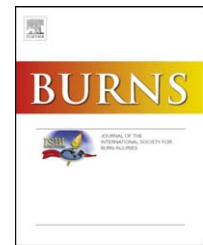


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Twenty-five year epidemiology of invasive methicillin-resistant *Staphylococcus aureus* (MRSA) isolates recovered at a burn center[☆]

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ABSTRACT

Over the past two decades, an epidemiologic emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) infections has occurred from that of primarily hospital-associated to community-associated. This emergence change has involved MRSA of different pulsed-field types (PFT), with different virulence genes and antimicrobial resistance patterns. In this study we, evaluate the changes in PFT and antimicrobial resistance epidemiology of invasive MRSA isolates over 25 years at a single burn unit. Isolates were tested by pulsed-field gel electrophoresis (PFGE), broth microdilution antimicrobial susceptibility testing, and PCR for the virulence factors Pantón–Valentine leukocidin (PVL) and arginine catabolic mobile element (ACME), and the resistance marker staphylococcal chromosomal cassette *mec* (*SCCmec*). Forty isolates were screened, revealing stable vancomycin susceptibility MIC without changes over time but decreasing susceptibility to clindamycin and ciprofloxacin. The majority of PFGE types were MRSA USA800 carrying the *SCCmec* I element and USA100 carrying the *SCCmec* II element. No strains typically associated with community-associated MRSA, USA300 or USA400, were found. USA800 isolates were predominately found in the 1980s, USA600 isolates were primarily found in the 1990s, and USA100 isolates were found in the 2000s. The PVL gene was present in only one isolate, the sole USA500 isolate, from 1987. The virulence marker ACME was not detected in any of the isolates. Overall, a transition was found in hospital-associated MRSA isolates over the 25 years, but no introduction of community-associated MRSA isolates into this burn unit. Continued active surveillance and aggressive infection control strategies are recommended to prevent the spread of community-acquired MRSA to this burn unit.

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1. Introduction

Staphylococcus aureus is a predominant cause of infections in patients with severe burns [1,2]. Between 1974 and 1998, the rate of *S. aureus* infections in patients in US burn units with burns was approximately 23% [2]. At the United States Army Institute of Surgical Research (USAISR), the rate of Gram-positive bacterial wound infections, primarily *S. aureus*, was 2% between 1982 and 1988 and 6% between 1989 and 1995 [3]. One of the major concerns with managing *S. aureus* infections is increasing antimicrobial resistance. Methicillin-resistant *S. aureus* (MRSA) was first noted in the United Kingdom in 1961 and soon thereafter around the rest of the world [4-6]. Although historically most MRSA isolates originated in the hospital (hospital-associated MRSA or HA-MRSA), more recently MRSA originating from the community (community-associated MRSA or CA-MRSA) has appeared [7-10]. Regional changes in antimicrobial resistance and molecular epidemiology have been noted in burn patient populations [11,12].

MRSA has been categorized into various PFT by the Centers for Disease Control and Prevention (CDC). These include the USA300 and USA400 types, which are strains typically associated with CA-MRSA. HA-MRSA strains are typically USA100, USA200, USA500, USA600, and USA800 [13]. Most resistance genes for *S. aureus* are carried on mobile genetic elements known as staphylococcal cassette chromosomes *mec* (SCC*mec*), of which there are various types (I-VII) of differing size and genetic composition. CA-MRSA typically carry SCC*mec* IV, V or VII elements while HA-MRSA typically carry the larger SCC*mec* I, II, III or VI elements and tend to carry resistance determinants in addition to *mecA*. In addition to varying antimicrobial resistance, the presence of certain virulence factors likely is associated with poorer clinical outcomes. The most notable protein possibly associated with increased virulence is Panton-Valentine leukocidin (PVL), which is cytotoxic to human monocytes, macrophages, and polymorphonuclear leukocytes through the formation of nonspecific pores in plasma membranes. PVL typically is associated with CA-MRSA isolates [14,15]. Arginine catabolic mobile element (ACME), which inhibits polymorphonuclear cell production, may also be associated with MRSA virulence and is typically found in USA 300 strains [14,16].

There are limited studies on the molecular epidemiology and impact of MRSA on medical facilities [17,18]. More recently burn units have reported increased rates of CA-MRSA USA300 isolates in association with wound infections and the presence of PVL genes [19,20]. In addition, evidence has shown the potential of aerosolization transmission of MRSA within a burn unit [21]. However, no systematic study has been performed in a single burn unit of invasive MRSA isolates over an extended period of time to assess changing molecular and antimicrobial resistance epidemiology. We evaluated MRSA isolates associated with invasive infections collected over a 25-year period for changes in antimicrobial resistance, PFT, and virulence factors to evaluate possible changing epidemiology.

2. Method

2.1. Burn unit

The USAISR Burn Center was established in 1947 at Fort Sam Houston, TX and has remained the only Department of Defense (DoD) burn referral center [22]. The USAISR burn center is currently a 40-bed unit located within Brooke Army Medical Center. In addition to serving DoD beneficiaries, it also serves a local civilian referral population from the southern Texas regional trauma system. In 1996, the USAISR Burn Unit relocated to a new facility with modern infection control measures to include private patient rooms, dedicated ante-rooms and sinks for handwashing. Aggressive infection control practices included contact isolation and strictly enforced hand hygiene practices especially after the move to the new facility. Varying topical antimicrobial creams were applied during hospitalizations at the discretion of the attending staff. Antimicrobial therapy has been variable during the history of the burn unit but vancomycin with an aminoglycoside have been the primary perioperative antimicrobial agents over the last 8 years with minimal to no use of cephalosporins. Care over the last 8 or more years has included early excision of full-thickness wounds with autograft coverage within 48 h of admission. If enough autograft was not available, remaining open wounds were covered with Integra (Integra Life Sciences, Plainsborough, NJ) or allograft skin until healing of donor sites for subsequent re-harvest. Grafted wounds were treated with vacuum dressings, mafenide acetate soaks, or silver impregnated dressings until postoperative day 4 when the wounds were inspected. Historically, skin touch preparation plates were used to assess for burn wound colonization but this has not been standard practice in the unit for the last 5 years. However, nasal swabs for MRSA colonization have been performed for approximately the last 5 years. Although nasal swab sampling for the presence of MRSA colonization was performed at the time of patient admission, colonized patients did not undergo decolonization or specific intervention as all patients were already placed within contact isolation upon admission.

2.2. MRSA isolates

The burn unit established a freezer storage system to maintain bacterial isolates recovered from burn patients as an infection control surveillance process. All *S. aureus* isolates recovered from clinical samples from patients treated in the USAISR Burn Center with bacteremia and/or pneumonia were stored in trypticase soy broth with 10% glycerol at -20°C . MRSA isolates from the initial positive blood or bronchoscopic pulmonary sample over the period 1982-2006 were evaluated for inclusion randomly selecting viable samples over the time period. All isolates were grown and subcultured once, identified by standard methodology, and screened by the disk diffusion method for resistance to cefoxitin according to CLSI guidelines to ensure they were MRSA. Cefoxitin-resistant isolates were then assessed for antimicrobial susceptibility by broth microdilution (BMD). Control organisms for the study were *S. aureus* ATCC 29213 for antimicrobial susceptibility testing, and *S. aureus* NCTC 8325 was used as reference standard for the Pulsed-field gel electrophoresis (PFGE).

2.3. Antimicrobial susceptibility testing

BMD testing was performed using CLSI criteria to determine antimicrobial susceptibility, broth microdilution performed in $\frac{1}{2}$ -log₂ dilutions [23]. The antimicrobial agents tested included vancomycin, daptomycin, linezolid, clindamycin, erythromycin, doxycycline, minocycline, trimethoprim-sulfamethoxazole, ciprofloxacin, rifampin, tigecycline and gentamicin. Wells containing daptomycin were supplemented with 50 µg/ml calcium, and panels were stored at -70 °C. Direct suspensions of log-phase colonies were made in cation-adjusted Mueller-Hinton broth to 0.5 McFarland standard, then diluted in 0.9% saline to achieve a final concentration of approximately 5×10^5 CFU/ml. Inoculated BMD panels were incubated at 35 °C for 20–22 h prior to visual interpretation of minimum inhibitory concentrations (MICs). A tigecycline Etest (AB Biodisk, Solna, Sweden) was performed for each isolate. Antimicrobial susceptibilities were assessed over four intervals: 1982–1987, 1988–1993, 1994–1999, and 2000–2006. For each interval, susceptibilities for each antimicrobial agent were compared by MIC₅₀ and MIC₉₀, the MIC for which 50% and 90% of isolates were susceptible, respectively; and by range of MICs observed. Inducible clindamycin resistance was tested by D-zone as previously reported [24].

2.4. Pulsed-field gel electrophoresis and molecular assessment of virulence and resistance

Isolates obtained during this study were assessed by PFGE following *Sma*I restriction enzyme digestion [13]. PFGE gels were interpreted and grouped into PFT using established criteria [13,25]. CDC epidemiologic database of *S. aureus* lineage was used for comparisons.

Genomic DNA was extracted from overnight cultures using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. All PCRs were conducted with Eppendorf Master Mix (Eppendorf, Hamburg, Germany) containing 1.25 U Taq DNA polymerase, 1.5 mM Mg²⁺ and 200 µM final concentration of each dNTP in a final volume of 25 µl.

A multiplex PCR, to simultaneously detect *mec* element types was performed as described elsewhere (control strains obtained from Herimina de Lencastre) [26]. This multiplex SCC*mec* PCR included eight loci and the *mecA* gene as an internal control and detects SCC*mec* I, II, III and IV. The following MRSA strains were used as positive amplification controls for all SCC*mec* PCR runs: COL for SCC*mec* I; HPV107 for SCC*mec* IA; ANS46 for SCC*mec* III; HU25 for SCC*mec* IIIA; HDG2 for SCC*mec* IIIB; BK2464 for SCC*mec* II; BM18 and MW2 for SCC*mec* IV.

The PVL gene was detected using 1 µM of primers PVL-1, 5'-CTGGTGCATTTCATGGTA-3', and PVL-2, 5'-CGATATCGTGGT-CATCACA-3', generating a 3524 bp product. The fragments were amplified using the following PCR conditions: 94 °C for 4 min followed by 35 cycles of 30 s at 94 °C, 3 min at 60 °C and 3 min at 72 °C followed by a final extension 4 min at 72 °C. *S. aureus* ATCC 49775 was used as positive control for the PVL PCR. The PVL PCR product was purified using the QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA) and sequenced using the Genome Lab DTCS Quick Start Kit and the automated CEQ8000 Genetic Analysis System (Beckman Coulter Inc., Fullerton, CA).

All isolates were screened for the presence of the *arcA* gene by PCR using the primer pair *arcA*-F 5'-GAGCCAGAAG-TACGCGAG-3' and *arcA*-R 5'-CACGTAACCTTGCTAGAACGAG-3'. The *arcA* gene belongs to the *arc* gene cluster which is a surrogate marker for type I ACME [14]. Amplification was carried out at 94 °C for 2 min followed by 30 cycles with denaturation at 94 °C for 20 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min using 400 nM of each primer resulting in a product of 724 bp. *S. epidermidis* ATCC 12228 was used as positive control for *arcA* PCR. All PCR products were resolved by electrophoresis through 1.2% or 2% agarose gels and visualized with ethidium bromide.

3. Results

3.1. Antimicrobial susceptibility testing

We tested 40 isolates representing the various time periods from 1982 to 2006. All but four isolates, two each from 1995 and 1996, were from a bloodstream source. Representative isolates from the various time periods did not demonstrate *in vitro* resistance or global increase in MIC to vancomycin, daptomycin, linezolid, tigecycline, or rifampin (Table 1). Resistance to clindamycin and ciprofloxacin increased over the study intervals from 61.5% to 100% and 7.7% to 100%, respectively, while gentamicin resistance decreased from 100% to 25%. Susceptibility to doxycycline and minocycline was demonstrated for all but two early isolates. Resistance to erythromycin was universal. The percentage of isolates exhibiting inducible resistance to clindamycin was 39% for the period 1982–1987, 20% for 1988–1993, 25% for 1994–1999, and 33% for 2000–2006.

3.2. Pulsed-field gel electrophoresis (PFGE) and molecular assessment of virulence and resistance

There were no CA-MRSA isolates based on PFGE pattern (Fig. 1). There were primarily 2 USA pulsed-field types: USA800 (21 isolates) and USA100 (11 isolates). All of the USA800 isolates were from 1982 to 1993 while the USA100 isolates included 1 isolate from 1982 to 1987, 2 isolates from 1994 to 1999 and 8 isolates from 2000 to 2006 (Fig. 2). There were 4 USA600 isolates from 1994 to 1999, 1 USA500 isolate from 1982 to 1987, and 2 USA200 isolates from 1994 to 1999. We were unable to categorize one isolate into a USA PFT.

The SCC*mec* types were assessed in all isolates. All of the USA800 isolates carried the SCC*mec* I element, while all the USA100 isolates carried the SCC*mec* II element. The SCC*mec* II element was also found in all USA600 isolates, all USA200 isolates and the unknown USA PFT isolate.

ACME was not found in any of the isolates and the PVL gene was detected only in the USA500 isolate from 1987. The presence of the PVL gene was confirmed by sequencing of the PCR product and comparison with the PVL gene region of USA300 FPP3757 (Accession # CP000255, data not shown). The USA500 isolate was notable as it carried the SCC*mec* IV element, the PVL gene and was also enterotoxin A (SEA) and SEB positive (data not shown).

Table 1 – MIC₅₀/MIC₉₀, MIC range, and percentage of isolates resistant by antimicrobial agent during each 5-year range, expressed in micrograms per milliliter.

Antimicrobial agent	1982–1987 (13 isolates)				1988–1993 (10 isolates)				1994–1999 (8 isolates)				2000–2006 (9 isolates)			
	MIC ₅₀	MIC ₉₀	MIC range	%R	MIC ₅₀	MIC ₉₀	MIC range	%R	MIC ₅₀	MIC ₉₀	MIC range	%R	MIC ₅₀	MIC ₉₀	MIC range	%R
Vancomycin	0.75	0.75	0.75–0.75	0	0.75	0.75	0.75–0.75	0	0.75	1	0.38–1	0	0.75	0.75	0.38–0.75	0
Daptomycin	0.5	1	0.5–1	0	0.5	0.5	0.5–1	0	0.5	1	0.5–1	0	0.5	1	0.5–1	0
Linezolid	2	4	2–4	0	2	4	2–4	0	4	4	2–4	0	4	4	4–4	0
Tigecycline	0.125	0.125	0.094–0.38	0	0.094	0.125	0.094–0.19	0	0.19	0.19	0.125–0.19	0	0.125	0.19	0.094–0.19	0
Rifampin	0.25	0.25	0.25–0.25	0	0.25	0.25	0.25–0.25	0	0.25	0.25	0.25–0.25	0	0.25	1	0.25–1	0
Clindamycin	>8	>8	0.12–>8	61.5	>8	>8	0.25–>8	90	>8	>8	16–16	100	>8	>8	16–16	100
Doxycycline	0.25	0.25	0.25–>16	7.7	0.25	0.25	0.25–0.25	0	0.25	4	0.12–4	0	0.25	0.25	0.12–0.25	0
Minocycline	0.06	0.06	0.06–>8	7.7	0.06	0.06	0.06–0.06	0	0.06	0.25	0.06–0.25	0	0.06	0.12	0.06–0.12	0
Trim–sulfa	0.12	>8	0.12–>8	15.4	0.25	0.25	0.12–0.25	0	>8	>8	0.12–>8	55.6	0.12	>8	0.12–>8	12.5
Ciprofloxacin	0.25	0.5	0.25–16	7.7	0.25	0.25	0.25–>16	10	>16	>16	>16–>16	100	>16	>16	>16–>16	100
Gentamicin	>8	>8	>8–>8	100	>8	>8	>8–>8	100	>8	>8	1–>8	66.7	1	>8	0.5–>8	25
Erythromycin	>64	>64	64–>64	100	>64	>64	>64–>64	100	>64	>64	>64–>64	100	>64	>64	>64–>64	100

Note: MIC₅₀ and MIC₉₀, MIC for which 50% and 90% of isolates are susceptible, respectively; %R, percentage of isolates resistant; Trim-sulfa, trimethoprim-sulfamethoxazole (based on trimethoprim component at a 1:19 ratio).

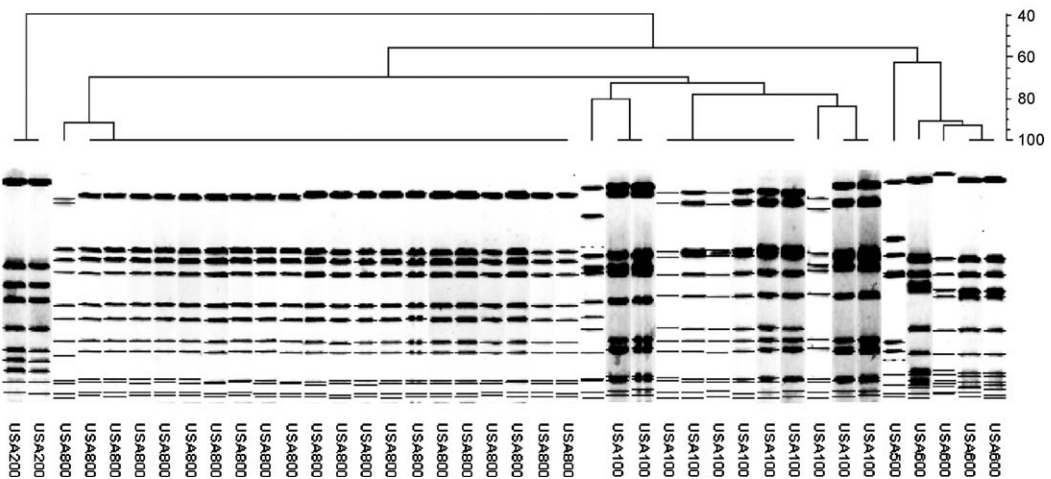


Fig. 1 – Dendrogram of USA PFTs of 40 MRSA isolates.

4. Discussion

The etiology of MRSA infections continues to emerge around the world from primarily HA-MRSA, traditionally known as less virulent strains with broader spectrum of antimicrobial resistance, to CA-MRSA, that are associated with more virulence factors but a narrower spectrum of antimicrobial resistance. Reports have described changing MRSA epidemiology in burn units, but this is the first study to assess this over an extended (25-year) period. Overall, MRSA strains associated with community acquisition were not found to have been introduced into this burn unit, but there were epidemiological changes in the types of HA-MRSA strains in the facility over the past 25 years with an associated change in antimicrobial resistance patterns.

The primary HA-MRSA strains identified in this study, USA800 and USA100, carry the resistance genes SCCmec I and II, respectively. The antimicrobial resistance patterns of the USA800 isolates in this study are consistent with those reported for other USA800 isolates including erythromycin, clindamycin and gentamicin resistance, similar to the first

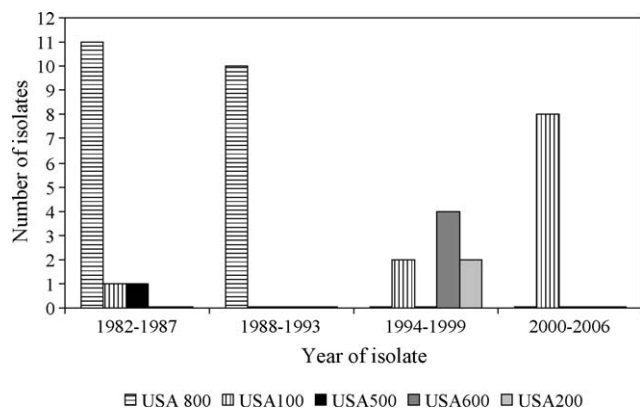


Fig. 2 – USA pulsed-field types by year.

MRSA isolates from the 1960s [13,27]. USA100 isolates in this study have antimicrobial resistance profiles similar to other USA100 isolates including erythromycin, clindamycin and fluoroquinolone resistance [13]. The presence of the SCCmec II element appears to have been introduced into healthcare facilities around 1982 [27]. SCCmec II first appeared in our isolates in 1987, and did not become common in this burn unit until the 2000s. Overall, it was encouraging that the vancomycin MIC was stable at 0.75 µg/ml over the entire study period, supporting other studies indicating a stable vancomycin MIC over time although this finding is not found in all studies [28].

It was not surprising that there was a lack of PVL or ACME genes present in the isolates tested as these are typically associated with CA-MRSA isolates. The PVL positive USA500 isolate was atypical as it was difficult to delineate the presence of PVL, as some primer sets detected the gene while others did not. But the presence of the PVL gene was finally confirmed by sequencing. Work is ongoing with the genetic structure of this isolate, but it did not become the predominant type in our facility or appear to transfer its virulence factors to other isolates. Studies from Japan have supported the hypothesis that PVL genes in CA-MRSA isolates could have originated from methicillin-susceptible *S. aureus* [27].

Although this is the first assessment of isolates from a burn unit over a 25-year-time period using state of the art technology, there were a number of limitations to the study. There were only 40 bacteria tested; however, there was relatively equal representation of isolates over the various time periods during the 25 years of the study. Although bloodstream and pulmonary isolates were studied together, the pulmonary samples both were from invasive bronchoscopically obtained samples and represented a minority of the total isolates. In addition, specific management strategies of burn wound care were not available. It is unclear what role current strategies of aggressive infection control, early escharotomy, and frequent exposure of patients to vancomycin or other broad-spectrum antimicrobial agents including use of topical antimicrobials influenced MRSA colonization or infection. Further studies in this area are required.

In conclusion, there has not been the introduction of strains associated with community onset MRSA infections into this burn unit, although the HA-MRSA strains have

changed over the 25 years studied. Without the introduction of CA-MRSA isolates into the facility, there has also been a lack of isolates with enhanced virulence. Further studies are necessary to compare the rate of CA-MRSA colonization at time of admission to the burn unit and the impact of ongoing interventions that appear to mitigate the development of CA-MRSA infections in the burn unit.

Conflict of interest

The authors have no financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work.

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