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Screening method for trimethoprim/sulfamethoxazole-resistant small colony variants of *Staphylococcus aureus*

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ABSTRACT

Objectives: Trimethoprim/sulfamethoxazole (SXT) is used to treat *Staphylococcus aureus* infections. However, the effect of treatment with SXT is sometimes not sufficient and there are patients whose treatment has to be prolonged. There are few reports of isolated strains of SXT-resistant *S. aureus*, but it is possible that some resistant strains cannot be detected by current testing methods We have therefore developed a tool to identify these resistant strains.
Methods: The mutant selection window (MSW) of SXT for 40 clinical isolates of *S. aureus*, including 20 methicillin-resistant *S. aureus* (MRSA), was determined. The optimum concentration of SXT and thymidine in agar for detecting SXT-resistant small colony variants (SCVs) of *S. aureus* was investigated.
Results: The MSW_{50} and MSW_{90} of SXT, presented as a multiple of the minimum inhibitory concentration (MIC), were 16× MIC and >256× MIC, respectively. SCVs were detected within the MSW in 32 (80%) of the 40 clinical isolates studied. To maintain the morphology of SCVs, the most suitable concentrations of SXT and thymidine for screening were 4 mg/L and 0.01 μg/mL, respectively. All 32 SCVs were resistant to SXT (MIC >32 mg/L). The sensitivity and specificity of this screening method was 100% and 88.9%, respectively.
Conclusions: SXT-resistant SCVs are not usually detected by routine laboratory tests performed in hospitals. However, the screening test described here can easily distinguish SXT-resistant SCVs among *S. aureus* isolated from specimens. This newly developed screening test could become an important tool to prevent inappropriate use of SXT.

1. Introduction

Trimethoprim/sulfamethoxazole (SXT) is used to treat infectious diseases caused by *Staphylococcus aureus*, including methicillin-resistant *S. aureus* (MRSA) [1,2]. No SXT-resistant strains of *S. aureus* were isolated in surveillance studies carried out in Japan, the USA and Greece [3–5]. However, it has been reported that treatment with SXT is sometimes not sufficiently effective and leads to relapse after treatment [6–8]. The emergence of in vivo SXT-resistant *S. aureus* has been noted as a reason for this relapse.

Pathogens that form small colonies have been isolated from patients with cystic fibrosis who have received prolonged treatment with SXT [9–12]. These pathogens are called small colony variants (SCVs) [13]. *S. aureus* SCVs show low pathogenicity, such as low toxicity and haemolytic activity, compared with *S. aureus* forming normal colonies [14–17]. In addition, *S. aureus* SCVs are hard to grow on Mueller–Hinton (MH) agar, which is conventionally used in bacteriological testing [13]. SXT inhibits the biosynthesis of folate, which is a functional cofactor of thymidylate synthase activity, and thereby depletes thymidine that is essential for bacterial DNA synthesis. Most SCVs emerging following exposure to SXT are thymidine-dependent SCVs (TD-SCVs) that uptake thymidine from the extracellular milieu by increasing the expression of nucleoside uptake protein NupC [18,19]. In other words, the mechanism of SXT resistance in *S. aureus* is considered to be mediated by hyperactivation of thymidine uptake, but this remains mostly unclear. Regarding potential causes of SXT treatment failure despite a low detection rate of SXT-resistant strains, one possibility is that SXT-resistant SCVs are missed by conventional bacteriological testing. We confirmed SXT resistance

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in *S. aureus* SCVs that emerged following exposure to SXT and have developed a screening method for these resistant strains.

2. Materials and methods

2.1. Bacterial strains

A total of 40 clinical isolates of *S. aureus*, including 20 MRSA, were collected from 14 general hospitals in the Tohoku Region of Japan. All of the strains were isolated from individual patient samples. The source of MRSA strains was mostly sputum (45%), followed by pus (20%), otorrhea (15%) and others (Fig. 1). On the other hand, methicillin-susceptible *S. aureus* (MSSA) strains were isolated mostly from pus (35%). *S. aureus* ATCC 29213 was used as the standard strain.

2.2. Minimum inhibitory concentration (MIC) of trimethoprim/sulfamethoxazole against clinical isolates

Sulfamethoxazole (Wako Pure Chemical Corp., Osaka, Japan) and trimethoprim (Nacalai Tesque, Inc., Kyoto, Japan) were used. The MIC of trimethoprim/sulfamethoxazole (19:1) was determined by the microdilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI) [20]. Briefly, MRSA and MSSA isolates were cultured on MH (Becton Dickinson, Tokyo, Japan) agar plates (Eiken, Tokyo, Japan) at 37 °C for 24 h, followed by suspension in MH broth to a cell density equivalent to a 0.5 McFarland standard. The bacterial suspension was added to MH broth containing different concentrations of SXT (0.025–25.6 mg/L, trimethoprim concentration only). The dilution series was then cultured at 37 °C for 20 h. Resistance was defined by an MIC $\geq$3.2 mg/L (trimethoprim concentration only) in consideration of 2017 CLSI breakpoints (MIC >2 mg/L, trimethoprim concentration only) [21].

2.3. Determination of the mutant prevention concentration (MPC) and mutant selection window (MSW)

The MPC of SXT was determined by the agar dilution method according to a modification of the method described above [22]. A total of 100 μL of bacterial suspension with a cell density of 1.0×10^{10} CFU/mL was spread on MH agar containing SXT at different concentrations, followed by culture at 37 °C for 120 h. The MPC was defined as the minimum concentration necessary to inhibit colony formation by the least susceptible single-step mutants. The MSW was determined as the difference between the MPC and MIC and is presented as a multiple of the MIC.

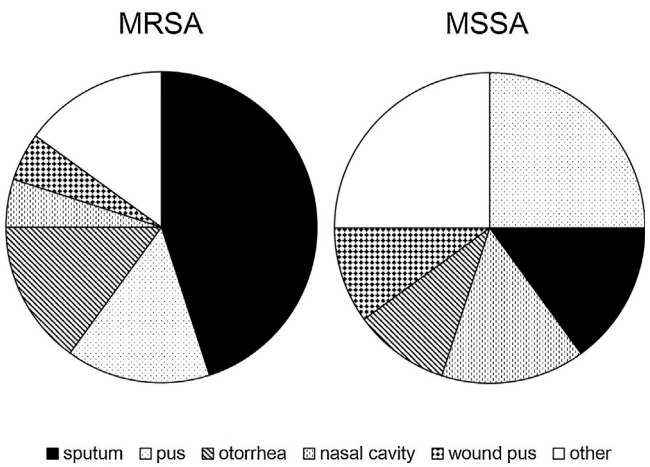

Fig. 1. Source of clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA).

2.4. Conditions for maintaining the morphology of small colony variants (SCVs) and minimum inhibitory concentration determination against SCVs

Next we attempted to establish the most suitable incubation conditions for maintenance of the morphology of selected SCVs within the MSW. The optimum concentration of SXT and thymidine was studied using each of the MRSA and MSSA strains for which SCVs were detected within the MSW of SXT. *S. aureus* ATCC 29213 strain was used as the negative control. Thymidine was added to SXT-containing agar (2 mg/L or 4 mg/L) to achieve thymidine concentrations of 0.001, 0.01 and 0.05 mg/L. The bacterial suspension was added to these agar plates and colony morphology was observed following incubation at 37 °C for 24 h or 48 h. The definition of a SCV phenotype was as described by Precit et al. [23]. The MIC of SXT for SCVs was determined by Etest using MH agar containing the most suitable SXT and thymidine concentration established as mentioned above. Because we could not distinguish colony morphology, the broth microdilution method was not performed.

2.5. Measurement of mRNA expression levels

The expression level of nucleoside permease *nupC* mRNA in SXT-resistant SCVs was compared with that of the parent strain. TRI Reagent® LS (Molecular Research Center, Inc., Cincinnati, OH) was used to extract RNA from bacteria. The expression level of mRNA was determined using a CFX Connect Real-Time System (Bio-Rad, Hercules, CA), a specific primer [18] (Table 1) and iTaq™ Universal SYBR® Green One-Step Kit (Bio-Rad). The mRNA expression level was standardised against expression of *yqiL* mRNA encoding acetyl-coenzyme A acetyltransferase. The expression level of mRNA was demonstrated as a multiple of the value obtained for *S. aureus* ATCC 29213.

2.6. Screening method for heteroresistant small colony variants

A screening method was developed to select the SCV heteroresistant strains among clinical isolates. Each optimum concentration of SXT and thymidine in MH agar considered to satisfy the condition for the formation of SCVs was maintained in the method mentioned above in Section 2.4. Another condition was that the standard *S. aureus* ATCC 29213 strain could not grow under the same conditions. The sensitivity and specificity of this screening method were determined using 32 SCV heteroresistant strains selected from within the MSW and another 9 SXT-susceptible strains including the *S. aureus* ATCC 29213 strain.

2.7. Statistical analysis

The detection rate of SCVs was compared by χ^2 test. The expression level of mRNA was compared by Student's *t*-test. Statistical significance was set at $P < 0.05$.

Table 1
Sequences of specific primers.

Primer	Sequence (5′ → 3′)	Reference
nupC-1	CTATTTAGCTCAGACAGGAAAAA	[18]
nupC-2	AGCAAGATAAATGCAAAGATAAG	[18]
yqiL-1	TGCGATGATGTTAGTCATGT	[18]
yqiL-2	TTCAAAGCCTTTTCTACAGC	[18]