

The Risk of Emerging Resistance to Trimethoprim/Sulfamethoxazole in *Staphylococcus aureus*

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Objective: Due to the spread of community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA), the demand for trimethoprim/sulfamethoxazole (SXT) is increasing in the world. It is not clear whether the resistant strain emerges by overuse of SXT. We investigated here the emergent risk of the SXT-resistant mutant in *S. aureus* by an in vitro SXT exposure experiment.

Methods: A total of 40 *S. aureus* clinical isolates (20 MSSA and 20 MRSA isolates) were exposed to sub-MIC of SXT for consecutive days, and MIC of SXT was determined every day. In addition, the *dfiB* DNA sequencing was performed to detect the mutation in the SXT-resistant strain.

Results: The SXT-resistant strain began to emerge on the eighth day and accounted for 45% (18/40 clinical isolates) after 14 days. Moreover, one half of these resistant strains showed F98Y mutation in DfrB to retain SXT-resistance without selective pressure.

Conclusion: The emergent risk was SXT exposure of 14 days or more.

Keywords: *Staphylococcus aureus*, antimicrobial resistance, trimethoprim/sulfamethoxazole, *dfiB*

Introduction

Community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) is spreading worldwide.¹⁻³ As a therapeutic option other than antibiotic agents, essential oil is considered⁴⁻⁶ against drug-resistant *S. aureus* such as MRSA; however, clinical evidence is limited. In actual clinical setting, trimethoprim/sulfamethoxazole (SXT) has been used to treat skin and soft tissue infections and bone and joint infections because the majority of *S. aureus* isolates, including CA-MRSA, are susceptible to this drug.^{7,8} However, there are some cases of failure and/or recurrence by the drug-resistant acquisition.^{9,10} *S. aureus* shows resistance to SXT due to the mutation of *dfi* gene where dihydrofolate reductase becomes the target of trimethoprim.¹¹ We investigate here the emergence risk of the SXT-resistant mutants through in vitro exposure of SXT.

Materials and Methods

Bacterial Strains

A total of 40 SXT-susceptible *S. aureus* clinical isolates, consisting of 20 MSSA and 20 MRSA isolates, were used. These strains were isolated, as a part of standard hospital protocol, from 16 general hospitals in the Tohoku region in Japan, their origins are as follows: sputum, 30%; pus, 20%; otorrhea, 13%; nasal cavity, 10%; and other, 27%. These isolates are collected and stocked by Tohoku Infectious Diseases society, and we received from them. In addition, these strains have confirmed not harbor mobile genetic elements including *dfiA*, *dfiK*, and/or *dfiG* by the conventional PCR method.¹²

Antimicrobial Susceptibility Testing and in vitro SXT Challenge

MIC of SXT was determined by the broth microdilution method according to the guideline of the Clinical and Laboratory Standard Institute,¹³ with resistance being in accordance with M100-S28.¹⁴ The SXT challenge to inoculate sub-MIC surviving strain into another dilution series was performed for a total of 14 days (Figure 1).¹⁵ Moreover, to investigate

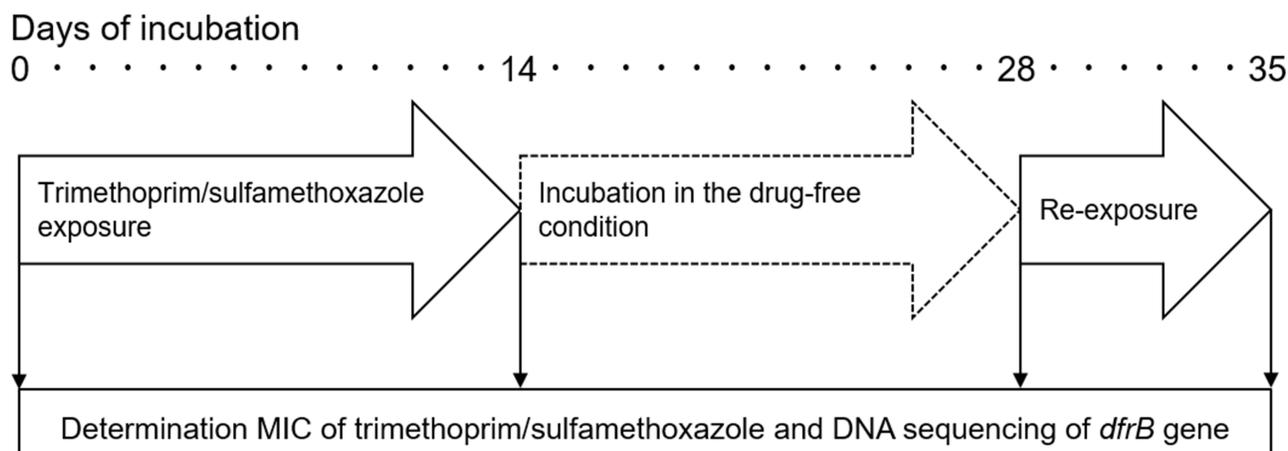


Figure 1 Experimental design. Allow indicated days of incubation with or without trimethoprim/sulfamethoxazole.

whether acquired resistance was maintained, strains were subsequently incubated using drug-free Mueller-Hinton broth (MHB) (Day 28). Then, these strains were re-challenged with SXT for 7 days (Day 35).

DNA Sequencing of *dfrB* Gene

The DNA sequence of the *dfrB* gene in the SXT-resistant strain was determined by the Dye Terminator Cycle Sequencing method using Genomelab GeXP and Quick Start Kit (Beckman Coulter Inc., CA, US) and the previously described primer.¹⁶

Growth Ability Assay

Further, to determine the growth ability of each strain, bacterial counts were measured.¹⁷ Briefly, the bacterial solution was inoculated into MHB following incubation for 48 hours. While the incubation, the bacterial amount was counted by plating each 0-, 3-, 6-, 9-, 12-, 24-, 36- and 48-hours later.

Statistical Analysis

The data were calculated as the mean and standard deviation per experimental group, and compared by an unpaired *t*-test. Statistical significance was set at $P < 0.05$. All experiments were performed in duplicate on another day.

Results

The first SXT-resistant strain was confirmed after 8 days; then a total of 18 strains (45%) had acquired resistance after 14 days (Figure 2). These 18 strains were confirmed as DfrB mutation, and the previously reported F98Y mutation was 11 out of 18 mutants (61%) (Table 1). In addition, previously unreported mutations L40I, F92L, T96I, and L141P were confirmed. The growth ability of 18 DfrB mutants after 6 hours was significantly low compared with the other 22 strains ($P < 0.05$) (Figure 3). Moreover, seven of the 18 strains recovered susceptibility by the incubation without the drug and lost mutation (Day 28 in Table 1). The growth ability of these 7 mutants had decreased more than its parental strains ($P < 0.05$) (Figure 4). Then, 7 strains that showed the same mutation by the second challenge of SXT (Day 35) acquired resistance again. In contrast, the other 11 strains-maintained resistance by the drug-free incubation. All mutations of DfrB in these 11 strains were F98Y, and they were maintained after 14 days of incubation (Day 28) (Table 1).

Discussion

Despite the SXT-resistance rate indicating approximately 1% in the United States and Japan,^{18,19} 45% of strains acquired resistance in this in vitro study. This was because the SXT challenge was performed at 14 days in vitro. SXT administration is generally completed from 5 to 7 days in skin infections caused by *S. aureus*, and therefore, DfrB

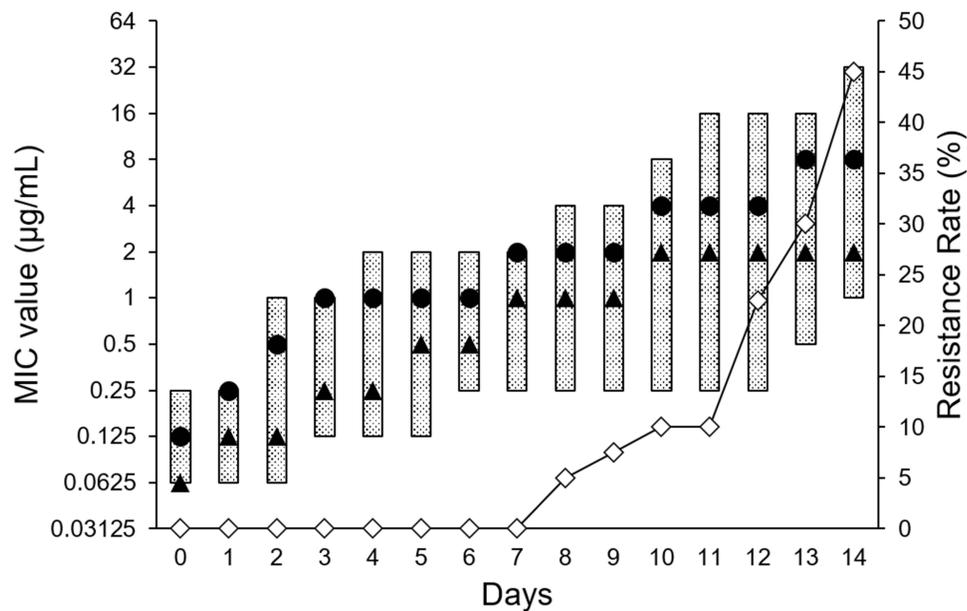


Figure 2 MIC values of trimethoprim/sulfamethoxazole and accumulative percentage of resistant *Staphylococcus aureus* strain. The left vertical axis indicates MIC of trimethoprim/sulfamethoxazole. The dot pattern box indicates the MIC range (n=40), the open triangle indicates MIC₅₀, and the open circle indicates MIC₉₀ by the day. The right vertical axis indicates the cumulative resistance rate (solid line and open square).

mutation may not occur. Moreover, it is difficult to isolate the causative organism in the bone and joint infection for which SXT is administered for a long period of time.²⁰ Therefore, these resistant strains might not be detected.

Because the 18 strains that acquired resistance in this study had significantly low growth ability 12 hours later, it is suggested that the initial stationary phase in the growth of the bacteria process was long. Trimethoprim has mutation inductivity,²¹ and its influence is dependent on the amount of exposure in the stationary phase.²² It was considered that

Table I MIC and DfrB Mutation Point in 18 Isolates of Trimethoprim/Sulfamethoxazole-Resistant *S. aureus*

Strain	MIC (µg/mL) of SXT				Mutation Point of DfrB		
	Day 0	Day 14	Day 28	Day 35	Day 14	Day 28	Day 35
MS-8	0.063	16	4	16	F98Y	F98Y	F98Y
MS-15	0.125	8	4	8	F98Y	F98Y	F98Y
MS-17	0.125	8	4	8	F98Y	F98Y	F98Y
MS18	0.125	16	4	16	F98Y	F98Y	F98Y
MS-19	0.125	8	4	8	F98Y	F98Y	F98Y
MR-9	0.063	32	8	32	F98Y	F98Y	F98Y
MR-10	0.125	4	4	4	F98Y	F98Y	F98Y
MR-11	0.063	16	4	16	F98Y	F98Y	F98Y
MR-14	0.063	8	4	8	F98Y	F98Y	F98Y
MR-17	0.125	4	4	4	F98Y	F98Y	F98Y
MR-18	0.125	8	4	8	F98Y	F98Y	F98Y
MS-3	0.063	4	2	4	F92L	-	F92L
MS-4	0.063	4	2	4	T96I	-	T96I
MS-7	0.125	4	1	4	L141P	-	L141P
MS-9	0.063	16	1	16	T96I, F98L	-	T96I, F98L
MR-2	0.125	8	2	8	L40I	-	L40I
MR-8	0.063	4	2	4	H149R	-	H149R
MR-12	0.063	4	2	4	H149R	-	H149R

Abbreviations: SXT, trimethoprim/sulfamethoxazole; MS, methicillin-susceptible; MR, methicillin-resistant.

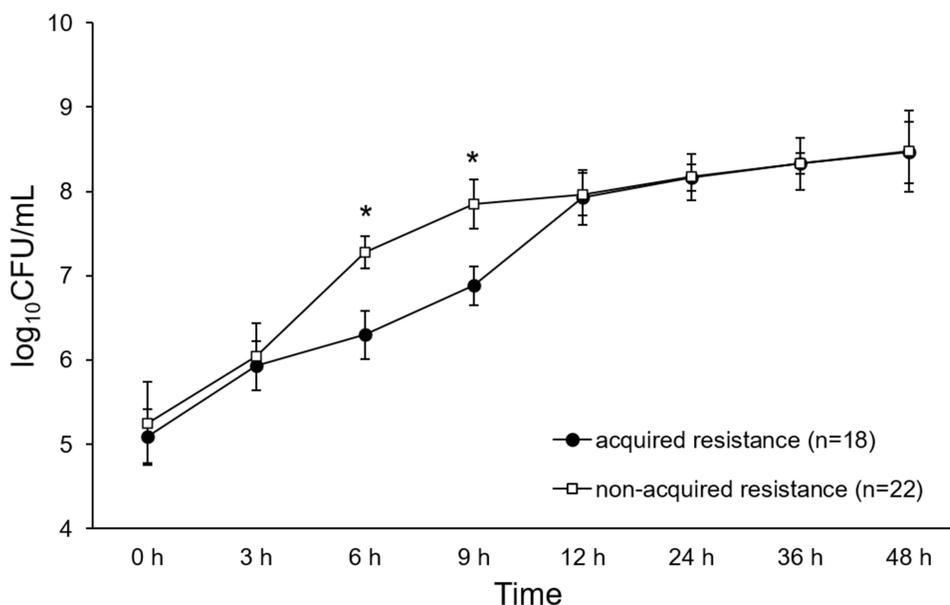


Figure 3 The comparison of the growth curve of *Staphylococcus aureus* strains. The vertical axis indicates the bacterial counts (\log_{10} CFU/mL) of parental strains that have acquired resistance (n=18) (open square) and parental strains that have not acquired resistance (n=22) (closed circle). * $P < 0.05$.

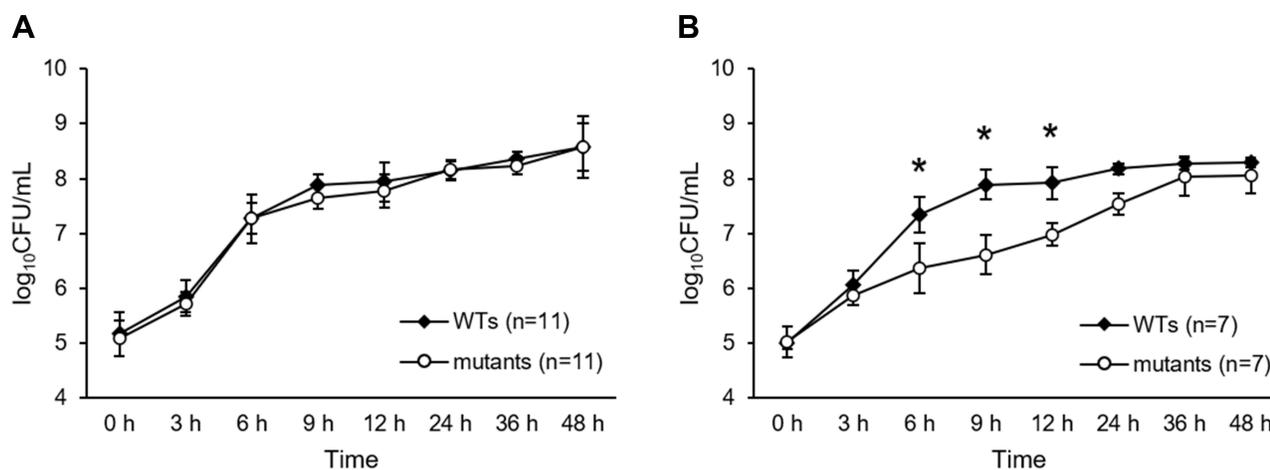


Figure 4 The change of the growth curve with the acquisition of SXT-resistance. The growth curve of *Staphylococcus aureus* strains that sustained resistance (n=11) (A) or that returned to susceptible (n=7) (B). The vertical axis indicates the optical density of wild type strains (WTs; closed square) and DfrB mutants (open circle). * $P < 0.05$.

the mutation was more likely to occur in the 18 strains because of continued SXT exposure in the long stationary phase. In addition, despite approximately 40% of DfrB mutants having recovered susceptibility, those showed resistance again by the second SXT challenge. Generally, the growth rate of the resistant strain is slower than that of the susceptible strain, which is the result of a gene mutation.²³ In fact, the growth ability of the susceptibility to recovery strain was significantly decreased compared with its parental strain ($P < 0.05$) (Figure 3). The resistant mutant will become dominant by the antibiotic selective pressure because only a mutant can survive. Moreover, as the wild type grows quickly if the selective pressure disappears, thus it seems to have recovered susceptibility.²⁴ However, caution is recommended as hetero-resistant strains can become easily resistant.

The SXT-resistance rate is high in some regions (eg, Taiwan, Nigeria, and a few towns in Africa),^{25–27} and it is involved in spreading the strain harboring the *dfrA*, *dfrG*, and/or *dfrK* gene via companion and livestock animals.^{12,28–30} At the moment, the human-derived strain is rarely reported in the United States and Japan, but it will be necessary to

monitor it closely. Furthermore, we reported that SXT-resistant small colony variants lurk in community strains,³¹ and these cannot be detected by a general bacterial test. These strains should also be kept in mind.

Conclusion

Forty-five percent of *S. aureus* clinical isolates became SXT-resistant through the 2-week in vitro challenge, and these strains have DfrB mutation. Thus, these mutants may be selected if SXT is administered over a 2-week period for *S. aureus* infection. To define these clinical problems, further clinical studies and trials including population analysis and AUC studies are required.

Ethical Approval

Not required, according to the Research Ethics Statement of the Ministry of Health, Labour and Welfare of Japan, because this study is basic research that used bacterial isolates only. Clinical isolates that were used in this study were collected and stocked by the Tohoku Infectious Diseases Society; therefore, we cannot access patient information.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis, and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

All authors have no conflict of interest to declare.

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