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PREVALENCE OF USA300 METHICILLIN-RESISTANT *STAPYLOCOCCUS AUREUS* AT THE HERSHEY MEDICAL CENTER AND THE DEVELOPMENT OF A NOVEL MULTIPLEX PCR METHOD TO DETECT USA300 MRSA

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ABSTRACT

The emergence and rapid spread of methicillin-resistant *S. aureus* (MRSA) epidemic clone USA300 has doubled the incidence of MRSA and *S. aureus* disease burden since the early 2000's. Although USA300 originated as a community-associated MRSA clone (CA-MRSA), it has been increasingly isolated from hospital-associated settings. Since hospital-associated and community-associated clones of MRSA have different resistance and infection profiles, antibiotics that work on HA-MRSA clones are often ineffective on CA-MRSA clones and vice versa. Analysis of clinical MRSA isolates from the Penn State Milton S. Hershey Medical Center (HMC) showed a dramatic increase in USA300 prevalence compared to previous analysis of MRSA isolates from the nasal passages of patients upon admission to HMC. A reliable method that differentiates between USA300 and other HA-MRSA clones may allow screening, tracking and control of USA300 in time to affect patient outcomes and potentially halt the spread between patients and staff. Use of multiplex-PCR methods as diagnostic aids would also significantly reduce the time spent administering ineffective antibiotics and potentially increase the possibility of positive outcomes.

A novel multiplex-PCR method was developed to target five genes unique to MRSA and USA300, *mecA* (methicillin resistance), *nuc* (*S. aureus* nuclease), *lukPV* (Panton-Valentine leukocidin), ACME (arginine catabolic mobile element), and SCC*mec*IVa (staphylococcal chromosomal cassette IVa). The electrophoretic pattern produced by the multiplex-PCR method can also differentiate several of the major epidemic clones of MRSA including USA100, USA400, and USA700. Known epidemic clone MRSA isolates previously analyzed using multi-virulence-locus sequence typing (MVLST) were used to validate this novel multiplex-PCR method.

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Chapter 1

The Evolution of Methicillin-Resistant *Staphylococcus aureus*

"One sometimes finds what one is not looking for."

Sir Alexander Fleming

When one thinks of the leading causes of death in developed nations such as the United States, infectious disease is not usually first to come to mind. However, as of the late 2000's, the death toll of one infectious disease is approximately equal to that of HIV/AIDs, tuberculosis, and hepatitis B combined (1). This pathogen is called methicillin-resistant *Staphylococcus aureus* (MRSA). One particular epidemic clone, USA300, is the most isolated clinical MRSA strain and is responsible for the dramatic increase in MRSA disease burden in the last decade (18).

Staphylococcus aureus

Staphylococcus aureus is a Gram-positive, non-motile, coccoid-shaped bacterium that forms grape-like clusters characteristic of the genus *Staphylococcus* (2). As a facultative anaerobe, *S. aureus* utilizes both lactic acid fermentation and aerobic respiration (2). It is mesophilic, halotolerant, and capable of growing at NaCl concentrations up to 15% (2, 3). Nearly all strains are coagulase positive, a phenotype used for identification (3). The production of this and other enzymes differentiates *S. aureus* from close relatives, including the usually non-pathogenic *S. epidermidis* (2, 3). When grown on blood agar, *S. aureus* typically has a beta hemolytic pattern, and all strains are considered potentially pathogenic. Its yellow appearance on rich medium has given it the nickname "golden staph" (3).

S. aureus colonizes the nasal passages, throats and skin of between 25 and 40% of healthy humans, although some studies have found much higher percentages (up to 60%) during single-time-point sampling (4-7). *S. aureus* can cause a large number of disease symptoms ranging from mild to life-

threatening. Superficial skin lesions include boils (furuncles), abscesses, cellulitis, staphylococcal scalded skin syndrome, pimples, and styes, while more serious disease syndromes include toxic shock syndrome (10), meningitis, sepsis, osteomyelitis, pneumonia (11, 12), septic phlebitis, necrotizing fasciitis (13), endocarditis (14), bacteremia (15), septic arthritis, mastitis, phlebitis, and urinary tract infections (2). Figure 1-1 shows the major sites of infections and diseases associated with *S. aureus* throughout the human body.

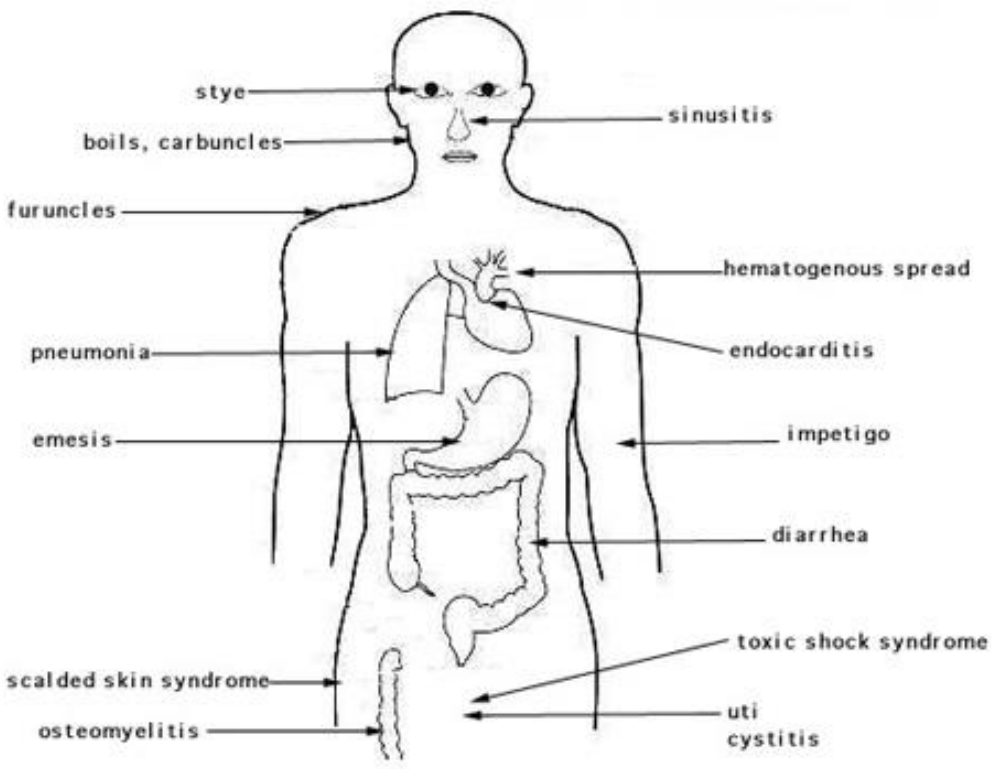

Figure 1-1: Sites of Infection and Diseases Caused by *S. aureus* (2)

As a major cause of nosocomial (hospital-associated) infections (17), *S. aureus* has been isolated from pagers, bed spaces, work stations, stethoscopes, and other places in hospitals (19-21). Food poisoning results when *S. aureus* grows in food and releases enterotoxins. TSST-1, a superantigen capable of nonspecifically activating T-cells, can lead to toxic shock syndrome if released into the blood

stream during an infection (10). Additionally, influenza is commonly complicated by staphylococcal pneumonia (11).

Since *S. aureus* colonizes endothelial layers on the outside of the human body, infection of deeper tissues typically requires a breach of this protective layer, which can be caused by needle sticks, surgical incisions, or destruction of cilia and mucus linings (2). *S. aureus* produces a number of endotoxins, exotoxins and virulence factors that aid in invasion, colonization, and evasion of host immune defenses (17). Table 1-1 lists the most important toxins and virulence factors associated with *S. aureus*.

Table 1-1: *S. aureus* virulence factors and toxins (2, 6, 10, 17)

Name	Type of Protein	Function
Clumping factor	Surface protein	Promotes colonization
Leukocidin, kinases, hylauronidase	Invasins	Promotes bacterial spread in tissues
Protein A	Surface protein	Inhibition of phagocytosis
Catalase; carotenoids	Enzyme; pigment	Enhances survival in phagocytes
Coagulase	Enzyme	Immunological disguise
Hemolysins, leukocidin	Membrane damaging toxins	Lysis of eukaryotic cell membranes
TSST-1, Exfoliating Toxin	Exotoxins	Damages host tissues
Mec	Alternative binding proteins	Resistance to methicillin

Emergence of MRSA

The emergence of drug-resistant *S. aureus* began shortly after the discovery and therapeutic use of antibiotics. In 1928 Sir Alexander Fleming is credited with accidentally discovering a mold that killed *S. aureus*. Ten years later Howard Florey and Earnest Chain successfully isolated penicillin from Fleming's strain of *Penicillium notatum*. It wasn't until 1941 that Dr. Charles Fletcher began using

penicillin to treat infections at a nearby hospital. Shortly thereafter, Florey was able to find a pharmaceutical company which could mass-produce the drug in time for WWII. In 1945 Fleming, Florey, and Chain received the Nobel Prize in Physiology or Medicine for the discovery, development and therapeutic use of penicillin.

With the discovery of penicillin, *S. aureus* infections that had once been death sentences now became easily treatable. Evolution brought about penicillin-resistant strains of *S. aureus*, which appeared by the late 1940's. In 1959 methicillin was developed as an alternative to penicillin and quickly became the most commonly used antibiotic; however, this resulted in the evolution of methicillin-resistant *Staphylococcus aureus* (MRSA). The first MRSA strains were identified by British scientists in 1961. Seven years later, the United States reported the first case of a human MRSA infection (23).

Much like *S. aureus*, the nasal carriage of MRSA varies. Though estimates differ, approximately 11-20% of *S. aureus* carriers have persistent MRSA predominantly in their nares and throats (34-36). While colonization research focuses primarily on the nose, a recent study showed that 80% of MRSA-colonized patients had at least one extranasal site and nearly half were colonized exclusively extranasally (37). The SENTRY Antimicrobial Surveillance Program surveyed hospitals globally and found MRSA prevalence ranged from 5% in the Philippines to 69% in Japan and Hong Kong (8). Countries with high prevalence included Singapore (62.3%) and Taiwan (59.6%) while lower MRSA prevalence was reported in South Africa (41.5%), United States (34.2%), China (27.8%), Australia (23.8%), and Canada (5.7%) (8, 9). MRSA prevalence was 45.9% in the Asian Pacific region, 35% in Latin America, and 26.3% in Europe (8, 9). A fourfold increased risk of infection is associated with persistent MRSA colonization (38).

MRSA infections are far more serious than a typical *S. aureus* infection due to three factors, 1) decreased effectiveness of antibiotics, 2) enhanced virulence of the antibiotic-resistant bacteria and 3) a delay in appropriate antibiotic treatment (121). While there may not be statistically significant differences in the outcomes of soft skin and tissue infections (SSTIs) between patients with either MRSA or MSSA (5, 88), the same is not true for more serious diseases like bacteremia. Patients with bacteremia caused by

MRSA are more than twice as likely to have acquired the infection while hospitalized for another primary cause (123), are more likely to die than patients with MSSA bacteremia (121, 123) and face significantly higher inpatient costs and longer hospital stays (123).

A problem inevitably arises when health-care workers (HCWs) are carriers of MRSA and potentially a means of infection. One study analyzed HCW MRSA colonization in a hospital in Sikkim, India and found significant MRSA prevalence in cleaners (51.35%), nurses and attendants (25.5%), technicians (21.62%), and doctors (3.12%) (146). Meta-analysis of nearly 200 MRSA outbreaks pinpointed 11 with strong epidemiological evidence supporting HCWs as a likely source of MRSA (147); furthermore, three of those were likely caused by asymptomatic MRSA carriers (147). While complicated privacy laws have hindered widespread screening, aggressive decolonization and prevention measures in the Netherlands and Denmark may have been responsible for the recent unprecedented drop in MRSA prevalence in these countries (145).

Thanks to the search-and-destroy method implemented by hospitals and health care centers, the Netherlands and Denmark report a prevalence of less than 1% (39). This highly aggressive policy screens patients with multiple risk factors and isolates them until culture results are known (39, 145). If positive for MRSA, they are cared for in isolation until decolonization protocols have eradicated MRSA in these patients. Health care workers in contact with MRSA patients are screened on a regular basis and are prohibited from working with patients directly until repeated screening cultures are MRSA-negative (39, 145). Although it is possible that the cost has kept this method from being implemented globally, a recent study found the cost of treating *S. aureus* infections outweighed the cost of the prevention program (39).

In the decades between 1961 and the present, the over-prescription of antibiotics resulted in MRSA strains resistant to an entire class of β-lactam antibiotics including amoxicillin and oxacillin. Individual strains were resistant to other classes of antibiotics including the so called “antibiotics of last resort”, which are almost as deadly as the bacteria they kill. Since MRSA and other highly resistant

bacteria can mutate much faster than drug companies can produce new antibiotics, alternative therapies are now being heavily researched.

The *mec* operon (SCC*mec*) is part of the staphylococcal chromosomal cassette (SCC) and contains *mecA*, which codes for an alternative binding protein (PBP2a) with reduced affinity for β-lactam antibiotics (22, 24). Two SCC*mec* encoded recombinases (*ccrA* and *ccrB*) excise SCC*mec* from a donor and stably integrate SCC*mec* into the recipient chromosome (28) at the 3' end of *orfX*, an open reading frame with an unknown function (25, 26). The *mecA* gene is 2.1 kb in length with the total size of SCC*mec* between 20.9 kb (type IV) and 66.9 kb (type III) (27, 28).

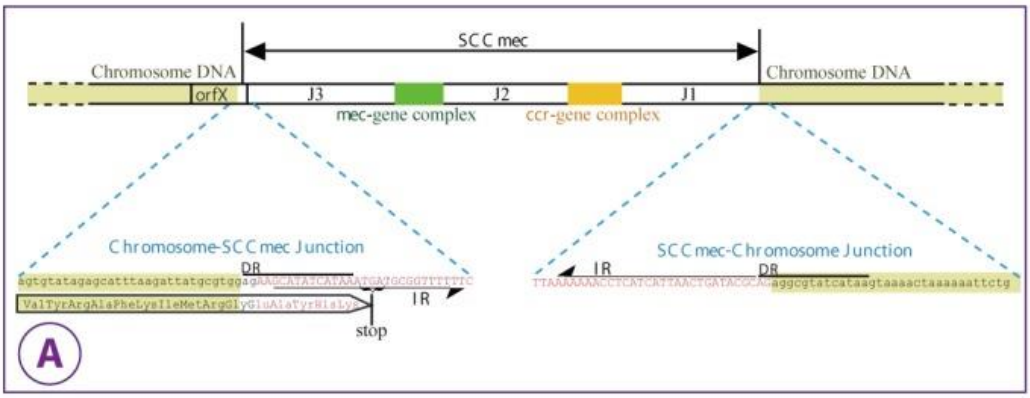

Figure 1-2: Basic Structure of SCC*mec*. Integration site sequences (ISS) found within Direct Repeats on either side of SCC*mec* are recognized by ccr. At the ends of SCCmec are two inverted repeats (IR) (27).

In the absence of β-lactam antibiotics, inhibitory gene *mecI* represses transcription of *mecA* through stable MecI dimers bound to the *mecA* promoter (22). The presence of β-lactam antibiotics activates MecRI which subsequently induces expression of *mecA* and *mecR1-mecI-mecR2* (22, 27, 28). MecR2 promotes the inactivation of MecI by proteolytic cleavage, sustaining *mecA* induction (22). Figure 1-3 shows the function of the SCC*mec*.

Traditionally, MRSA strains are distinguished based on the arrangement of *mec* and *ccr* (cassette chromosome recombinase) in the operon. More recently, MRSA strains are differentiated based on

sequence type (ST), antibiotic resistance, and the presence of extra-chromosomal elements conferring additional resistance, and/or virulence factors such as Panton-Valentine leukocidin (PVL).

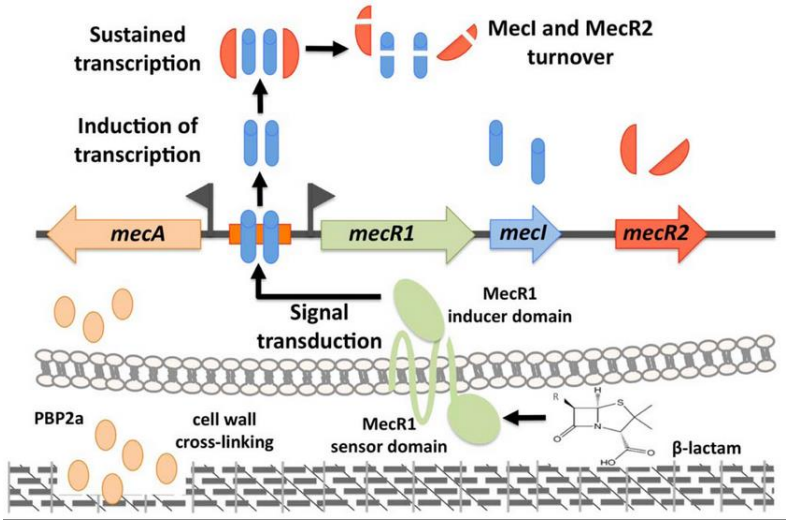

Figure 1-3: Mechanism of Methicillin Resistance. β-lactam antibiotics activate MecR1 which induces *mecA* and *mecR1-mecI-mecR2*. MecR2 is required to sustain the mecA induction by promoting the inactivation of MecI through proteolytic cleavage (22).

Most MRSA strains are also resistant to aminoglycosides such as streptomycin, gentamicin, and kanamycin by three common mechanisms 1) modifying enzymes, 2) ribosomal mutations, and 3) active efflux of the drug (30). Antibiotic resistance to vancomycin and other glycopeptides is conferred by several gene clusters (*vanA* through *vanG)* which code for alternative vancomycin binding sites (31). The first *S. aureus* clone with decreased susceptibility to vancomycin was isolated in Japan in the late 1990's (32). Vancomycin-resistant *S. aureus* (VRSA) first appeared in the U.S. in 2002 (33). Additional plasmids and transposons confer resistance to a variety of exogenous xenobiotics and natural contaminants. Of the integrated plasmids, pT181 confers tetracycline resistance, pI258 gives resistance to penicillins and heavy metals, and pUB110 carries the *ant(4')* gene which confers resistance to aminoglycosides including kanamycin, bleomycin, and tobramycin (28). Tn554 is a transposon that carries the *ermA* gene which carries constitutive and inducible resistance to macrolides, lincosamides, and streptogramin (MLS) (27, 28). Since most MRSA strains are resistant to multiple antibiotics, treatment usually involves non-β-

lactam antibiotics and broad spectrum anti-Gram positive antibiotics. Glycopeptides (i.e. vancomycin) are only prescribed as the first line of attack in highly invasive MRSA infections.

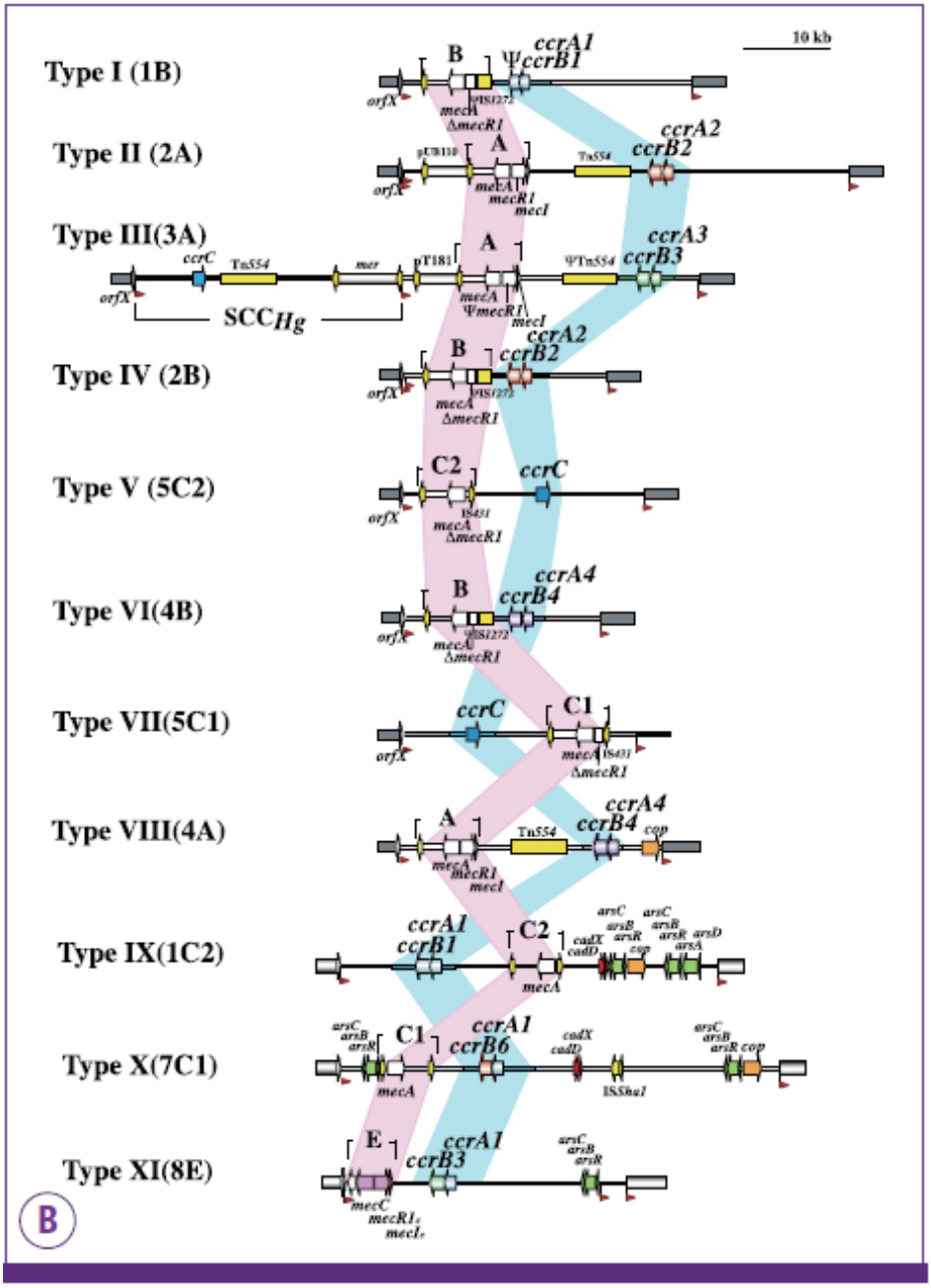

Figure 1-4: Schematic of Common SCC*mec* Types. The location of five (A-E) classes of *mec* gene complexes is indicated by pink belt. The blue belt shows the location of the *ccr*-gene complexes. Insertion sequences and transposons are represented in yellow boxes (28).

Classification Systems for MRSA

Phage-typing, named because of the use of bacteriophage, was one of the first systematic methods of differentiating MRSA strains. The International Subcommittee on Phage-Typing of staphylococci standardized a recommended protocol and set of 23 phages to test strains of *S. aureus* (40). Lawn cultures of bacteria were tested against the phage set with areas of lysis (plaques) determining a strong or weak reaction through a phage dilution series. While reproducible and cost effective, it is technically demanding, time consuming and requires maintenance of active phage. However, the major limitation is that a large percentage of MRSA strains cannot be typed using this method (41). MRSA-specific phages have been utilized to increase typeability; however the use of phage typing has been discarded in favor of more discriminatory techniques (42).

Pulsed-field gel electrophoresis (**PFGE**) is similar to conventional gel electrophoresis, except that the electric field orientation is changed periodically, which minimizes the overlapping of large fragments (Figure 1-5). *SmaI*, the standard restriction enzyme for *S. aureus*, digests the DNA of all strains of MRSA (43). The resulting gel bands are analyzed using software (Dice comparison and Unweighted Pair Group Method with Arithmetic Mean [UPGMA]) based on the standard of Tenover et al. (44).

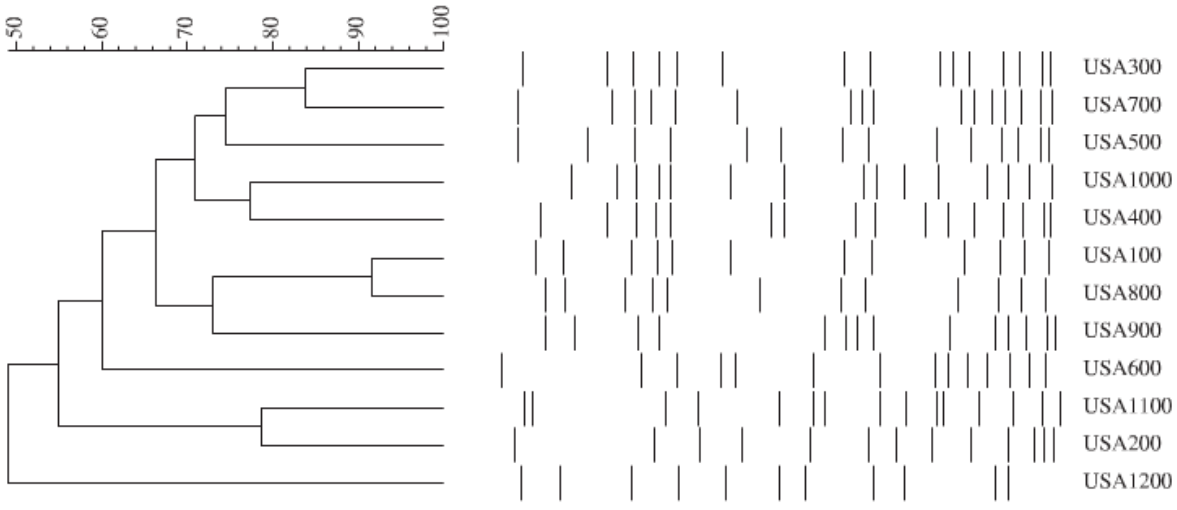

Figure 1-5: Dendrogram and PFGE types of CDC-defined "USA" S. aureus clones (47)

Although more discriminatory than phage typing, PFGE is expensive, ambiguous, procedurally challenging, and time consuming (45). Attempts to create an international, standardized procedure and nomenclature were unsuccessful, making large-scale comparisons cumbersome. Within the United States, McDougal et al. (46) established a standardized PFGE system based on analysis of nearly 1000 *S. aureus* isolates and designated the lineages USA100 through USA800, which has since been enlarged.

Another popular technique, **SCC*mec* typing,** assigns nomenclature designations based on the SCC*mec* cassette. Several methods have been developed including a multiplex-PCR method by Milheririco et al. (48) that identify SCC*mec* I through VI and *mecA* and one that analyzes *ccr* gene variations and the *mec* complex structural differences (49). Conflicting results occasionally occur since these techniques analyze different regions of the same genes. While SCC*mec* typing can be rapid and simple, it lacks discriminatory power and consistency. As a result, it is often combined with other subtyping techniques.

Single-locus sequencing typing became popular following the advent of sequencing technology. This technique analyzes polymorphisms across a single stretch of a gene, often at a region featuring repeats. While it can be utilized for many species, Frenay et al. (50) developed a method known as ***spa* typing** for *S. aureus* that analyzes variation in the *S. aureus* protein A (*spa*) locus. The *spa* gene has a polymorphic region X featuring 24 bp repeats with point mutations as the primary source of diversity, although deletions and repeat duplications occasionally occur (28). The use of two separate classification systems (51, 52) hampered comparisons until Ridom GmbH, a DNA sequencing software company based in Germany, released StaphType as a universal comparison method. The company also maintains one of the largest *S. aureus* typing databases (http://www.ridom.de/spa-server) to which its software synchronizes. *Spa* typing has numerous advantages including high discriminatory power, reliability, and compared to other methods it is inexpensive and relatively rapid (50).

Multilocus sequence typing (MLST) is one of the most common genetic methods to analyze bacterial pathogens including MRSA. Depending on the species studied, it analyzes approximately 500 bp

of seven-to-ten housekeeping genes and assigns allelic profiles based on the variation in sequences (53, 54). The seven housekeeping genes analyzed in MRSA-targeted MLST are listed in Table 1-2. The designated sequence types (STs) are analyzed using BURST (algorithm Based Upon Related Sequence Types). STs with identical DNA sequences across five or more genes are grouped into related clonal complexes (CCs) (54). The ST with the most single locus variants is assigned as the ancestral clone (29).

An international nomenclature has recently been established using MLST and SCC*mec* type by Enright et al. (54) and was accepted as an international standard by a subcommittee of the International Union of Microbiology Societies in Tokyo in 2002. For example, EMRSA-15 (epidemic-MRSA) is assigned an MLST allelic profile of 7-6-1-5-8-8-6 across the seven housekeeping genes (www.mlst.net), giving it the designation ST22. Since it contains the SCC*mec*IV element, its name based on this system is ST22-MRSA-IV (a methicillin susceptible clone would be labeled ST22-MSSA-IV) (54). While MLST provides comparable sequence data and is more discriminatory than previous methods, the housekeeping genes are limited to global epidemiologic relationships and are often not diversified enough on a local level to discriminate between closely related strains that have recently diverged over the course of an outbreak (53, 55).

Table 1-2: Gene Targets for MLST of *S. aureus* (54)

Gene	Name	Function
arcC	Carbamate kinase	Purine, glutamate, nitrogen metabolism
aroE	Shikimate dehydrogenase	Amino acid biosynthesis
glpF	Glycerol kinase	Triglyercide and phospholipid synthesis
gmk	Guanylate kinase	GMP recycling, purine metabolism
pta	Phosphate acetyltransferase	Pyruvate metabolism
tpi	Triosephosphate isomerase	Glycolysis and energy production
yqiL	Acetyl coenzyme A acetyltransferase	Beta oxidation of fatty acids

One of the newest methods of identifying isolates using sequencing techniques is closely related to MLST, but targets virulence genes rather than housekeeping genes essential for survival. **Multi-virulence-locus sequence typing** (MVLST) was developed by Zhang et al. (56) to investigate the global epidemiology of *L. monocytogenes*, but was adapted for MRSA by Verghese et al (53). MVLST targets ~500 bp of virulence genes whose exposure to the immune system and environmental stressors may result in faster rates of evolution and thus serve as better predictors of evolutionary relatedness during outbreaks (53). Table 1-3 lists the gene targets used in MVLST. Given that it is capable of separating out closely related clones that MLST clumps into the same sequence type, MVLST is a better technique to analyze recently diverged epidemic clones (53).

Table 1-3: Gene Targets for MVLST of *S. aureus* (53)

Gene	Name	Function
alt	Autolysin	Catalyzes breakdown of peptidoglycan matrix
essC	Type VII secretion system protein	Aids secretion of proteins
geh	Lipase	Lipid metabolism
hlgA	γ-hemolysin	Lyse red blood cells
htrA	Serine protease	Degrades misfolded proteins in periplasm
srdC	Ser-Asp rich protein	Cell adhesion

Although there are many other phenotypic, genetic, and epidemiologic techniques to identify MRSA, those listed are the most common methods. As the cost and time of sequencing continues to decrease, there has been a rise in whole-genome sequencing which is by far the most discriminatory and expensive technique available.

The number of different techniques, each with a unique nomenclature system, presents the science community with the problem of keeping all the names straight. Two similar clones may have identical phage types, *spa* types, and PFGE banding patterns, but can be differentiated by MLST or MVLST; however, the reverse is also occasionally true, particularly with closely related and recently

diverged strains (53, 56). Also, while standard protocols may have been established, many countries still follow old naming schemes, so the same clone or strain may have half a dozen names. From this point on, MRSA clones will be referred to by the accepted standard proposed by Enright et al. (54), i.e. ST6-MRSA-II, or their PFGE "USA" designation, i.e. USA300 (46) (Table 1-4).

Table 1-4: Standard MLST and SCC*mec* Epidemic Clone Names and PFGE Epidemic Clone Names (25, 46).

PFGE "USA" Name	MLST Name	Other Historical Names
N/A	**ST250-MRSA-I***	Archaic Clone, COL
N/A	**ST239-MRSA-III**	Brazilian/Hungarian Clone
N/A	**ST247-MRSA-I**	Iberian Clone
USA100	ST5-MRSA-II	New York/Japan Clone
USA200	ST36-MRSA-II	UK EMRSA-16
USA300**	ST8-MRSA-IV	Community-Associated MRSA
USA400	ST1-MRSA-IV	Midwest Clone
USA500	ST8-MRSA-IV	UK EMRSA-2/-6
USA600	ST45-MRSA-IV	Berlin Clone
USA700	ST72-MRSA-IV	
USA800	ST5-MRSA-IV	Pediatric Clone
USA900	ST15-MSSA	
USA1000	ST59-MRSA-IV or VII	Pacific Clone
USA1100	ST30-MRSA-IV	Southwest Pacific Clone

*Even though sequence types will have more than one SCC*mec* associated with them, the table shows the accepted standard SCC*mec*.
**USA300 is traditionally referred to by the PFGE naming system rather than the MLST/SCC*mec* combination.

Evolutionary History of MRSA

While the original donor of the *mecA* gene is still unknown, *S. sciuri* contains an intrinsic penicillin binding protein (PBP) with 87.8% amino acid homology with MRSA's PBP2a, so it was once proposed as a potential original source of this genetic element (58). However, a recent study analyzed additional staphylococcal species and found a *mecA* gene with 99% sequence identity in *S. fleuretti*, one of the oldest members of the *sciuri* family (28). Furthermore, whole genome sequencing revealed that instead of being carried by a mobile element, the *mecA* homologue in *S. fleuretti* is part of the core genome (58, 59). This genomic structure would be a plausible explanation for why truncated portions of both operons are sometimes seen flanking SCC*mec* regions in *S. aureus* (28, 59).

Although it was first believed that all current MRSA clones descended from the first MRSA isolated in 1961, genetic analysis has since revealed extensive genomic differences that suggest acquisition of the *mecA* gene arose independently several times, leading to five separate clonal complexes containing epidemic MRSA clones (10, 54, 57, 60). Based on sequence analysis of nearly 1000 *S. aureus* and MRSA isolates, ST250-MRSA-I or the "Archaic Clone" is the presumed original MRSA clone (54). This first MRSA strain carried a type I SCC*mec* complex and diverged from the ST8 lineage following a single point mutation in the *yqiL* gene (57). Since ST8 and its clonal complex CC8 does not contain any early MRSA isolates, ST250-MRSA-I likely diverged from ST8, a successful MSSA clone, before it acquired methicillin resistance through horizontal gene transfer (29).

Genomic analysis of over 5000 isolates collected in Denmark between 1957 and 1973 further elucidated the early evolutionary relationships between major MRSA clones (60) (Figure 1-6). ST8-MSSA is the presumptive ancestor of the Archaic Clone (ST250-MRSA-I), as well as the expected ancestor of CC8, which includes two highly successful pandemic MRSA clones, the Iberian Clone (ST247-MRSA-Ia) and the Brazilian/Hungarian Clone (ST239-MRSA-III), in addition to the Hannover Clone (ST254-MRSA-IV) (60-62). ST239-MRSA-III likely arose as a result of homologous

recombination between an approximately 557 kb region of ST30-MSSA and ST8-MSSA and acquisition of type III SCC*mec* (29). Since the ‘60s, ST8 has acquired types I, II, and IV SCC*mec* (29, 61).

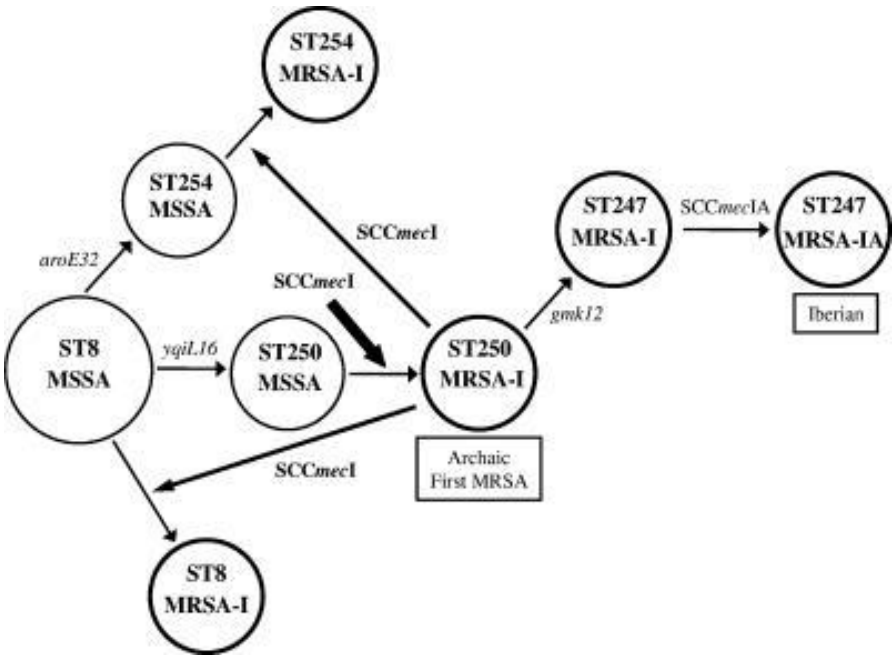

Figure 1-6: Model for early evolution of MRSA in CC8. It is based on genomic analysis of 5000 MRSA and MSSA isolates collected between 1957 and 1973 in Denmark. Arrows indicate the direction and changes between genotypes with genetic acquisitions marked (60).

Representatives from four of the five epidemic MRSA lineages (ST5, ST8, ST30, & ST45) were identified from the Denmark isolates, indicating 1) they originated as dominant MSSA lineages with a superior ability to colonize and evade host immune defenses and 2) they independently acquired SCC*mec* complexes facilitated their emergence as major MRSA clones (55, 60). USA100 (New York/Japan Clone) and USA800 were likely derived from ST8-MSSA following acquisition of SCC*mec* II and IV, respectively (25). ST8-MSSA likewise is thought to have diverged into multiple lineages including the Archaic Clone (ST250-MRSA-I), USA500 (ST8-MRSA-IV) and USA300 (ST8-MRSA-IVa) (62). USA300 likely arose from USA500 after acquisition of PVL, ACME, *sek2,* and *seq2* (Figure 1-7) (62). ST22 may have evolved into EMRSA-15 after SCC*mec* type IV acquisition, while ST30 probably

diverged into ST36, USA200 (SCC*mec*II), and USA1100 (PVL and SCC*mec*IV) (55). ST45 includes USA600 (the Berlin Clone) which carries a type IV SCC*mec* complex (25).

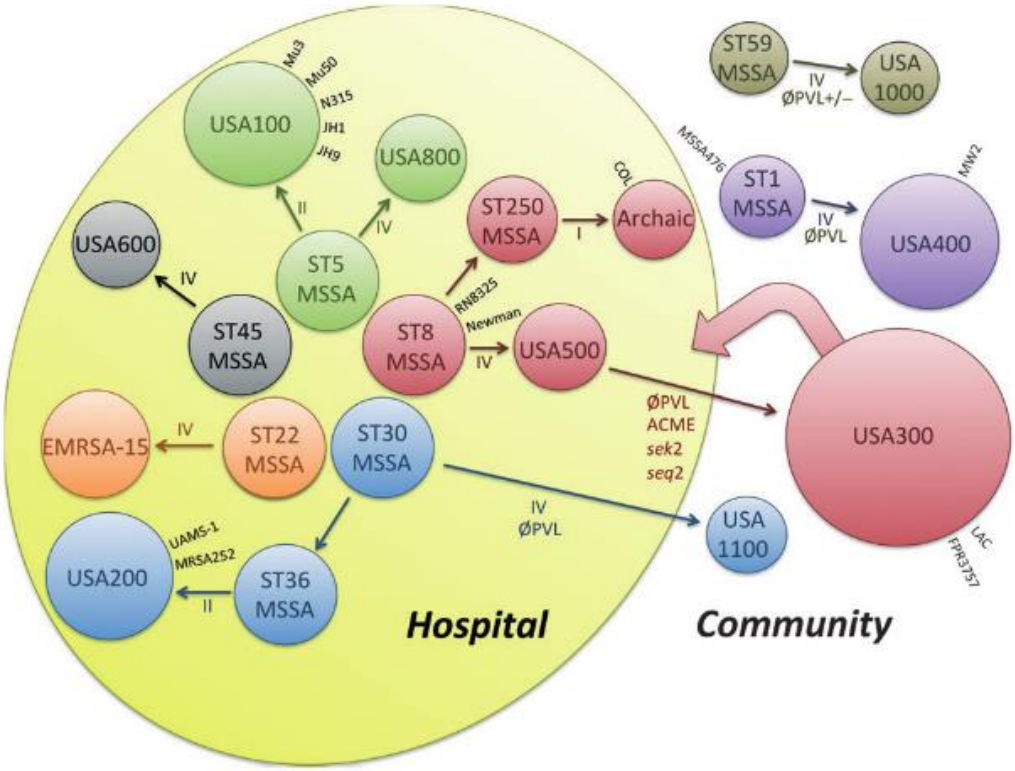

Figure 1-7: Schematic Showing the Evolution of the Five Main Lineages of MRSA (USA100, 200, 300, 400, and 500) from MSSA. CC1: purple, CC5: green, CC8: red, CC22: orange, CC30: blue, CC45: black. Roman numerals depict acquisitions of SCC*mec* types. Gene acquisition is noted below SCC*mec* acquisition (i.e. USA400 acquired PVL and SCC*mec*IV). Additional designations of strains are listed on the outside of the relevant circles (25).

Emergence of Community-Associated MRSA

For roughly four decades following its emergence, MRSA has been synonymous with nosocomial infections. That changed when the CDC released a report in 2003 concerning several outbreaks of MRSA involving a fencing team in Colorado (February 2003), high school wrestlers in Indiana (January 2003), high school and college football teams in Indiana (2003-2002), Los Angeles County (September 2002), and Pennsylvania (September 2000) and a prison outbreak in Mississippi in 2000 (63). Since none of the

affected individuals had any of the traditional risk factors associated with MRSA infections, officials realized a new community-associated MRSA pathogen had likely emerged. While there have been several published definitions of CA-MRSA (64-66), the CDC Active Bacterial Core Surveillance Program now defines a CA-MRSA case as a positive MRSA culture isolated from a patient more than 48 hours after admission to a hospital with the following, 1) no history of dialysis, hospitalization, surgery, or residence in a long term care facility within one year of the positive MRSA culture, 2) no prior positive MRSA culture, and 3) no permanent catheter or percutaneous medical device (67). Additionally, the presence of genetic markers such as PVL or SCC*mec* types IV, V, or VII can define an isolate as CA-MRSA (29). Distinguishing features of early CA-MRSA isolates were their susceptibility to older antibiotics and frequent carriage of SCC*mec* type IV (68-70).

Shortly after its emergence, CA-MRSA appeared repeatedly in predominantly young and healthy populations with outbreaks reported in religious groups (71), prison inmates (72), men who have sex with men (73), high school, college and professional athletes (74, 116) and soldiers (75, 76). Critical risk factors identified included sharing of personal items, poor hygiene and wound management, overcrowding, frequent skin-to-skin contact, and exposure to antimicrobials (65, 76, 77). Additional factors such as diabetes, incarceration, and playing contact sports are all associated with at least a 3-fold increased risk of MRSA colonization, while HIV is associated with an increased risk of nearly 14-fold (37). While studies have analyzed the virulence of CA-MRSA, HA-MRSA, and MSSA (17, 62, 87, 121-124), the hierarchy is still not clear as virulence differs between infection models and epidemic clones. Aside from the mentioned differences, CA-MRSA and HA-MRSA clones display extremely divergent genetic traits, antibiotic resistance patterns, and infection sites. As a result, treatments vary, with HA-MRSA clones displaying more resistance to antibiotics and CA-MRSA less so (78).

Table 1-5: Differences Between CA-MRSA and HA-MRSA Epidemic Clones (78, 79, 120)

Differences	HA-MRSA	CA-MRSA
Genetic Elements	SCC*mec* I, II, III (most common)	SCC*mec* IV, V, VI (most common) Panton-Valentine leukocidin ACME (USA300)
Dominant Clones	USA100	USA300, USA400
Main Infection Sites	Blood Surgical site & implants	Skin and soft tissue Lungs
Populations at Risk	Immunocompromised Surgery and dialysis patients Recent hospitalizations Long term care facility residents	Young, healthy individuals Anyone
Transmission	Skin-to-skin Contact with contaminated surfaces Poor hand hygiene Contaminated equipment	Fomites Skin to skin High density living conditions Poor hygiene
Antibiotic Resistance	β-lactams Glycopeptides, ie. Vancomycin Sulfa drugs, Tetracyclines, Clindamycin, Teicoplanin (some)	β-lactams Erythromycin (often)
Treatment of Choice*	Vancomycin as first attempt If not responsive then one or more of the following: Linezolid, Daptomicin, Tigecycline	Doxycycline, Clindamycin, and/or Bactrim as first attempt Vancomycin if not responsive
Prevention	Alcohol-based hand sanitizer CDC infection control guidelines Education of staff Careful use of antimicrobial agents Surveillance and follow up	Alcohol-based hand sanitizer Proper wound care Not sharing personal items (towels, razor blades, etc.) Education
Screening	Skin or nasal swab PCR rapid screen	Testing of wound drainage PCR rapid screen

*Treatment of each infection differs between patients depending on severity and infection site.

Changing Distribution of MRSA

Since the mid-late 90's, the distribution and prevalence of MRSA has changed drastically (18, 121). Prior to the emergence of USA300, multidrug resistant USA100 (ST5-MRSA-II, New York/Japan Clone) was the predominant MRSA epidemic clone within the U.S. (11). USA100 caused the majority of nosocomial MRSA infections, outnumbering community-associated MRSA infections three-to-one (121, Figure 1-8). Until USA300 evolved, the principal CA-MRSA strain within the U.S. and Canada was USA400 (83, 84). Over the course of a decade, the appearance of USA300 caused a fifteen-fold increase in CA-MRSA disease and a two-fold increase in *S. aureus* disease (18). This trend has reversed the distribution of MRSA incidence such that by 2010, CA-MRSA cases outnumbered HA-MRSA cases by approximately three-to-one (121, Figure 1-9).

Globally, ST8 and ST1 are predominantly found in the Americas, and ST80, ST22, and ST5 predominate in Europe, Northern Africa and the Mediterranean. (144). ST30 has caused outbreaks in Australia, Ocreania, Southesast Asia, Europe, and the America (144). A global distribution of the most common MRSA epidemic clones is displayed in Figure 1-10.

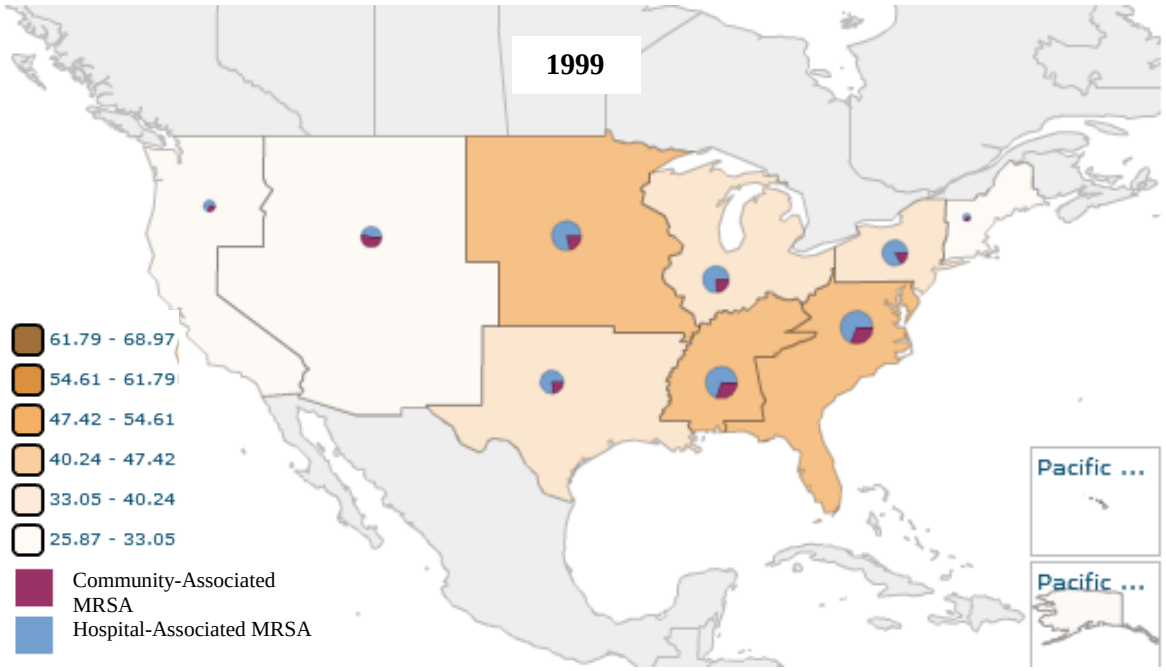

Figure 1-8: Regional Trends of HA-MRSA and CA-MRSA in the USA in 1999. In 1999, Hospital-Associated MRSA outnumbered Community-Associated MRSA by about three-to-one (121).

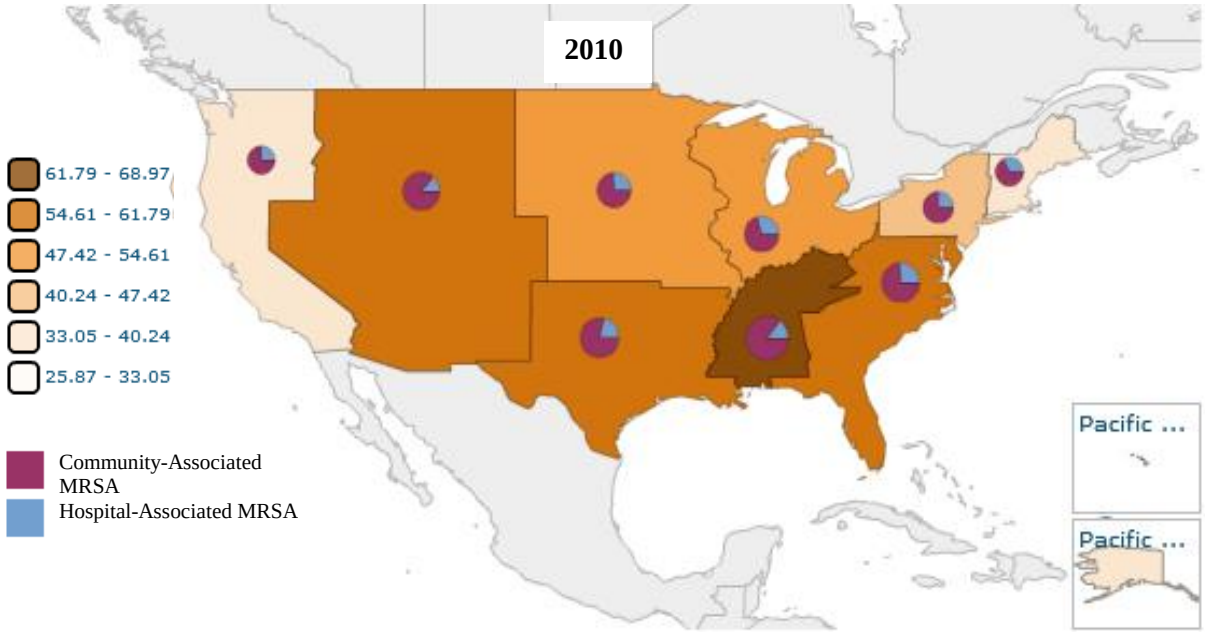

Figure 1-9: Regional Trends of HA-MRSA and CA-MRSA in the U.S. in 2010. In 2010, Community-Associated MRSA outnumbered Hospital-Associated MRSA by about three-to-one, reversing the trend seen just ten years prior (121).

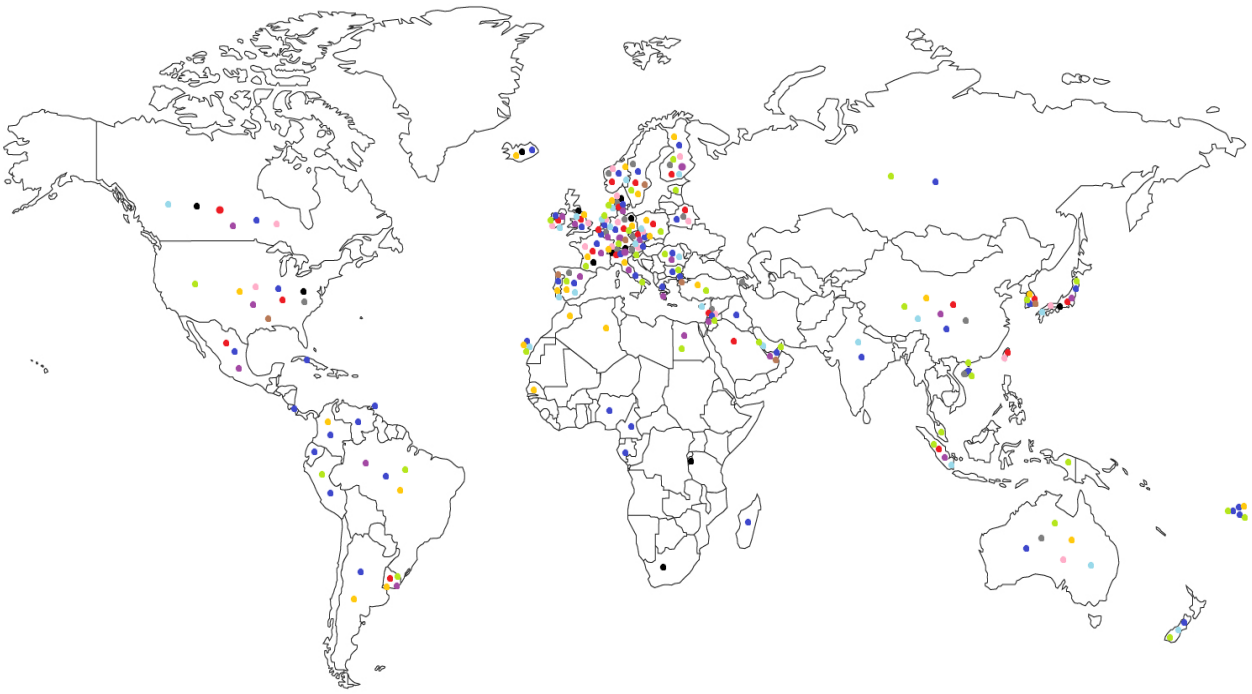

Figure 1-10: Global Distribution of MRSA Epidemic Clones. Color scheme: black (ST250/Archaic Clone), red (ST5/USA100), **blue (ST8/USA300**), purple (ST1/USA400), pink (ST8/USA500), grey (ST25/USA600), brown (ST72/USA700), gold (USA800/Pediatric Clone), lime (ST30/USA1100), light blue (ST22/EMRSA-15). Adapted from 29, 137.

Chapter 2

The Emergence of USA300

"It is not the strongest or the most intelligent who will survive but those who can best manage change."

Charles Darwin

Hidden among the other community-associated MRSA outbreaks during the end of the '90s and the early '00s, a new family of isolates emerged, causing disease in prisons (80) and among football players (63); however, it was not given the name USA300 until years later. USA300 was identified as one of the eight original MRSA strains by McDougal et al. (46). However, while USA300 strains yield slightly different PFGE patterns, they are greater than 80% similar when analyzed using BioNumerics software (47). During early outbreaks, one of its characteristics was the lack of nasal carriage. While it was first speculated that USA300 could not colonize nasal passages, one possible reason is that some individuals were already undergoing antibiotic therapy, which can prevent microbial recovery from external sites (74). A National Health and Nutrition Examination Survey between 2001 and 2004 showed a statistically significant increase in both USA300 and USA300-0114 nasal carriage (81). However, subsequent studies showed that USA300 preferentially colonizes extranasal sites such as the rectum, genitals, and auxilla, providing it with a competitive colonization advantage over other MRSA clones (18, 82).

Since its emergence, USA300 has replaced the previously dominant CA-MRSA clone USA400 (83, 84) to become the leading CA-MRSA epidemic clone in the U.S. (85-87). By 2004, USA300 had caused a fifteen-fold increase in CA-MRSA cases which doubled the overall burden of *S. aureus* disease (18). USA300 alone now accounts for over 50% of all *S. aureus* infections in the U.S. (87-89). Having successfully established itself as endemic within the United States, USA300 is rapidly advancing globally

and is already the dominant community-associated strain in Canada (CMRSA-10) (90). One notable characteristic of this highly successful epidemic clone was the low sequence divergence between USA300 strains, suggesting minor genomic changes can profoundly affect virulence (91).

USA300 is capable of causing multiple types of *S. aureus* infections; however, skin and soft tissue infections (SSTIs) have historically been the most common. In U.S. emergency rooms, up to 98% of all MRSA presenting as SSTIs are caused by USA300 (85). Although USA300 is a community-associated epidemic clone, it has recently also become endemic in hospital and health-care settings throughout the U.S., displacing ST5-MRSA-II (USA100), the primary bloodstream isolate in certain regions (92). Some studies reported at least half of the hospital-associated MRSA infections were due to USA300 (86, 93) and it may now account for over half of invasive CA-MRSA infections (11). The environment may serve as a key reservoir and may play a role in pathogenesis since USA300 is transmissible from fomites such as cotton towels and bed sheets for over two weeks and over eight weeks in the case of plastic and vinyl surfaces after initial inoculation (96). The ability of USA300 to cause significant HA-MRSA diseases like bacteremia (15), necrotizing fasciitis (13), fatal necrotizing pneumonia (94), and endocarditis (14) is blurring the boundaries between CA-MRSA and HA-MRSA (86, 93, 95).

Molecular Evolution of USA300

Clonal Complex 8 is thought to have branched into several lineages, one of which is the ancestral MRSA clone (29, 47, 60, 62). Another prominent lineage involves a significant genome recombination event between ST30 MSSA and the ST8 progenitor to form ST239 (Figure 2-1) (47, 62). In the third branch, USA300 evolved from ST8 through an intermediate, thought to be USA500, a somewhat related hospital-associated clone (25, 60, 62). USA500 MRSA has no known enterotoxin genes encoded on prophages and pathogenicity islands and is nearly identical in *in vivo* infection models compared to USA300 (62). Multiple genetic element acquisition events occurred in USA300 during its evolution from USA500, including acquisition of a pathogenicity island with two enterotoxins K (*sek*) and Q (*seq*), a

prophage with PVL encoded by the *lukSF-PV* genes, and ACME, an arginine catabolic mobile element unique to USA300 MRSA but common in *S. epidermidis* strains, a nonpathogenic distant relative (28, 97).

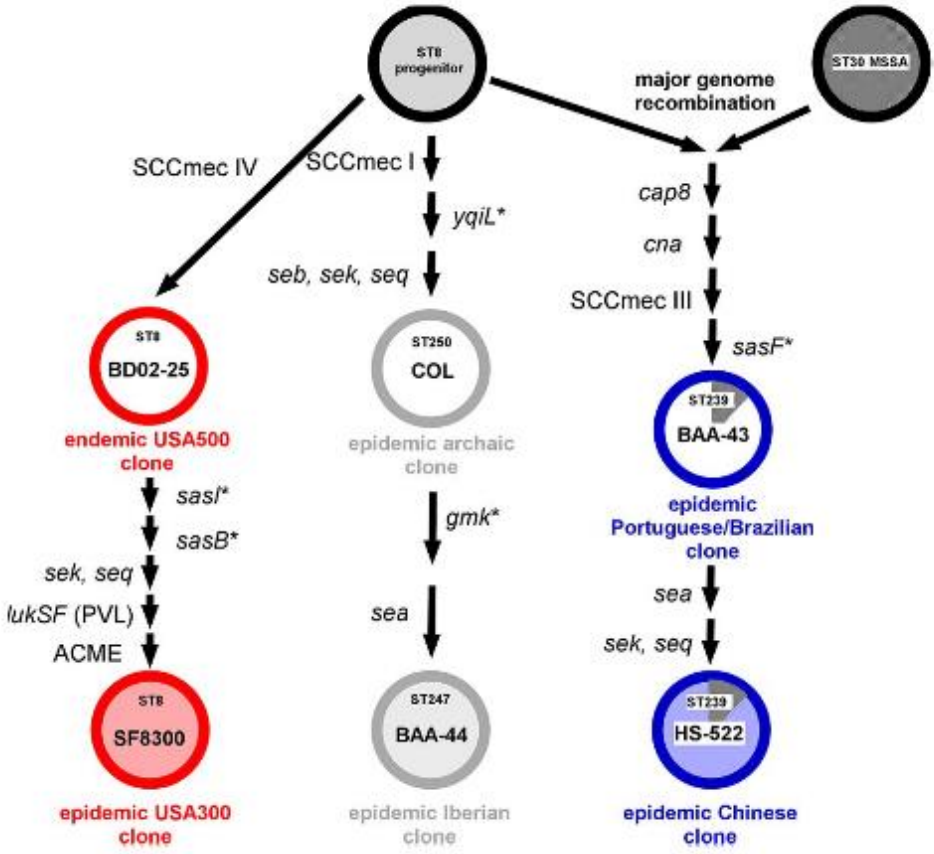

Figure 2-1: Evolutionary Relationship between CC8 MRSA Lineages. Sublineages are shaded in red, grey, and blue and labeled with clone designations (62).

USA300 Virulence Determinants are Responsible for Hypervirulence

USA300 MRSA is hypervirulent in animal models compared to other CA- and HA-MRSA clones (62, 96, 98). USA300 is more lethal in rat septic (62) and pneumonia (99) models than other epidemic clones of MRSA. Bacteremia, pneumonia and a co-infection with influenza and pneumonia are strongly correlated with severe patient outcomes including high morbidity and mortality (94, 100-102). USA300

causes approximately one-third of MRSA bloodstream infections (BSIs) (15) which are associated with more severe sepsis and greater mortality, than the previously predominant HA-MRSA USA100 (120).

However, some population studies have recorded better outcomes associated with USA300 relative to HA-MRSA clones (92, 94, 102). Given the inherent susceptibility of USA300 to antibiotics, these treatments can reduce the outcome severity in population studies relative to HA-MRSA (103). Regardless of the outcome, one stark fact is clear: in just over a decade, USA300 has been able to displace the dominant CA-MRSA and HA-MRSA epidemic clones in America (47, 93). While the exact mechanism remains to be seen, the combination of its colonization and virulence determinants may explain its rapid transmission and predominance.

USA300 contains several intrinsic virulence determinants that are by no means unique; however, overexpression of these determinants may aid colonization, invasion, and pathogenesis. One such molecule is an **exotoxin** called alpha-hemolysin. Exotoxins are peptides, proteins, or small molecules secreted by bacteria that result in structural, cellular, and metabolic damage. USA300 produces many exotoxins including alpha-hemolysin (*hla*) which codes for a potent heptameric 100 angstrom pore-forming protein that inserts in the plasma membrane inducing lysis in the target cell (105). This toxin is crucial in nearly every known MRSA disease model and is highly expressed in USA300 (62, 97, 98).

The peptide-based **accessory gene regulation (*agr*) quorum sensing system** is a master virulence factor regulator (106, 107) and is highly up-regulated within USA300 (25). This two-component signal transduction system is activated by accumulation of an autocrine pheromone peptide (109, 110). This activates expression of toxins, surface adhesions, pathogen-associated molecular patterns, phenol soluble modulins (PSMs) (108) and other genes (109, 110). Agr activates neutrophils via the formyl peptide receptor 2 (107); inactivation of the system leads to significantly decreased neutrophil response (107), and attenuation in murine sepsis, pneumonia, and skin abscess models (98, 111, 112). Hyperactivity in USA300 can account at least in part for increased production of PSMs, proteases, RNAIII (111), α-hemolysin, and toxin production (98). Agr is important for necrotizing pneumonia and

skin infections in mouse models (111); however, given that USA400 displays a similar increased expression of *agr* (110), it is unlikely to explain USA300's success as a global pathogen.

Encoded on the unique pathogenicity island SaPI5 are pyrogenic superantigen **enterotoxin Q** (*seq2)* and **K** (*sek2*), which stimulate T cell receptor Vβ-expressing T cells (97). These two enterotoxins share nearly identical homology (98%) to *sek* and *seq* found on the plasmid SaPI3 carried by the archaic ST250-MRSA-I clone (113). While it is possible that *seq2* and *sek2* have a role in pathogenesis, this has not been demonstrated *in vivo*.

Like all MRSA clones, USA300 contains a SCC*mec* cassette containing *mecA*. Nearly all strains of USA300 carry **SCC*mec*IVa**, the shortest version at around 20 kb; unlike I, II, and III, SCC*mec*IVa is not associated with a fitness cost (17, 114-116). SCC*mec*IV is the most common SCC*mec* form, having been identified in divergent lineages including ST8 (USA300, USA500), ST1 (USA400), ST80, and ST72 (USA700) among others (25, 117, 118). One likely reason for its prevalence is that its small size may allow phage to transfer it between bacteria (27). Although SCC*mec*IV does not carry any additional resistance genes, this apparent disadvantage may, in fact, be beneficial since there is an inverse relationship between resistance level and growth rate (119). Aside from the growth advantage, SCC*mec*IV is likely not the major contributor to USA300's success given the plethora of clones with SCC*mec*IV that are not a serious global health threat.

The bi-component toxin, **Panton-Valentine leukocidin**, coded by the *lukS-PV* and *lukF-PV* genes on the prophage SaPI5 (97) is a possible candidate for USA300's success. As a leukocidin, the pore-forming toxin targets white blood cells and induces necrosis and apoptosis (125). PVL is strongly correlated with CA-MRSA (126) and is used as a marker in determining CA-MRSA disease (29) as nearly all strains of USA300, USA400 and USA1000 carry the PVL prophage (126, 127). Clinical data support a strong association between PVL production and severe outcomes in SSTIs, necrotizing pneumonia and fasciitis (70, 128). Furthermore, PVL has been isolated directly from skin abscesses in leukocidal concentrations (129). However, PVL's influence on virulence is controversial (29, 62, 87,

130) as clones lacking PVL retain the capacity to cause outbreaks (131, 132). Furthermore, species differences have complicated efforts to elucidate the role of PVL since the toxin is much less active against rat, murine and non-human primate neutrophils (133). Like SCC*mec*IV and *agr* quorum sensing system, the ubiquitous nature of PVL within CA-MRSA strains including non-epidemic clones suggests a minor role in pathogenesis.

Currently, the only known genetic element unique to USA300 MRSA is ACME, arginine catabolic mobile element, which is commonly found in strains of *S. epidermidis*, a non-pathogenic distant relative (97). USA300 likely acquired ACME, which is linked adjacent to SCC*mec*IV via horizontal gene transfer, from *S. epidermidis* (126, 134). The vast majority of USA300 clones carrying SCC*mec*IV also have ACME; however, few clones carrying other *mec* cassettes contain ACME (118). Among the genes encoded on this mobile element is *arc*, a complete arginine deaminase system responsible for the conversion of L-arginine to L-ornithine and *speG*, a spermine/spermidine acetyltransferase (29, 97). Spermidine is a bactericidal compound produced in toxic quantities during inflammation, wound healing, and keratinocyte proliferation in the skin (134, 135). Thanks to *speG*, USA300 is the only known clone to be resistant to polyamines like spermidine (29, 125). This tolerance has been shown to decrease antibiotic susceptibility, decrease killing by human keratinocytes, and enhance biofilm formation (135), which strongly suggests a powerful colonization advantage over other epidemic clones. However, this idea is tempered by the fact that some USA300 clones that lack ACME (and thus *speG*) cause significant CA-MRSA in Latin America (136). Thus, while it is likely that ACME and *speG* play a role in colonization, they are not solely responsible for USA300's dominance.

Enterotoxins Q and K, the *agr* quorum sensing system, SCC*mec*IVa, exotoxins, PVL, and ACME all appear to have some role in the colonization and pathogenesis of USA300 MRSA; however, even without any one of these components, USA300 still retains the ability to cause infections and outbreaks. In order to be a successful epidemic clone, both enhanced transmission and virulence are required. ACME and the *agr* quorum sensing system may play roles in colonization and potentially in transmission, while

PVL, enterotoxins and exotoxins may fulfill the later role. Rather than a particular virulence factor serving as the magic ingredient, it appears that the unique and possibly synergistic combination of these particular virulence determinants is what has allowed USA300 to establish itself as a global pathogen, out-competing much older epidemic MRSA clones.

Chapter 3

USA300 Prevalence at the Penn State Milton S. Hershey Medical Center

"Evolution on the large scale unfolds, much like human history, as a succession of dynasties."

Edmund Beecher Wilson

Introduction

USA300, the most common MRSA strain in America, is now responsible for approximately one third of all bloodstream infections caused by MRSA (15, 137). In some emergency departments, all but a fraction of the staphylococcal skin and soft tissue infections and over half of invasive staph infections are caused by USA300 (11, 85-87). Furthermore, USA300 is increasingly causing infections in nosocomial settings (15, 138). Some hospitals now report at least half of their HA-MRSA infections are caused by USA300 (86, 93). With new outbreaks reported monthly, it has been suggested that CA-MRSA and USA300 will replace HA-MRSA clones in hospitals and other healthcare settings to become the leading cause of nosocomial MRSA infections (47, 93, 139)

This troublesome trend has created the need for epidemiologic techniques to track outbreaks of USA300 and other epidemic clones within healthcare settings. While multilocus sequence typing (MLST) is commonly used in combination with other assays like spa-typing and PFGE, Dr. Knabel's laboratory decided to use a newer technique developed in his laboratory, multi-virulence-locus sequence typing (MVLST), to analyze the prevalence of USA300 in isolates from the Penn State Milton S. Hershey Medical Center (HMC). In contrast to MLST, which targets ~500 bp regions of seven housekeeping genes, MVLST evaluates ~500 bp of six virulence genes (*alt*, *essC*, *geh*, *hlgA*, *hrtA*, *srdC*) that better predict evolutionary and genomic relatedness (53).

Verghese et al. (53) previously analyzed MRSA prevalence in the nasal passages of incoming patients at HMC using a combined MVLST/SCC*mec* subtyping scheme; interestingly, the majority of presumptive MRSA isolates were MLST ST5, ST8, and ST105 with much greater SCC*mec* diversity than previously reported using solely MLST subtyping (53). Furthermore, MVLST analysis showed 27 virulence types (VTs) present with three accounting for over half, VT6 (32.8%), VT9 (8.9%), and VT2 (8.9%), which corresponds to USA300 (53).

Materials and Methods

To identify the prevalence of USA300 in infected patients, 22 clinical MRSA isolates were collected from MRSA infected patients in 2012 at the Pennsylvania State University Milton S. Hershey Medical Center (HMC). MRSA cultures were transported to the Foodborne Pathogen Laboratory of Dr. Stephen Knabel in the Food Science Department at the Pennsylvania State University, University Park, PA. The isolates 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 overnight at 35°C. Isolated, presumptive positive colonies were inoculated into tryptic soy broth (Difco, Sparks, MD) and grown overnight at 35°C. The UltraClean Microbial DNA Isolate Kit (MoBio Laboratories, Solona Beach, CA) was used to isolate chromosomal DNA. Isolates were compared to 12 reference strains provided by the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) (strains USA100, USA200, USA300, USA500, USA600, USA700, USA800, USA1000, and USA1100) and the Centers for Disease Control and Prevention (CDC) (strains USA 300 and USA400).

For each of the 22 MRSA isolates, MVLST protocol was conducted as described by Zhang et al. (56). PCR primers for amplifying the desired fragments were based on Verghese et al. (53) (Table 3-1) and were synthesized at the Penn State University Genomics Core Facility. PCR-cycling conditions for *alt*, *essC*, *hlgA*, and *htrA* were 95°C for 3 min, then 35 cycles (95°C for 30 s, 60°C for 30 s, 72°C for 2 min) followed by final extension at 72°C for 10 min in a Mastercycler thermocycler (Eppendorf Scientific, Hamburg, Germany). PCR-cycling conditions for *sdrC* and *geh* were 95°C for 3 min, then 35

cycles (95°C for 30 s, 53°C for 30 s, 72°C for 2 min) followed by final extension at 72°C for 10 min. Amplified products were electrophoresed in 2% agarose gels at 120V to confirm the presence of a single product. Unincorporated primers and free nucleotides were digested by 0.5 µl of exonuclease I (10 U/µl; USB Corp., Cleveland OH) and 0.5 µl of shrimp alkaline phosphatase (1 U/µl; USB Corp.) per 10 µl of PCR product. The mixture was incubated at 37°C for 45 min to degrade the primers and then at 80°C for 15 min to inactivate the enzymes. The Genomics Core Facility at the Pennsylvania State University carried out DNA sequencing on an ABI Hitachi 3730XL DNA Analyzer (Applied Biosystems, Inc., Foster City, CA). Sequence alignments were performed using molecular evolutionary genetic analysis software (MEGA version 4.0). The neighbor-joining tree algorithm in MEGA was used to construct a dendrogram to determine evolutionary relationships between isolates.

Table 3-1: Virulence Gene Primer Sequences for MVLST

Virulence gene Targeted	Primer Sequence (5'-3')	Amplicon Size (bp)	Reference
alt	(F) CCGACTTCGCCAATTTTATC (R) TCCCATTAGGTTGGTTCAATG	866	Verghese et al. 2012 (53)
essC406 *essC1082*	(F) GCATTGTGCACGATAAAAATACA (R) GACTTTTCTACATTCGCAATACC	649	Verghese et al. 2012 (53)
geh	(F) AGCACAAGCCTCGGAAAAA (R) CCAAATGCACTTACACTTGCTT	875	Verghese et al. 2012 (53)
hlgA	(F) CCCCTTTAGCCAATCCATTT (R) CTGGACCAGTTGGGTCTTGT	480	Verghese et al. 2012 (53)
htrA	(F) TCAATTGATAAGCACGAACG (R) GCCCTTTGTTCAATTTTGATG	778	Verghese et al. 2012 (53)
srdC	(F) TCAAAATTTATATAATGCCCAAGGT (R) TCCCTTTTTCATTGGCATCT	573	Verghese et al. 2012 (53)

Results

Of the 22 clinical MRSA isolates from the Hershey Medical Center, 82% matched the reference strain USA300; 17 had 100% sequence identity at all six virulence genes and one (Isolate 15) had one single nucleotide polymorphism (SNP) difference, a non-synonymous mutation in the *alt* gene. The remaining four isolates did not match any of the remaining eleven control strains.

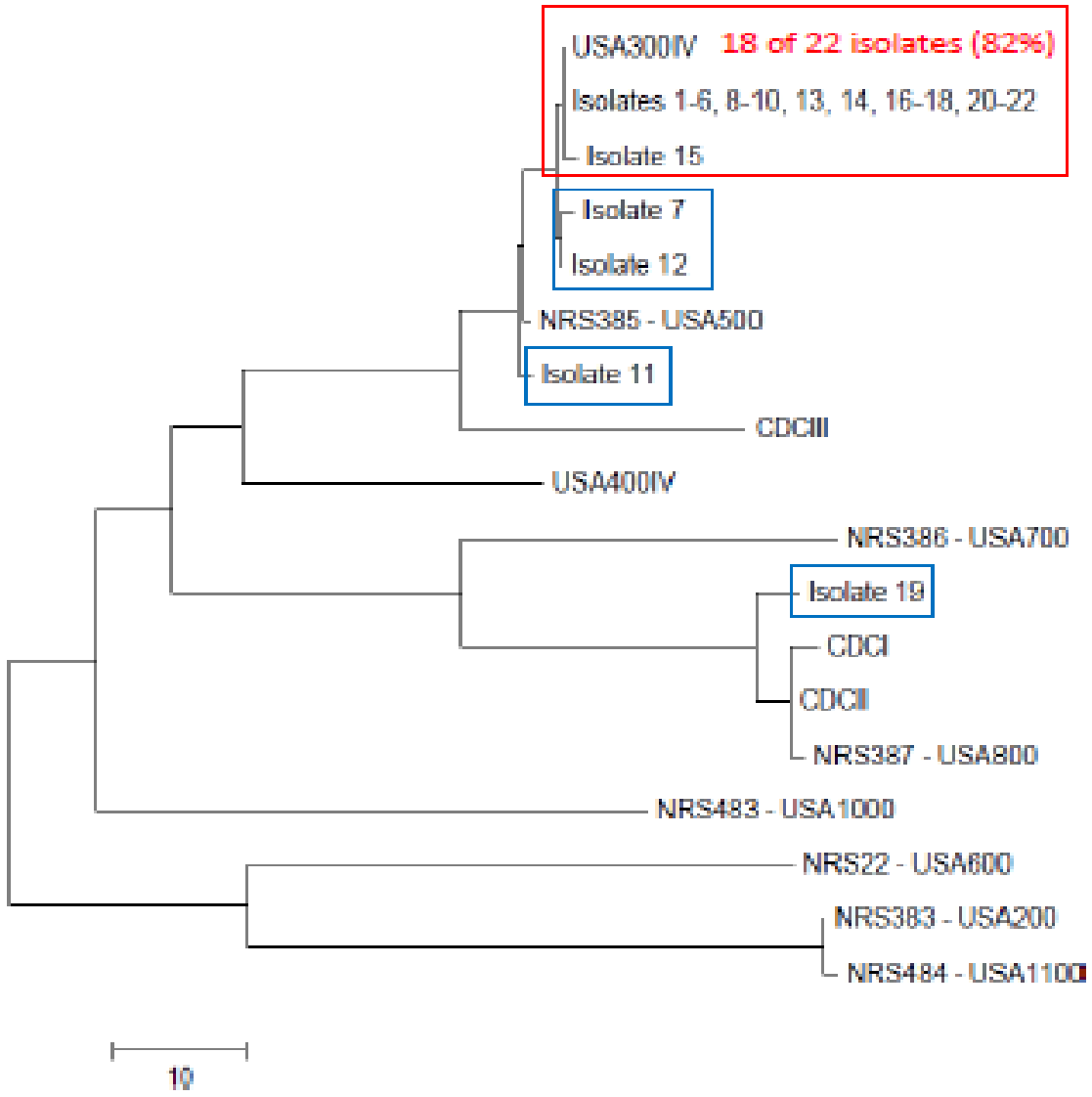

Figure 3-1: Dendrogram Depicting Evolutionary Relationships of the 22 HMC Clinical Isolates Compared to 12 CDC and NARSA Reference Strains

Discussion

Over 80% of the clinical isolates from MRSA-infected patients from the HMC Intensive Care Units were USA300, which is troubling given that many of these patients typically present with underlying conditions and complications, which can significantly increase the chances of a more severe infectious disease outcome. Just a few years prior, Verghese et al. (53) found approximately 9% of nasal isolates from incoming patients were USA300. The high prevalence found in this study parallels with the growing trend of USA300 increasingly causing in nosocomial infections (85-87, 93).

Given that the sample size was relatively small, we cannot draw too many conclusions from this data; however, it suggests that USA300 may have become endemic within HCM. Although the samples came from a random distribution within the ICUs, we do not have the exact ward and room locations and cannot predict the spread of the potential outbreak over the course of the sample time. Epidemiologic data aside, it suggests that USA300 is highly prevalent at HMC.

Since USA300 is highly transmissible (96), an endemic strain within HMC could potentially cause a catastrophic number of infections and complications in already ill patients who can little afford a complicating infection. Therefore, the need to screen patients rapidly, reliably and cheaply to specifically detect USA300 MRSA epidemic clones is paramount for preventing transmission between patients, staff, and visitors.

Chapter 4

Development of a Novel Multiplex PCR Method to Detect USA300

"It is failure that guides evolution; perfection provides no incentive for Improvement, and nothing is perfect."

Colson Whitehead

Introduction

The prevalence of USA300 in nosocomial settings has highlighted the need for rapid detection methods that can differentiate between the more aggressive but antibiotic-sensitive USA300 and the more resistant but less fit epidemic MRSA clones like USA100 and USA500. While MLST and MVLST can provide a vast amount of genomic data, they are too cumbersome, expensive, and time consuming for rapid diagnostic and screening purposes. PCR has traditionally been a highly utilized technique to differentiate between strains as it is relatively rapid and inexpensive. By combining multiple primers into a single multiplex-PCR, identification of clonal complexes, SCC*mec* types, and PFGE types is simple. Based on the above, a multiplex-PCR method was developed that differentiates USA300 from several of the other major MRSA epidemic clones.

Materials and Methods

PCR primers for amplifying the desired fragments were based on Zhang et al. (SCC*mec*IVa) (140), Zhang et al. (*arcA*) (141), Lina et al. (*lukS-PV* and *lukF-PV*) (142), Zhang et al. (*nuc*) (56), and Arakere et al. (*mecA*) (143) (Table 4-1). Primers were synthesized at the Penn State University Genomics Core Facility. Primers were mixed with 12.5 µl of Qiagen MasterMix (Qiagen, Inc., Valencia, CA) and 0.5 µl of extracted genomic DNA. PCR-cycling conditions were 95°C for 3 min, then 35 cycles (95°C for 45 s, 53°C for 45 s, 72°C for 1 min 30 s) followed by final extension at 72°C for 10 min in a Mastercycler thermocycler (Eppendorf Scientific, Hamburg, Germany). Positive controls consisted of NARSA and

CDC reference strains USA100, USA300, USA400, USA700 and USA800. The negative control consisted of the reaction mixture lacking template DNA. Five µl of the PCR products were mixed with 1.5 µl of loading buffer and electrophoresed on a 2% agarose gel. PCR products were stained with ethidium bromide and visualized on an UVP EC3 Imaging System (UVP; Upland, CA) after electrophoresis.

Table 4-1: Gene Primers for Novel Multiplex-PCR Method

Gene Target	Sequence (5'-3')	Amplicon Size (bp)	Concentration (µM)
SCC*mec*IVa	(F) GCCTTATTCGAAGAAACCG (R) CTACTCTTCTGAAAAGCGTCG	776	5
***arcA* gene on ACME**	(F) GCAGCAGAATCTATTACTGAGCC (R) TGCTAACTTTTCTATTGCTTGAGC	513	7.5
lukS-PV* and *lukF-PV	(F) ATCATTAGGTAAAATGTCTGGACATGATCCA (R) GCATCAAGTGTATTGGATAGCAAAAGC	433	5
nuc	(F) GCGATTGATGGTGATACGGTT (R) AGCCAAGCCTTGACGAACTAAAGC	219	2
mecA	(F) ACTGCTATCCACCCTCAAAC (R) CTGGTGAAGTTGTAATCTGG	169	5

Results

When tested on the reference strains, the novel multiplex-PCR method differentiated USA300 from all other MRSA epidemic clones including USA100, USA400, USA700, and USA800. Figure 4-1 shows the characteristic banding pattern for each of the reference strains tested. Table 4-2 details the markers used for each clone. The multiplex-PCR was validated using previously subtyped MRSA-positive isolates obtained from the Hershey Medical Center.

Table 4-2: Genetic Markers Present in Different MRSA Epidemic Clones

Epidemic MRSA Clone	Genetic Marker
USA100	*mecA, nuc*
USA300	*mecA, nuc, arcA, lukS-PV/lukF-PV, mec*IVa
USA400	*mecA, nuc, lukS-PV/lukF-PV, mec*IVa
USA700	*mecA, nuc, mec*IVa
USA800	*mecA, nuc*

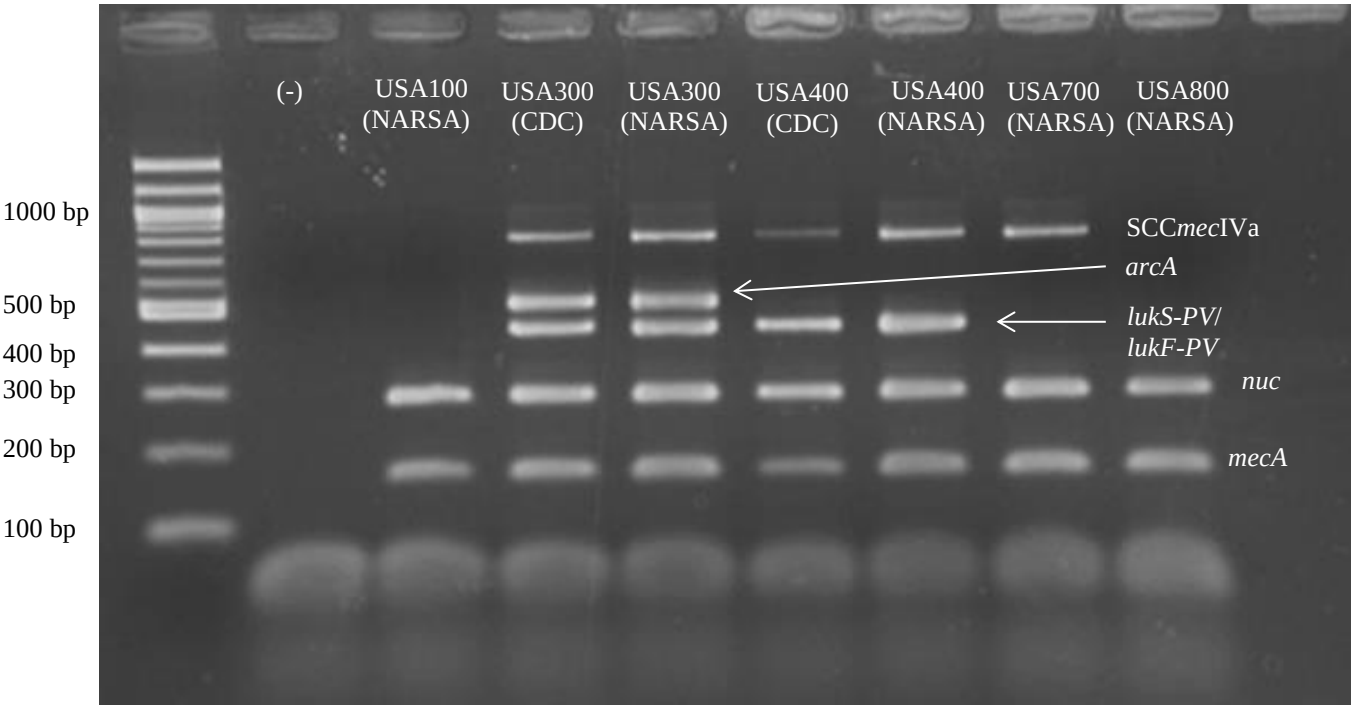

Figure 4-1: Novel Multiplex PCR Method Differentiates USA300 from other Epidemic Clones of MRSA. Genetic markers were detected at the following lengths: SCC*mec*IVa (776 bp), *arcA* (513 bp), *lukS-PV/lukF-PV* (433 bp), *nuc* (279), *mecA* (169 bp).

Discussion

The development of a novel multiplex-PCR method to detect USA300 from other epidemic MRSA clones will allow rapid screening, identification, and tracking of this MRSA clone within nosocomial settings. The novel multiplex-PCR differs from other standard typing methods including SCC*mec* typing, PFGE, and MLST in a variety of ways. From a practical standpoint, multiplex-PCR is by far the cheapest and fastest method of all four as it does not require sequencing. However, it is the least discriminatory method and is not capable of differentiating between strains within the same sequence type or clone, but it can provide real-time diagnostic and screening information to allow a more rapid response to USA300 infection.

There have been several multiplex-PCR methods developed in the past specifically for MRSA; however, many have focused on *mec* typing. Zhang et al. (140) developed a novel multiplex-PCR assay that targeted SCC*mec* types I through V in combination with the *ccr* gene complex. McClure et al. (148) developed a novel multiplex-PCR method to simultaneously detect PVL and differentiate between MRSA and MSSA. Schwalm et al. (149) developed a novel multiplex-PCR to detect MRSA clonal complexes. Other multiplex-PCR methods have been developed to detect a broad combination of genes (141). To date there are other PCR methods developed that can specifically differentiate USA300 MRSA from other epidemic clones (141, 150); both used *arcA* to test for the presence of USA300.

The novel multiplex-PCR method differs from previous methods in that the primary focus is not on SCC*mec* types or clonal clusters but rather on major MRSA epidemic clones. One of the USA300 PCR methods was developed as a singleplex to identify USA300 strains in order to later sequence a polymorphism in *pbp3* (150). As a result, it is not ideal for screening. The other USA300 specific PCR method is a complicated multiplex-PCR method developed to identify PVL-positive and –negative USA300 and USA400 strains (141). The novel multiplex-PCR method developed in this study is similar to the second; however, it is less specific and more focused on differentiated USA300 from other MRSA epidemic clones. Furthermore, PVL and SCC*mec*IV are strongly correlated with community-associated

MRSA clones so even though this novel multiplex-PCR method cannot provide detailed resistance profiles, it can separate community-associated MRSA isolates from hospital-associated MRSA based on electrophoresis staining patterns. This information may serve as a springboard for treatment options.

The novel multiplex-PCR method is much more discriminatory than many of the current epidemiological diagnostic techniques that often rely on plate assays (i.e. growth on selective media). It differentiates specific epidemic clones such including USA100 and USA300 but also provides important general information. In differentiating USA300 from the most common community-associated MRSA strains, as well as the multi-drug resistant hospital-associated MRSA strains, this novel multiplex-PCR method reduces diagnostic time and thus may increases positive health outcomes.

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ACADEMIC VITA

Elisabet Bjanes

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Education

Pennsylvania State University - Schreyer Honors College May 2014
B.S. Immunology & Infectious Diseases
B.S. Toxicology

Peking University; Beijing, China Summer 2012
- Cell, Cancer, & Developmental Biology - Three weeks of intensive study in Beijing. Class included trips to sites of cultural and historical significance: the Great Wall, Forbidden City, Summer Palace, etc.

Internships

Penn State Milton Hershey Medical Center
Summer Undergraduate Research Internship Program (SURIP) Summer 2013
- Researched necessary cell components for viral replication and trafficking

Pennsylvania Institute for Conservation Education Summer 2011
- Helped with administration, planning and running of two weeklong summer schools, and wrote grants
- Wrote an 82-page manual for future interns

Research Experience

Penn State Department of Food Science; Knabel Lab Group September 2012-2014
- Determined population evolution of clinical MRSA isolates using multi-virulence-locus-sequence typing
- Developed, optimized, and validated a multiplex PCR to detect USA300 MRSA strains

Penn State Milton Hershey Medical Center May 2013-August 2013
Department of Medicine, Division of Infectious Disease; Parent Lab Group
- Investigation into host cellular proteins that interact with and facilitate Mouse Mammary Tumor Virus (MMTV) replication, packaging and secretion using murine cell cultures and immunofluorescence.
- Determination of the interaction between MMTV svRNA and the endogenous retrotransposon LINE-1 using PCR mutagenesis and confocal microscopy

Penn State Department of Biochemistry & Molecular Biology; Tan Lab Group
Fall 2011-Fall 2012
- Tested the solubility of an over-expressed NSD1 protein domain implicated in childhood Sotos syndrome.
- Created truncations in hMSL1v1-MOFv1 complex to test strength of association with nucleosomes
- Optimized the expression growth of a number of protein complexes

Biology 220W & 230W - Phagehunter Lab Section; Thomas Lab Group
Spring and Fall 2011

- Isolated and purified a bacteriophage to infect a close relative of *Mycobacterium tuberculosis*.
- Sequenced and annotated the genome. Completed project sent to HHMI and NGRI for further testing.

Awards and Scholarships

- Dean's List — Fall 2010 through Fall 2013
- Fred Fotis Award — 2014
- Morrell Smith Scholarship — 2013-2014
- Horace T. Woodward Scholarship in College of Agriculture — 2013-2014
- NRHH Student of the Month (March) — 2013
- Gamma Sigma Delta's (College of Agriculture Honors Society) Outstanding Sophomore — 2011
- The President's Freshman Award — 2011
- STARS recipient — 2011
- Lancaster County Conservation District Counselor of the Year Award — 2011

Honors Societies

- National Society of Leadership and Success
- National Residence Hall Honorary
- Golden Key International Honor Society
- Phi Eta Sigma
- Gamma Sigma Delta
- National Society of Collegiate Scholars

Teaching Assistantship

MICROB 202: Introductory Microbiology Laboratory — Spring 2014
Instructor: Dr. Keiler

MICRB 410: **Principles of Immunology** — Fall 2013
Instructors: Dr. Sarkar & Dr. Kalia

KINES 17: Introduction to Ballroom — Spring and Fall 2013, Spring 2014
Instructor: Jolene Nickols

Activities

Lion Ambassador — 2013-2014
New Member Education Committee: Sourcebook Co-Chair — 2013-2014
Embassy Education Ad-Hoc Chair — 2013-2014
Guard the Lion Shrine Entertainment Subhead — Fall 2013

Association of Residence Hall Students
LateNight Cinemas Director — 2012-2014
Hired/managed weekly work schedule of seven employees, drafted budgets, handled all advertising and social media for the movies, coordinated logistics and managed relationship with vendors
South Halls Representative — 2011-2012

University Park Undergraduate Association
S-Book Committee: Content Editor 2014

Ballroom Dance Club
Keystone Classic Dance Competition Judging Coordinator 2012-2013
Drafted official contracts, coordinated housing and logistics for judges

Fencing Club
Secretary 2011-2012

South Halls Residence Association
Fundraising Chair 2011-2012
Building Representative 2010-2011

Biomedical Sciences Club
Undergraduate Conference Outreach Chair 2011-2012
Coordinated advertising, publicity, and awareness of conference

Schreyer Honors College Student Council
Merchandise Committee 2011-2012

Volunteerism

Schreyer Honors College Career Development Program Fall 2012, 2013-2014
Mentor

Welcome Week Crew Captain Fall 2012, 2013

Penn State IFC/Pan-Hellenic **Dance Marathon**, Public Relations 2011-2012
Photography Committee

Pennsylvania Institute for Conservation Education
Program Assistant July 2012 & 2013
Intern Summer 2011

Lancaster County Youth Conservation School
Senior Counselor July 2010 & 2011

Relevant Skills

Coursework: Mechanisms of Disease
The Immune System
Cancer Research and Medicine
Cell, Developmental, and Cancer Biology
Principles of Immunology
Bacterial Pathogenesis
Principles of Epidemiology
Introduction to Molecular Pharmacology
Principles of Toxicology
Immunotoxicology