Published in final edited form as:

Pediatr Infect Dis J. 2011 May ; 30(5): 418–421. doi:10.1097/INF.0b013e31820d7fd5.

Molecular Distinctions Exist between Community-Associated Methicillin Resistant *Staphylococcus aureus* Colonization and Disease-Associated Isolates in Children

ISAAC THOMSEN, MD, BRIAN D. MCKENNA, BS, ELIZABETH J. SAYE, BS, NATALIA JIMENEZ, MSCI, KATHRYN M. EDWARDS, MD, and C. BUDDY CREECH, MD, MPH

Department of Pediatrics, Pediatric Infectious Diseases and the Vanderbilt Vaccine Research Program, Vanderbilt University School of Medicine and the Monroe Carell, Jr. Children's Hospital at Vanderbilt, Nashville, Tennessee

Abstract

Objective—To define the molecular epidemiology of colonization and disease-associated isolates of CA-MRSA.

Design—Laboratory-based comparative study of clinical staphylococcal isolates.

Methods—We analyzed 255 pediatric CA-MRSA isolates for molecular characteristics associated with colonization and disease. We used PCR to determine the presence of Pantone-Valentine Leukocidin (PVL) and the lantibiotic element, *bsaB*, and to characterize the *SCCmec* type and accessory gene regulator locus. Pulsed-field gel electrophoresis (PFGE) was used to determine genetic relatedness between strains.

Results—150 isolates were obtained from patients with clinical disease (37 invasive infections, 113 non-invasive infections) and 105 from subjects with nasal colonization alone. 123/150 (82%) of disease-associated isolates belonged to PFGE group USA300 while only 19/105 (18%) of colonization isolates were of the USA300 lineage. Colonization isolates were less likely to possess *SCCmec* type IV, Pantone-Valentine Leukocidin (PVL), or *agr* Type 1 ($p < 0.001$).

Conclusions—Colonization strains of CA-MRSA in children differ significantly from those strains recovered from patients with staphylococcal infections. This suggests that only colonization with specific strain types, rather than MRSA colonization, in general, increases the risk for CA-MRSA disease.

Keywords

CA-MRSA; MRSA; Molecular Epidemiology; Colonization; aureus; *S. aureus*

Introduction

Each year in the US, nearly 20,000 individuals die as a result of infection with methicillin-resistant *Staphylococcus aureus* (MRSA).¹ These infections, once limited to the healthcare setting, have now emerged as one of the most common community-acquired bacterial

Correspondence: C. Buddy Creech, MD, MPH, Assistant Professor, Pediatric Infectious Diseases, Assistant Director, Vanderbilt Vaccine Research Program, Program Co-Director, Pediatric Infectious Diseases Fellowship, Vanderbilt University School of Medicine and the Monroe Carell, Jr. Children's Hospital at Vanderbilt, 1161 21st Avenue South, CCC-5311 MCN Nashville, Tennessee 37232-2573, Phone: 615-343-0332 Fax: 615-343-4738, buddy.creech@vanderbilt.edu.

Conflicts of Interest: None

infections, complicating treatment strategies and taxing our infection control practices. At the same time, there has been an increase in the frequency of asymptomatic MRSA nasal carriage. Previous data from our group estimate an MRSA carriage frequency of nearly 10% in otherwise healthy children,² though rates as high as 22% have been reported in hospitalized children.³ What remains unclear is whether molecular distinctions exist between strains more likely to result in asymptomatic carriage and strains more likely to cause disease.

The primary aim of this study was to expand on previous work by determining the molecular characteristics of MRSA isolated from asymptomatic carriers and from patients with acute MRSA disease in the same geographic region. The study was designed to test the hypothesis that the molecular characteristics of colonization strains of MRSA are different than disease-associated strains, particularly those virulence factors associated with colonization fitness, disease pathogenesis, and regulation of virulence factor expression. This study focused on Panton-Valentine Leukocidin (PVL), a potent cytolytic toxin highly associated with CA-MRSA,⁴⁻⁷ but whose pathogenic role in MRSA infections is uncertain;⁸⁻¹² *bsaB*, a lantibiotic-encoding element (antimicrobial peptide) and putative virulence factor in CA-MRSA strains;^{13, 14} assessment of variations in the accessory gene regulatory locus (*agr*), a global regulator of staphylococcal virulence factor expression; and determination of the presence of secreted toxins such as toxic shock syndrome toxin 1 (TSST-1), the staphylococcal enterotoxins, and the exfoliative toxins.

Methods

Since 2003, we have maintained an archival collection of CA-MRSA isolates, confirmed by PCR to contain *mecA*. The collection includes colonization isolates from previously reported studies^{2, 15} and clinical isolates from pediatric patients cared for at Monroe Carell, Jr. Children's Hospital at Vanderbilt since 2003. Approved by the Vanderbilt Institutional Review Board, this collection is maintained as a de-identified repository with only the source of the isolate retained.

For this study, 255 distinct isolates were evaluated. This included 150 disease-associated isolates and 105 nasal colonization isolates (55 isolates from 2003–2006, 50 isolates from 2006–2008). The two time periods for colonization isolates were selected *a priori* to determine if significant epidemiologic changes occurred during the study period. All isolates were numbered sequentially and a random number generation scheme (Microsoft Excel) was used to select isolates for inclusion in the study. Of the 150 disease-associated isolates, 37 were from invasive infections. Sites of infection included septic arthritis (18), pneumonia or parapneumonic effusion (6), osteomyelitis without arthritis (4), bacteremia (3), meningitis (2), CVL-related bacteremia (3), and pelvic abscess (1). Non-invasive isolates (n=113) were isolated from patients with localized skin and soft tissue infections without evidence of invasion or other organ system involvement. No duplicate isolates were included in the study.

Confirmation of MRSA from archived samples was performed by plating samples onto paired mannitol salt agar plates, with and without 4 µg/ml of oxacillin (Hardy Diagnostics, Santa Maria, CA). After incubation at 37 C for 48 hours and at room temperature for 18 hours, plates were inspected for yellow colonies indicative of mannitol fermentation, characteristic of *S. aureus*. After subculturing onto tryptic soy agar with 5% sheep blood (Hardy Diagnostics, Santa Clara, CA), rapid latex agglutination testing for clumping factor and protein A was performed on all isolates (Staphaurex Plus; Remel, Lenexa, KS). Following phenotypic confirmation of *S. aureus*, crude genomic DNA was prepared by incubating the isolates with lysostaphin for one hour at 37°C and heating the samples to

95°C for 15 minutes. This template DNA was used to detect the presence of the *mecA* gene using previously described oligonucleotide primer sequences.¹⁶

Purified genomic DNA (Promega, Madison, WI) was used as the template for PCR detection of genes encoding Panton-Valentine Leukocidin (PVL),⁴ determination of the accessory gene regulator (*agr*) locus type,¹⁷ and detection of *bsaB* (using novel oligonucleotide primers developed from BLAST sequences of CA-MRSA strains, forward 5'-CGCCAATGAGTGCAGATG-3'; reverse 5'-CGGGTCCTCGATAAGATG-3').

Assignment of SCC*mec* type was determined using the multiplex strategy of Oliveira and de Lencastre.¹⁸ For those strains unable to be characterized by the multiplex strategy, *ccr* and *mec* complex typing were performed as previously described.¹⁹ To assess genetic relatedness between isolates, the Tennessee State Microbiology Laboratory performed pulsed-field gel electrophoresis (PFGE). Assignment of PFGE type was provided by the Centers for Disease Control and Prevention using the criteria of McDougal et al.²⁰ For colonization isolates in the later time period (2006–8), repetitive element, sequence-based polymerase chain reaction (DiversiLab System; Biomerieux, Durham, North Carolina) was used to determine genetic relatedness between strains.²¹

When comparing disease-associated strain characteristics with colonization strains, invasive and non-invasive isolates were combined. Differences between groups were calculated using Pearson's chi-square or Fisher's exact test, with p-values <0.05 considered to be statistically significant. For situations in which multiple comparisons were made, a Bonferroni correction was applied. Stata 8.0 for Windows was used for statistical analysis.

Results

Invasive and non-invasive disease-associated isolates were first compared with each other based on their SCC*mec* region and targeted virulence factor repertoire. These results are presented in Table 1. There were no significant differences in SCC*mec* type, presence of PVL (though neither invasive CSF isolate was positive for PVL), *agr* locus type, or presence of *bsaB* between invasive and non-invasive disease associated isolates. There were also no significant differences in the frequency of specific exotoxins except for enterotoxin G, found more frequently in invasive isolates than non-invasive isolates (46% vs. 21%, p=0.003, Table 2). Due to the overall homogeneity of the invasive and non-invasive disease-associated isolates, these isolates were considered "disease-associated" isolates and grouped for comparison to colonization isolates.

To determine whether colonization isolates were the same as disease-associated isolates, the same series of experiments was performed. When compared with the disease-associated isolates, colonization isolates were significantly less likely to possess SCC*mec* IV, PVL, *bsaB*, or *agr* Type I (Table 1). In disease-associated isolates, 126/130 (97%) of organisms possessing *agr* type 1 also contained *bsaB*; all 33 invasive isolates that were *agr* type 1 possessed *bsaB*. In contrast, only 13/23 (57%) of the colonization-associated isolates that possessed *agr* type 1 contained *bsaB* (p<0.001).

To determine whether USA300 colonization isolates possessed a different virulence factor repertoire than the predominantly USA300 disease-associated cohort, the same analysis was performed. With the exception of one isolate that harbored TSST-1, enterotoxin C, and enterotoxin G, no USA300 colonization isolate contained genes for any of the toxins assessed (Table 2).

Genetic Relatedness

To determine if disease-associated and colonization-associated isolates belonged to the same genetic background, genotyping was performed by pulsed-field gel electrophoresis. The majority of disease-associated isolates (123 of 150) belonged to USA300 (Supplemental Figures 1a and 1b), the most commonly circulating CA-MRSA lineage in the United States; however, there was significant heterogeneity in colonization isolates (Supplemental Figure 1c). Overall, colonization isolates were less likely to belong to the USA300 pulsed-type ($p < 0.001$)

Differences in Colonization Isolates Over Time

To assess whether disease-associated strain types might become the predominant nasal flora in our community, we studied an additional 50 isolates, obtained two years after the initial collection of colonization strains (2006–2008). Colonization strains remained diverse, with multiple strain types represented (USA300 [14%], USA400 [16%], USA500 [24%], and USA 700 [28%], Supplemental Figure 1d). There was a significant increase in the presence of *SCCmec* IV in the colonization isolates from 2006–8 (91% vs. 47%, $p < 0.001$); however, there were no significant differences in the other factors studied, when compared with isolates from the original sample (2003–6, Table 1).

Discussion

In this study, we demonstrate that the molecular characteristics of disease-associated and colonization-associated MRSA were distinct. Disease-associated isolates were more likely to be USA300, contain an *SCCmec* IV cassette, and possess PVL, *bsaB*, and *agr* type I. Invasive USA300 isolates were more likely to possess SEG than either non-invasive USA300 isolates or USA300 colonization isolates. With time, the characteristics of colonization isolates changed significantly, with more isolates harboring an *SCCmec* IV cassette; however, these isolates continued to be less likely to possess PVL, *agr* type I, and *bsaB* than disease-associated isolates.

These data expand to pediatrics the findings of Ellis *et al.*,²² who recently described the molecular epidemiology of CA-MRSA isolated from skin and soft tissue infections and the anterior nares in US soldiers. Of 3,447 soldiers enrolled, 134 (3.9%) had MRSA colonization; 39 subjects developed MRSA skin and soft tissue infections. Isolates belonging to the USA300 PFGE type were recovered from 97% of abscesses, compared with only 53% of colonization isolates ($p < 0.001$). Additionally, colonization isolates were less likely to contain PVL (49% vs. 97%, $p < 0.001$) and *SCCmec* IV (89% vs. 100%, $p = 0.03$).

The heterogeneity observed within colonization isolates generates the hypothesis that certain strain-types, possessing a specific combination of virulence determinants (e.g., USA300, *SCCmec* IV), may be more adept at promoting staphylococcal disease. This may provide an explanation for why some individuals have asymptomatic colonization with *S. aureus* while others develop clinical disease, as colonization with less virulent strain-types might be associated with a reduced risk of subsequent infection. This has significant implications for strategies aimed at preventing or reducing colonization in those in whom MRSA is detected. Since some individuals are more likely to be carriers than others,²³ understanding the strain types they harbor may be more important for disease prevention than the simple denotation of carrier/non-carrier. For instance, in a subject with stable long-term colonization of a non-USA300 strain and no significant risk factors for MRSA disease, decolonization may be unnecessary. Conversely, the identification of an individual colonized with a USA300, *SCCmec* IV, PVL+ strain may provide an opportunity for pre-emptive decolonization,

particularly if the individual belongs to a group with increased risk for CA-MRSA disease (e.g., military personnel, athletes, neonates).

One limitation in our study is the lack of colonization isolates from extranasal sites, such as the axilla, perineum, or oropharynx. Recent work by Chen et al²⁴ demonstrates discordance between MRSA wound isolates and MRSA nasal colonization isolates. Of 95 children enrolled with SSTI, 40 children (42%) grew *S. aureus* from anterior nares cultures. While MRSA nasal colonization was more common in children with concomitant MRSA SSTI vs. methicillin-susceptible *S. aureus* SSTI (31% vs. 5%, $p=0.021$), PFGE concordance was present in only 67% of paired nasal/wound cultures. This suggests that either colonization was not detected due to sampling techniques (e.g., lack of broth enrichment of nasal swabs prior to primary plating),^{25, 26} MRSA nasal colonization does not always precede infection, or that extranasal sites of colonization play a more important role in CA-MRSA skin and soft tissue infection than previously estimated. Regardless, it is clear that substantial heterogeneity exists within pediatric CA-MRSA isolates and future work should focus on defining the relationship between nasal and extranasal sites of colonization.

This was a retrospective study; as a result, it is unknown whether individuals colonized with USA300 CA-MRSA developed infection with a USA300 CA-MRSA strain. Therefore, we cannot assess whether carriage of a non-USA300 strains is protective against colonization or infection from other MRSA strain-types. In addition, colonization isolates were randomly selected from a staphylococcal isolate library in which not all subjects were followed over time. As a result, we cannot confirm in all cases that a specific colonization isolate did not eventually lead to staphylococcal disease; however, for approximately half of the isolates, at least 6 months of follow-up data were available and no suspected staphylococcal infections occurred. Last, there are many virulence factors predicted to be important in CA-MRSA disease that were not assessed; instead, we chose characteristics that were representative of specific properties of *S. aureus*. There are undoubtedly additional staphylococcal virulence determinants, as well as host factors, that promote the transition from colonization to disease.

In summary, in this collection of pediatric MRSA isolates, there were significant molecular differences between colonization and disease-associated isolates of MRSA. Nearly all MRSA infection isolates belonged to the USA300 PFGE type while colonization isolates were considerably heterogeneous. Among the USA300 MRSA colonization isolates, virulence markers such as PVL and the staphylococcal enterotoxins were less common. This could have important implications in both infection control and vaccine development. Additional prospective studies of staphylococcal colonization and infection are critically needed to determine the most effective ways to combat this pathogen.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial Support: National Institutes of Health Contract N01-AI-25462 to K.M.E.

References

1. Klevens RM, Morrison MA, Nadle J, et al. Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. JAMA. 2007; 298:1763–71. [PubMed: 17940231]

2. Creech CB 2nd, Kernodle DS, Alsentzer A, Wilson C, Edwards KM. Increasing rates of nasal carriage of methicillin-resistant *Staphylococcus aureus* in healthy children. *Pediatr Infect Dis J*. 2005; 24:617–21. [PubMed: 15999003]
3. Alfaro C, Mascher-Denen M, Fergie J, Purcell K. Prevalence of methicillin-resistant *Staphylococcus aureus* nasal carriage in patients admitted to Driscoll Children's Hospital. *Pediatr Infect Dis J*. 2006; 25:459–61. [PubMed: 16645516]
4. Lina G, Piemont Y, Godail-Gamot F, et al. Involvement of Pantone-Valentine leukocidin-producing *Staphylococcus aureus* in primary skin infections and pneumonia. *Clin Infect Dis*. 1999; 29:1128–32. [PubMed: 10524952]
5. Hsu LY, Koh TH, Anantham D, Kurup A, Chan KP, Tan BH. Pantone-Valentine leukocidin-positive *Staphylococcus aureus*, Singapore. *Emerg Infect Dis*. 2004; 10:1509–10. [PubMed: 15503401]
6. Baggett HC, Hennessy TW, Rudolph K, et al. Community-onset methicillin-resistant *Staphylococcus aureus* associated with antibiotic use and the cytotoxin Pantone-Valentine leukocidin during a furunculosis outbreak in rural Alaska. *J Infect Dis*. 2004; 189:1565–73. [PubMed: 15116291]
7. Voyich JM, Otto M, Mathema B, et al. Is Pantone-Valentine leukocidin the major virulence determinant in community-associated methicillin-resistant *Staphylococcus aureus* disease? *J Infect Dis*. 2006; 194:1761–70. [PubMed: 17109350]
8. Bubeck Wardenburg J, Palazzolo-Ballance AM, Otto M, Schneewind O, DeLeo FR. Pantone-Valentine leukocidin is not a virulence determinant in murine models of community-associated methicillin-resistant *Staphylococcus aureus* disease. *J Infect Dis*. 2008; 198:1166–70. [PubMed: 18729780]
9. Cremieux AC, Dumitrescu O, Lina G, et al. Pantone-valentine leukocidin enhances the severity of community-associated methicillin-resistant *Staphylococcus aureus* rabbit osteomyelitis. *PLoS One*. 2009; 4:e7204. [PubMed: 19779608]
10. Varshney AK, Martinez LR, Hamilton SM, et al. Augmented production of Pantone-Valentine leukocidin toxin in methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* is associated with worse outcome in a murine skin infection model. *J Infect Dis*. 2010; 201:92–6. [PubMed: 19929693]
11. Loffler B, Hussain M, Grundmeier M, et al. *Staphylococcus aureus* pantone-valentine leukocidin is a very potent cytotoxic factor for human neutrophils. *PLoS Pathog*. 2010; 6:e1000715. [PubMed: 20072612]
12. Diep BA, Otto M. The role of virulence determinants in community-associated MRSA pathogenesis. *Trends Microbiol*. 2008; 16:361–9. [PubMed: 18585915]
13. Baba T, Takeuchi F, Kuroda M, et al. Genome and virulence determinants of high virulence community-acquired MRSA. *Lancet*. 2002; 359:1819–27. [PubMed: 12044378]
14. Daly KM, Upton M, Sandiford SK, et al. Production of the Bsa lantibiotic by community-acquired *Staphylococcus aureus* strains. *J Bacteriol*. 2010; 192:1131–42. [PubMed: 20023032]
15. Creech CB 2nd, Johnson BG, Alsentzer AR, Hohenboken M, Edwards KM, Talbot TR 3rd. Vaccination as infection control: a pilot study to determine the impact of *Staphylococcus aureus* vaccination on nasal carriage. *Vaccine*. 2009; 28:256–60. [PubMed: 19799842]
16. Nakamura MM, Rohling KL, Shashaty M, Lu H, Tang YW, Edwards KM. Prevalence of methicillin-resistant *Staphylococcus aureus* nasal carriage in the community pediatric population. *Pediatr Infect Dis J*. 2002; 21:917–22. [PubMed: 12394812]
17. Shopsin B, Mathema B, Alcades P, et al. Prevalence of agr specificity groups among *Staphylococcus aureus* strains colonizing children and their guardians. *J Clin Microbiol*. 2003; 41:456–9. [PubMed: 12517893]
18. Oliveira DC, de Lencastre H. Multiplex PCR strategy for rapid identification of structural types and variants of the mec element in methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother*. 2002; 46:2155–61. [PubMed: 12069968]
19. Kondo Y, Ito T, Ma XX, et al. Combination of multiplex PCRs for staphylococcal cassette chromosome mec type assignment: rapid identification system for mec, ccr, and major differences in junkyard regions. *Antimicrob Agents Chemother*. 2007; 51:264–74. [PubMed: 17043114]

20. McDougal LK, Steward CD, Killgore GE, Chaitram JM, McAllister SK, Tenover FC. Pulsed-field gel electrophoresis typing of oxacillin-resistant *Staphylococcus aureus* isolates from the United States: establishing a national database. *J Clin Microbiol.* 2003; 41:5113–20. [PubMed: 14605147]
21. Tenover FC, Gay EA, Frye S, Eells SJ, Healy M, McGowan JE Jr. Comparison of typing results obtained for methicillin-resistant *Staphylococcus aureus* isolates with the DiversiLab system and pulsed-field gel electrophoresis. *J Clin Microbiol.* 2009; 47:2452–7. [PubMed: 19553588]
22. Ellis MW, Griffith ME, Jorgensen JH, Hospenthal DR, Mende K, Patterson JE. Presence and molecular epidemiology of virulence factors in methicillin-resistant *Staphylococcus aureus* strains colonizing and infecting soldiers. *J Clin Microbiol.* 2009; 47:940–5. [PubMed: 19213694]
23. van Belkum A, Verkaik NJ, de Vogel CP, et al. Reclassification of *Staphylococcus aureus* nasal carriage types. *J Infect Dis.* 2009; 199:1820–6. [PubMed: 19419332]
24. Chen AE, Cantey JB, Carroll KC, Ross T, Speser S, Siberry GK. Discordance between *Staphylococcus aureus* nasal colonization and skin infections in children. *Pediatr Infect Dis J.* 2009; 28:244–6. [PubMed: 19165132]
25. van Ogtrop ML. Effect of broth enrichment cultures on ability to detect carriage of *Staphylococcus aureus*. *Antimicrob Agents Chemother.* 1995; 39:2169. [PubMed: 8540742]
26. Nonhoff C, Denis O, Brenner A, et al. Comparison of three chromogenic media and enrichment broth media for the detection of methicillin-resistant *Staphylococcus aureus* from mucocutaneous screening specimens: Comparison of MRSA chromogenic media. *Eur J Clin Microbiol Infect Dis.* 2009; 28:363–9. [PubMed: 18855028]

TABLE 1
Comparison of Targeted Staphylococcal Virulence Factors by Source of Isolate (Invasive, Noninvasive, Colonization)

Virulence Factor	Invasive (n . 37)	Noninvasive (n . 113)	Colonization (n . 55)	Colonization Subsequent 2 Years (n . 50)
SCCmec IV	31 (84%)	102 (90%)	26 (47%)*	45 (91%)
PVL	31 (84%)	99 (88%)	13 (24%)*	4 (8%)*
agr type I	33 (89%)	97 (86%)	23 (42%)*	18 (36%)*
bsaB	33 (89%)	102 (90%)	26 (47%)*	11 (22%)*

* Significant differences between groups (P . 0.001), using invasive/noninvasive isolates as the comparator.
SCCmec IV indicates staphylococcal cassette chromosome mec; PVL, Panton-Valentine Leukocidin; agr, accessory gene regulator; bsaB, bacteriocin of *S. aureus*.

TABLE 2
Comparison of Targeted Virulence Factors Invasive and Noninvasive MRSA Isolates

Virulence Factor	Invasive (n . 37)	Noninvasive (n . 113)	USA300 Colonization (n . 12)
TSST-1 SEA SEB SEC SEG SEH ETA ETB	1 (2.7%) 2 (5.4%) 0 1 (2.7%) 17 (46%)* 1 (2.7%) 0	10 (8.8%) 5 (4.4%) 1 (0.9%) 10 (8.8%) 24 (21%)* 2 (1.8%) 0 0	1 (8.3%) 0 0 1 (8.3%) 1 (8.3%) 0 0 0

The gene encoding SEG was detected more commonly in invasive isolates than noninvasive or colonization isolates (*P . 0.003). One USA300 colonization isolate harbored genes for TSST-1, SEC, and SEG; none of the virulence determinants were detected in USA300 colonization isolates. TSST-1 indicates toxic shock syndrome toxin-1; SEA-SEH, staphylococcal entero-toxin A-H; ETA-B, exfoliative toxin A-B.