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NOTES

Bacterial and Host Factors Implicated in Nasal Carriage of Methicillin-Resistant *Staphylococcus aureus* in Mice

Bruno González-Zorn,[1]† Jose P. M. Senna,[1]‡ Laurence Fiette,[2] Spencer Shorte,[3] Aurélie Testard,[1] Michel Chignard,[4] Patrice Courvalin,[1] and Catherine Grillot-Courvalin[1]*

Unité des Agents Antibactériens,[1] Unité de Recherche et d'Expertise en Histotechnologie et Pathologie,[2] Plate-forme d'Imagerie Dynamique,[3] and Unité de Défense Innée et Inflammation, INSERM E336,[4] Institut Pasteur, Paris, France

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Nasal carriage is a major risk factor for *Staphylococcus aureus* infection, especially for methicillin-resistant strains (MRSA). Using a mouse model of nasal carriage, we have compared several *S. aureus* strains and demonstrated increased colonization levels by MRSA in cystic fibrosis transmembrane conductance regulator-deficient mice and Toll-like receptor 2 (TLR2)-deficient mice but not TLR4-deficient mice.

Methicillin-resistant *Staphylococcus aureus* (MRSA) has spread worldwide, primarily as a nosocomial pathogen (2). Due to multiresistance of these strains, including recent acquisition of high-level glycopeptide resistance (3), few antibiotics remain effective for MRSA infections. Recent emergence of community-acquired MRSA infections is of particular concern, with some clinical isolates harboring the Panton-Valentine leukocidin determinant responsible for lethal necrotizing pneumonia (5).

Anterior nares are the major reservoir of *S. aureus*: 20% of humans are persistently and asymptomatically colonized, 60% are colonized intermittently, and 20% are noncarriers (13). Subgroups, such as cystic fibrosis (CF) patients, have higher carriage rates (10). Nasal carriage is a major risk factor for staphylococcal infection (7, 25). Commensal carriage of MRSA in healthy individuals remains low (from 0.2 to 2.8%) (6) but constitutes a greater risk for subsequent infection than methicillin-susceptible *S. aureus* carriage (13).

Mice constitute a good animal model for *S. aureus* nasal colonization; most mice inoculated with 10^8 CFU maintain carriage for at least 20 days (12). Recently, a cotton rat model in which *S. aureus* persists at higher CFU for up to 6 weeks (14) demonstrated the role of *S. aureus* wall teichoic acid in colonization (26). A mouse model, however, permits the study of inbred and knockout strains. We have modified the previous mouse model (12) and studied various MRSA isolates and knockout mice to dissect interactions between MRSA and the nasal tissue.

To assess anatomical location of intranasally introduced *S. aureus*, we used the stably bioluminescent MRSA Xen31 with a chromosomal copy of the modified *Photorhabdus luminescens luxABCDE* operon (Table 1). These bacteria were grown overnight in brain heart infusion medium at 37°C with shaking, washed twice, and resuspended in phosphate-buffered saline (Dulbecco) at 10^9 CFU/10 μl. Five- to eight-week-old female BALB/c mice (Harlan, Gannat, France) were anesthetized, and 10 μl of the bacterial suspension was introduced into both nares without touching the tip of the nose. The bioluminescent signal was strictly intranasal (Fig. 1A) and not detectable in the trachea or lungs. Bioluminescence monitored at 0, 12, 24, and 36 h after inoculation diminished during the first 24 h, disappearing by 36 h postinoculation (data not shown). Mice instilled intranasally with Xen31 were sacrificed after 12 h. The lower jaws were cut, the palate containing the nasal associated lymphoid tissue (NALT) was excised, and the nasal mucosa was exposed by cutting the head sagitally. The entire bioluminescent signal was located in the nasal mucosa, and no emission could be detected from the NALT (Fig. 1B). This is in contrast to bioluminescent group A streptococci, which were predominantly found in the NALT (16). The nasal mucosa dissected under a section microscope was exposed for bioluminetric detection; the whole bioluminescent signal was recorded from the dissected nasal tissue (Fig. 1C). Hence, this microsurgical method was used for the enumeration of CFU in the nasal cavity of mice inoculated with nonbioluminescent MRSA.

For histological study, mice inoculated intranasally with strain COL (Table 1) were sacrificed after 24 h; the heads, decalcified in 4% neutral formalin buffer with 10% trichloroacetic acid for 2 weeks, were coronally cut into five to six fragments and embedded in paraffin and 5-μm-thick sections were stained with hematoxylin-eosin or Gram stain. Cellular clumps with neutrophils and necrotic cells, undetectable in control mice (Fig. 2A), were present within the nasal lumen in

* Corresponding author. Mailing address: Unité des Agents Antibactériens, Institut Pasteur, 25 rue du Docteur Roux, 75724 Paris Cedex 15, France. Phone: (33) 1 45 68 83 20. Fax: (33) 1 45 68 83 19. E-mail: ccourval@pasteur.fr.

† Present address: Departemento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, 28040 Madrid, Spain.

‡ Present address: Laboratorio de Tecnologia Recombinante, Instituto Oswaldo Cruz, Rio de Janeiro, RJ, Brazil.

TABLE 1. Properties of the strains

S. aureus strain	Relevant characteristics	Source or reference
COL	*mecR1*Δ, *mecI*Δ, SCC *mec* typeIB, Tc^r	1
COL *mecA*	*mecA* Ω*tet*(M)	8
BM4559	Iberian clone, SCC *mec* type IA	19
BM4560	*luk*S-PV, *luk*F-PV, SCC *mec* type IV	5
Xen31	*mecA,* clinical isolate with chromosomal modified *luxABCD*	Xenogen

COL-inoculated mice (Fig. 2B$_1$). At higher magnification, a large number of bacteria appeared tightly attached to or inside cells (Fig. 2B$_3$).

A recently described mouse model of *S. aureus* nasal colonization allows quantification of bacteria in the nasal mucosa of mice (12). However, this method yields highly dispersed bacterial counts ranging from 0.5×10 to 1×10^5 CFU/mouse at day 5 postinoculation, thus requiring many mice. In this model, the nasal tissue is excised with scissors and vortexed. Since we observed cell-associated bacteria (Fig. 2), CFU were determined on thoroughly homogenized nasal tissue obtained by careful dissection. Twenty-four BALB/c mice were inoculated with 10^9 CFU of strain COL and sacrificed at days 3, 7, and 14. The dissected nasal mucosa was collected in 1 ml of sterile saline and homogenized, and serial dilutions were plated on Baird-Parker agar. After overnight incubation, colonies were enumerated and replica plated on brain heart infusion agar containing 10 μl of oxacillin/ml to ensure methicillin resistance. At day 3, a mean of 4.7×10^4 CFU/mouse was obtained, with small dispersion between counts; at day 7, all mice were colonized with a mean of 1.6×10^2 CFU/mouse (Table 2 and Fig. 3). At day 14, all the mice were still colonized, but at this late time point the CFU counts were low, ranging from 1.2×10 to 1.8×10/mouse. An inoculum of 10^8 CFU of strain COL or of 10^9 CFU of strain Xen31 led to colonization of all mice at days 3 and 7 but yielded lower CFU counts than those obtained with 10^9 CFU (Table 2). These results indicate that, although mice are not colonized with MRSA in the long term, this model is sufficiently reproducible to study bacterial and host factors involved in MRSA nasal colonization by using small numbers of animals.

To study if MRSA strains differ in their colonization ability, we inoculated two epidemiologically successful clinical isolates

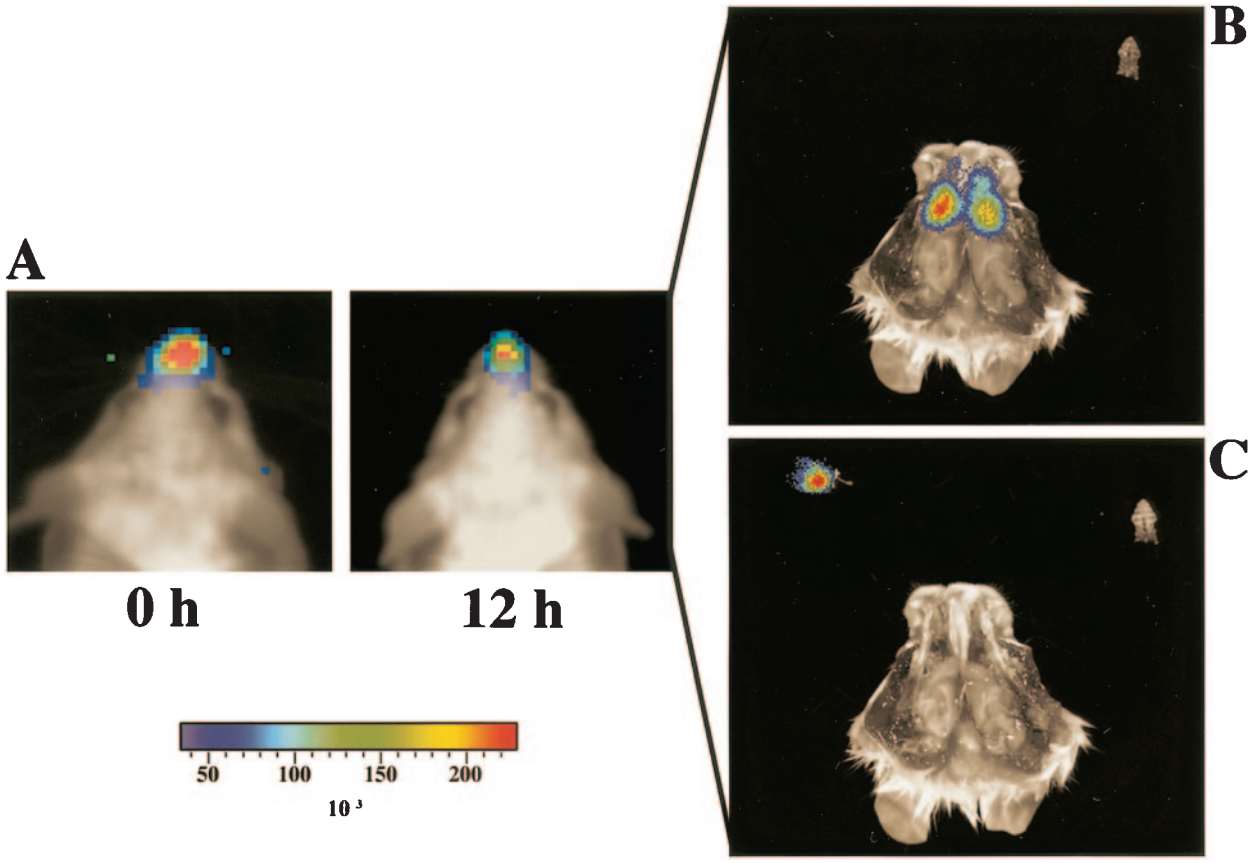

FIG. 1. Bioluminescence from mice inoculated nasally with MRSA Xen31. (A) Bioluminescence from the entire mouse was recorded for 15 min with a backside Hamamatsu ORCA-3 camera, and the images were processed with Compix software (Compix Inc., Brandywine, Pa.). A strong bioluminescent signal was recorded from the nose of the mice upon inoculation and 12 h postinoculation. (B) Anatomical location of MRSA during colonization. Twelve hours after inoculation, mice were sacrificed and dissected as described in the text, and the palate and dissected NALT (white ovals) were exposed for bioluminetric analysis with a Xenogen 100 IVIS system. The entire bioluminescent signal was emitted from the nasal mucosa, and no signal could be detected from the NALT. (C) Upon microdissection, the whole photonic signal was recorded from the excised mucosal tissue.

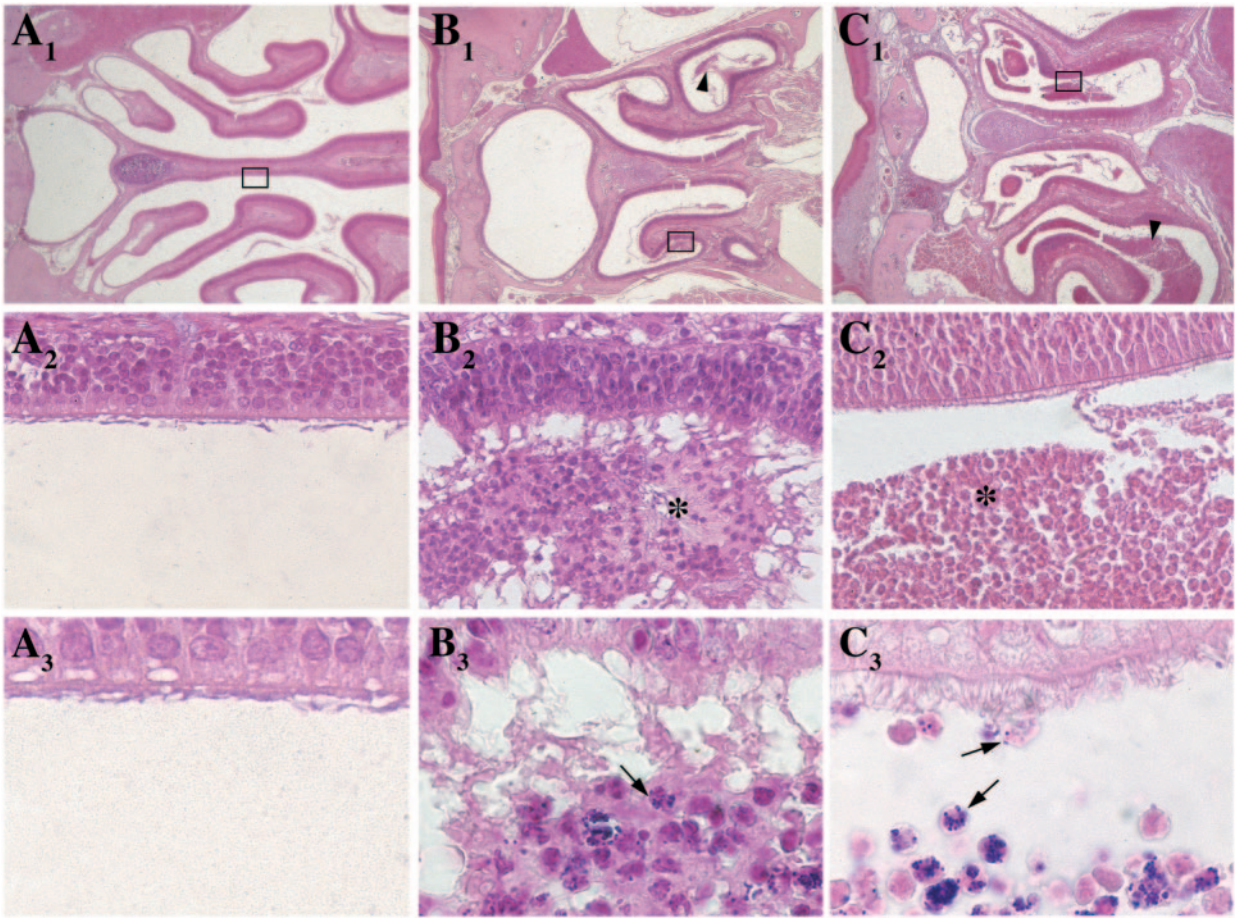

FIG. 2. Histological sections of the nasal cavities of control BALB/c mice (A) and BALB/c and CFTR$^{-/-}$ mice 24 h after inoculation of strain COL (B and C, respectively). Hematoxylin-eosin (upper panel, 25× enlargement; middle panel, 400× enlargement of selected area) and Gram staining (lower panel, 1,000×) were performed with 5-μm-thick sections. Luminal cellular clumps spread along the nasal cornets are indicated with arrowheads (B_1 and C_1) and asterisks (B_2 and C_2). Bacteria that appeared to be located intracellularly within the cellular clumps composed of neutrophils and degenerated cells are denoted with an arrow (B_3 and C_3). In CFTR$^{-/-}$ mice, bacteria were present along the nasal epithelium (C_3).

(Table 1). Strain BM4559 is a representative of the Iberian clone, a hospital-restricted, worldwide-spread MRSA, and BM4560 is a French isolate responsible for community-acquired infections and which produces the Panton-Valentine leukocidin. On day 3, lower counts of both strains were recovered, whereas at day 7 there was no significant difference between the three MRSA strains (Fig. 3).

Methicillin resistance in *S. aureus* is mediated by *mecA*, a gene encoding additional penicillin-binding protein PBP2a with low affinity for β-lactam antibiotics. When bacteria are grown in the presence of β-lactams, the production of PBP2a is induced; PBP2a is exposed at the bacterial surface and can interact with host tissues (unpublished data). To test if PBP2a is implicated in nasal colonization, we used a *mecA* null COL derivative (8); colonization (mean, 4.5×10^4 CFU/mouse at day 3 and 2.3×10^2 CFU/mouse at day 7) was not significantly different from that by parental strain COL. Similarly, colonization was not significantly enhanced when strain COL was grown in the presence of oxacillin (mean, 4.9×10^4 CFU/mouse at day 3 and 8.5×10^2 CFU/mouse at day 7), indicating that PBP2a is not a colonizing factor.

S. aureus is often the first pathogen to infect CF patients,

TABLE 2. Nasal colonization by MRSA strains COL and Xen31

Strain (inoculum)	Colonization at:			
	Day 3		Day 7	
	No. of carriers/ total no. assayed	Mean CFU (range)	No. of carriers/ total no. assayed	Mean CFU (range)
COL (10^8)	5/5	2.3×10^4 (6.3×10^3–2.5×10^4)	5/5	1.5×10^2 (1×10^1–6.3×10^2)
COL (10^9)	9/9	4.7×10^4 (2×10^3–3.2×10^5)	8/8	1.6×10^2 (3.2×10^1–4×10^2)
Xen31 (10^9)	5/5	1.3×10^3 (2.5×10^2–4×10^3)	5/5	5×10^1 (2×10^1–2×10^2)

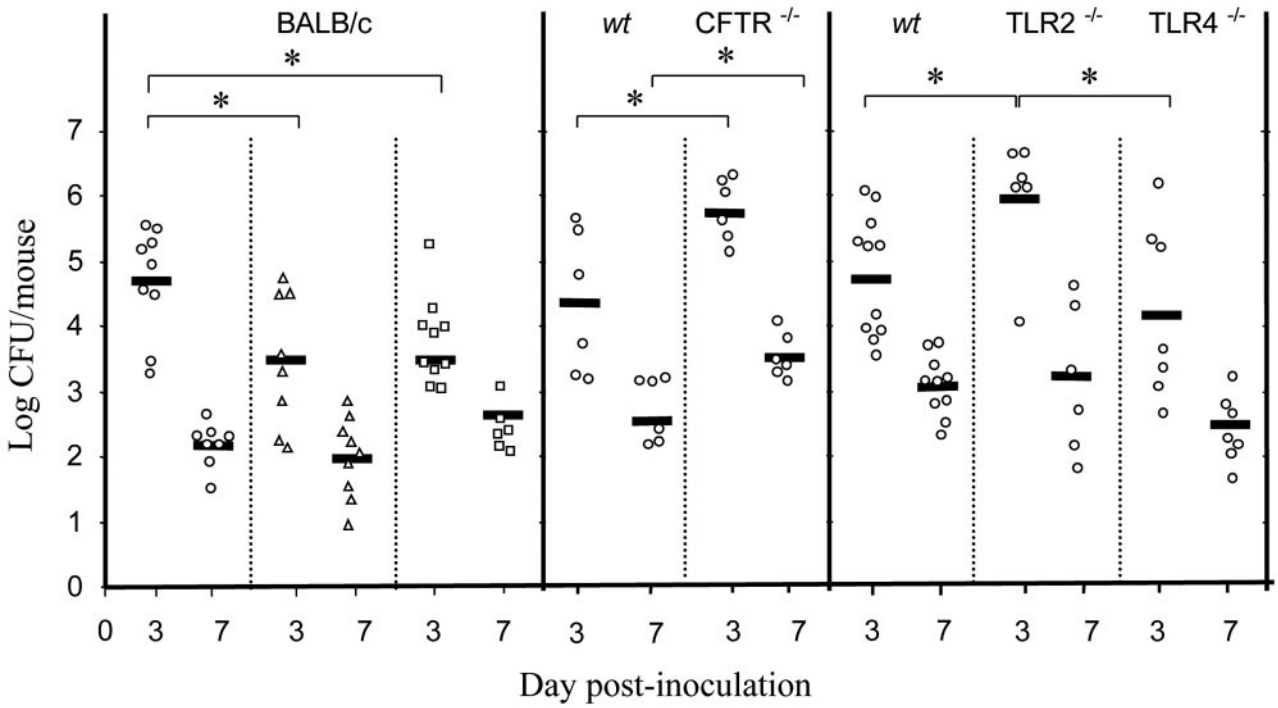

FIG. 3. Nasal colonization of mice by MRSA. Colonization by strain COL (open circles), BM4559 (open triangles), and BM4560 (open squares) was recorded on days 3 and 7 after inoculation. Each symbol represents CFU counts in a single mouse. The mean $\log_{10}$ CFU per nose for each group is represented by a horizontal line. Results are from a minimum of two independent experiments. Pairwise comparison of bacterial counts from the various groups of animals was performed with the Mann-Whitney U test. A P value of <0.05 was considered significant and is denoted with an asterisk.

who can be colonized with the same strain for years (as reviewed in reference 18). Using the CF transmembrane conductance regulator (CFTR) knockout mouse model (21), we inoculated intranasally $CFTR^{-/-}$ and parental $CFTR^{+/+}$ C57BL/6 mice (CNRS, Orleans, France) with MRSA COL. The $CFTR^{-/-}$ mice harbored increased numbers of MRSA (mean, 4.5×10^5 CFU/mouse at day 3 and 3.7×10^3 CFU/mouse at day 7) compared to the $CFTR^{+/+}$ parent (mean, 2.5×10^4 CFU/mouse at day 3 [$P = 0.022$] and 1.9×10^2 CFU/mouse at day 7 [$P = 0.01$]) (Fig. 3). The difference between the two mouse strains is an underestimate, since the $CFTR^{-/-}$ mice are much smaller than their $CFTR^{+/+}$ counterparts. Histological preparations of infected $CFTR^{-/-}$ mice showed bacteria entrapped in a cell-loaded intraluminal mucus mostly present within the nasal cornets (Fig. 2C). Reduced mucociliary clearance of CF epithelia (27) and reduced bactericidal activity of the airway surface fluid (20) may account for increased survival of bacteria in the mucus of these animals.

Toll-like receptors 2 and 4 (TLR2 and TLR4) mediate an inflammatory response against several gram-positive and gram-negative pathogens, respectively, and TLR2-deficient mice are highly susceptible to *S. aureus* sepsis (22). Although it has been shown that both receptors are expressed in the human upper airway epithelium (4), little is known about their role in the nasal mucosa. We inoculated homozygous 8- to 10-week-old $TLR2^{-/-}$ and $TLR4^{-/-}$ knockout C57BL/6 (23) and age-matched wild-type mice. At day 3, 10 times more MRSA CFU were isolated from the nasal tissue of $TLR2^{-/-}$ mice (mean, 9.1×10^5 CFU/mouse) than from C57BL/6 mice (mean, 6.3×10^4 CFU/mouse; $P = 0.016$) or from $TLR4^{-/-}$ mice (mean, 1.4×10^4 CFU/mouse; $P = 0.032$) (Fig. 3). However, at day 7, there was no significant difference between the three groups of mice. These results suggest that TLR2, but not TLR4, is involved in the early stages of the innate immune response against nasal *S. aureus* colonization and that other TLR/interleukin-1 family members may play a role, as demonstrated in other infection models of *S. aureus* (22).

Direct in vivo monitoring of biofilm formation for up to 20 days by using bioluminescent *S. aureus* and *Pseudomonas aeruginosa* in mice has been reported (11). These studies modeled foreign-body infection and used surgical subcutaneous implantation of catheters in the flanks, where bioluminescence detection is easier than through nasal bones. Under our technical conditions, bioluminescence could not be followed more than 24 h, yet strain Xen31 was still present 36 h after inoculation at a mean of 10^4 CFU/mouse (range, 2.4×10^3 to 1.8×10^4). Since bioluminescence production by bacteria depends on their metabolic activity (17), the constant decrease in the signal suggests that *S. aureus* did not multiply actively in the nasal cavity of the mice. Bioluminescence imaging under the present technical conditions will be useful to study factors involved in the early steps of colonization or to assess prevention of colonization, as shown previously in a thigh model of infection with *S. aureus* (9).

Humans can be colonized by a single *S. aureus* strain which can persist in the nasal cavity for months (10). We have demonstrated that PBP2a, responsible for methicillin resistance, does not play a role in colonization. However, only a small

number of MRSA clones has been studied. Comparison of representatives of the five major pandemic clones (15) and of community-acquired MRSA (24) will allow determination of the role of other determinants encoded within or outside the staphylococcal chromosomal cassette (SCC*mec*) in colonization.

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