Clinical and Molecular Epidemiology of Methicillin-Resistant *Staphylococcus aureus* in a Neonatal Intensive Care Unit in the Decade following Implementation of an Active Detection and Isolation Program

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Methicillin-resistant *Staphylococcus aureus* (MRSA) is a frequent source of infection in the neonatal intensive care unit (NICU), often associated with significant morbidity. Active detection and isolation (ADI) programs aim to reduce transmission. We describe a comprehensive analysis of the clinical and molecular epidemiology of MRSA in an NICU between 2003 and 2013, in the decade following the implementation of an MRSA ADI program. Molecular analyses included strain typing by pulsed-field gel electrophoresis, *mec* and accessory gene regulator group genotyping by multiplex PCR, and identification of toxin and potential virulence factor genes via PCR-based assays. Of 8,387 neonates, 115 (1.4%) had MRSA colonization and/or infection. The MRSA colonization rate declined significantly during the study period from 2.2 to 0.5/1,000 patient days (linear time, $P = 0.0003$; quadratic time, $P = 0.006$). There were 19 cases of MRSA infection (16.5%). Few epidemiologic or clinical differences were identified between MRSA-colonized and MRSA-infected infants. Thirty-one different strains of MRSA were identified with a shift from hospital-associated to combined hospital- and community-associated strains over time. Panton-Valentine leukocidin-positive USA300 strains caused 5 of the last 11 infections. Staphylococcal cassette chromosome *mec* (SCC*mec*) types II and IVa and *agr* groups 1 and 2 were most predominant. One isolate possessed the gene for toxic shock syndrome toxin; none had genes for exfoliative toxin A or B. These results highlight recent trends in MRSA colonization and infection and the corresponding changes in molecular epidemiology. Continued vigilance for this invasive pathogen remains critical, and specific attention to the unique host, the neonate, and the distinct environment, the NICU, is imperative.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a common etiology of life-threatening, health care-associated (HA) infection in neonatal intensive care units (NICU) (1, 2). The National Nosocomial Infections Surveillance System observed a >300% increase in the incidence of late-onset MRSA infections in NICUs, from 0.7 to 3.1 infections/10,000 patient days, between 1995 and 2004 (3). Unique host and environmental factors, including immature immune systems, high frequency of contact with health care providers (4), exposure to numerous invasive procedures, NICU overcrowding and understaffing (5, 6), and prolonged hospitalization (7), make NICU patients at especially high risk of becoming colonized and infected with MRSA. Since colonization with MRSA is a risk factor for development of MRSA infection, prevention of MRSA transmission within the NICU is critical (7, 8). Many NICUs have implemented active detection and isolation (ADI) programs, which involve surveillance to rapidly identify affected patients, followed by cohorting and isolation with standard contact precautions, in attempts to prevent MRSA transmission and reduce infection rates.

Several large NICUs have published reports regarding the clinical epidemiology of neonatal MRSA following implementation of surveillance, transmission prevention, and/or decolonization strategies (7, 9–12). Some also incorporated molecular analyses in their studies, including antibiotic susceptibility testing (9, 10), strain typing (9, 11, 12), genotyping (9, 12), and/or Panton-Valentine leukocidin gene detection (9, 12). No studies have compre-

hensively examined all of those molecular features of MRSA and additionally assessed the presence or absence of genes encoding proteins for a variety of staphylococcal toxins and potential virulence factors over an extensive time period following implementation of an ADI program in an NICU.

In response to the appearance of MRSA in the NICU at Yale-New Haven Children's Hospital (YNHCH) in July 2002 and a cluster of MRSA infections between November 2002 and February

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FIG 1 Flow diagram of study, 1 March 2003 through 28 February 2013. NICU, neonatal intensive care unit. MRSA, methicillin-resistant *Staphylococcus aureus*; ADI, active detection and isolation; PFGE, pulsed-field gel electrophoresis; SCCmec, staphylococcal cassette chromosome *mec*; agr, accessory gene regulator.

2003 (see Text S1 in the supplemental material), an MRSA ADI program with universal surveillance screening and isolation of MRSA-positive patients was implemented. The objective of this study was to comprehensively analyze the clinical and molecular epidemiology of MRSA colonization and infection during the 10 years following ADI program implementation. (This work was presented in part at the Pediatric Academic Societies Annual Meeting, Washington, DC, May 2013.)

MATERIALS AND METHODS

Study design and setting. This retrospective cohort study and laboratory evaluation took place in the YNHCH NICU in New Haven, CT, USA, from 1 March 2003 to 28 February 2013 (Fig. 1). The NICU is a 54-bed, level IV quaternary care referral center for neonates with complex medical and surgical needs. This study was approved by the Human Investigation Committee of the Yale University School of Medicine.

MRSA ADI program. In March 2003, an MRSA ADI program was initiated in the NICU at YNHCH. Staff education and training regarding active surveillance and infection control measures occurred at the time of program implementation. Surveillance nares cultures were obtained at admission and weekly for every admitted patient. Patients with MRSA colonization or infection were isolated and cohorted under standard contact precautions. No decolonization strategies were employed.

Case identification and data collection. A search of the Yale-New Haven Hospital (YNHH) microbiology database identified every case of MRSA colonization and/or infection that occurred within the NICU during the study period. Retrospective reviews of medical records were conducted.

Definitions. MRSA colonization was defined as a positive MRSA surveillance nares culture. MRSA infection was defined as a positive culture from blood, urine, cerebrospinal fluid (CSF), wound, tracheal aspirate, or other bodily fluid culture in the setting of clinical signs of infection and subsequent treatment with appropriate antimicrobial therapy. A patient with multiple MRSA-positive cultures was counted only once in the analyses. An outbreak was defined as >6 MRSA in-

fections within a 12-month period. Prolonged rupture of membranes (13), chorioamnionitis (13), bronchopulmonary dysplasia (BPD) (14), necrotizing enterocolitis (NEC) (15), and late-onset sepsis were defined as described previously (16).

MRSA identification and confirmation. Initially, MRSA surveillance screening specimens were cultured on Columbia 5% sheep blood agar (Remel, Lenexa, KS, USA), and colonies were identified as *S. aureus* with the Staphaurex (Remel, Lenexa, KS, USA) rapid latex agglutination test. Methicillin sensitivity versus methicillin resistance was initially determined by disk diffusion with a 30-microgram cefoxitin (FOX) disk. Later, MRSA surveillance screening was determined by Spectra MRSA agar (Remel, Lenexa, KS, USA), which is a selective and differential chromogenic medium for MRSA detection. Multiplex PCR confirmed the presence of the *mecA* gene as described previously (17, 18).

Antibiotic susceptibility testing. Antibiotic susceptibility testing was performed by the disk diffusion method (19) and later by an automated instrument system (Vitek 2 System; bioMérieux, Durham, NC, USA) (20). Throughout the study period, there was variation in which antibiotics were utilized for antibiotic susceptibility testing in the YNHCH microbiology laboratory, so strains were not uniformly subjected to testing with the same panel of antibiotics.

Strain typing. Strain typing was performed by pulsed-field gel electrophoresis (PFGE) of *Sma*I-digested bacterial DNA as described previously (21). Genomic DNA was prepared via proteinase K digestion of bacteria using standard methodology. Genomic DNA was digested with *Sma*I restriction enzyme (Boehringer Mannheim, Indianapolis, IN, USA) and separated by electrophoresis with either a CHEF-DR II or CHEF-DR III apparatus (Bio-Rad, Hercules, CA, USA). Parameters included the following: temperature, 15°C; run time, 20 h; switch time, 5 to 40 s; and voltage, 198 V (CHEF-DR II) or 6 V/cm (CHEF-DR III). PFGE patterns were compared with those from all other MRSA isolates observed at YNHCH as well as those from positive-control strains representing the major circulating MRSA clonal types. Isolates with PFGE patterns that were identical without any band differences were considered to belong to the same strain. Isolates with PFGE patterns with 2 or 3 band differences were considered closely related and subclassified as “strain-CR.” Isolates

TABLE 1 Oligonucleotide primers for staphylococcal cassette chromosome *mec* (SCC*mec*) typing, accessory gene regulator (*agr*) group designation, and PCR analyses for staphylococcal toxins and potential virulence factors

Relevant staphylococcal characteristic	Gene(s)	Oligonucleotide primer(s)	Amplicon size (bp)	Reference
<i>Staphylococcus</i> genus-specific	16S rRNA	Staph756F (5'-AATCTGTTATTAGGGAAGAACA-3')	756	McClure et al. (17)
16S rRNA gene		Staph750R (5'-CCACCTTCTCCGGTTTGTACCC-3')		
<i>mecA</i>	<i>mecA</i>	MecA1 (5'-GTAGAAATGACTGAACGTCCGATAA-3')	310	McClure et al. (17)
		MecA2 (5'-CCAATTCCACATTGTTTCGGTCTAA-3')	147	Zhang et al. (18)
		MecA147-F (5'-GTGAAGATATACCAAGTGATT-3')		
		MecA147-R (5'-ATGCGCTATAGATTGAAAGGAT-3')		
SCC <i>mec</i> type I		Type I-F (5'-GCTTTAAAGAGTGTCTGTACAGG-3')	613	Zhang et al. (18)
		Type I-R (5'-GTTCTCTCATAGTATGACGTCC-3')		
SCC <i>mec</i> type II		Type II-F (5'-CGTTGAAGATGATGAAGCG-3')	398	Zhang et al. (18)
		Type II-R (5'-CGAAATCAATGGTTAATGGACC-3')		
SCC <i>mec</i> type III		Type III-F (5'-CCATATTGTGTACGATGCG-3')	280	Zhang et al. (18)
		Type III-R (5'-CCTTAGTTGTCGTAACAGATCG-3')		
SCC <i>mec</i> type IVa		Type IVa-F (5'-GCCTTATTCGAAGAAACCG-3')	776	Zhang et al. (18)
		Type IVa-R (5'-CTACTCTTCTGAAAAGCGTCG-3')		
SCC <i>mec</i> type IVb		Type IVb-F (5'-TCTGGAATTACTTCAGCTGC-3')	493	Zhang et al. (18)
		Type IVb-R (5'-AAACAATATTGCTCTCCCTC-3')		
SCC <i>mec</i> type IVc		Type IVc-F (5'-ACAATATTTGTATTATCGGAGAGC-3')	200	Zhang et al. (18)
		Type IVc-R (5'-TTGGTATGAGGTATTGCTGG-3')		
SCC <i>mec</i> type IVd		Type IVd-F (5'-CTCAAAATACGGACCCCAATACA-3')	881	Zhang et al. (18)
		Type IVd-R (5'-TGCTCCAGTAATTGCTAAAG-3')		
SCC <i>mec</i> type V		Type V-F (5'-GAACATTGTGTTAAATGAGCG-3')	325	Zhang et al. (18)
		Type V-R (5'-TGAAAGTTGTACCCTTGACACC-3')		
<i>agr</i>	<i>agr</i>	Pan (5'-ATGCACATGGTGCACATGC-3')		Gilot et al. (22)
<i>agr</i> group 1		<i>agr</i> 1 (5'-GTCACAAGTACTATAAGCTGCGAT-3')	441	Gilot et al. (22)
<i>agr</i> group 2		<i>agr</i> 2 (5'-TATTACTAATTGAAAAGTGGCCATAGC-3')	575	Gilot et al. (22)
<i>agr</i> group 3		<i>agr</i> 3 (5'-GTAATGTAATAGCTTGTAATAATACCCAG-3')	323	Gilot et al. (22)
<i>agr</i> group 4		<i>agr</i> 4 (5'-CGAATATGCCGTAATACCCG-3')	659	Gilot et al. (22)
Panton-Valentine leukocidin (PVL)	<i>lukS/F-PV</i>	Luk-PV-1 (5'-ATCATTAGGTAAATGTCTGGACATGATCCA-3')	433	McClure et al. (17)
		Luk-PV-2 (5'-GCATCAAGTGTATTGGATAGCAAAAGC-3')		
Arginine catabolic mobile element (ACME)	<i>arcA</i>	<i>arcA</i> -F (5'-GCAGCAGAATCTATTACTGAGCC-3')	513	Zhang et al. (23)
		<i>arcA</i> -R (5'-TGCTAACTTTTCTATTGCTTGAGC-3')		
Toxic shock syndrome toxin (TSST-1)	<i>tst</i>	GTSSSTR-1 (5'-ACCCCTGTTCCCTTATCATC-3')	326	Mehrotra et al. (24)
		GTSSSTR-2 (5'-TTTTTCAGTATTGTAAACGCC-3')		
Exfoliative toxin A (ETA)	<i>eta</i>	GETAR-1 (5'-GCAGGTGTTGATTTAGCATT-3')	93	Mehrotra et al. (24)
		GETAR-2 (5'-AGATGTCCCTATTTTGTCTG-3')		
Exfoliative toxin B (ETB)	<i>etb</i>	GETBR-1 (5'-ACAAGCAAAAGAATACAGCG-3')	226	Mehrotra et al. (24)
		GETBR-2 (5'-GTTTTTGGCTGCTTCTCTTG-3')		
LukE-LukD leukocidin	<i>lukE-lukD</i>	LUKDE-1 (5'-TGAAAAAGGTTCAAAGTTGATACGAG-3')	269	Jarraud et al. (25)
		LUKDE-2 (5'-TGATTTCGATAGCAAAAGCAGTGCA-3')		
Beta-hemolysin	<i>hly</i>	HLB-1 (5'-GTGCACTTACTGACAATAGTGC-3')	309	Jarraud et al. (25)
		HLB-2-2 (5'-GTTGATGAGTAGCTACCTTCAGT-3')		

with PFGE patterns with 4 to 6 band differences were considered possibly related and subclassified as “strain-PR,” and PFGE patterns with >7 band differences were considered different. Unique strains were categorized based on their serial identification at YNHH (i.e., the first strain was categorized as 1, the second as 2, etc.).

Genotyping. (i) Genomic DNA extraction and purification for use in PCR assays. MRSA isolates were plated on BBL Columbia agar with 5% sheep blood (Becton, Dickinson and Company, Sparks, MD, USA). Genomic DNA was extracted and purified from a single colony grown from the isolate with MasterPure Gram Positive DNA purification kit (epicenter, Madison, WI, USA) according to the manufacturer's instructions.

(ii) **SCC*mec* typing.** Multiplex PCR was utilized for staphylococcal cassette chromosome *mec* (SCC*mec*) typing with primers designed for SCC*mec* types and subtypes (types I, II, III, IVa, IVb, IVc, IVd, V) and the *mecA* gene, as described (Table 1) (18). The PCR mixture included 2 µl of template DNA in a 50-µl final reaction volume containing 50

mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM MgCl₂, 0.2 mM each deoxynucleoside triphosphate, 2.0 units of *Taq* DNA polymerase, and primer mix. The respective concentrations of primers were as follows: 0.048 µM type I primers, 0.032 µM type II primers, 0.04 µM type III primers, 0.104 µM type IVa primers, 0.092 µM type IVb primers, 0.078 µM type IVc primers, 0.28 µM type IVd primers, 0.06 µM type V primers, and 0.046 µM *mecA* primers. Thermocycling conditions were 94°C for 5 min, followed by 10 cycles of 94°C for 45 s, 65°C for 45 s, and 72°C for 90 s, followed by 25 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 90 s, followed by 72°C for 10 min. The following *S. aureus* isolates were provided by the NARSA program for use as control strains (Table 2): NRS 108 (SCC*mec* type I), NRS 382 (SCC*mec* type II), NRS 65 (SCC*mec* type III), NRS 384 (SCC*mec* type IVa), NRS 484 (SCC*mec* type IVc), NRS 385 (SCC*mec* type IVd). The PCR amplicons were electrophoresed in a 2% agarose gel containing GelGreen nucleic acid gel stain (Biotium, Hayward, CA USA) and visualized under UV light as distinct bands corresponding to the expected molecular sizes (Table 1).

TABLE 2 *Staphylococcus aureus* isolates utilized as control strains for PCR analyses

Isolate	Staphylococcal characteristic(s)
ATCC 29213	Positive for 16S rRNA
ATCC 33591	Positive for 16S rRNA, positive for <i>mecA</i>
ATCC 25923	Positive for 16S rRNA, positive for <i>lukS/F-PV</i>
NRS 382 ^a	USA100, SCCmec type II, <i>agr</i> group 2
NRS 383 ^a	USA200, SCCmec type II, <i>agr</i> group 3, positive for <i>tst</i>
NRS 384 ^a	USA300, SCCmec type IVa, <i>agr</i> group 1, positive for <i>lukS/F-PV</i> , positive for <i>arcA</i>
NRS 123 ^a	USA400, SCCmec type IVa, <i>agr</i> group 3
NRS 385 ^a	USA500, SCCmec type IV, <i>agr</i> group 1
NRS 22 ^a	USA600, SCCmec type II, <i>agr</i> group 1
NRS 386 ^a	USA700, SCCmec type IVa, <i>agr</i> group 1
NRS 387 ^a	USA800, SCCmec type IV, <i>agr</i> group 2
NRS 483 ^a	USA1000, SCCmec type IV
NRS 484 ^a	USA1100, SCCmec type IV, <i>agr</i> group 2
NRS 645 ^a	Iberian, SCCmec type IV
NRS 65 ^a	SCCmec type III
NRS 108 ^a	SCCmec type I
NRS 164 ^a	Positive for <i>eta</i> , positive for <i>lukE-lukD</i>
NRS 165 ^a	<i>agr</i> group 4, positive for <i>eta</i> , positive for <i>etb</i> , positive for <i>lukE-lukD</i>
NRS 166 ^a	<i>agr</i> group 4, positive for <i>eta</i> , positive for <i>etb</i> , positive for <i>lukE-lukD</i>
NRS 167 ^a	Positive for <i>eta</i>

^a Isolate was obtained through the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) program supported under NIAID/NIH contract no. HHSN272200700055C.

(iii) ***agr* group designation.** Multiplex PCR with primers designed to detect the four different accessory gene regulator (*agr*) groups of the *agr* operon was performed as described previously (Table 1) (22). The PCR mixture included 2 μ l of template DNA in a 50- μ l final reaction volume containing 0.03 μ M each primer (Pan, *agr1*, *agr2*, *agr3*, *agr4*). Thermocycling conditions were 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 60°C for 90 s, and 72°C for 60 s, followed by 68°C for 10 min. The following *S. aureus* isolates were provided by the NARSA program for use as control strains (Table 2): NRS 385 (*agr* group 1), NRS 382 (*agr* group 2), NRS 123 (*agr* group 3), NRS 166 (*agr* group 4). The PCR amplicons were electrophoresed in a 1% agarose gel containing GelGreen nucleic acid gel stain (Biotium, Hayward, CA, USA) and visualized under UV light as distinct bands corresponding to the expected molecular sizes (Table 1).

(iv) **Assessment for PVL.** Multiplex PCR was performed with primers designed to detect the *Staphylococcus* genus-specific 16S rRNA gene (internal positive control), the *lukS-PV* and *lukF-PV* (*lukS/F-PV*) genes, which encode Panton-Valentine leukocidin (PVL), and the *mecA* gene, which confers methicillin resistance, as described previously (Table 1) (17). The PCR mixture included 2 μ l of template DNA in a 50- μ l final reaction volume containing 0.07 μ M 16S rRNA primers, 0.08 μ M *lukS/F-PV* primers, and 0.24 μ M *mecA* primers. Thermocycling conditions were set at 94°C for 10 min, followed by 10 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 75 s, followed by 25 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 75 s. The following *S. aureus* isolates were used as control strains (Table 2): ATCC 29213 (PVL-negative methicillin-susceptible *S. aureus* [MSSA]), ATCC 25923 (PVL-positive MSSA), and ATCC 33591 (PVL-negative MRSA). The PCR amplicons were electrophoresed in a 2% agarose gel containing GelGreen nucleic acid gel stain (Biotium, Hayward, CA, USA) and visualized under UV light as distinct bands corresponding to the expected molecular sizes (Table 1).

(v) **Assessment for ACME.** Multiplex PCR with primers designed to detect the *arcA* gene on the arginine catabolic mobile element (ACME), which is a genetic element specific to USA300, was performed as described previously (Table 1) (23). The PCR mixture included 2 μ l of template

DNA in a 50- μ l final reaction volume containing 0.3 μ M *arcA* primers. Thermocycling conditions were set at 94°C for 4 min, followed by 10 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s, followed by 25 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 45 s. The following *S. aureus* isolate was provided by the NARSA program for use as a control strain (Table 2): NRS 384 (USA300). The PCR amplicons were electrophoresed in a 2% agarose gel containing GelGreen nucleic acid gel stain (Biotium, Hayward, CA, USA) and visualized under UV light as a distinct band corresponding to the expected molecular size of 513 bp (Table 1).

(vi) **Assessment for TSST-1, ETA, and ETB.** Multiplex PCR with primers designed to detect the toxic shock syndrome toxin (TSST-1) and the exfoliative toxins A and B (ETA and ETB) was performed as described previously (Table 1) (24). The PCR mixture included 2 μ l of template DNA in a 50- μ l final reaction volume containing 500 mM KCl, 100 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 0.2 mM each deoxynucleoside triphosphate, 2.5 units of *Taq* DNA polymerase, and primer mix. The respective concentrations of primers were 50 pmol of *eta* primers, 20 pmol *etb* primers, and 20 pmol of *tst* primers. The following *S. aureus* isolates were provided by the NARSA program for use as control strains (Table 2): NRS 383 (TSST-1 positive), NRS 167 (ETA positive), NRS 165 (ETA positive, ETB positive). The PCR amplicons were electrophoresed in a 2% agarose gel containing GelGreen nucleic acid gel stain (Biotium, Hayward, CA, USA) and visualized under UV light as distinct bands corresponding to the expected molecular sizes (Table 1).

(vii) **Assessment for LukE-LukD leukocidin.** PCR with primers designed to detect the *lukE-lukD* gene that encodes LukE-LukD leukocidin was performed (Table 1) (25). The PCR mixture included 2 μ l of template DNA in a 50- μ l final reaction volume containing 0.2 μ M each *lukE-lukD* primer. Thermocycling conditions were set at 94°C for 30 s, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by 72°C for 60 s. The following *S. aureus* isolates were provided by the NARSA program for use as control strains (Table 2): NRS 164 (LukE-LukD positive), NRS 165 (LukE-LukD positive), NRS 166 (LukE-LukD positive), NRS 167 (LukE-LukD negative). The PCR amplicons were electrophoresed in a 2% agarose gel containing GelGreen nucleic acid gel stain (Biotium, Hayward, CA, USA) and visualized under UV light as a distinct band corresponding to the expected molecular size of 269 bp (Table 1).

(viii) **Assessment for beta-hemolysin.** PCR with primers designed to detect the *hlyB* gene that encodes beta-hemolysin was performed (Table 1) (25). The PCR mixture included 2 μ l of template DNA in a 50- μ l final reaction volume containing 0.2 μ M each *lukE-lukD* primer. Thermocycling conditions were set at 94°C for 30 s, followed by 45 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 30 s, followed by 72°C for 60 s. The PCR amplicons were electrophoresed in a 2% agarose gel containing GelGreen nucleic acid gel stain (Biotium, Hayward, CA, USA) and visualized under UV light as a distinct band corresponding to the expected molecular size of 309 bp (Table 1).

Statistical analysis. Univariate comparisons of continuous data were made utilizing the independent samples Student's *t* test. Dichotomous data were compared using Pearson's chi-square or Fisher's exact test for any cell containing values of <5. A *P* value of <0.05 based on two-sided tests was considered statistically significant (SPSS, Inc., Chicago, IL, USA). Trends in MRSA colonization were assessed from 2003 to 2013 with the number of colonized infants per year assumed to be Poisson distributed. Changes in the rate of colonization over time were analyzed using a Poisson regression with the number of patient days in a given year used as an offset variable (SPSS, Inc., Chicago, IL, USA). The time effect was not assumed to be linear, and a quadratic effect was also tested in the regression model. Statistical significance was established with a *P* value of <0.05.

RESULTS

Subjects and MRSA case rates. There were 8,387 neonates admitted during the study period, totaling 163,136 patient days. A total of 27,375 surveillance cultures were collected. Of the 8,387 admis-

FIG 2 (A) Methicillin-resistant *Staphylococcus aureus* (MRSA) colonization and infection rates per 1,000 patient days by year in the Yale-New Haven Children's Hospital (YNHCH) neonatal intensive care unit (NICU) during the study period. (B) MRSA colonization and infection rates per 1,000 patient admissions by year in the YNHCH NICU during the study period.

sions, 115 cases of MRSA colonization and/or infection (1.4%) occurred. Eight of 115 cases involved patients who were transferred to the YNHCH NICU with MRSA-positive status; these cases were either identified as MRSA positive at an outside hospital prior to transfer or diagnosed upon admission to the YNHCH NICU. For the 115 cases, the median gestational age was 30 weeks (range, 22 to 41 weeks), the median birth weight was 1,310 g (range, 440 to 4,620 g), and 57% of the infants were male. Deliveries occurred at YNHCH 75% of the time, and 71% were by cesarean section. The median length of stay was 45 days (range, 3 to 259 days).

Surveillance cultures yielded 112 cases of MRSA colonization (97% of 115 MRSA cases; 3 cases involved infection without evi-

dence of colonization). The overall MRSA colonization rate was 0.7/1,000 patient days (Fig. 2). A statistically significant decrease in the rate of MRSA colonization was observed from 2.2/1,000 patient days at the start of the study period to 0.5/1,000 patient days at study end (linear time, $P = 0.0003$; quadratic time, $P = 0.006$). The median day of life at which colonization was identified was 17 days (range, 4 to 159 days). Almost two-thirds of neonates became MRSA colonized during the first 3 weeks of life; a few became colonized later in admission (Fig. 3). There was an overlap of hospital length of stay for several patients with similar MRSA strains in 2003-2004, whereby potential MRSA transmission could have occurred within the NICU.

Nineteen of 115 cases were diagnosed with at least one MRSA

FIG 3 Frequency of cases by week of life during which methicillin-resistant *Staphylococcus aureus* (MRSA) colonization was first identified by surveillance culture.

infection (16.5%), and 5 cases experienced multiple MRSA-related infections. Infections included bacteremia (10 cases), skin/soft tissue infection (5 cases), pneumonia or tracheitis (5 cases), perforated otitis media (1 case), omphalitis (1 case), and peritonitis (1 case). The overall infection rate was 0.1/1,000 patient days, ranging between 0 and 0.3/1,000 patient days annually (Fig. 2). Ten infants were identified as MRSA colonized prior to developing MRSA infection. The median interval between colonization and infection was 6 days (range, 1 to 42 days). Two cases were diagnosed with MRSA infection before admission to YNHCH. Two cases were simultaneously identified as colonized and infected. Two cases were diagnosed with infection before colonization. Three infants with MRSA infection never had documented MRSA colonization.

Comparison of MRSA-colonized and MRSA-infected cases. Unadjusted, univariate comparisons of MRSA-infected and MRSA-colonized infants were performed to determine if the two groups differed clinically, but few statistically significant differences were identified. MRSA-infected infants were more often intubated and mechanically ventilated ($P < 0.001$) and receiving total parenteral nutrition (P value 0.006) and had more late-onset sepsis ($P < 0.001$) than MRSA-colonized infants (Table 3). Infants with MRSA infection were exposed to more days of systemic antimicrobial treatment ($P = 0.001$). There was increased mortality in MRSA-infected infants, which approached statistical significance ($P = 0.059$).

Antibiotic susceptibility testing. Antibiotic susceptibility testing results were based on reports from the YNHCH microbiology laboratory ($n = 46$) as well as confirmatory testing of available isolates with an automated instrument system at the study conclusion ($n = 66$). Results indicated that no isolates were resistant to vancomycin, rifampin, trimethoprim-sulfamethoxazole, linezolid, quinupristin-dalfopristin, or tigecycline (Table 4). Some isolates were resistant to erythromycin (88%), ciprofloxacin (65%), levofloxacin (65%), moxifloxacin (58%), clindamycin

(57%), tetracycline hydrochloride (36%), and gentamicin (30%). Some isolates were intermediately resistant to moxifloxacin (8%), tetracycline hydrochloride (1%), and nitrofurantoin (3%).

Strain characterization. PFGE analysis of 105 of the 115 MRSA isolates (91%) identified 31 different strains. HA-MRSA strains, i.e., USA100/USA100-closely related (CR)/USA100-possibly related (PR) (45%) and USA500/USA500-CR (20%), and community-associated MRSA (CA-MRSA) strain USA300/USA300-CR (12%) were the most frequently identified strain types (Fig. 4; see also Table S1 in the supplemental material). USA100/USA100-CR strains were prevalent throughout the study period, mainly in cases of colonization. USA500/USA500-CR strains were observed between 2003 and 2005 in cases of colonization and infection. USA300/USA300-CR strains appeared from 2006 onward. PCR analysis of 66 of the 115 MRSA isolates (57%) available for additional molecular typing confirmed that all isolates carried the *mecA* gene (Table 5; see also Table S1 in the supplemental material). Staphylococcal cassette chromosome *mec* (SCC*mec*) types II and IVa were most commonly identified (Table 5; see also Table S1 in the supplemental material). Multiplex PCR analyses revealed that *S. aureus* accessory gene regulator (*agr*) groups 1 and 2 were most predominant (Table 5; see also Table S1 in the supplemental material).

The presence or absence of genes encoding the proteins for PVL, ACME, TSST-1, ETA, ETB, Luke-LukD leukocidin, and beta-hemolysin was determined via PCR (Table 5; see also Table S1 in the supplemental material). Most USA300 strains carried the genes for PVL. Although the genes for PVL were present in only one-quarter of the isolates, strains with PVL were responsible for 5 of the last 11 infections. Not surprisingly, the gene for ACME was detected in USA300/USA300-CR strains. None of the isolates possessed the gene for ETA or ETB, and only one had the gene for TSST-1 (not a USA300 strain) (Table 5; see also Table S1 in the supplemental material). Most isolates carried the genes for beta-

TABLE 3 Comparison of MRSA-colonized, noninfected cases with MRSA-infected cases in the Yale-New Haven Children's Hospital (YNHCH) neonatal intensive care unit (NICU) during the study period

	No. (%) of cases ^a		
Characteristics	Colonized, noninfected (<i>n</i> = 96)	Infected (<i>n</i> = 19)	<i>P</i> value
Demographics			
Gestational age (wk)	30 (22–41) ^b	26 (23–40)	0.765
Birth wt (grams)	1,355 (440–4,620) ^b	850 (510–1,300)	0.225
Male sex	58 (60)	8 (42)	0.140
Inborn	73 (76)	13 (68)	0.485
Cesarean delivery	70 (73)	12 (63)	0.390
Day of life MRSA colonization identified	17 (4–159) ^b	12 (6–101) ^c	0.191
Day of life MRSA infection identified	NA	17 (5–101)	NA
Exposures			
Prolonged rupture of membranes	12 (13)	2 (11)	0.566
Chorioamnionitis	6 (6)	1 (5)	0.804
Intrapartum antibiotic exposure	57 (59)	9 (47)	0.605
Intubation and mechanical ventilation at colonization or infection ^c	20 (29)	12 (80)	<0.001
Central venous catheter at colonization or infection ^c	39 (48)	13 (72)	0.064
Total parenteral nutrition at colonization or infection ^c	39 (46)	14 (82)	0.006
Gavage feeding at colonization or infection ^c	65 (77)	10 (63)	0.241
Breast milk and/or breastfeeding at colonization or infection ^c	54 (70)	7 (64)	0.662
Surgery prior to colonization or infection ^c	28 (62)	7 (58)	0.806
Antimicrobial exposure prior to colonization or infection (days)	4 (0–82) ^b	4 (0–38)	0.529
Outcomes			
Bronchopulmonary dysplasia (BPD) ^c	20 (27)	6 (55)	0.065
Necrotizing enterocolitis (NEC)	13 (14)	5 (26)	0.161
Late-onset sepsis (>72 h of life)	18 (19)	13 (68)	<0.001
Total antimicrobial exposure (days)	5 (0–97) ^b	26 (3–153)	0.001
Length of stay (days)	45 (3–259) ^b	55 (12–236)	0.427
Death	6 (6)	4 (21)	0.059

^a Unless otherwise specified, values are numbers (%). NA, not applicable.^b Median (range).^c Total number differs based on subset designation.**TABLE 4** Overall antibiotic susceptibility of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates from the study period^a

Antibiotic	No. of susceptible isolates/total no. tested (%)
Vancomycin	112/112 (100)
Rifampin	112/112 (100)
Trimethoprim-sulfamethoxazole	68/68 (100)
Linezolid	66/66 (100)
Quinupristin-dalfopristin	66/66 (100)
Tigecycline	66/66 (100)
Nitrofurantoin	64/66 (97) ^b
Gentamicin	78/112 (70)
Tetracycline HCl	61/97 (37) ^b
Moxifloxacin	23/66 (35) ^b
Levofloxacin	23/66 (35)
Ciprofloxacin	23/66 (35)
Clindamycin	36/111 (32)
Erythromycin	13/107 (12)
Oxacillin	0/112 (0)

^a These antibiotic susceptibility testing results should not be used to inform clinical treatment. Appropriateness for patient safety must be considered. For example, trimethoprim-sulfamethoxazole should not be used to treat infants less than 2 months of age due to potential risk of kernicterus.^b One or more strains were intermediately resistant to antibiotic; percentage of strains intermediately resistant to moxifloxacin, 8%; to tetracycline-HCl, 1%; to nitrofurantoin, 3%.

hemolysin (97%) and LukE-LukD leukocidin (95%) (Table 5; see also Table S1 in the supplemental material).

DISCUSSION

Neonatal MRSA colonization and infection continue to be a significant concern in the NICU. Accordingly, many NICUs have employed ADI programs to attempt to decrease rates of neonatal MRSA and reduce the transmission of this formidable pathogen. Our study reports the most comprehensive analysis of the clinical and molecular epidemiology of neonatal MRSA following ADI implementation to date. The MRSA colonization rate had a statistically significant decrease following the introduction of the ADI program. This low rate was maintained, and no MRSA epidemics were observed. Few significant differences were observed between MRSA-colonized and MRSA-infected infants. Cases of MRSA colonization and infection occurred intermittently, with 31 different MRSA strains detected during the study period. A shift from hospital-associated to combined hospital- and community-associated strains of MRSA was observed. Community-associated strain USA300 was particularly virulent, with 5 of the last 11 infections attributable to PVL-positive varieties of this strain. One isolate possessed the gene for TSST-1, and no isolates had genes for ETA or ETB. Overall, these results describe the recent trends in MRSA colonization and infection in one NICU following ADI implementation as well as the corresponding changes in molecular ep-

FIG 4 Prevalence of the most common methicillin-resistant *Staphylococcus aureus* (MRSA) strain types identified by pulsed-field gel electrophoresis (PFGE) of bacterial isolates from cases of MRSA colonization and/or infection in the Yale-New Haven Children's Hospital (YNHCH) neonatal intensive care unit (NICU) by study year.

idemiology and highlight the continued presence of this virulent pathogen within the NICU and its effect on our most ill and vulnerable neonatal patients.

One of the most notable findings of this study was the large variety of strains detected during the study period. Thirty-one different strains were identified, suggesting that the introduction of new strains into the NICU, despite the ADI program, likely represents MRSA that is endemic to the health care and community setting. The sources of these strains were mostly unknown, except for those neonates with known MRSA colonization or infection admitted from outside hospitals. However, the introduction of these new strains, not preventable by an ADI program, did not result in significant MRSA transmission in the NICU. It is likely that sustained and successful efforts to reduce MRSA within the NICU contributed to low MRSA colonization pressure and decreased transmission risk. Whether MRSA infection rates would have been higher had the program not been in place is speculative. However, rates of colonization, a known risk factor for infection (8, 26), significantly declined and remained low. Since Healy et al. published the first report of CA-MRSA infections in NICU patients in 2004 (27), other NICUs have observed similar shifts from HA-MRSA strains to CA-MRSA strains (9, 10, 12). In our NICU, HA-MRSA strains were initially observed, and CA-MRSA strains abruptly appeared in 2004. PFGE and SCCmec typing showed a shift from the HA-MRSA strains USA100 and USA500 with SCCmec type II to CA-MRSA USA300 and USA400 with SCCmec type IVa. The observation that neonates admitted to the NICU developed colonization and/or infection with CA-MRSA strains despite never having left the hospital highlights the increasingly blurred distinction between HA-MRSA and CA-MRSA.

Antibiotic susceptibility monitoring within individual NICUs is imperative to highlight the most appropriate empirical antimicrobial therapies for use in patients with suspected infection. While vancomycin remains the mainstay of treatment for MRSA infections, MRSA strains resistant to vancomycin have been reported, including vancomycin-intermediate *S. aureus* (VISA), described in 1996 (28, 29), and vancomycin-resistant *S. aureus* (VRSA), described in 2002 (30), that acquired the *vanA* resistance gene from strains of vancomycin-resistant enterococci (VRE)

(31). No strains from the study period were resistant to vancomycin, rifampin, trimethoprim-sulfamethoxazole, linezolid, quinupristin-dalfopristin, or tigecycline. Frequent resistance to erythromycin (88%), quinolones (65%), and clindamycin (57%) and occasional resistance to tetracycline (37%) and gentamicin (30%) were observed. Several isolates had intermediate resistance to moxifloxacin, tetracycline, and nitrofurantoin. Fortunately, there have been no reports of neonates with VISA or VRSA infections to date, but continued vigilance remains critical.

Our study provides the most extensive analysis of staphylococcal toxins and other potential virulence factors in NICU MRSA isolates (9, 12). Seybold et al. and Carey et al. observed USA300 strains positive for PVL, a staphylococcal toxin that can lead to extensive necrosis, especially in skin and soft tissue infections (9, 12, 17). In our study, genes encoding PVL were detected in most USA300 strains, and those strains frequently caused invasive infections, including 5 of the last 11, which were primarily cases of pneumonia and abscesses. We were surprised to find an MRSA strain carrying the superantigen TSST-1 in an isolate from an asymptomatic, colonized infant. TSST-1 produced by MRSA has been linked to neonatal toxic shock syndrome-like exanthematous disease, an emerging neonatal illness with fever, systemic ex-

TABLE 5 Prevalence of genes encoding staphylococcal toxins and potential virulence factors of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates as determined by PCR analysis

Genes, toxins, and potential virulence factors	Prevalence ^a in:		
	Isolates of colonization (n = 57) ^b	Isolates of infection (n = 9) ^b	MRSA isolates (n = 66) ^b
<i>mecA</i>	57 (100)	9 (100)	66 (100)*
SCCmec type (I to V)			
I	0	0	0
II	31 (54)	2 (22)	33 (50)
III	1 (2)	0	1 (2)
Iva	11 (19)	5 (56)	16 (24)
IVb	0	1 (11)	1 (2)
IVc	1 (2)	0	1 (2)
IVd	0	0	0
V	3 (5)	0	3 (5)
Undetermined	10 (18)	1 (11)	11 (17)
<i>agr</i> group (<i>agr</i> 1 to 4)			
<i>agr</i> 1	17 (30)	5 (56)	22 (33)
<i>agr</i> 2	38 (67)	4 (44)	42 (64)
<i>agr</i> 3	2 (4)	0	2 (3)
<i>agr</i> 4	0	0	0
Panton-Valentine leukocidin (PVL)	12 (21)	5 (56)	17 (26)
Arginine catabolic mobile element (ACME)	10 (18)	5 (56)	15 (23)
Toxic shock syndrome toxin (TSST-1)	1 (2)	0	1 (2)
Exfoliative toxin A (ETA)	0	0	0
Exfoliative toxin B (ETB)	0	0	0
LukE-LukD leukocidin	54 (95)	9 (100)	63 (95)
Beta-hemolysin	55 (96)	9 (100)	64 (97)

^a Values are numbers (%). SCCmec, staphylococcal cassette chromosome *mec*; *agr*, accessory gene regulator.

^b Total number of isolates available for PCR analysis.

anthema, and thrombocytopenia in Japan (32). No neonates had symptoms of neonatal toxic shock syndrome-like exanthematous disease or toxic shock syndrome during the study period. The staphylococcal exfoliative toxins ETA and ETB cause staphylococcal scalded skin syndrome in patients of all ages, including neonates (33). None of our MRSA isolates carried genes for these toxins, and no cases of staphylococcal scalded skin syndrome were observed.

Despite the successful reduction in the rate of MRSA colonization following the ADI program implementation, the MRSA infection rate remained stably low throughout the study period without a similar decline. Whether infection rates would have been higher had the program not been in place is unknown. MRSA colonization is a known risk factor for subsequent development of infection (8, 26). Factors other than the ADI program may have influenced infection rates. Ineffective infection prevention measures and the immature immune system of premature neonates probably played a role. Certainly, numerous strategies, including and in addition to ADI programs, are needed to target the reduction of MRSA infection rates in NICU patients.

This study has a number of limitations. This was an observational, retrospective study performed in a single-center NICU in a university-affiliated medical center, and therefore results might not be generalizable to all other NICUs. MRSA surveillance screening was not performed prior to ADI program implementation, so rates before and after the intervention cannot be compared. MRSA surveillance included the assessment of only a single anatomical site, the patients' nares, which could have reduced sensitivity compared to multiple-site sampling. While many other studies of MRSA in the NICU have performed surveillance similarly (7, 11, 12), multiple-site sampling (nares, umbilicus, conjunctiva, groin) could have potentially improved detection (34, 35). MRSA swabs were not incubated in broth, which might have resulted in reduced sensitivity. Surveillance screening of family members and health care personnel was not performed, and decolonization strategies were not utilized, so the effectiveness of these interventions cannot be assessed. MRSA-negative controls were not assessed, so comparisons cannot be made to identify potential risk factors for MRSA acquisition. Although the intended approach of the microbiologic analyses and antibiotic susceptibility testing was extensive, the retrospective nature of this study, with variation in microbiology lab methodologies over time and the reduced number of isolates available for subsequent analysis, limits the scope of the results.

In conclusion, the problem of MRSA colonization, transmission, and infection in the NICU is complex. Our single-center study observed a long-term statistically significant reduction in MRSA colonization rates following the implementation of an ADI program. MRSA was not completely eradicated from the NICU. The identification of 31 different MRSA strains suggests that the constant introduction of new strains into the NICU, despite the ADI program, likely represents MRSA strains endemic to the health care and community setting. PVL-positive USA300 strains were particularly virulent and caused 5 of the last 11 infections. Molecular analyses of the observed strains helped elucidate trends in the appearance of new strains, changing antibiotic susceptibility patterns, and the presence of potential virulence factors, which can assist in the formulation of new approaches to prevent MRSA from harming NICU patients. Tailored strategies are needed to reduce MRSA colonization, infection, and transmission in hospi-

talized neonates. Efforts to control MRSA in the NICU need to combine data obtained from prospective randomized multicenter trials with ongoing local surveillance of trends in MRSA clinical and molecular epidemiology. Continued vigilance for this invasive pathogen remains critical, and specific attention to the unique host, the neonate, and the distinct environment, the NICU, is imperative.

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