Acta Medica et Biologica
Vol. 51, No.3, 103-109, 2003

Molecular DNA Analysis of Methicillin-resistant *Staphylococcus aureus* (MRSA) Infection in a Neonatal Intensive Care Unit

Yoko TAKIZAWA[1,2], Ikue TANEIKE[1], Saori NAKAGAWA[1], Shiro ISHII[3], Takumi KURABAYASHI[3], Kenichi TANAKA[3], Fumitake GEJYO[2] and Tatsuo YAMAMOTO[1]

[1]Division of Bacteriology, Department of Infectious Disease Control and International Medicine, [2]Division of Respiratory Medicine, Department of Homeostatic Regulation and Development, [3]Division of Obstetrics and Gynecology, Department of Obstetrics and Gynecology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan

Received May 22 2003; accepted September 9 2003

Summary. **Methicillin-resistant *Staphylococcus aureus* (MRSA) infection in neonatal intensive care units (NICUs) has become a serious problem in Japan. MRSA nosocomial transmissions occurring in an NICU during 2001 and 2002 were investigated by pulsed-field gel electrophoresis and PCR. MRSA from a nosocomial transmission with incidences of neonatal toxic shock syndrome-like exanthematous disease (NTED) produced type II coagulase and possessed multiple superantigenic toxin genes for toxic shock syndrome toxin (TSST)-1, staphylococcal enterotoxin (SE) C, SEG, and SEI. MRSA from a nosocomial transmission with incidences of staphylococcal scalded skin syndrome (SSSS) was clonally distinct from the NTED-associated MRSA, produced type I coagulase, and possessed only the exfoliative toxin B gene (no previous superantigenic toxin genes were detected).**

Key words—methicillin-resistant *Staphylococcus aureus* (MRSA), neonatal intensive care unit (NICU), neonatal toxic shock syndrome-like exanthematous disease (NTED), staphylococcal scalded skin syndrome (SSSS), nosocomial transmission.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major agent in nosocomial infections. It produces a number of exotoxins, such as superantigenic toxins (e.g., toxic shock syndrome toxin-1 [TSST-1] and staphylococcal enterotoxins [SEs]) and exfoliative toxin[1]. TSST-1 is the major causative toxin of toxic shock syndrome (TSS), which is a life-threatening staphylococcal infection[1] and neonatal TSS-like exanthematous disease (NTED)[2]. Exfoliative toxin is the causative toxin of staphylococcal scalded skin syndrome (SSSS) which in many cases, is seen in infants and children[1]. MRSA infection in a neonatal intensive care unit (NICU) has been increasingly noted in Japan. However, the coagulase type and toxin type of MRSA have not been reported with MRSA in NICUs. In this study, we investigated MRSA nosocomial transmissions in an NICU at the molecular DNA level.

METHODS

Screening for MRSA infection in the NICU

Nasal mucosal swabs or sputum aspirates from newborns were examined for MRSA by cultivation in routine practice to monitor MRSA infection. Intubation was administered to infants who required respiratory assistance, and the tubes were subjected to MRSA test after extubation.

Correspondence: Dr. Tatsuo Yamamoto, Division of Bacteriology, Department of Infectious Disease Control and International Medicine, Niigata University Graduate School of Medical and Dental Sciences, 757 Ichibanchou, Asahimachidori, Niigata, Japan

Abbreviations—ET, exfoliative toxin; MRSA, methicillin-resistant *Staphylococcus aureus*; NICU, neonatal intensive care unit; NTED, neonatal toxic shock syndrome-like exanthematous disease, PCR, polymerase chain reaction, SE, staphylococcal enterotoxin; SSSS, staphylococcal scalded skin syndrome; TSS, toxic shock syndrome; TSST, toxic shock syndrome toxin.

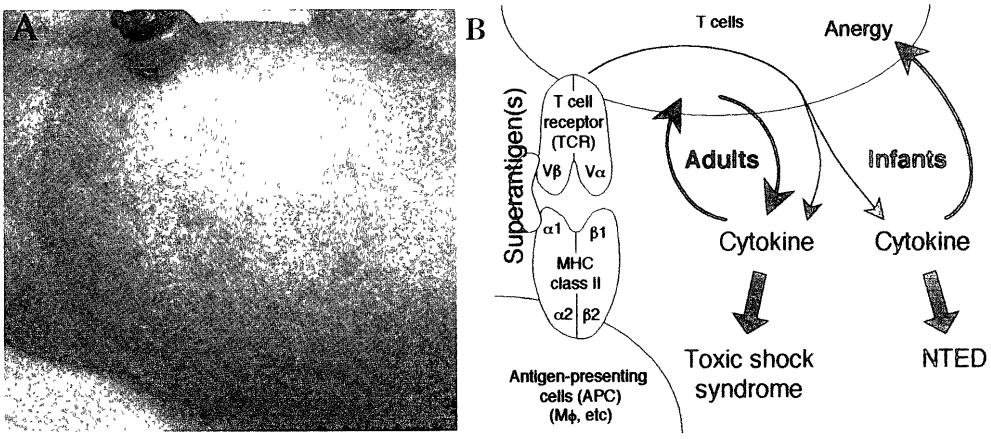

Fig. 1. Exanthema spreading over the body of an infant infected with MRSA (**A**) and the mechanism (hypothesis) of NTED development in infants via a staphylococcal superantigen (**B**). In (**B**), the model was constructed based on data from references 7) and 9).

Nosocomial transmission - Case 1

MRSA infection occurred in seven infants (aged 5-30 days old) in an NICU in Niigata in 2001. Among the infants, five (aged 6-30 days old) developed no symptoms and MRSA was isolated from the nasal mucosa. Two infected infants developed symptoms. One infant (female) was delivered by cesarean section (day 1). Her platelet count was $222\times10^9/l$. Fever developed on day 2 (38-38.6°C) and the platelet count decreased to $112\times10^9/l$ on day 3. The peak CRP was 7.5 mg/*l*. Fever persisted on day 4 (38-38.8°C), exanthema appeared on the skin, and umbilical blennorrhoea was observed. On day 5, exanthema spread over the body (Fig. 1A), the platelet count decreased to 71 X $10^9/l$, and MRSA (strain E6) was isolated from the umbilical blennorrhoea. Exanthema improved on day 6. This patient was diagnosed with NTED. The other patient (male) was also delivered by cesarean section (day 1). On day 2, CRP was 5.9 mg/*l* and the platelet count was $90\times10^9/l$. On day 3, his CRP increased to 13 mg/*l*, the platelet count decreased to $16\times109/l$, and eruption-like exanthema appeared, suggesting NTED. Fever did not develop. MRSA (strain E7) was isolated from the navel and exanthema on day 9. During the examination period, MRSA was isolated from the nasal mucosa of five medical care staff members associated with the NICU. Those persons were all asymptomatic.

Nosocomial transmission - Case 2

MRSA infection occurred in six infants (aged 7-81 days old) in an NICU in Niigata in 2002. Among the infants, five (aged 7-81 days old) developed no symptoms, and MRSA was isolated from the nasal mucosa or sputum. One infected infant (female) who developed SSSS was an extremely premature infant born at a gestational age of 24 weeks (700 g). The infant was resuscitated by intubation two minutes after delivery and admitted to the NICU. Exfoliative toxin B-producing MRSA (strain H5) was isolated from the inserted tube on day 10 after birth. Epidermal abrasions developed on day 16 and became aggravated the next day, and a diagnosis of MRSA-induced SSSS was made. The skin of the infant completely peeled off. Chemotherapy with arbekacin and ceftazidime was initiated. Gamma-globulin was administered for three days and steroid ointment was applied. The symptoms gradually subsided and antibiotic administration was discontinued on day 21 after birth.

DNA analysis

Total bacterial DNA was purified and subjected to pulsed-field gel electrophoresis, in which bacterial DNA was digested with *Sma*I or *Sac*II, electrophoresed in 1.2% agarose with the molecular size standards-lambda ladder (Bio-Rad, Hercules, CA, USA), and stained with ethidium bromide.

PCR assay

The primer sets used for PCR assay were: TST-3 and TST-6 (generating a 445-bp product) for the TSST-1 gene[3], SEA-3 and SEA-4 (generating a 127-bp product) for the SEA gene[3], SEB-1 and SEB-4 (generating a 477-bp product) for the SEB gene[3], SEC-3 and

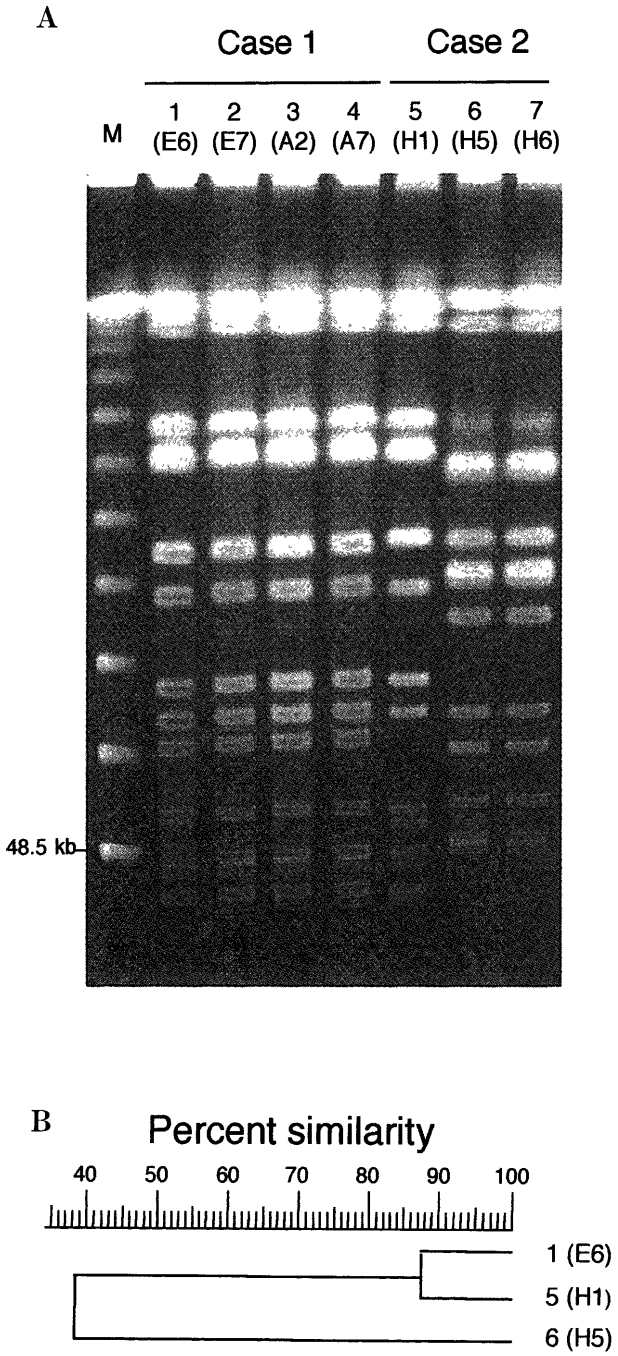

Fig. 2. DNA analysis of the MRSA strains derived from nosocomial transmissions in an NICU. In (**A**), MRSA DNA was analyzed by pulsed-field gel electrophoresis. Lanes: M, molecular size standards (lambda ladder); 1, strain E6 isolated from omphalitis in an infant diagnosed with NTED; 2, strain E7 isolated from the navel of an infant suspected of NTED; 3, strain A2 isolated from the nasal mucosa of an asymptomatic infant; 4, strain A7 isolated from the nasal mucosa of an asymptomatic medical care worker (carrier); 5, strain H1 isolated from the nasal mucosa of an asymptomatic infant; 6, strain H5 isolated from the inserted tube of an infant diagnosed with SSSS; 7, strain H6 isolated from the sputum of an asymptomatic infant. Bacterial DNA was digested with *Sma*I. Similar data were obtained even when DNA was digested with *Sac*II. In (**B**), a dendrogram was constructed by the computer-assisted comparison of pulsed-field gel electrophoresis data of strains E6, H1, and H5 shown in (**A**).

Table 1. Summary of coagulase and toxin type of NTED and SSSS strains in an NICU[a)]

Coagulase or toxin	*S. aureus* from an NICU		
	NTED-associated		SSSS-associated
	E6	E7	H5
Coagulase	Type 2	Type 2	Type 1
TSST-1	+ (512)[b)]	+ (512)[b)]	− (<1)[b)]
SEA	− (<1)[c)]	− (<1)[c)]	− (<1)[c)]
SEB	− (<1)[d)]	− (<1)[d)]	− (<1)[d)]
SEC	+ (2048)[e)]	+ (4086)[e)]	− (<1)[e)]
SED	− (<1)[f)]	− (<1)[f)]	− (<1)[f)]
SEE	−	−	−
SEG	+	+	+
SEH	−	−	−
SEI	+	+	−
SEJ	−	−	−
ETA	− (<1)[g)]	− (<1)[g)]	− (<1)[g)]
ETB	− (<1)[h)]	− (<1)[h)]	+ (4194304)[h)]

[a)]The toxin genes were assayed by PCR. The toxin production levels (titers representing the highest dilution [fold] to yield positive results) are shown in parentheses. ETA, exfoliative toxin A; ETB, exfoliative toxin B.

[b)]Titer 512 (1 : 512) corresponds to a concentration of ca. 256 ng of TSST-1 per m*l* in the original culture supernatant. Titer <1 corresponds to a concentration of < ca. 1ng of TSST-1 per m*l* in the original culture supernatant.

[c)]Titer <1 corresponds to a concentration of < ca. 1ng of SEA per m*l* in the original culture supernatant.

[d)]Titer <1 corresponds to a concentration of < ca. 1ng of SEB per m*l* in the original culture supernatant.

[e)]Titer 2048 (1 : 2048) corresponds to a concentration of ca. 1024 ng of SEC per m*l* in the original culture supernatant. Titer <1 corresponds to a concentration of < ca. 1ng of SEC per m*l* in the original culture supernatant.

[f)]Titer <1 corresponds to a concentration of < ca. 1ng of SED per m*l* in the original culture supernatant.

[g)]Titer <1 corresponds to a concentration of < ca. 1ng of ETA per m*l* in the original culture supernatant. The amount of ETA in the culture supernatants was determined by passive latex agglutination using EXT-RPLA kit (Denka Seiken), as described in the text.

[h)]Titer 4194304 (1 : 4194304) corresponds to a concentration of ca. 4.2 mg of ETB per m*l* in the original culture supernatant. Titer <1 corresponds to a concentration of < ca. 1ng of ETB per m*l* in the original culture supernatant. The amount of ETB in the culture supernatants was determined by passive latex agglutination using an EXT-RPLA kit (Denka Seiken), as described in the text. In the case of the MRSA strains from asymptmatic infants (including strain H6), the ETB production levels were ca. 2.1 to 8.4 mg per m*l*.

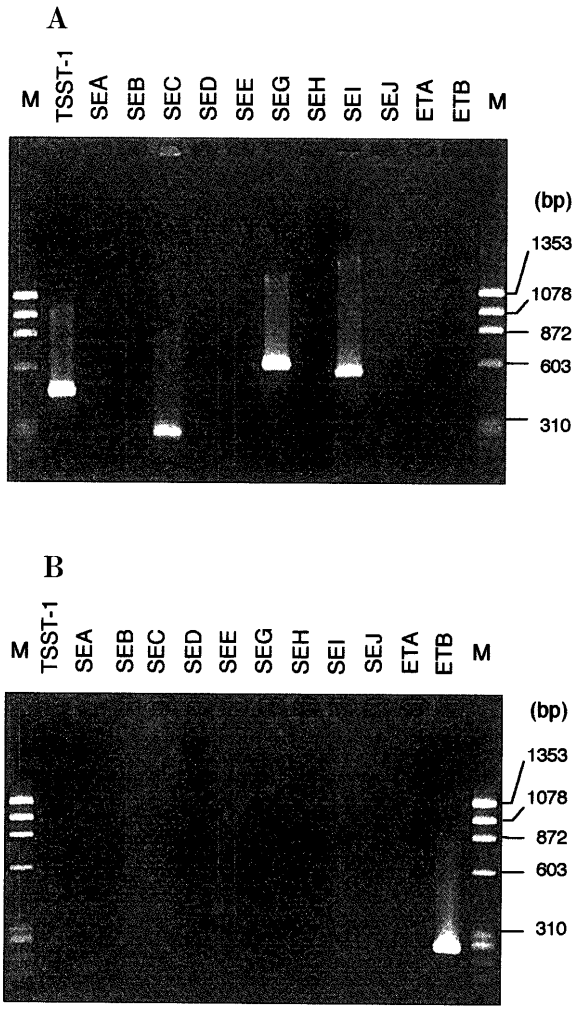

Fig. 3. Superantigenic toxin genes and exfoliative toxin genes were analyzed by PCR for strain E6 (**A**) and for strain H5 (**B**). Lane M, molecular size standards (fX174 RF DNA/*Hae*III fragments).

SEC-4 (generating a 271-bp product) for the SEC gene[3], SED-3 and SED-4 (generating a 319-bp product) for the SED gene[3], SEE-3 and SEE-2 (generating a 178-bp product) for the SEE gene[3], SEG-1 and SEG-2 (generating a 642-bp product) for the SEG gene[4], SEH-1 and SEH-2 (generating a 375-bp product) for the SEH gene[4], SEI-1 and SEI-2 (generating a 576-bp product) for the SEI gene[4], ZID and ZDR (generating a 1731-bp product) for the SEJ gene[5], ETA-3 and ETA-4 (generating a 119-bp product) for the exfoliative toxin A gene[3], and ETB-3 and ETB-4 (generating a 262-bp product) for the exfoliative toxin B gene[3]. Cycling conditions were denaturation for 45 s at 94°C, annealing for 45 s at 55°C, and polymerization for 45 s at 72°C (35 cycles). Amplified PCR products were analyzed by gel electrophoresis with 2% agarose and stained with ethidium bromide. ϕX174 RF DNA/*Hae*III fragments (Life Technologies, Gaithersburg, MD, USA) were used as molecular size standards.

Toxin production assay

Bacteria were grown for 18 h at 37°C in Brain Heart Infusion broth (Difco Laboratories, Detroit, Mich.), and adjusted to a bacterial concentration of 2.0×10^9 cfu/m*l*. The amount of toxin in the culture supernatants was determined by passive latex agglutination using TST-RPLA, SET-RPLA, and EXT-RPLA kits (Denka Seiken Co., Tokyo). The assay kits for SEG and SEI were not available in this study.

Coagulase typing

The coagulase type of the MRSA strains was examined using a staphylococcal coagulase antiserum kit (Denka Seiken) in accordance with the manufacturer's instructions.

Computer analysis

Computer-assisted analysis of the pulsed-field gel electrophoresis patterns was performed using a program called Molecular Analyst Fingerprinting Plus (Bio-Rad), according to the UPGMA clustering algorithm[6].

RESULTS

Pulsed-field gel electrophoresis showed that all the 12 MRSA strains isolated from the infants and medical care staff in a nosocomial transmission with NTED (case 1) were indistinguishable from each other, as shown in Fig. 2A (lanes 1 to 4). In another nosocomial transmission with SSSS (case 2), two types of MRSA were isolated. One type (e.g., strain H1) was clonally similar to the case 1 MRSA, with an 87.5% homology (Fig. 2A and B), and all three infected infants were asymptomatic. The other type (e.g., strain H5) was clonally divergent from the case 1 MRSA (with a homology of only 38.1%; Fig. 2A and B). Of the three infected infants, one developed SSSS, and the remaining two were asymptomatic.

The NTED-associated, case 1 MRSA (e.g., strain E6) produced type 2 coagulase and was positive for TSST-1, SEC, SEG and SEI, and negative for SEA, SEB, SED, SEE, SEH, SEJ, and exfoliative toxin A or B in the PCR assay (Fig. 3A and Table 1). The

determined sequences of the TSST-1 gene (705 bp) and SEC gene (801 bp) of the MRSA strains (GenBank accession number AB 084255 and AB 084256, respectively) from the infants were in complete agreement with the previously described gene sequences for MRSA from adults[7]. The toxin production levels were 0.5 $\mu g/ml$ for TSST-1 and 2.0-4.1 $\mu g/ml$ for SEC.

The SSSS-associated, case 2 MRSA (e.g., strain H5), produced type 1 coagulase, and was positive for only exfoliative toxin B in the PCR assay (Fig. 3B, Table 1). The exfoliative toxin B production level was 4.2 mg/ml (Table 1). Another type of the case 2 MRSA (e.g., strain H1) shared the same coagulase type and toxin type of the NTED-associated MRSA (case 1).

DISCUSSION

MRSA infection in the NICU was detected in 46.2% of patients in 1999, 34.6% in 2000, 14.4% in 2001, and 16.5% in 2002. This study analyzed infants with symptoms in a nosocomial transmission and revealed two major types of MRSA. One was associated with NTED, produced type 2 coagulase, and had multiple superantigenic toxin genes for TSST-1, SEC, SEG and SEI. The other type was associated with SSSS, produced type 1 coagulase, and had only the exfoliative toxin B gene.

NTED is caused by MRSA in neonates and was first recognized in the 1990 s in Japan[2]. It frequently occurs at 2-4 days of age, and the major symptoms are fever and subsequent exanthema. The fever persists for about one day and decreases spontaneously. The exanthema is systemic erythema (2-3 mm erythemas later fuse) spreading over the face, trunk, all four limbs, palms, and soles, spontaneously diminishing within 2-3 days.

A superantigen, TSST-1, produced by MRSA, was reported to be the major cause of NTED[2]. In adults, TSST-1 binds to MHC class II-positive cells and abnormally activates T cells expressing a specific Vβ region to overproduce cytokines (e.g., tumor necrosis factor-α, interleukin-2, interferon-γ) inducing an abnormal reaction in the whole body[8], as summarized in Fig. 1B. In contrast, NTED spontaneously heals without causing shock in neonates[2]. It is thought that anergy is induced and cytokine overproduction is unlikely to occur in neonates (Fig. 1B)[2]. Another possibility is that the TSST-1 of MRSA from NTED patients is divergent from that of MRSA from adults. However, this possibility was completely ruled out because the TSST-1 of MRSA from infants (analyzed in this study) was the same as the previously described TSST-1 of MRSA from adults in terms of the gene sequence.

In this study, the MRSA isolated from the NTED patients was positive for SEC, SEG, and SEI in addition to TSST-1. These toxins may also have a role in the onset of NTED. A combination of TSST-1 and SEC shows a higher association with the syndrome[9], and SEG and SEI have been suggested to cause the syndrome in cases of TSST-1-negative cases[10].

In a nosocomial transmission with NTED, the DNA of the MRSA strains isolated from all seven infants infected in an NICU was the same as that of the MRSA strains isolated from the nasal mucosa of five medical care staff (asymptomatic carriers) on pulsed-field gel electrophoresis analysis, strongly suggesting that MRSA was transmitted from the medical care staff to the infants.

In the case of SSSS-associated MRSA, no previous superantigenic toxin genes were detected. In Japan, exfoliative toxin B-positive MRSA infection has tended to increase recently in NICUs.

Acknowledgments. This work was supported by the Yujin Memorial Grant, Japan (I. Taneike).

REFERENCES

1) Bohach GA, Foster TJ: *Staphylococcus aureus* exotoxins, Fischetti VA, Novick RP, Ferretti JJ, Portnoy DA, Rood JI (eds): Gram- Positive Pathogens, American Society for Microbiology, Washington, DC, 2000, p 367-378.
2) Takahashi N, Nishida H, Kato H, Imanishi K, Sakata Y, Uchiyama T: Exanthematous disease induced by toxic shock syndrome toxin 1 in the early neonatal period. *Lancet* **351:** 1614-1619, 1998.
3) Becker K, Roth R, Peters G: Rapid and specific detection of toxigenic *Staphylococcus aureus:* use of two multiplex PCR enzyme immunoassays for amplification and hybridization of staphylococcal enterotoxin genes, exfoliative toxin genes, and toxic shock syndrome toxin 1 gene. *J Clin Microbiol* **36:** 2548-2553, 1998.
4) Jarraud S, Cozon G, Vandenesch F, Bes M, Etienne J, Lina G: Involvement of enterotoxins G and I in staphylococcal toxic shock syndrome and staphylococcal scarlet fever. *J Clin Microbiol* **37:** 2446-2449, 1999.
5) Zhang S, Iandolo JJ, Stewart GC: The enterotoxin D plasmid of *Staphylococcus aureus* encodes a second enterotoxin determinant (*sej*). *FEMS Microbiol Lett* **168:** 227-233, 1998.
6) Nei M, Li WH: Mathematical model for studying

genetic variation in terms of restriction endonucleases. *Proc Natl Acad Sci USA* **76:** 5269-5273, 1979.

7) Kuroda M, Ohta T, Uchiyama I, Baba T, Yuzawa H, Kobayashi I, Cui L, Oguchi A, Aoki K, Nagai Y, Lian J, Ito T, Kanamori M, Matsumaru H, Maruyama A, Murakami H, Hosoyama A, Mizutani-Ui Y, Takahashi NK, Sawano T, Inoue R, Kaito C, Sekimizu K, Hirakawa H, Kuhara S, Goto S, Yabuzaki J, Kanehisa M, Yamashita A, Oshima K, Furuya K, Yoshino C, Shiba T, Hattori M, Ogasawara N, Hayashi H, Hiramatsu K: Whole genome sequencing of meticillin-resistant *Staphylococcus aureus*. *Lancet* **357:** 1225-1240, 2001.

8) Li H, Llera A, Mariuzza RA: Structure-function studies of T-cell receptor-superantigen interactions. *Immunol Rev* **163:** 177-186, 1986.

9) Crass BA, Bergdoll MS: Toxin involvement in toxic shock syndrome. *J Infect Dis* **153:** 918-926, 1986.

10) Jarraud S, Cozon G, Vandenesch F, Bes M, Etienne J, Lina G: Involvement of enterotoxins G and I in staphylococcal toxic shock syndrome and staphylococcal scarlet fever. *J Clin Microbiol* **37:** 2446-2449, 1999.