MICROBIAL DRUG RESISTANCE
Volume 18, Number 2, 2012

DOI: 10.1089/mdr.2011.0182



# *Staphylococcus aureus* Reservoirs and Transmission Routes in a Portuguese Neonatal Intensive Care Unit: A 30-Month Surveillance Study

Teresa Conceição,[1] Marta Aires de Sousa,[2] Maria Miragaia,[1] Elsa Paulino,[3] Rosalina Barroso,[3] Maria João Brito,[3] Teresa Sardinha,[3] Luísa Sancho,[3] Helena Carreiro,[3] Germano de Sousa,[3] Maria do Céu Machado,[3] and Hermínia de Lencastre[1,4]

Although *Staphylococcus aureus* is a major cause of outbreaks in neonatal intensive care units (NICUs), there are no studies on the epidemiology of *S. aureus* isolates responsible for infection in Portuguese NICUs. Between July 2005 and December 2007, a total of 54 methicillin susceptible *S. aureus* (MSSA) isolates were recovered from 16 infected infants, parents, health care workers (HCWs), and the environment in a level III NICU. Isolates were characterized by pulsed-field gel electrophoresis (PFGE), *spa* typing, and multilocus sequence typing. Virulence determinants were detected by multiplex polymerase chain reaction. Three major MSSA clones were endemic in the NICU, representing 70% ($n=38$) of the isolates: PFGE type A-ST5 ($n=17$); type B-ST30 ($n=12$); and type C-ST1 ($n=9$). Leukotoxins and hemolysins were present in all isolates, although none of them carried PVL. HCWs, plastic folders protecting clinical files, and mothers' nipples were identified as potential reservoirs and/or vehicles of dissemination of *S. aureus*. Consequently, additional infection control measures were implemented in this NICU.

## Introduction

Nosocomial infection rates in neonatal intensive care units (NICUs) increased over the past decade, reaching 25.5% in premature and very-low-birth-weight neonates.[10–12,29] This group is particularly susceptible to infections due to their immature immune system, which is reflected in the high rates of mortality and morbidity, and in a significant increase in the length of hospital stay and associated costs.[14,29,46] *Staphylococcus aureus* remains the second major cause of hospital acquired infections in NICUs being responsible for 16.7% of pneumonias and 22.2% of surgical-site infections reported in neonates.[12,24,46]

In Portugal, the last survey of hospital infection rates showed that *S. aureus* was responsible for 21.3% of total hospital acquired infections, which are more common in ICUs (33.2%) and pediatric units (9.3%).[34] Moreover, this is a country that has one of the highest rates of methicillin resistant *S. aureus* (MRSA) in Europe (49.1%).[16]

Surveillance of nosocomial infections is essential to improve the quality of patient care. Episodes of *S. aureus* infection in neonates are frequently related to health care workers (HCWs), parents, and environment colonization.[5,8,9,18,23,24,44] Preventing MRSA transmission within an NICU has been shown to be achievable through implementation of optimal infection control strategies, namely hand hygiene practices; prevention of central venous catheter-related bloodstream infections; judicious use of antimicrobials; skin care, as it is the first line of defense against infection; and early enteral feeding with human milk.[10,45]

Although several studies have been focused on the molecular characterization of *S. aureus* isolates recovered from different hospitals in Portugal over time,[2,4] none of them specifically analyzed the molecular epidemiology of *S. aureus* in NICUs. The primary aim of the present study was to identify the incidence, reservoirs, and routes of transmission of *S. aureus* isolates responsible for infection in one of the largest Portuguese NICUs, in order to implement additional adequate infection control strategies in the unit and reduce infection rates. A second aim of the study was to describe the population structure of the *S. aureus* clones circulating in this NICU.

[1]Laboratory of Molecular Genetics, Instituto de Tecnologia Química e Biológica, Oeiras, Portugal.
[2]Escola Superior de Saúde da Cruz Vermelha Portuguesa, Lisbon, Portugal.
[3]Hospital Fernando Fonseca, Amadora, Portugal.
[4]Laboratory of Microbiology, The Rockefeller University, New York, New York.

## Materials and Methods

### *Setting*

Hospital Fernando Fonseca is a large tertiary care hospital (670 beds) located in the suburbs of Lisbon, Portugal, that services an outpatient population of about 600,000, and has the second largest maternity ward in the country with ~5,400 deliveries per year. The NICU is a level III unit with 26 beds and an admission of about 450 neonates per year, out of which around 17% have very low birth weight (≤1,500 g). One hundred HCWs work in this NICU.

### *Study design and case definition*

From July 2005 to December 2007, all neonates admitted for at least 72 hours at the NICU and presenting clinical symptoms of *S. aureus* infection, namely positive hemocultures, were included in the study. Neonates were considered infected if *S. aureus* was isolated from a normally sterile site (*e.g.,* blood) or from other cultures obtained for clinical purposes (*e.g.,* umbilical exudates) when there was suspicion of infection. Whenever an infected infant was identified, an active surveillance screening was performed to detect eventual *S. aureus* carriers and reservoirs: nasal swabs from parents of the infected infant and HCWs that worked in the NICU during the 4 days preceding each infection case; swabs from the skin of mothers' nipples in case of breast feeding; and swabs from the environment of the NICU (cardio-respiratory monitors, incubators, milk pumps, stethoscopes, plastic folder protecting the infants clinical records hanging on the incubators, and telephones). In the case of multiple infection cases occurring within the same 45-day period since the detection of the first case, HCWs were screened only once, and the infection cases were considered as a part of the same infection episode. The infection episodes and the infected neonates were chronologically identified by roman and arabic numbers, respectively.

The Hospital Ethic Committee approved the study, and an informed consent was obtained from each individual screened, or from the parents in the case of infants.

### *Statistical analysis*

Descriptive statistics such as frequency and percentage, or mean ± standard deviation were used to characterize the population. Infection rates were calculated considering the number of infection cases per 1,000 patient days per month and the number of infection cases per 100 admitted neonates (cumulative incidence rate) during the study period.

### *Bacterial identification*

Blood samples were cultured in BD BACTEC Plus Aerobic/F media and incubated in BACTEC 9000 MB apparatus (Becton and Dickinson) for a maximum of 7 days and then, if positive, plated onto PolyViteX Chocolate agar selective media (bioMérieux). Screening swabs were inoculated onto Columbia Blood agar plates (bioMérieux) and incubated at 37°C overnight. All *S. aureus* isolates were identified by coagulase agglutination test using the STAPH AUREUS FUMOUZE kit (Fumouze Diagnostics) and by production of catalase using the ID Color Catalase kit (bioMérieux), followed by a semiautomatic identification through the VITEK2 system, using Gram-positive identification cards (GP-card) (bioMérieux SA). All isolates were conserved at −80°C in tryptic soy broth with 15% of glycerol.

### *Antimicrobial susceptibility testing and* mecA *screening*

Susceptibility testing to a panel of eight antibiotics (penicillin, amoxicillin-clavulanic acid, flucloxacillin, ciprofloxacin, erythromycin, tetracycline, gentamicin, and trimethoprim-sulfamethoxazole) was performed with the semiautomatic VITEK2 system, according to the manufacturer's instructions. Detection of *mecA* was performed on all *S. aureus* isolates by polymerase chain reaction (PCR).[38]

### *Molecular typing*

Pulsed-field gel electrophoresis. Pulsed-field gel electrophoresis (PFGE) was performed on all isolates after *Sma*I digestion as described by Chung *et al*.[13] The resulting patterns were analyzed by visual inspection using the criteria of McDougal *et al.*,[32] followed by an automated analysis with BioNumerics software version 4.61 (Applied Maths) for relatedness evaluation. Dendrograms were generated as previously described,[17] except that a tolerance value of 1.3% for band pattern comparisons was used. Dice coefficient similarity cutoff at 85% and 95% were used for PFGE type and subtype clusters definition, respectively.

*spa* typing and multilocus sequence typing. A representative isolate of each PFGE type, preferably recovered from infants, was selected for further characterization by *spa* typing and multilocus sequence typing (MLST) as described.[1,15] *spa* types were assigned using the Ridom web server (http://spaserver.ridom.de).[1] MLST allelic profiles, sequence types, and clonal complexes were defined using the MLST online database (www.mlst.net).

### *Detection of virulence determinants*

The presence of 21 staphylococcal virulence genes, including 3 leukocidins (*lukS-lukF*, *lukE-lukD*, and *lukM*), 3 hemolysins (*hlb*, *hlg*, and *hlgv*), and 15 super-antigenic toxins (*eta*, *etb*, *etd*, *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, *sel*, *sep*, and *tst*) were determined by PCR in all isolates, as described.[35,47] The detection of the accessory gene regulator (*agr*) group (I–IV) was performed on representative isolates of each PFGE type, as described.[21]

## Results

### *Incidence of* S. aureus *infection in the NICU and clinical characteristics of the infection episodes*

From July 2005 through December 2007, 1,021 high-risk infants were admitted in the NICU, of whom 16 developed an invasive infection due to methicillin susceptible *S. aureus* (MSSA), which corresponds to a rate of 0.86 infection cases per 1,000 patient days per month (ranging from 0 to 4.84 infection cases per 1,000 patient days per month) or 1.57 infection cases per 100 admissions. No invasive MRSA infections were detected in the NICU during the 30-month study period.

Fifteen out of the 16 infected infants were delivered in the maternity ward (Caesarean section, $n = 12$) and were directly

transferred to the NICU due to prematurity (mean gestational age of 31 weeks, ranging from 24 to 39 weeks) and/or low birth weight (mean of 1,187.5 g, and standard deviation of 649.8 g). Male accounted for 64.3% of the infants, and 71.4% were Caucasians. All the infants had venous central catheters and/or peripheral punctures at sepsis diagnosis, and 11 received parenteral nutrition. The mean time to acquire an *S. aureus* infection since admittance to the NICU was 15 days (ranging from 2 to 39 days) with the exception of infant 6 who got infected 187 days after admission. Two infants infected during the same episode died of multiorgan failure.

### S. aureus *isolates collected in the NICU*

The 16 infection cases reported were distributed over 10 infection episodes, resulting in a total of 54 *S. aureus* isolates: 30 isolates recovered from the 16 infants and 24 isolates recovered from surveillance screenings (Table 1). In four cases, different infants were infected within the same period of 45 days and were, therefore, included in the same infection episode (Fig. 1). The 30 *S. aureus* isolates from infants were recovered from hemocultures ($n=19$, 66.3%), catheters ($n=5$), umbilical exudates ($n=2$), wounds ($n=1$), pus of intestinal abscess ($n=1$), pus of skin abscess ($n=1$), and urine ($n=1$).

Among the 154 swabs obtained from the surveillance screenings, 24 *S. aureus* isolates were recovered from 18 HCWs ($n=19$, including two isolates from a re-colonized individual), two fathers ($n=2$), nipples of a mother ($n=2$), and the environment ($n=1$).

A total of 64 out of the 100 HCWs working in this NICU were screened during the study period showing a prevalence of *S. aureus* nasal colonization of 28% (18 out 64). We emphasize that 53% of the screened HCWs (34 out of 64) were in contact with an infected infant in different episodes and for this reason, they were screened more than once (number of screenings: one [$n=30$ HCWs], two [$n=25$], three [$n=7$], four [$n=1$], and five [$n=1$]).

Both mother and father of seven infants were available for nasal screening. Only two fathers (infants 3 and 7) were nasal carriers for MSSA. The nipples of the mother of the single breastfed infant (infant 16) were screened, and two MSSA isolates were recovered.

Regarding the NICU environment, different inanimate surfaces were swabbed: cardio-respiratory monitors ($n=3$), plastic folders hanging on the incubators to protect the clinical paper files ($n=3$), incubators ($n=6$), milk pumps ($n=4$), stethoscopes ($n=3$), and telephones ($n=9$). The unique MSSA isolate recovered from the NICU environment was isolated from a plastic folder.

### *Antimicrobial resistance*

A common nonmultiresistant antibiotic profile characterized all the isolates from this study: none was resistant to amoxicillin-clavulanic acid, flucloxacillin, gentamicin, tetracycline, and trimethoprim-sulfamethoxazole; four (from three infants) were susceptible to penicillin; three showed resistance to erythromycin, and one isolate (from an infant) was resistant to ciprofloxacin; and none of the isolates carried the *mecA* gene.

### *Molecular characterization and virulence factors*

The molecular characterization distributed the 54 MSSA isolates into 11 PFGE types (A to H and J to L) (Table 1). The majority of the isolates ($n=38$, 70.4%) belonged to three major clonal types: A-ST5 ($n=17$); B-ST30 ($n=12$); and C-ST1 ($n=9$). Representatives of each of these clonal types were characterized by *spa* types t1228, t012, and t922, respectively.

Isolates belonging to PFGE types A, B, C, and H were recovered during different episodes from infants and HCW (Fig. 1): A (seven episodes), B (six episodes), C (four episodes), and H (two episodes), while isolates with PFGE types K and J were identified in both infants and HCW in the same episode. PFGE types D and E contained isolates recovered from infants only, while types F, G, and L were exclusively associated to HCW.

The prevalent clonal types, PFGE A-ST5 and PFGE B-ST30 were recovered during the study period, while PFGE C-ST1 appeared sporadically in 2006 during episode III and then reappeared in May 2007 (Fig. 1). The remaining clonal types were recovered sporadically in isolated episodes.

Table 2 presents the virulence determinants found among the 54 MSSA isolates. Although none of the isolates was positive for PVL, *lukM*, *eta*, *etb*, *etd*, *seb*, *sej*, and *see*, all amplified *sel*. Isolates other than those belonging to PFGE types B and J were positive for *lukE-lukD* and γ-hemolysin variant. PFGE types B, D, and E and the single isolate type L, harbored β-hemolysin. Only isolates belonging to PFGE type B presented both β- and γ-hemolysins. Genes *sec*, *seh*, and *sep* were exclusively found among isolates of type C, while only isolates of types B and F were positive for the *tst* gene, which encodes the toxic shock syndrome toxin.

Concerning the auxiliary gene regulator (*agr*), *agr* type I was associated to seven PFGE types found in this study (C-E, G, and J-L). Isolates belonging to PFGE types B and F showed *agr* type III, while isolates with PFGE types A and H presented *agr* types II and IV, respectively (Table 1).

### *MSSA reservoirs and transmission routes*

In three infection cases, the same strain was recovered from the infant and at least one HCW (infant 1/HCW58- episode I; infant 13/HCWs 16, 34, and 52 and infant 15/HCW28- episode IX) (Table 1 and Fig. 1).

In episode VI, the infant was infected with the same strain (PFGE type A3) recovered in previous episodes from a HCW (episode II) and from the environment (plastic folder for clinical records hanging on an infant incubator - episode IV).

In four infants (infants 2, 5, 9, and 11), the same strain was recovered from the blood and the catheter (Table 1), suggesting the catheters as the possible source of infection.

In episode X, the strain responsible for the infant's infection was found on the mother's nipples. In no other episode did we find identical isolates shared by the infant and any of the parents.

*S. aureus* with three distinct PFGE types were responsible for infection in multiple infants in distinct episodes (PFGE type A2: infants 5 and 13; PFGE type B1: infants 1 and 8; PFGE type C3: infants 14 and 16). Moreover, strains A2 and B1 were detected in several HCWs (Fig. 1). Interestingly, HCW52, colonized with strain A2 in episode IX, had been previously colonized with a different strain (PFGE F1) in episode II. This was the only HCW that showed an

Table 1. Molecular Characterization of the 54 MSSA Isolates Recovered During 10 Infection Episodes in a Portuguese NICU from July 2005 to December 2007

| | | | | | | | *MLST* | | |
|---|---|---|---|---|---|---|---|---|---|
| *Episode* | *Isolate ID* | *Isolation date* | *Source* | *Individual* | *PFGE type* | spa *type*[a] | *ST* | *CC* | agr *type* |
| I | HFF191 | Jul-05 | Exudate | Infant1 | B1 | t012 | 30 | 30 | III |
| I | HFF192 | Aug-05 | Catheter | Infant2 | A1 | | | | |
| I | HFF193 | Aug-05 | Blood | Infant2 | A1 | t1228 | 5 | 5 | |
| I | HFF196 | Sep-05 | Nasal | HCW24 | A2 | | | | |
| I | HFF194 | Sep-05 | Nasal | HCW58 | B1 | | | | |
| II | HFF198 | Nov-05 | Blood | Infant3 | E | t377 | 630 | 8 | |
| II | HFF199 | Nov-05 | Exudate | Infant3 | E | | | | |
| II | HFF190 | Nov-05 | Blood | Infant4 | E | | | | I |
| II | HFF203 | Nov-05 | Nasal | Father3 | B2 | | | | |
| II | HFF200 | Nov-05 | Nasal | HCW51 | A3 | | | | |
| II | HFF202 | Nov-05 | Nasal | HCW52 | F | t1537 | 707 | sing | III |
| II | HFF201 | Nov-05 | Nasal | HCW53 | G | t008 | 1508 | 8 | I |
| III | HFF206 | Jan-06 | Catheter | Infant5 | A2 | | | | |
| III | HFF207 | Jan-06 | Blood | Infant5 | A2 | | | | |
| III | HFF208 | Jan-06 | Urine | Infant5 | A2 | | | | |
| III | HFF226 | Feb-06 | Blood | Infant6 | D | t2152 | 188 | 15 | I |
| III | HFF227 | Feb-06 | Blood | Infant6 | D | | | | |
| III | HFF225 | Jan-06 | Nasal | HCW15 | H1 | | | | |
| III | HFF209 | Jan-06 | Nasal | HCW55 | C1 | t922 | 1 | 15 | |
| IV | HFF274 | Apr-06 | Blood | Infant7 | B3 | | | | |
| IV | HFF276 | May-06 | Nasal | Father7 | A4 | | | | |
| IV | HFF275 | May-06 | Surface | Environment | A3 | | | | |
| V | HFF280 | Jul-06 | Blood | Infant8 | B1 | | | | |
| VI | HFF427 | Mar-07 | Catheter | Infant9 | A3 | | | | |
| VI | HFF428 | Mar-07 | Blood | Infant9 | A3 | | | | |
| VII | HFF507 | May-07 | Blood | Infant10 | C2 | | | | |
| VIII | HFF500 | Jun-07 | Blood | Infant11 | B4 | | | | |
| VIII | HFF501 | Jun-07 | Catheter | Infant11 | B4 | | | | |
| VIII | HFF502 | Jun-07 | Blood | Infant11 | B4 | | | | |
| VIII | HFF503 | Jun-07 | Nasal | HCW54 | B5 | | | | |
| IX | HFF613 | Oct-07 | Catheter | Infant12 | K1 | t091 | 7 | 7 | I |
| IX | HFF620 | Oct-07 | Blood | Infant12 | H2 | t2155 | 121 | 121 | IV |
| IX | HFF614 | Oct-07 | Blood | Infant13 | A2 | | | | II |
| IX | HFF617 | Oct-07 | Exudate | Infant13 | A2 | | | | |
| IX | HFF618 | Oct-07 | Blood | Infant14 | C3 | | | | |
| IX | HFF619 | Oct-07 | Exudate | Infant14 | C3 | | | | |
| IX | HFF621 | Nov-07 | Blood | Infant15 | J1 | t065 | 45 | 45 | I |
| IX | HFF622 | Nov-07 | Blood | Infant15 | J1 | | | | |
| IX | HFF629 | Nov-07 | Nasal | HCW34 | A2 | | | | |
| IX | HFF631 | Nov-07 | Nasal | HCW52 | A2 | | | | |
| IX | HFF636 | Dec-07 | Nasal | HCW61 | A2 | | | | |
| IX | HFF627 | Nov-07 | Nasal | HCW26 | B1 | | | | |
| IX | HFF630 | Nov-07 | Nasal | HCW48 | B6 | | | | |
| IX | HFF628 | Nov-07 | Nasal | HCW32 | B7 | | | | |
| IX | HFF625 | Nov-07 | Nasal | HCW28 | J1 | | | | |
| IX | HFF635 | Dec-07 | Nasal | HCW27 | J2 | | | | |
| IX | HFF632 | Nov-07 | Nasal | HCW4 | K2 | | | | |
| IX | HFF626 | Nov-07 | Nasal | HCW61 | L | t514 | 101 | 101 | I |
| X | HFF637 | Dec-17 | Nasal | HCW56 | A2 | | | | |
| X | HFF633 | Dec-07 | Blood | Infant16 | C3 | | | | I |
| X | HFF634 | Dec-07 | Blood | Infant16 | C3 | | | | |
| X | HFF638 | Dec-17 | Nipple | Nipple16-R | C3 | | | | |
| X | HFF639 | Dec-17 | Nipple | Nipple16-L | C3 | | | | |
| X | HFF640 | Dec-07 | Pus | Infant16 | C3 | | | | |

[a]Ridom nomenclature (http://spaserver.ridom.de/)

MLST, multilocus sequence typing; ST, sequence type; CC, clonal complex, defined by eBurst v3 accessed on 28 September 2011; sing, singleton; HCW, health care worker; nipple16, mother's nipples from infant 16; R, right; L, left; MSSA, methicillin susceptible *S. aureus*; NICU, neonatal intensive care units.

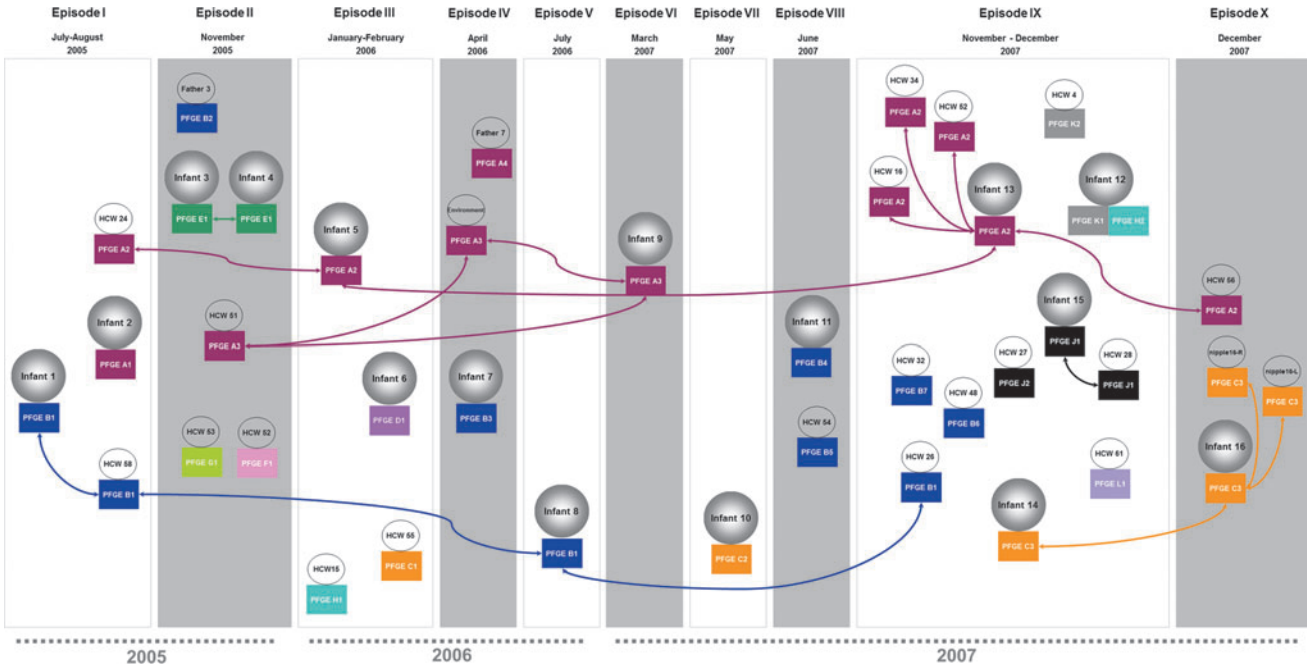


**FIG. 1.** Transmission routes and reservoirs of MSSA identified in the NICU from July 2005 to December 2007. Continuous arrows indicate the same PFGE subtype between isolates. Clonal types are color differentiated. HCW, health care worker; NICU, neonatal intensive care units; nipple16, mother's nipples from infant 16; R, right; L, left.

TABLE 2. REPRESENTATIVE ISOLATES OF EACH PFGE SUBTYPE FOUND AMONG THE MSSA COLLECTION AND RELATED VIRULENCE DETERMINANTS

| | | | *Virulence determinants*[a] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Isolate ID* | *Individual* | *PFGE subtype* | lukE-lukD | hlb | hlg | hlgv | sea | sec | sed | seg | seh | sei | sep | tst |
| HFF193 | Infant2 | A1 | + | − | − | + | − | − | − | + | − | + | − | − |
| HFF207 | Infant5 | A2 | + | − | − | + | − | − | − | + | − | + | − | − |
| HFF428 | Infant9 | A3 | + | − | − | + | − | − | − | + | − | + | − | − |
| HFF276 | Father7 | A5 | + | − | − | + | − | − | − | + | − | + | − | − |
| HFF191 | Infant1 | B1 | − | + | + | − | − | − | − | + | − | − | − | + |
| HFF203 | Father3 | B2 | − | − | − | − | − | − | − | + | − | − | − | + |
| HFF274 | Infant7 | B3 | − | + | + | − | − | − | − | + | − | − | − | + |
| HFF500 | Infant11 | B4 | − | + | + | − | − | − | − | + | − | − | − | +/− |
| HFF503 | HCW54 | B5 | − | + | + | − | − | − | − | + | − | − | − | + |
| HFF630 | HCW48 | B6 | − | + | + | − | + | − | − | + | − | − | − | + |
| HFF628 | HCW32 | B7 | − | + | + | − | + | − | − | + | − | − | − | + |
| HFF209 | HCW55 | C1 | + | − | − | + | + | − | − | − | + | − | − | − |
| HFF507 | Infant10 | C2 | + | − | − | + | − | − | − | − | − | − | − | − |
| HFF618 | Infant14 | C3 | + | − | − | + | − | + | − | − | − | − | + | − |
| HFF226 | Infant6 | D | + | + | − | + | − | − | − | − | − | − | − | − |
| HFF198 | Infant3 | E | + | + | − | + | − | − | − | − | − | − | − | − |
| HFF202 | HCW52 | F | + | − | − | + | − | − | + | − | − | − | − | + |
| HFF201 | HCW53 | G | + | − | − | + | − | − | + | − | − | − | − | − |
| HFF225 | HCW15 | H1 | + | − | − | + | − | − | − | + | − | − | − | − |
| HFF620 | Infant12 | H2 | + | − | − | + | − | − | − | + | − | − | − | − |
| HFF621 | Infant15 | J1 | − | − | + | − | − | − | − | +/− | − | + | − | − |
| HFF635 | HCW27 | J2 | − | − | + | − | − | − | − | + | − | + | − | − |
| HFF613 | Infant12 | K1 | + | − | − | + | − | − | − | − | − | − | − | − |
| HFF632 | HCW4 | K2 | + | − | − | + | − | − | − | − | − | − | − | − |
| HFF626 | HCW61 | L | + | + | − | + | − | − | − | − | − | − | − | − |

[a]All isolates were tested for 21 virulence genes. Only virulence determinants that showed variance in presence/absence are listed. The totality of the isolates were positive for *sel* encoding staphylococcal enterotoxin L and negative for *lukF-lukS-PV* and *lukM* encoding the Panton Valentine leukocidin and leukocidin M, respectively; *eta*, *etb*, and *etd*, encoding exfoliative toxins A, B, and D and *seb*, *see*, and *sej*, encoding staphylococcal enterotoxins B, E, and J. *lukE-lukD*—leukocidins D and E genes; *hlb*, *hlg*, and *hlgv*—β-hemolysin, γ-hemolysin, and γ-hemolysin variant genes, respectively; *sea*, *sec*, *sed*, *seg*, *seh*, *sei*, *sel*, and *sep*—staphylococcal enterotoxins A, C, D, G, H, I, L, and P genes, respectively; *tst*—toxic shock syndrome toxin gene.

HCW, health care worker; +, gene presence; −, gene absence; +/−, variable result within the same PFGE subtype.

intermittent carriage of two clonally distinct strains, suggesting the occurrence of re-colonization.

## Discussion

*S. aureus* infections in NICUs are a matter of great concern, as the newborn population is particularly vulnerable. In the present study, we assessed the epidemiology of *S. aureus* infections in a level III Portuguese NICU during a 30-month period. During this time, no MRSA infections occurred; however, 16 infants were infected with MSSA, corresponding to a rate of 0.86 infection cases per 1,000 patient-days per month or 1.57 infection cases per 100 total admissions. These values are considerably lower than the estimated values in a large NICU in the United States or in Brazil (2.18 and 4.4 MSSA infection cases per 100 admissions, respectively).[11,44]

Molecular typing showed that three predominant MSSA clonal types A (ST5), B (ST30), and C (ST1), were responsible for infection in 11 out of the 16 infants, and were maintained in the unit during the whole study period (30 months), suggesting that these clones were not only endemic but had high epidemicity as well. The pathogenic potential of these clonal types is apparent in a study by Grundmann *et al.* where these clonal types were common among the most frequent MSSA responsible for invasive disease in Europe.[26] Moreover, ST5 and ST30 strains were found to be the most common among nosocomial MSSA isolates from Portugal recovered between 1992 and 2003, suggesting that they should be endemic in many Portuguese hospitals.[3] Interestingly, the ST5 genetic background appears to be also common among the MRSA population, not only in the hospital where this study was undertaken—where the New York/Japan MRSA clone (ST5-SCC*mec* II) was the major MRSA clone in adult wards in 2006—but also disseminated among all Portuguese hospitals, being the second most prevalent clone in the country, following the EMRSA-15 clone (ST22-IVh).[2]

It is well known that HCWs who are nasal carriers play an important role as sources of *S. aureus* transmission, and neonatal outbreaks due to HCWs colonizing strains have been previously reported.[8,27,33,42] In the present study, most HCWs were colonized with MSSA clonal types that were endemic in the unit and, therefore, may be acting as reservoirs of potential pathogenic MSSA strains inside the NICU. CA-MRSA outbreaks have been also described in NICUs due to vertical transmission from colonized parents.[37,43] In the present study, in a single episode, the strain responsible for sepsis and abscess infection in the infant (PFGE C3-ST1) also colonized the mother's nipples. Although nowadays expressed breast milk should be pasteurized before use, previous studies have shown vertical transmission of MRSA through contaminated breast milk.[7,19] In our study, the mother was not nasally colonized, which prevents us from suggesting that the direction of the transmission was from the mother to the child. Since clone C was endemic in the NICU and *S. aureus* nasal colonization frequently occurs soon after birth,[25,28] the infant might have been first colonized, developing the infection later from an endogenous source and subsequently colonized the mother's nipples that became a reservoir for the infection strain. Evaluation of the nasal carriage among the infants could have provided insights on the possible endogenous transmission.

HCWs and infants' parents may constitute a bridge between the hospital and the community, and represent possible vehicles for the introduction of community-related isolates in the NICU.

NICU environmental surfaces are potential growing settings for nosocomial pathogens, and numerous fomites have been reported as reservoirs for nosocomial transmission.[14,18] Moreover, it is known that *S. aureus* can be stable in dry environments with a median survival time of 12 days (1 to >60 days) on inanimate surfaces in ICUs.[20,30] In the present study, a single MSSA isolate recovered from one plastic folder showed a PFGE indistinguishable from the strains colonizing a HCW and infecting an infant several months apart. The fact that the plastic folders hanging on each incubator were not replaced between consecutive infants could act as an *S. aureus* reservoir in this NICU and, therefore, increased the risk of cross-transmission.

The majority (13 out of 16) of the infants were infected with isolates belonging to clonal types endemic in the NICU (clonal types A, B, and C) and/or colonizing HCWs (clonal types J and K). However, three infants were infected by unique clones, nonrelated to any other MSSA isolate recovered during the study period, and the source of these isolates could not be determined. Transient contamination of HCWs hands has been documented on many occasions[6,22,40] and could have been the way through which *S. aureus* was transmitted to patients. Nevertheless, it was not possible to evaluate it retrospectively.

Nosocomial MSSA isolates are usually susceptible to most antimicrobial agents, and their success is probably due to their increased virulence, rather than multiresistance.[36] The MSSA isolates circulating in the NICU, in fact, showed a common nonmultiresistant profile, but carried several virulence factors including leukocidins, hemolysins, and super antigens. The MSSA isolates that simultaneously infected infants 3 and 4 (PFGE type E isolates), who died after intestinal lesions and consequently multiorgan failure, produced several different toxins (hemolysins, one leukocidin, and one enterotoxin), which could have been responsible for necrosis of the intestinal host cells, as reported for the pathogenesis of enterocolitis due to *S. aureus*.[31]

After episode II (November 2005), when two infected babies died and the unit was closed for disinfection, several infection control measures have been implemented in the unit: (1) After each episode, all medical material that was in contact with an infected infant, including incubators, was carefully disinfected (tablets containing 50% of sodium dichloroisocyanurate followed by ethanol); (2) Implementation of a routine replacement of the plastic folder hanging on the incubators at each new admission; (3) Educational workshops on staphylococcal infections in NICUs were performed in the unit for all HCW with emphasis on correct handwashing procedures, a key factor for the infection control.[10]

The study presents some limitations, especially concerning the surveillance screening process. In the majority of the episodes, the screening was incomplete, as some HCWs were not available or did not agree to being screened. Therefore, some reservoirs and/or transmission routes may have been underestimated or even not identified. Data regarding the noninfected infants admitted to the NICU were not available, and risk factors for MSSA infection could not be traced. Nevertheless, some particular traits, already reported as

major risk factors for infection, were also present in the infected infants included in this study, namely the low gestational age, the low birth weight, and the use of central catheters and/or peripheral punctures. Since colonization with commensal bacteria in infants admitted at an NICU cannot be avoided, a periodical screening of all infants would not only establish global *S. aureus* carriage rates, but also identify exactly the time of colonization as well, which is known to be a risk factor for infection.[39,41]

In the present study, we showed that nationally and internationally disseminated MSSA clones were endemic in a major Portuguese NICU, and we provided evidence for the role of HCWs as reservoirs and vehicles of transmission of *S. aureus* in this high-risk setting. Plastic clinical folders that are usually hanging on the infants' incubators were shown for the first time to act as a potential reservoir of *S. aureus*. The study led to the improvement of infection control measures in the NICU, and may serve as a model to other NICUs in Portugal.

## Acknowledgments

This work was partially supported by Fundação para a Ciência e a Tecnologia (FCT) through Grant No. PEst-OE/EQB/LA0004/2011 and Project POCTI/SAU-ESP/57841/2004, and by funding from the European Community, Project TROCAR (FP7-HEALTH-2007-B Project No. 223031). T. Conceição was supported by Grant SFRH/BD/21424/2005 from FCT, Portugal.

The authors thank the staff of the NICU and Microbiology Laboratory of Hospital Fernando Fonseca and the caregivers of the infants for their willingness to participate in this study.

## Disclosure Statement

The authors have declared that no competing interests exist.

## References

1. **Aires-de-Sousa, M., K. Boye, H. de Lencastre, A. Deplano, M.C. Enright, J. Etienne, A. Friedrich, D. Harmsen, A. Holmes, X.W. Huijsdens, A.M. Kearns, A. Mellmann, H. Meugnier, J.K. Rasheed, E. Spalburg, B. Strommenger, M.J. Struelens, F.C. Tenover, J. Thomas, U. Vogel, H. Westh, J. Xu, and W. Witte.** 2006. High interlaboratory reproducibility of DNA sequence-based typing of bacteria in a multicenter study. J. Clin. Microbiol. **44:**619–621.
2. **Aires-de-Sousa, M., B. Correia, and H. de Lencastre.** 2008. Changing patterns in frequency of recovery of five methicillin-resistant *Staphylococcus aureus* clones in Portuguese hospitals: surveillance over a 16-year period. J. Clin. Microbiol. **46:**2912–2917.
3. **Aires de Sousa, M., T. Conceicao, C. Simas, and H. de Lencastre.** 2005. Comparison of genetic backgrounds of methicillin-resistant and -susceptible *Staphylococcus aureus* isolates from Portuguese hospitals and the community. J. Clin. Microbiol. **43:**5150–5157.
4. **Aires de Sousa, M., and H. de Lencastre.** 2004. Bridges from hospitals to the laboratory: genetic portraits of methicillin-resistant *Staphylococcus aureus* clones. FEMS Immunol. Med. Microbiol. **40:**101–111.
5. **Al-Tawfiq, J.A.** 2006. Father-to-infant transmission of community-acquired methicillin-resistant *Staphylococcus aureus* in a neonatal intensive care unit. Infect. Control Hosp. Epidemiol. **27:**636–637.
6. **Albrich, W.C., and S. Harbarth.** 2008. Health-care workers: source, vector, or victim of MRSA? Lancet Infect. Dis. **8:**289–301.
7. **Behari, P., J. Englund, G. Alcasid, S. Garcia-Houchins, and S.G. Weber.** 2004. Transmission of methicillin-resistant *Staphylococcus aureus* to preterm infants through breast milk. Infect. Control Hosp. Epidemiol. **25:**778–780.
8. **Bertin, M.L., J. Vinski, S. Schmitt, C. Sabella, L. Danziger-Isakov, M. McHugh, G.W. Procop, G. Hall, S.M. Gordon, and J. Goldfarb.** 2006. Outbreak of methicillin-resistant *Staphylococcus aureus* colonization and infection in a neonatal intensive care unit epidemiologically linked to a healthcare worker with chronic otitis. Infect. Control Hosp. Epidemiol. **27:**581–585.
9. **Bertini, G., P. Nicoletti, F. Scopetti, P. Manoocher, C. Dani, and G. Orefici.** 2006. *Staphylococcus aureus* epidemic in a neonatal nursery: a strategy of infection control. Eur. J. Pediatr. **165:**530–535.
10. **Borghesi, A., and M. Stronati.** 2008. Strategies for the prevention of hospital-acquired infections in the neonatal intensive care unit. J. Hosp. Infect. **68:**293–300.
11. **Carey, A.J., J. Duchon, P. Della-Latta, and L. Saiman.** 2010. The epidemiology of methicillin-susceptible and methicillin-resistant *Staphylococcus aureus* in a neonatal intensive care unit, 2000–2007. J. Perinatol. **30:**135–139.
12. **Carey, A.J., L. Saiman, and R.A. Polin.** 2008. Hospital-acquired infections in the NICU: epidemiology for the new millennium. Clin. Perinatol. **35:**223–249.
13. **Chung, M., H. de Lencastre, P. Matthews, A. Tomasz, I. Adamsson, M. Aires de Sousa, T. Camou, C. Cocuzza, A. Corso, I. Couto, A. Dominguez, M. Gniadkowski, R. Goering, A. Gomes, K. Kikuchi, A. Marchese, R. Mato, O. Melter, D. Oliveira, R. Palacio, R. Sa-Leao, I. Santos Sanches, J.H. Song, P.T. Tassios, and P. Villari.** 2000. Molecular typing of methicillin-resistant *Staphylococcus aureus* by pulsed-field gel electrophoresis: comparison of results obtained in a multilaboratory effort using identical protocols and MRSA strains. Microb. Drug Resist. **6:**189–198.
14. **Clark, R., R. Powers, R. White, B. Bloom, P. Sanchez, and D.K. Benjamin, Jr.** 2004. Nosocomial infection in the NICU: a medical complication or unavoidable problem? J. Perinatol. **24:**382–388.
15. **Crisostomo, M.I., H. Westh, A. Tomasz, M. Chung, D.C. Oliveira, and H. de Lencastre.** 2001. The evolution of methicillin resistance in *Staphylococcus aureus*: similarity of genetic backgrounds in historically early methicillin-susceptible and -resistant isolates and contemporary epidemic clones. Proc. Natl. Acad. Sci. U. S. A. **98:**9865–9870.
16. **[EARSS] European Antimicrobial Resistance Surveillance System.** 2010. EARSS Annual Report 2009. EARSS, Bilthoven, The Netherlands, pp. 28–30. Available at www.earss.rivm.nl (online.) Accessed on September 28, 2011.
17. **Faria, N.A., J.A. Carrico, D.C. Oliveira, M. Ramirez, and H. de Lencastre.** 2008. Analysis of typing methods for epidemiological surveillance of both methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* strains. J. Clin. Microbiol. **46:**136–144.
18. **Fujimura, S., S. Kato, M. Hashimoto, H. Takeda, F. Maki, and A. Watanabe.** 2004. Survey of methicillin-resistant *Staphylococcus aureus* from neonates and the environment in the NICU. J. Infect. Chemother. **10:**131–132.

19. **Gastelum, D.T., D. Dassey, L. Mascola, and L.M. Yasuda.** 2005. Transmission of community-associated methicillin-resistant *Staphylococcus aureus* from breast milk in the neonatal intensive care unit. Pediatr. Infect. Dis. J. **24:**1122–1124.
20. **Gastmeier, P., F. Schwab, S. Bärwolff, H. Rüden, and H. Grundmann.** 2006. Correlation between the genetic diversity of nosocomial pathogens and their survival time in intensive care units. J. Hosp. Infect. **62:**181–186.
21. **Gilot, P., G. Lina, T. Cochard, and B. Poutrel.** 2002. Analysis of the genetic variability of genes encoding the RNA III-activating components Agr and TRAP in a population of *Staphylococcus aureus* strains isolated from cows with mastitis. J. Clin. Microbiol. **40:**4060–4067.
22. **Girou, E., G. Pujade, P. Legrand, F. Cizeau, and C. Brun-Buisson.** 1998. Selective screening of carriers for control of methicillin-resistant *Staphylococcus aureus* (MRSA) in high-risk hospital areas with a high level of endemic MRSA. Clin. Infect. Dis. **27:**543–550.
23. **Gomez-Gonzalez, C., C. Alba, J.R. Otero, F. Sanz, and F. Chaves.** 2007. Long persistence of methicillin-susceptible strains of *Staphylococcus aureus* causing sepsis in a neonatal intensive care unit. J. Clin. Microbiol. **45:**2301–2304.
24. **Graham, P.L., 3rd, A.S. Morel, J. Zhou, F. Wu, P. Della-Latta, D. Rubenstein, and L. Saiman.** 2002. Epidemiology of methicillin-susceptible *Staphylococcus aureus* in the neonatal intensive care unit. Infect. Control Hosp. Epidemiol. **23:**677–682.
25. **Gries, D.M., T.F. Zemzars, K.A. Gibson, E. O'Hern, M. Iyer, M. Myers, M.J. Pultz, Y. Li, and C.J. Donskey.** 2009. A pilot study to assess frequency of carriage and routes of acquisition of *Staphylococcus aureus* by healthy infants. Am. J. Infect. Control **37:**598–600.
26. **Grundmann, H., D.M. Aanensen, C.C. van den Wijngaard, B.G. Spratt, D. Harmsen, and A.W. Friedrich.** 2010. Geographic distribution of *Staphylococcus aureus* causing invasive infections in Europe: a molecular-epidemiological analysis. PLoS Med. **7:**e1000215.
27. **Heinrich, N., A. Mueller, P. Bartmann, A. Simon, G. Bierbaum, and S. Engelhart.** 2011. Successful management of an MRSA outbreak in a neonatal intensive care unit. Eur. J. Clin. Microbiol. Infect. Dis. **30:**909–913.
28. **Huang, Y.C., Y.H. Chou, L.H. Su, R.I. Lien, and T.Y. Lin.** 2006. Methicillin-resistant *Staphylococcus aureus* colonization and its association with infection among infants hospitalized in neonatal intensive care units. Pediatrics **118:**469–474.
29. **Hudome, S.M., and M.C. Fisher.** 2001. Nosocomial infections in the neonatal intensive care unit. Curr. Opin. Infect. Dis. **14:**303–307.
30. **Lin, Y.C., T.L. Lauderdale, H.M. Lin, P.C. Chen, M.F. Cheng, K.S. Hsieh, and Y.C. Liu.** 2007. An outbreak of methicillin-resistant *Staphylococcus aureus* infection in patients of a pediatric intensive care unit and high carriage rate among health care workers. J. Microbiol. Immunol. Infect. **40:**325–334.
31. **Lin, Z., D. Kotler, P. Schlievert, and E. Sordillo.** 2010. Staphylococcal enterocolitis: forgotten but not gone? Dig. Dis. Sci. **55:**1200–1207.
32. **McDougal, L.K., C.D. Steward, G.E. Killgore, J.M. Chaitram, S.K. McAllister, and F.C. Tenover.** 2003. Pulsed-field gel electrophoresis typing of oxacillin-resistant *Staphylococcus aureus* isolates from the United States: establishing a national database. J. Clin. Microbiol. **41:**5113–5120.
33. **Mean, M., M.R. Mallaret, P. Andrini, C. Recule, T. Debillon, P. Pavese, and J. Croize.** 2007. A neonatal specialist with recurrent methicillin-resistant *Staphylococcus aureus* (MRSA) carriage implicated in the transmission of MRSA to newborns. Infect. Control Hosp. Epidemiol. **28:**625–628.
34. **Melo-Cristino, J., L. Marques-Lito, and E. Pina.** 2002. The control of hospital infection in Portugal. J. Hosp. Infect. **51:**85–88.
35. **Monday, S.R., and G.A. Bohach.** 1999. Use of multiplex PCR to detect classical and newly described pyrogenic toxin genes in staphylococcal isolates. J. Clin. Microbiol. **37:**3411–3414.
36. **Moore, P.C., and J.A. Lindsay.** 2001. Genetic variation among hospital isolates of methicillin-sensitive *Staphylococcus aureus*: evidence for horizontal transfer of virulence genes. J. Clin. Microbiol. **39:**2760–2767.
37. **Morel, A.S., F. Wu, P. Della-Latta, A. Cronquist, D. Rubenstein, and L. Saiman.** 2002. Nosocomial transmission of methicillin-resistant *Staphylococcus aureus* from a mother to her preterm quadruplet infants. Am. J. Infect. Control **30:**170–173.
38. **Okuma, K., K. Iwakawa, J.D. Turnidge, W.B. Grubb, J.M. Bell, F.G. O'Brien, G.W. Coombs, J.W. Pearman, F.C. Tenover, M. Kapi, C. Tiensasitorn, T. Ito, and K. Hiramatsu.** 2002. Dissemination of new methicillin-resistant *Staphylococcus aureus* clones in the community. J. Clin. Microbiol. **40:**4289–4294.
39. **Peacock, S.J., A. Justice, D. Griffiths, G.D. de Silva, M.N. Kantzanou, D. Crook, K. Sleeman, and N.P. Day.** 2003. Determinants of acquisition and carriage of *Staphylococcus aureus* in infancy. J. Clin. Microbiol. **41:**5718–5725.
40. **Pessoa-Silva, C.L., S. Dharan, S. Hugonnet, S. Touveneau, K. Posfay-Barbe, R. Pfister, and D. Pittet.** 2004. Dynamics of bacterial hand contamination during routine neonatal care. Infect. Control Hosp. Epidemiol. **25:**192–197.
41. **Regev-Yochay, G., R. Dagan, M. Raz, Y. Carmeli, B. Shainberg, E. Derazne, G. Rahav, and E. Rubinstein.** 2004. Association between carriage of *Streptococcus pneumoniae* and *Staphylococcus aureus* in children. JAMA **292:**716–720.
42. **Saiman, L., A. Cronquist, F. Wu, J. Zhou, D. Rubenstein, W. Eisner, B.N. Kreiswirth, and P. Della-Latta.** 2003. An outbreak of methicillin-resistant *Staphylococcus aureus* in a neonatal intensive care unit. Infect. Control Hosp. Epidemiol. **24:**317–321.
43. **Sax, H., K. Posfay-Barbe, S. Harbarth, P. Francois, S. Touveneau, C.L. Pessoa-Silva, J. Schrenzel, S. Dharan, A. Gervaix, and D. Pittet.** 2006. Control of a cluster of community-associated, methicillin-resistant *Staphylococcus aureus* in neonatology. J. Hosp. Infect. **63:**93–100.
44. **Silva Hde, A., E.M. Pereira, R.P. Schuenck, R.C. Pinto, V.O. Abdallah, K.R. Santos, and P.P. Gontijo-Filho.** 2009. Molecular surveillance of methicillin-susceptible *Staphylococcus aureus* at a neonatal intensive care unit in Brazil. Am. J. Infect. Control **37:**574–579.
45. **Song, X., S. Cheung, K. Klontz, B. Short, J. Campos, and N. Singh.** 2010. A stepwise approach to control an outbreak and ongoing transmission of methicillin-resistant *Staphylococcus aureus* in a neonatal intensive care unit. Am. J. Infect. Control **38:**607–611.
46. **Stoll, B.J., N. Hansen, A.A. Fanaroff, L.L. Wright, W.A. Carlo, R.A. Ehrenkranz, J.A. Lemons, E.F. Donovan, A.R. Stark, J.E. Tyson, W. Oh, C.R. Bauer, S.B. Korones, S. Shankaran, A.R. Laptook, D.K. Stevenson, L.A. Papile, and W.K. Poole.** 2002. Late-onset sepsis in very low birth

weight neonates: the experience of the NICHD Neonatal Research Network. Pediatrics **110:**285–291.

47. **Vandenesch, F., T. Naimi, M.C. Enright, G. Lina, G.R. Nimmo, H. Heffernan, N. Liassine, M. Bes, T. Greenland, M.E. Reverdy, and J. Etienne.** 2003. Community-acquired methicillin-resistant *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes: worldwide emergence. Emerg. Infect. Dis. **9:**978–984.

Address correspondence to:
*Hermínia de Lencastre, Ph.D.*
*Laboratory of Microbiology*
*The Rockefeller University*
*1230 York Ave.*
*New York, NY 10065*

*E-mail:* lencash@mail.rockefeller.edu