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Antimicrobial Agent of Susceptibilities and Antiseptic Resistance Gene Distribution among Methicillin-Resistant *Staphylococcus aureus* Isolates from Patients with Impetigo and Staphylococcal Scalded Skin Syndrome

Norihisa Noguchi,[1]* Hidemasa Nakaminami,[1] Setsuko Nishijima,[2] Ichiro Kurokawa,[3] Hiromu So,[2] and Masanori Sasatsu[1]

Department of Microbiology, School of Pharmacy, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan[1]; Department of Dermatology, Kori Hospital, Kansai Medical University, 8-45 Korihondori, Neyagawa, Osaka 572-8551, Japan[2]; and Department of Dermatology, Graduate School of Medicine, Mie University, 2-174 Edobashi, Tsu, Mie 514-8507, Japan[3]

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The susceptibilities to antimicrobial agents of and distributions of antiseptic resistance genes in methicillin-resistant *Staphylococcus aureus* (MRSA) strains isolated between 1999 and 2004 in Japan were examined. The data of MRSA strains that are causative agents of impetigo and staphylococcal scalded skin syndrome (SSSS) were compared with those of MRSA strains isolated from patients with other diseases. The susceptibilities to antiseptic agents in MRSA isolates from patients with impetigo and SSSS were higher than those in MRSA isolates from patients with other diseases. The distribution of the *qacA*/*B* genes in MRSA strains isolated from patients with impetigo and SSSS (1.3%, 1/76) was remarkably lower than that in MRSA strains isolated from patients with other diseases (45.9%, 95/207). Epidemiologic typings of staphylococcal cassette chromosome *mec* (SCC*mec*) and pulsed-field gel electrophoresis (PFGE) showed that MRSA strains isolated from patients with impetigo and SSSS had type IV SCC*mec* (75/76), except for one strain, and 64.5% (49/76) of the strains had different PFGE types. In addition, the patterns of restriction digestion of all tested *qacA*/*B* plasmid in MRSA isolates having different PFGE types were identical. The results showed that a specific MRSA clone carrying *qacA*/*B* was not prevalent, but *qacA*/*B* was spread among health care-associated MRSA strains. Therefore, it was concluded that the lower distribution rate of *qacA*/*B* resulted in higher susceptibilities to cationic antiseptic agents in MRSA isolated from patients with impetigo and SSSS.

Methicillin-resistant *Staphylococcus aureus* (MRSA), which produces a penicillin-binding protein 2′ (PBP2′) with a low affinity to β-lactam antibiotics (11, 41, 48), is a major nosocomial pathogen throughout the world. The PBP2′ is encoded by the *mecA* gene that is located on a genetic element called the staphylococcal cassette chromosome (SCC) in *Staphylococcus aureus* (14, 17). SCC*mec* has been classified into five major types according to gene structure (15). Types I, II, and III of SCC*mec* are found in health care-associated MRSA (H-MRSA) strains, whereas types IV and V are found in community-associated MRSA (C-MRSA) strains (10, 15, 27). An increase in the number of C-MRSA strains carrying type IV SCC*mec* has became a matter of public concern.

Many antiseptic agents are used to prevent infections (25). Overuse of antiseptic agents has led to the emergence of MRSA with decreased antiseptic susceptibility, i.e., antiseptic-resistant MRSA (1, 2, 24, 32, 35). At least 12 antiseptic resistance genes (*qacA* to *qacJ*, *smr*, and *norA*) have been identified in *Staphylococcus* species (4, 8, 12, 13, 38, 43). Four antiseptic resistance genes, *qacA*, *qacB*, *smr*, and *norA*, are found mainly in clinical isolates of *S. aureus* (1, 2, 24, 35) and are associated with resistance to monovalent cationic agents such as quaternary ammonium compounds and ethidium bromide (1, 2, 23, 35, 38, 40). The *qacA*, *qacB*, and *smr* are mainly found on plasmids (22, 45), and *norA* is located on the *S. aureus* chromosome (30, 50). Antiseptic resistance in *S. aureus* is caused by proton motive force-dependent multidrug efflux (9, 29, 43). The *qacA* and *qacB* genes encode a 14-transmembrane-segment protein that belongs to the major facilitator superfamily (40, 43). Although *qacA* also confers more resistance to divalent cationic agents than *qacB*, the sequence of *qacB* is identical to that of *qacA* except for only seven or nine bases (2, 38). Therefore, it is difficult to distinguish *qacA* and *qacB* by simple PCR, and *qacA* and *qacB* are considered to be the same. The *smr* gene, which is identical to *qacC*, *qacD*, and *ebr*, encodes a small protein that belongs to a small multidrug resistance family (20, 22, 40). Therefore, the plasmid-borne antiseptic resistance genes are classified structurally into two families, *qacA*/*B* and *smr* (40). The chromosomal antiseptic resistance gene *norA* confers low-level resistance to hydrophilic fluoroquinolones such as norfloxacin and levofloxacin as well as to antiseptic agents (33, 50). The resistance of *norA* seemed to be due to the mutation(s) in the 5′-untranslated region that led to increases in *norA* transcription (7, 16, 33). At least seven mutations conferring antiseptic resistance have been identified (33). However, compared to *qacA*/*B*, the *norA* mutation(s) is considered to have a very low contribution to the resistance of

* Corresponding author. Mailing address: Department of Microbiology, School of Pharmacy, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan. Phone: 81-426-76-5619. Fax: 81-426-76-5647. E-mail: noguchin@ps.toyaku.ac.jp.

cationic antiseptic agents. The *qacA/B* gene has been reported to be associated with high-level resistance to antiseptic agents and to be widely prevalent among MRSA isolates found in Europe and Asia (2, 24, 32, 35). Therefore, the *qacA/B* gene(s) has become a major antiseptic resistance gene in MRSA.

Impetigo and staphylococcal scalded skin syndrome (SSSS), which are diseases primarily of young children and neonates, are blistering skin diseases that are caused by exfoliative toxins (ETs) produced by *S. aureus* (19, 39). Serologically, ETs involved in human diseases consist of two types: ETA and ETB proteins (19, 39). The *eta* gene, encoding ETA, is located on a chromosome, whereas the *etb* gene, encoding ETB, is found on a plasmid (39). The *etb* gene might be transferred horizontally by transduction (42). Recently, an increasing number of refractory patients with impetigo and SSSS caused by ET-producing MRSA has became a serious problem (49). Almost all MRSA strains (i.e., C-MRSA) isolated from outpatients with impetigo and SSSS were reported to carry type IV SCC*mec* and were susceptible to various antibiotics except β-lactam antibiotics (3, 31, 36). However, the decreased susceptibility of C-MRSA to macrolides and aminoglycosides has also been reported (49). The spread of antiseptic resistance genes into MRSA leads to decreased susceptibility to antiseptic agents (35). The prevalence of antiseptic resistance genes, such as *qacA/B*, in C-MRSA is a cause for public health concern.

The aim of this study was to understand the susceptibility of MRSA strains isolated from patients with impetigo and SSSS to antimicrobial agents, including antiseptics. A secondary aim of our study was to find potential effective antiseptic agents to MRSA that cause impetigo. To this end, we determined the susceptibilities of antimicrobial agents including antiseptics and distribution of antiseptic resistance genes in MRSA isolated between 1999 and 2004 and compared the data of MRSA strains isolated from patients with impetigo and SSSS with those of MRSA strains isolated from patients other diseases. In addition, we performed molecular epidemiological typings of SCC*mec* and pulsed-field gel electrophoresis (PFGE).

MATERIALS AND METHODS

Bacterial strains. A total of 283 isolates of MRSA were collected at Kori Hospital (250 isolates) and Rakusai Newtown Hospital (14 isolates), both associated with Kansai Medical University, and Hyogo Prefecture Tsukaguchi Hospital (19 isolates) in Japan between July 1999 and December 2004. Seventy-six strains were isolated from outpatients with impetigo (66 strains) and SSSS (10 strains), and 207 strains were isolated from the following clinical departments: otolaryngology, 70 strains; dermatology, 49 strains; pediatrics, 36 strains; urology, 21 strains; surgery, 16 strains; internal medicine, 13 strains; and gynecology, 2 strains. All strains were isolated from different patients. *S. aureus* N315 (18) was used for a typical strain of MRSA, and JCM2874 (ATCC 29213) was used as a reference strain and for quality control during susceptibility testing. The following strains were used as SCC*mec* type strains: *S. aureus* NCTC10442 (type I), N315 (type II), 85/2082 (type III), and JCSC4744 (type IV) (15).

Bacterial identification. All clinical isolates were identified as *S. aureus* by a positive Gram stain, the utilization of mannitol salt agar (Oxoid, Hampshire, England), and a production of coagulase (PS LATEX; Eiken Chemical, Tokyo, Japan). MRSA strains were identified by proliferation on Muller-Hinton agar (Oxoid) including 6 μg/ml of oxacillin and 4% of NaCl and the detection of the *mecA* gene by PCR (47). The MRSA strains isolated from patients with impetigo and SSSS were judged by the production of ET and detection of an ET gene(s), in addition to the identification of MRSA (39).

Antimicrobial susceptibility testing. MICs were determined by the agar doubling dilution method according to the CLSI (formerly the National Committee for Clinical Laboratory Standards) guidelines (28). Cefmetazole, clarithromycin, levofloxacin, and arbekacin were kindly provided by their manufacturers. Vancomycin, gentamicin, minocycline, benzalkonium chloride, benzethonium chloride, chlorhexidine digluconate, cetyltrimethylammonium bromide, and ethidium bromide were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), and oxacillin was from Sigma-Aldrich (Tokyo, Japan), and oxacillin was from Sigma-Aldrich (Tokyo, Japan). The breakpoints of these antimicrobial agents were determined by the interpretation criteria of the CLSI (28).

PCR amplification. PCR assays for detection of various genes were performed using the modified colony direct method (35). Briefly, the point of a toothpick was gently placed into 100 μl of H_2O. A small sample of cells was suspended, and 1 μl of the cell suspension was added directly into 20 μl of the PCR mixture, containing primers and 10 μl of PCR master mix (Promega). Searches for *mecA*, *eta*, *etb*, *qacA/B*, and *smr* were performed with the following sets of primers: for *mecA*, 5′-GTGGAAGTTAGATTGGGATCATAGC-3′ and 5′-GTCAACGATTGTGACACGATAGC-3′ (product size, 544 bp) (GenBank accession no. X52593); for *eta*, 5′-ATATCAACGTGAGGGCTCTAGTAC-3′ and 5′-ATGCAGTCAGCTTCTTACTGCTA (product size, 1,155 bp) (GenBank accession no. AP001553); for *etb*, 5′-CACACATTACGGATAATGCAAG-3′ and 5′-TCAACCGAATAGAGTGAACTTATCT-3′ (product size, 604 bp) (GenBank accession no. AP003088); for *qacA/B*, 5′-GCAGAAAGTGCAGAGTTCG-3′ and 5′-CCAGTCCAATCATGCCTG-3′ (product size, 361 bp); and for *smr*, 5′-GCCATAAGTACTGAAGTTATTGGA-3′ and 5′-GACTACGGTTGTTAAGACTAAACCT-3′ (product size, 195 bp). PCR was performed in the following cycles: for *mecA*, 25 cycles (30 s of denaturation at 95°C, 30 s of annealing at 58°C, and 30 s of extension at 72°C); and for *eta*, *etb*, *qacA/B*, and *smr*, 25 cycles (30 s of denaturation at 95°C, 30 s of annealing at 52°C, and 1.5 min of extension at 72°C). PCR products were analyzed by agarose gel electrophoresis. All results were confirmed by at least two independent experiments.

SCC*mec* and PFGE typings. SCC*mec* typing was performed by the multiplex PCR method described by Ito et al. and by Oliveira and de Lencastre (15, 37). PFGE of SmaI-digested chromosomal DNA was performed as described previously (26, 34, 35, 46). The DNA patterns obtained by PFGE were analyzed with BioNumerics software (Applied Maths, Saint-Martens-Latem, Belgium) using the Dice coefficient (34, 35). *S. aureus* N315 was used as a DNA reference standard because the genome of N315 has been determined (18).

Statistical analysis. Differences in distribution of *qacA/B* in MRSA between patients with impetigo and SSSS and those with other diseases were tested by the χ^2 test, with P values of <0.05 considered to be statistically significant.

RESULTS

Antimicrobial susceptibility. The MIC_{50}s and MIC_{90}s of the antimicrobial agents for the MRSA strains isolated from patients with impetigo and SSSS were compared with those of MRSA strains isolated from patients with other diseases, excluding impetigo and SSSS (Table 1). All MRSA strains isolated from patients with impetigo and SSSS, except for one stain, were susceptible to levofloxacin and minocycline, although 17 and 26% of MRSA strains isolated from patients with other diseases were susceptible to levofloxacin and minocycline, respectively. However, the rate of gentamicin-resistant MRSA strains isolated from impetigo and SSSS patients (95%) was higher than that of MRSA strains isolated from patients with other diseases. The rates of clarithromycin-resistant MRSA strains isolated from patients with impetigo and SSSS and from patients with other diseases were 80 and 91%, respectively. The MRSA strains isolated from impetigo and SSSS patients were more susceptible to the antiseptic agents tested than MRSA strains isolated from patients with other diseases (Table 1).

Distributions of *qacA/B* and *smr*. Although the antiseptic resistance gene *qacA/B* was detected in 45.9% (95/207) of MRSA strains isolated from patients with diseases other than impetigo and SSSS, only one MRSA (1.3%) strain isolated from a patient with impetigo and SSSS carried *qacA/B* (Table 2). The *smr* gene was detected in only four strains of MRSA isolated from patients with impetigo but not found among

TABLE 1. Antimicrobial susceptibility of MRSA strains in this study

Antimicrobial agent[a]	MIC (μg/ml) for strains isolated from patients:					
	With impetigo and SSSS			Without impetigo and SSSS		
	Range	50%	90%	Range	50%	90%
Oxacillin	4–≥256	64	128	4–≥256	≥256	≥256
Cefmetazole	4–≥64	32	≥64	4–≥64	≥64	≥64
Clarithromycin	≤0.125–≥256	≥256	≥256	≤0.125–≥256	≥256	≥256
Levofloxacin	≤0.125–≥256	≤0.125	≤0.125	≤0.125–≥256	≥256	≥256
Gentamicin	0.5–≥64	≥64	≥64	0.25–≥64	≥64	≥64
Arbekacin	0.5–8	2	4	0.25–≥32	1	2
Vancomycin	≤0.5–2	1	1	≤0.5–2	1	1
Minocycline	≤0.125–16	≤0.125	0.25	≤0.125–32	16	16
Benzalkonium-Cl	2–4	2	2	1–8	4	8
Benzethonium-Cl	1–8	1	2	0.5–16	4	8
Chlorhexidine-Glu	1–4	2	2	1–8	4	4
Cetyltrimethylammonium-Br	≤0.5–16	4	8	2–16	8	8
Ethidium-Br	1–32	8	8	1–≥256	8	≥256

[a] Benzalkonium-Cl, benzalkonium chloride; benzethonium-Cl, benzethonium chloride; chlorhexidine-Glu, chlorhexidine digluconate; cetyltrimethylammonium-Br, cetyltrimethylammonium bromide; ethidium-Br, ethidium bromide.

MRSA isolates from patients with diseases other than impetigo and SSSS. The prevalence of *qacA/B* in MRSA isolates from patients with impetigo and SSSS was significantly lower than that in MRSA isolates from patients with other diseases ($P < 0.0001$).

Molecular and epidemiological analysis. To study the genotypic characteristics and genetic relatedness of MRSA isolates, 283 MRSA strains were analyzed by SCC*mec* and PFGE typings. The SCC*mec* type of MRSA strains isolated from patients with impetigo and SSSS was type IV, with the exception of one strain. In 207 MRSA strains isolated from patients with other diseases, types I, II, III, and IV were detected in 5, 164, 1, and 37 strains, respectively. The numbers of SCC*mec* types of MRSA strains carrying *qacA/B* were 1 of type I, 91 of type II, 0 of type III, and 4 of type IV. One strain carrying both *qacA/B* and *etb* had type IV SCC*mec*. The antimicrobial susceptibilities of H-MRSA and C-MRSA were compared (Fig. 1). Although the susceptibilities of H-MRSA to oxacillin, levofloxacin, and minocycline were different from those of C-MRSA, no distinction in the resistance profiles between C-MRSA strains isolated from patients with impetigo and SSSS and C-MRSA isolated from patients with other diseases was present.

On the other hand, the PFGE type of MRSA carrying SCC*mec* type IV differed significantly from that of MRSA carrying SCC*mec* type II (Fig. 2). Seventy-six MRSA strains which had SCC*mec* type IV, except one strain, isolated from patients with impetigo and SSSS were classified into 49 PFGE types, and 96 MRSA isolates carrying *qacA/B* were classified into 44 PFGE types. When the *qacA/B* plasmids carrying six MRSA strains with different PFGE types were tested, the patterns of the restriction digestion of all of the *qacA/B* plasmids were identical (data not shown). These genetic findings suggest that a specific MRSA clone carrying *qacA/B* was not prevalent, but *qacA/B* was horizontally transferred among various MRSA clones.

TABLE 2. Distributions of *qacA/B* and *smr*

Gene(s) or parameter	No. (%) of strains isolated from patients		
	With impetigo and SSSS ($n = 76$)	Without impetigo and SSSS ($n = 207$)	Total ($n = 283$)
qacA/B	1 (1.3)	95 (45.9)	96 (33.9)
smr	4 (5.3)	0	4 (1.4)
ND[a]	71 (93.4)	112 (54.1)	183 (64.7)

[a] ND, not detected.

DISCUSSION

In recent studies, the incidence rate of C-MRSA among patients with skin infections, including impetigo and SSSS, has been increasing, and most C-MRSA strains carried type IV SCC*mec*. Unlike H-MRSA, C-MRSA is frequently susceptible to non-β-lactam antibiotics, such as aminoglycosides, tetracyclines, and fluoroquinolones (3, 31). In this study, 99% of the MRSA strains isolated from patients with impetigo and SSSS had type IV SCC*mec*. However, most C-MRSA strains isolated from patients with impetigo and SSSS were resistant to clarithromycin and gentamicin, although those strains were susceptible to levofloxacin and minocycline. The results suggest that the C-MRSA strains isolated from patients with impetigo and SSSS have evolved not only β-lactam resistance but also multidrug resistance. Recently, skin and soft tissue infections and severe necrotizing pneumonia caused by C-MRSA strains carrying the Panton-Valentine leukocidin gene (*pvl*) have become a serious problem (6, 21, 44, 51). Although screening for *pvl* was carried out by PCR, no *pvl* was detected among the C-MRSA strains used in this study (data not shown).

Cationic antiseptic agents such as quaternary ammonium compounds and chlorhexidine digluconate and iodine compounds, such as povidon iodine, are commonly used for the disinfection of MRSA on skin and hands (5). The *qacA/B* gene that confers resistance to cationic antiseptic agents was detected in 46% of MRSA isolates cultured from patients with diseases other than impetigo and SSSS. This frequency (44%) of *qacA/B* was very similar to that previously reported in Japan (35). Almost all MRSA isolates with *qacA/B* had SCC*mec* type

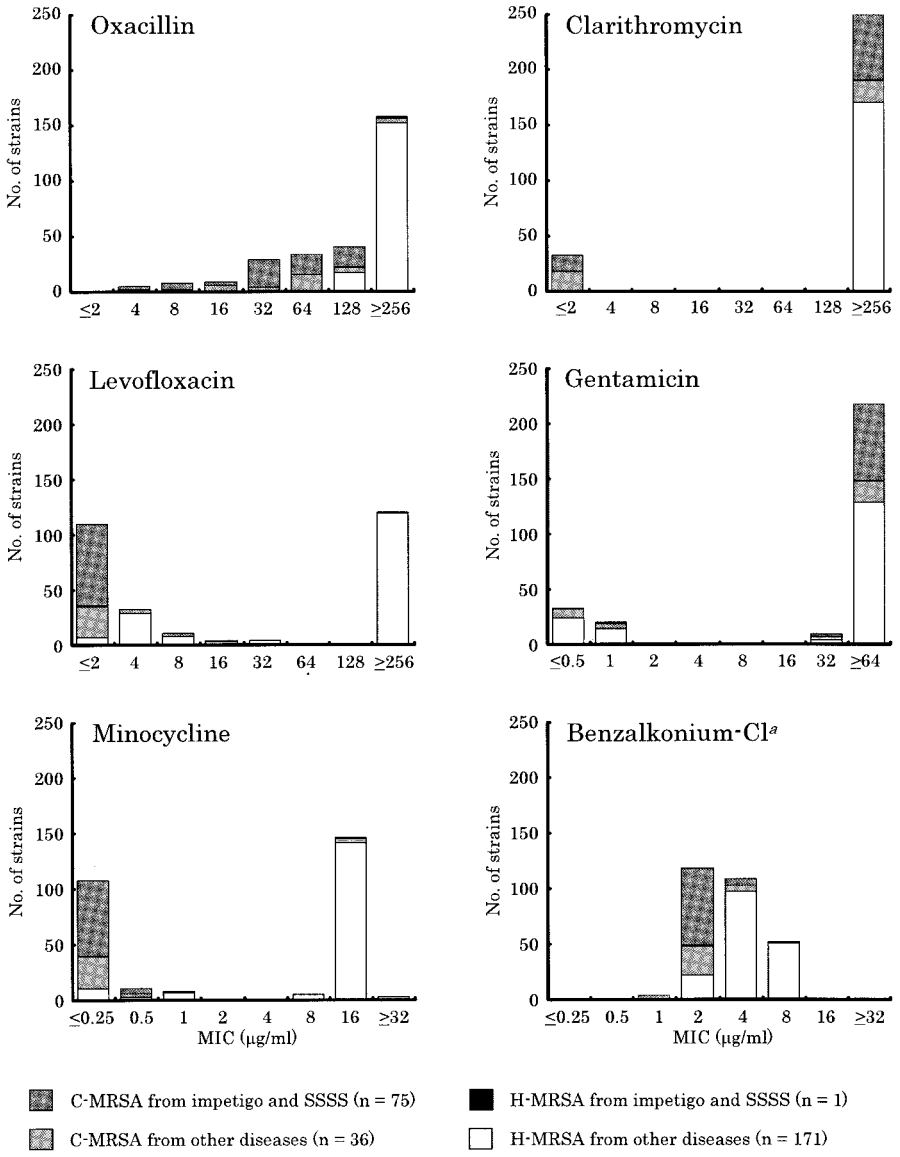

FIG. 1. Comparison of antimicrobial susceptibilities for H-MRSA and C-MRSA strains used in this study. *a*, benzalkonium chloride.

II (91/96). This seems to be due to the significant exposure of H-MRSA isolates to antiseptics in hospital environments. Therefore, our data predict that the *qacA/B* plasmid has been further dispersed among various H-MRSA by horizontal transfer.

SCC*mec* type IV MRSA strains have been frequently isolated from patients with skin infections. In SCC*mec* type IV MRSA isolates, *qacA/B* was detected in 8.1% (3/37) of strains isolated from patients with other diseases. These data showed that *qacA/B* was able to maintain SCC*mec* type IV MRSA, whereas the genes encoding exfoliative toxin are the causative genes of impetigo and SSSS and the *etb* gene is located on a plasmid (19, 39). Although the ET genes were found in nine C-MRSA strains (SCC*mec* type IV) isolated from patients without impetigo and SSSS, no *qacA/B* was detected in the nine strains. Only one strain carrying both *qacA/B* and *etb* was found in patients with SSSS. This SCC*mec* type IV strain had a PFGE pattern which resembled that of the SCC*mec* type II MRSA group (Fig. 2) and was resistant to levofloxacin, minocycline, and gentamicin. Therefore, the C-MRSA carrying both *qacA/B* and *etb* seemed to be a rare MRSA clone among the MRSA carrying the ET genes. When the plasmid was purified and analyzed by using restriction enzymes, the strain with *qacA/B* and *etb* carried a single plasmid that had the same restriction pattern as the *qacA/B* plasmid (data not shown). Therefore, the *qacA/B* plasmid seemed to be maintained in the strain with *etb* by integration of *etb* into the chromosome. These data suggest that the *qacA/B* plasmid may be incompatible with the *etb* plasmid. Further studies of the plasmids encoding *qacA/B* and *etb* are necessary to demonstrate the incompatibility between the *qacA/B* and *etb* plasmids.

On the contrary, the *smr* gene was found in 5% (4/76) of the MRSA strains isolated from patients with impetigo and SSSS but not detected in MRSA strains isolated from patients with

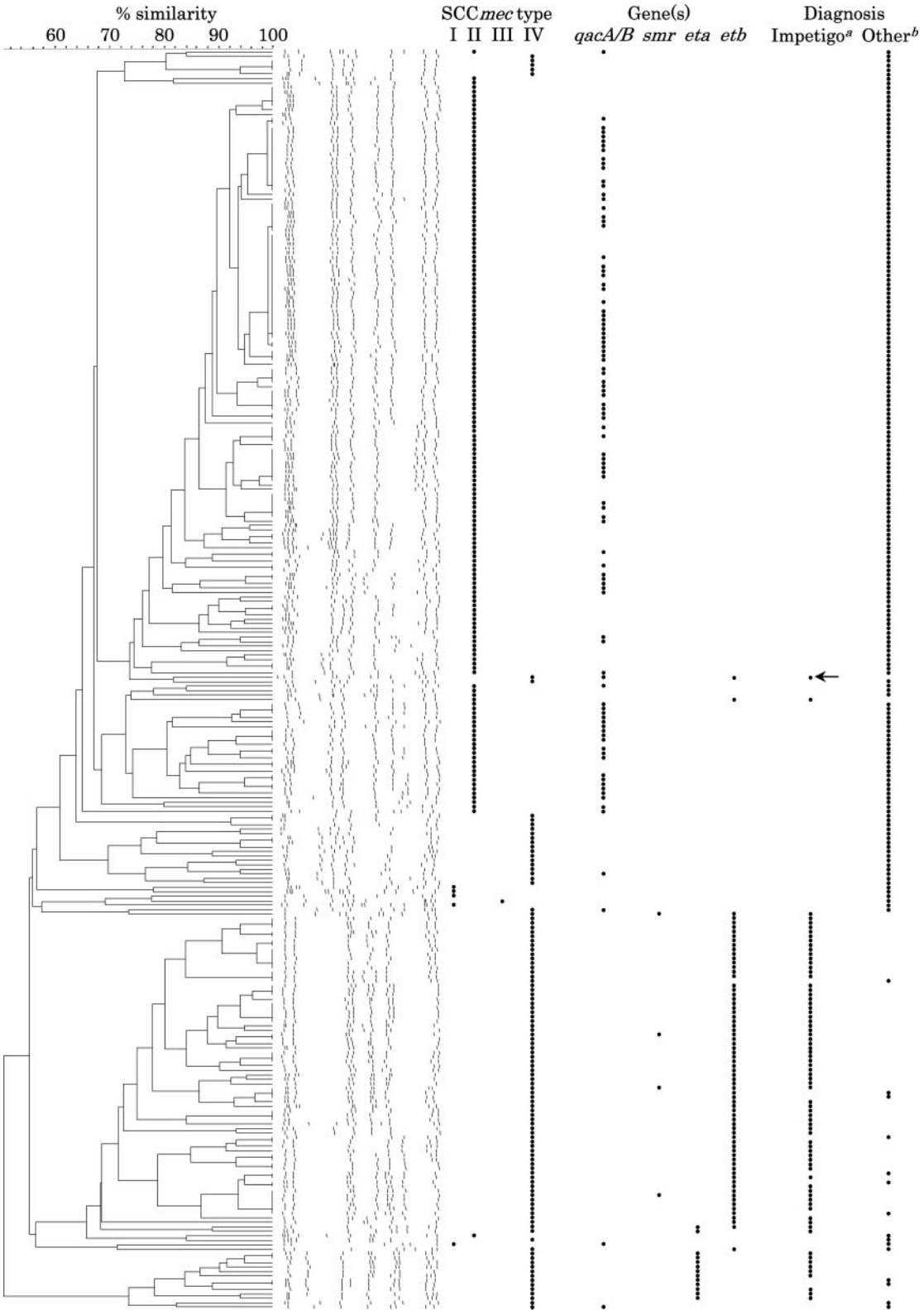

FIG. 2. Comparison of PFGE patterns, SCC*mec* types, and antiseptic resistance genes of MRSA isolated from patients with impetigo and SSSS and those of MRSA strains isolated from patients with other diseases. The arrow indicates the strain carrying both *qacA*/*B* and *etb* isolated from patients with SSSS. *a*, strain isolated from patients with impetigo and SSSS; *b*, strain isolated from patients without impetigo and SSSS.

other diseases. This frequency of this gene in patients with impetigo and SSSS (3.4%) was similar to that of *smr* previously reported in Japan (35). The *smr* plasmid was also detected in MRSA strains carrying the *etb* plasmid from patients with impetigo. It might be possible to maintain *smr* plasmid in the cells carrying the *etb* plasmid, because the *smr* plasmid is compatible with the *qacA*/*B* plasmid (24, 35).

In summary, the *qacA*/*B* gene, which is a dominant plasmid-borne antiseptic resistance gene in MRSA, was not prevalent in C-MRSA strains isolated from patients with impetigo and SSSS, although *qacA*/*B* was likely transferred horizontally among various H-MRSA clones. Consequently, our results suggest that antiseptic agents have a higher potential to prevent the infection of impetigo and SSSS caused by C-MRSA.

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