HMC	66 isolates	Presumptive MRSA isolates from MRSA-positive nasal swabs taken upon admission to HMC ICUs.

MLST. MLST was performed on all isolates as previously described using the *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqi* genetic markers (21). MLST eBURST analysis was subsequently used to determine the clonal complex of each isolate (<http://www.mlst.net>).

PCR. SCC*mec* typing was performed on all isolates as described previously (105) using modified *mecA* primers as shown in Table 2.2. Clonal complex (CC) M-PCR primers including the *mecA* primers (Table 2.2) were designed using NCBI PrimerBLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi>) and were synthesized at the Pennsylvania State University Genomics Core Facility. PCR reaction mixtures (25 μ l total volume) contained 10 μ l Multiplex PCR Master Mix (QIAGEN, Valencia, CA), 10 μ g/ml template DNA, and primers at the concentrations shown in Table 2.2. Amplifications were carried out in an Eppendorf Mastercycler thermocycler (Eppendorf NA, New York, NY) with an initial activation step of 95°C for 15 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 90 s, and extension at 72°C for 90 s, followed by a final extension at 72°C for 10 min.

Amplified products were separated by agarose gel electrophoresis (2% UltraClean Agarose [MoBio Laboratories, Carlsbad, CA]) in 0.5x Tris-borate-EDTA buffer at 105 volts for 1 hour then subsequently stained in ethidium bromide. Gels images were subsequently captured using a UVP Benchtop 2UV transilluminator (UVP, Upland, CA).

TABLE 2.2. Genetic markers, primer sequences, PCR amplicon sizes, and primer concentrations in the clonal complex multiplex PCR assay

Genetic Marker *	Primer Sequence	Amplicon Size (bp)	Primer Concentration (μ M)
<i>clfB</i>	(F) AACAGAGCCAGCTTCAACAAATGA (R) GTCTTTCGGATTTACTGCTGAATC	1309	3.2
MW_1924	(F) TCACGAAGTCGAACGAAGAA (R) GCTGAACGCTCTTCTGCTTC	956	2
<i>seg</i>	(F) AGAATCAACAACCTTTATTATCTCCG (R) TATGTGAATGCTCAACCCGA	675	0.8
<i>arcD</i>	(F) TGCCATTGATGGATTAGCAA (R) GTTTTTCAAGTGCTTGGGGA	566	1.2
<i>cap8</i>	(F) GGAGGAAATGACGATGAGGA (R) TGTCACCCTGCTAGCATCAA	439	2.4
<i>cna</i>	(F) TGCTGTCCACCTTGAATCTG (R) GTTATTACGCCAGACGGAGC	335	2.4
<i>lukE</i>	(F) GCATTATGCTTTTCTTTCTGCG (R) AATGGTCCAACAGGTTCAGC	247	1.2
<i>mecA</i>	(F) TGGAACCTTGTTGAGCAGAGG (R) CCACCCTCAAACAGGTGAAT	138	1.6

* See text for description of genetic markers.

Method Comparison. Simpson's index of diversity was used to quantify the discriminatory power of the SCC*mec*, MLST, and CC M-PCR typing methods as previously described (44). A strain was defined as an isolate or group of isolates that exhibited a distinctive combination of results for SCC*mec* typing, MLST and CC M-PCR (90). Non-typable strains were not used in the calculation of discriminatory power.

Results

SCCmec typing. SCCmec types for 66 of 82 isolates/reference strains could be determined.

SCCmec type II was the most common SCCmec type, being found in 43 (67%) of the isolates/reference strains. The other SCCmec types found were type IVa (8 isolates and 5 reference strains), type III (2 isolates and 1 reference strain), type I (2 reference strains), type IVc (2 isolates), type IVd (1 isolate and 1 reference strain), and type IVb (1 isolate). Of the 13 HMC isolates and 3 reference strains for which a SCCmec type could not be determined, 7 HMC isolates and 1 reference strain lacked the *mecA* gene, while 6 HMC isolates and 2 reference strains contained the *mecA* gene, but were non-typable by the SCCmec typing method used in the present study. The discriminatory power of SCCmec typing was 0.787.

MLST. Multilocus sequence types could be determined for 77 of 82 isolates/reference strains.

The remaining 5 HMC isolates were not typeable using the *S. aureus* MLST genetic markers described above. These isolates were later found not to contain the *clfB* genetic marker, confirming they were not *S. aureus*. eBURST analysis revealed the presence of 7 clonal complexes as shown in Table 2.4. The discriminatory power of MLST was 0.938.

TABLE 2.3. Genetic markers used to identify the major MRSA clonal complexes in the present study.

Clonal Complex	Identifying Genetic Markers
1	<i>cap8, cna, lukE</i>
5	<i>seg</i> and <i>lukE</i>
8	<i>arcD</i> and <i>lukE</i> or <i>lukE</i> *
30	<i>seg, cap8, cna</i>
45	<i>seg</i> and <i>cna</i>
59	<i>cap8</i> without <i>seg</i> or <i>lukE</i>
133	<i>cap8</i> and <i>lukE</i>

*The presence of *lukE* without *seg*, *cap8*, or *cna*.

Novel CC M-PCR. The results of the CC M-PCR for the MRSA reference strains and a *S. epidermidis* strain isolated from an HMC nasal swab identified in the present study are shown in Figure 2.1. The combination of genetic markers present in each reference strain was defined as the multiplex type (MT) for that strain. Each MT was associated with the clonal complex of the reference strain determined by the MLST eBURST analysis. Isolates which shared major identifying genetic markers with a reference strain were grouped in the same clonal complex as the reference strain (Table 2.3). Each MT is designated by the number of the clonal complex to which the reference strain belongs, and an arbitrary lowercase letter to denote different CC M-PCR genetic marker combinations within a clonal complex. The discriminatory power of CC M-PCR was 0.943.

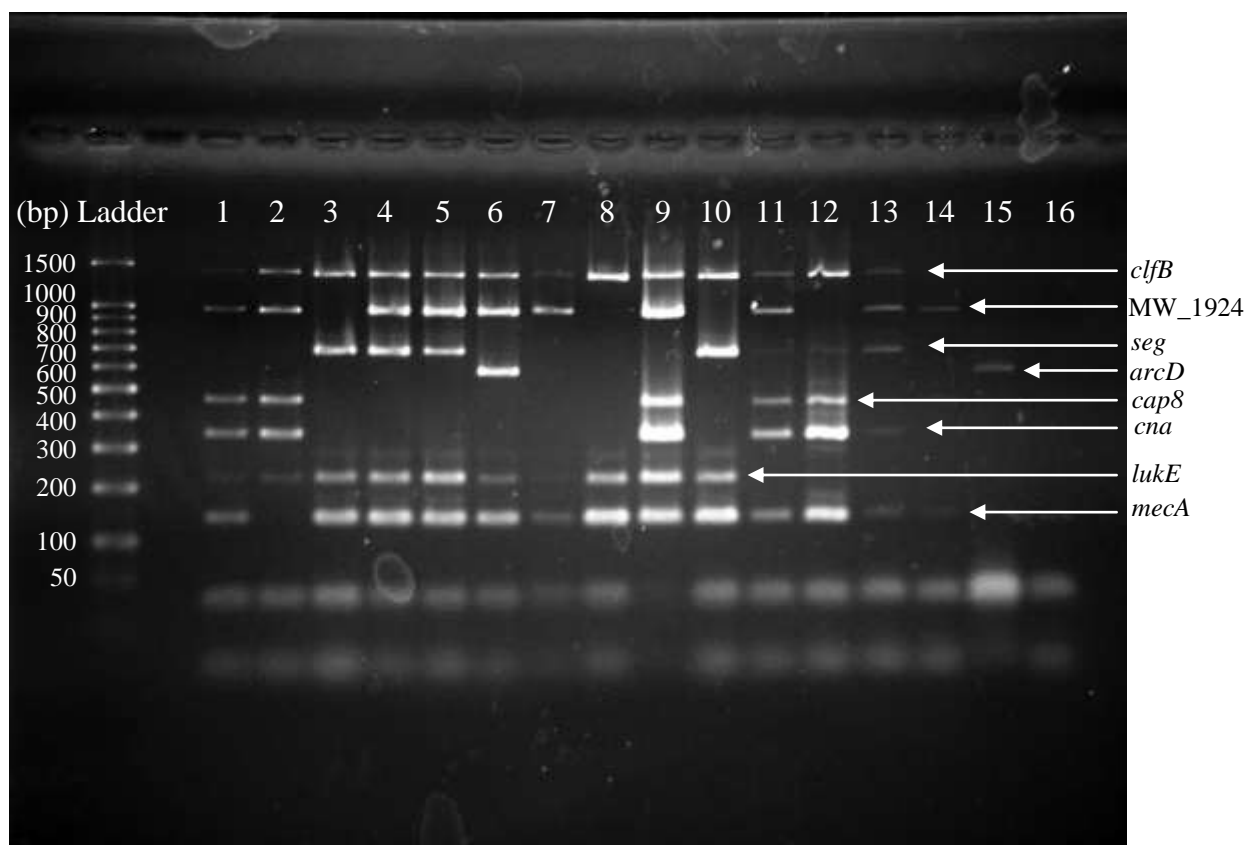


FIG 2.1. Multiplex PCR assay results for the *S. aureus* reference strains analyzed in the present study. Lanes 1-16 *S. aureus* reference strains 1- CDC USA400, 2- NRS72 (MSSA476), 3- CDC SCCmec I, 4- NRS70 (N315), 5- NRS387 (USA800), 6- NRS384 (USA300), 7- NRS385 (USA500), 8- NRS100 (COL), 9- CDC SCCmec III, 10- NRS386 (USA700), 11- NRS383 (USA200), 12- NRS484 (USA1100), 13- NRS22 (USA600), 14- NRS483 (USA1000), 15- *S. epidermidis*, and 16- H₂O, negative control.

Discussion

MRSA is an increasingly common source of infection in community and healthcare settings. While molecular subtyping methods exist for determining the identity, phylogeny and epidemiology of MRSA isolates, there is a need for a single method that accomplishes these goals more rapidly and in a more cost effective manner. The CC M-PCR developed in the present study rapidly identified CDC and NARSA MRSA reference strains and accurately determined which CC they belonged to. Therefore, CC M-PCR was subsequently used to analyze isolates from HMC to further evaluate the effectiveness of the method and to determine the clonal structure of MRSA in intensive care units (ICUs) within HMC.

The following genes were identified as molecular markers for each MRSA clonal complex and thus were selected for inclusion in the CC M-PCR (Table 2.2). Clumping factor B encoded by *clfB* is a virulence gene found in virtually all *S. aureus* isolates (55). The inclusion of *clfB* as a marker allowed the confirmation of isolates as *S. aureus* and excluded other non-*S. aureus* methicillin-resistant species which initially appeared to be *S. aureus* on the screening media. Detection of the *mecA* gene, which confers methicillin-resistance as part of the *SCCmec* element (63), was used to confirm the presence of the *SCCmec* element in all isolates (Table 2.4). The *arcD* gene is a part of ACME, which is regularly associated with the USA300 PFT belonging to CC8 (4). The collagen-adhesion factor encoded by *cna* was recently shown to be associated with CCs 1, 12, 22, 30, 45, 51 and 239 (16). The type 8 capsular polysaccharide is encoded by *cap8* found in CC1, CC30 and CC59 (2, 18). The Staphylococcal enterotoxin G (*seg*) is found as part of the enterotoxin gene cluster (*egc*) associated with CC5, CC22, CC30 and CC45 (26). The type E leukocidin gene *lukE* has been shown to be associated with CC1, CC5 and CC8 (2, 18). Locus MW_1924 is a prophage gene found in several MRSA strains. Sequence

variation exists within the targeted region which caused MW_1924 to be a possible locus for a multi-virulence-locus sequence typing (MVLST) method (Verghese, unpublished data).

However, the targeted region was not found in all isolates, which allowed further differentiation within CCs 1, 5, 8 and 30 by the CC M-PCR. The criteria for assigning MT clonal complex was based on the genetic markers previously identified in the literature. A summary of the markers previously shown to be present in the CCs detected is presented in Table 2.3. Genetic markers that were considered not major identifying genetic markers for an isolate were denoted by the arbitrary lowercase letter in each MT to signify the existence of multiple combinations of CC M-PCR genetic markers within a single CC.

TABLE 2.4. Number of HMC isolates in each multiplex type (number of NARSA or CDC reference strains in parentheses, where applicable), the MLST clonal complex of each multiplex type, and the genetic markers amplified in each multiplex type (major genetic markers amplified are in bold).

Multiplex Type	No. of Isolates	MLST CC	Genetic Markers Amplified in CC M-PCR
1a	0 (1)	1	<i>clfB</i> , MW_1924 , <i>cap8</i> , <i>cna</i> , <i>lukE</i> , <i>mecA</i>
1b	1 (1)	1	<i>clfB</i> , MW_1924 , <i>cap8</i> , <i>cna</i> , <i>lukE</i>
1c	1	1	<i>clfB</i> , <i>cap8</i> , <i>cna</i> , <i>lukE</i>
1d	1	1	<i>clfB</i> , <i>cap8</i> , <i>cna</i> , <i>lukE</i> , <i>mecA</i>
5a	38 (3)	5	<i>clfB</i> , MW_1924 , <i>seg</i> , <i>lukE</i> , <i>mecA</i>
5b	1	5	<i>clfB</i> , MW_1924 , <i>seg</i> , <i>lukE</i>
5c	1	5	<i>clfB</i> , MW_1924 , <i>seg</i> , <i>arcD</i> , <i>lukE</i> , <i>mecA</i>
5d	0 (2)	5, 8	<i>clfB</i> , <i>seg</i> , <i>lukE</i> , <i>mecA</i>
8a	7 (2)	8	<i>clfB</i> , MW_1924 , <i>arcD</i> , <i>lukE</i> , <i>mecA</i>
8b	3 (2)	8	<i>clfB</i> , MW_1924 , <i>lukE</i> , <i>mecA</i>
8c	0 (1)	8	<i>clfB</i> , <i>lukE</i> , <i>mecA</i>
30a	1 (1)	30	<i>clfB</i> , MW_1924 , <i>seg</i> , <i>cap8</i> , <i>cna</i> , <i>mecA</i>
30b	2	30	<i>clfB</i> , MW_1924 , <i>seg</i> , <i>cap8</i> , <i>cna</i>
30c	0 (1)	30	<i>clfB</i> , <i>cap8</i> , <i>cna</i> , <i>mecA</i>
30d	1	30	<i>clfB</i> , MW_1924 , <i>seg</i> , <i>cap8</i> , <i>cna</i> , <i>lukE</i> , <i>mecA</i>
45a	0 (1)	45	<i>clfB</i> , MW_1924 , <i>seg</i> , <i>cna</i> , <i>mecA</i>
45b	1	45	<i>clfB</i> , MW_1924 , <i>seg</i> , <i>cna</i>
59a	0 (1)	59	<i>clfB</i> , MW_1924 , <i>cap8</i> , <i>mecA</i>
59b	2	59	<i>clfB</i> , MW_1924 , <i>cap8</i> , <i>cna</i> , <i>mecA</i>
133	1	133	<i>clfB</i> , <i>cap8</i> , <i>lukE</i> , <i>mecA</i>
<i>S. epidermidis</i>	3	N/A ²	<i>arcD</i>
MRS ¹	2	N/A	<i>mecA</i>
Total	66 (16)		

1 - Methicillin-resistant species.

2 – Not applicable.

The CC M-PCR successfully detected the correct clonal complex in 81 of 82 isolates/reference strains. The ST72 CC8 NARSA386 reference strain was misidentified by the CC M-PCR as a CC5 strain. A unique target was not able to be generated for this strain due to the lack of whole genome sequence data for ST 72. Primers specific to *SCCmec* type IVa could potentially be added to the CC M-PCR in the future to differentiate ST72 from the CDC *SCCmec* type I strain, and potentially other members of CC5. However, part of the aim of the present study was to develop a CC M-PCR method independent of *SCCmec* type, therefore these primers were not included.

Isolates and reference strains were classified into 34 strains based on the combination of result from *SCCmec* typing, MLST, and CC M-PCR. As shown in Table 2.3, the strains identified belonged to 6 major clonal complexes, CCs 1, 5, 8, 30, 45 and 59, in the northeastern United States and the animal-associated ST133 (85). Epidemiologic concordance could not be calculated for CC M-PCR because the strains tested were not known to be from well-defined epidemics (90). Assuming CC M-PCR assigned 33 of 34 strains to the correct CC and HMC CCs represented individual epidemics, then the epidemiologic concordance of CC M-PCR would be 33/34 or 0.97.

HMC isolates accounted for 40 of the 45 isolates identified as CC5 by the CC M-PCR. CC 5 was previously identified as the predominant MRSA clone in Pennsylvania (79). Nearly all CC5 isolates belonged to either ST5 or ST105, which differ only at the *yqiL* locus. CC8 was the second most commonly observed CC in HMC nasal swab isolates. A majority of the CC8 isolates from HMC contained ACME and 7 of 10 were classified as MT8a. MT8a isolates all contained *SCCmec* type IVa and thus were inferred to be USA300. The remainder of the isolates identified as CC8 strains were determined to have other *SCCmec* types, while the USA300,

USA400, and USA700 reference strains were the only others that were identified as *SCCmec* type IVa. MT 5c was positive for *arcD*, identified as ST 5 by MLST, and non-typable by the *SCCmec* typing method. This agrees with a previously published report which indicated the movement of ACME into a CC5 strain (32). Genetic exchange between HA-MRSA strains, such as those in CC 5, and USA300 strains that typically carry ACME has led to a highly virulent USA300 strain with the vancomycin-intermediate resistance observed in some HA-MRSA strains (40). The proliferation of this or a similar strain could lead to the predominance of a strain that very rapidly causes severe disease and is resistant to the best antibiotics available for treating MRSA infections.

The CC M-PCR exhibited a higher discriminatory power (0.943) than *SCCmec* typing (0.787) and MLST (0.938), as well as potentially high epidemiologic concordance (0.97). However, the CC M-PCR method developed in the present study was only validated in one healthcare-associated facility, HMC. The CC M-PCR is likely to retain its validity in other healthcare facilities in the northeastern United States where a similar MRSA clonal structure was shown to be present (79). However, in other parts of the United States or other countries where a different MRSA clonal structure is prevalent, the CC M-PCR may not have as high a discriminatory power or epidemiologic concordance. Additional studies using a large and diverse collection of epidemic-associated isolates from other healthcare and community settings is needed to determine the epidemiologic concordance of the CC M-PCR method developed in the present study. The majority of isolates identified in nasal swabs from HMC belonged to CC5, while several isolates were also identified as CC8. Although the healthcare history of patients from whom nasal swab isolates were taken is unknown, it can be inferred that a majority of the patients had some prior healthcare exposure, because of the majority of isolates from HMC were

CC5, a clonal complex common in healthcare facilities in the northeastern U. S.. In a healthcare setting, the CC M-PCR could be used to rapidly detect the clonal structure of clinical isolates and identify the emergence of new CCs. Further investigation involving multiple healthcare facilities is needed to verify this. The novel CC M-PCR screening method developed in the present study might provide an important screening tool to facilitate rapid tracking and control of CCs of HA-MRSA and CA-MRSA within both healthcare and community settings.

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