1 **Addition of thymidine to culture media for accurate**
2 **examination of thymidine-dependent small-colony variants of**
3 **methicillin-resistant *Staphylococcus aureus*: a pilot study**

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13 Running title: Modified media for detection of TD-SCVs of MRSA

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23 Abbreviations: TD-SCVs, thymidine-dependent small-colony variants

Abstract

Small-colony variants (SCVs) are slow-growing subpopulations of various auxotrophic bacterial strains. Thymidine-dependent SCVs (TD-SCVs) are unable to synthesize thymidine; hence, these variants fail to grow in a medium without thymidine. In this study, we used 10 TD-SCVs of *Staphylococcus aureus*, of which four strains possessed *mecA*. We compared the efficacy of a newly modified medium containing thymidine for the detection of TD-SCVs of methicillin-resistant *S. aureus* (MRSA) to the efficacy of routinely used laboratory media. We observed that none of the 10 TD-SCVs of *S. aureus* grew in Mueller–Hinton agar, and four TD-SCVs of MRSA failed to grow on all MRSA screening media, except for the ChromID™ MRSA medium. Laboratory tests conducted using medium with thymidine incorporated showed that thymidine did not affect the minimum inhibitory concentrations of oxacillin and cefoxitin for clinical isolates of *S. aureus*, and was able to detect MRSA, including TD-SCVs. These findings showed that thymidine-incorporated media are able to detect TD-SCVs of MRSA without altering the properties of other clinically isolated MRSA strains.

1 Keywords: MRSA screening media, thymidine, TD-SCVs of MRSA

1 Introduction

Small-colony variants (SCVs) are slow-growing subpopulations of bacteria that are characterized by distinctive phenotypic traits, including atypical colony morphology and auxotrophy for various growth factors such as menadione, hemin, and thymidine (described for *Staphylococcus aureus* by Melter and Radojevič, 2010). Clinically, SCVs are detected mainly in patients with chronic infections, undergoing long-term antibiotic treatment (Gilligan et al., 1987). They are less susceptible to antibiotics than their wild-type counterparts and are associated with persistent and recurrent infections (Kahl et al., 1998; Kahl et al., 2003a; Proctor et al., 2006). SCVs of *S. aureus* are isolated from airway infections, including cystic fibrosis, osteomyelitis, and device-related infections (Gilligan et al., 1987; Kahl et al., 1998; Kahl et al., 2003a; von Eiff et al., 1997; Spanu et al., 2005; Seifert et al., 2003), and they form characteristic tiny, translucent, and less β -hemolytic “pinpoint” or “fried egg” colonies, due to their decreased metabolic activity (Kahl et al., 2003b). SCVs of *S. aureus* often have altered electron transport activity (Proctor et al., 1994; Proctor et al., 1998) or thymidine synthesis ability (Besier et al., 2007; Chatterjee et al., 2008). SCVs are frequently thymidine-dependent, and nearly all thymidine-dependent SCVs (TD-SCVs) emerge due to long-term treatment with trimethoprim-sulfamethoxazole (SXT) (Gilligan et al.,

19 1987; Proctor et al., 2006; von Eiff et al., 2004). The mutants are deficient in
20 thymidylate synthetase, which catalyzes the conversion of deoxyuridine monophosphate
21 to deoxythymidine monophosphate (Besier et al., 2007; Chatterjee et al., 2008). Thus,
22 they are SXT-resistant (Gilligan et al., 1987; Proctor et al., 2006). TD-SCVs fail to
23 grow on commercially prepared Mueller–Hinton agar because of the low thymidine
24 content of this agar (Gilligan et al., 1987).

25 Clusters of SCVs of *S. aureus* can contain populations of methicillin-resistant
26 *Staphylococcus aureus* (MRSA); for example, Kipp et al. (2004) reported the presence
27 of SCVs of MRSA strains. MRSA is one of the most problematic bacterial pathogens
28 for hospital-acquired infections; TD-SCVs of MRSA are also occasionally known to
29 cause infections (Kipp et al., 2003; Seifert et al., 1999; Coman et al., 2008). Therefore, a
30 reliable, accurate, and rapid test that detects MRSA, including TD-SCVs of MRSA, is
31 necessary for appropriate antibiotic therapy and infection control. Screening tests for
32 MRSA have been developed and improved (Apfalter et al., 2002; Perry et al., 2004;
33 Smyth and Kahlmeter, 2005; Stoakes et al., 2006; 23. Zadik et al., 2001); however, no
34 reports have been published concerning the laboratory tests available for the detection
35 of TD-SCVs of MRSA.

In this study, we show the usefulness of a newly modified medium supplemented with thymidine for detecting TD-SCVs of MRSA.

2 Materials and methods

2.1 Bacterial strains

In total, 10 SCVs of *S. aureus* strains (SH1–SH10) were isolated from nine patients at the Shinshu University Hospital within a period of five years. These strains detected on TSAII with 5% Sheep Blood/Drigalski Agar, Modified (Becton Dickinson, Tokyo, Japan) after incubation for 24–48 hours at 37°C under aerobic conditions. None of the strains could be cultured on Mueller–Hinton agar; however, they grew on trypticase soy agar with 5% sheep blood (Becton Dickinson) and exhibited tiny, translucent, and less β -hemolytic “pinpoint” or “fried egg” colonies, which corresponded with the characteristic features of SCV colonies (Kahl et al., 2003b). They were determined to be different clones, except the SH1 (*mecA* negative) and SH2 (*mecA* positive) strains which were isolated from a single patient, by pulsed-field gel electrophoresis using *Sma*I for total DNA digestion (data not shown). To evaluate their modified susceptibility to antibiotic test medium, 10 additional wild-type MRSA and 10

additional Methicillin-susceptible *Staphylococcus aureus* (MSSA) strains were used. These MRSA and MSSA strains were clinically isolated from different patients, and each showed a distinct antibiotic susceptibility pattern.

2.2 Molecular identification

The 16S ribosomal RNA gene of each strain was amplified using universal primers (Neilan et al., 1997). The partial DNA sequences (approximately 500 bp) were determined using a BigDye® Terminator v1.1 Cycle Sequencing Kit with an Applied Biosystems 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The sequence data were compared with those available in the International Nucleotide Sequence Database by BLAST algorithm or by using the Ribosomal Database Project (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp).

2.3 Evaluation of auxotrophisms

Auxotrophy testing was performed on Mueller–Hinton agar (Becton Dickinson) using sterilized filter paper disks containing 10 µg of thymidine (Nakalai Tesque, Kyoto, Japan), hemin (Wako Pure Chemical Industries, Ltd., Osaka, Japan), or 0.1 µg of

menadione (Nakalai Tesque). The appearance of SCV growth only around the sterilized filter paper disks after incubation for 48 h at 35°C under aerobic conditions indicated the respective auxotrophisms.

2.4 Detection of *mecA*

mecA was detected by polymerase chain reaction (PCR) using *mecA*-specific primers (forward primer: 5'-AAAATCGATGGTAAAGGTTGGC-3' and reverse primer: 5'-AGTTCTGCAGTACCGGATTTGC-3') (Murakami et al., 1991). A 533-bp region of *mecA* (nucleotides 1282–1814 of the PBP 2' coding frame) was amplified.

2.5 Growth characteristics depending on thymidine concentration

TD-SCVs of *S. aureus* were subcultured at least three times on trypticase soy agar with 5% sheep blood (TSA II) before examination. We cultured 10 TD-SCVs of *S. aureus* strains and *S. aureus* ATCC 29213 on trypticase soy agar with 5% sheep blood (TSA II) overnight at 35°C. The strains were suspended in 10 mL of Mueller–Hinton broth (Becton Dickinson), and adjusted to an optical density at 600 nm (OD₆₀₀) of 0.1. Subsequently, we added thymidine to the broth, at final concentrations of 0, 0.1, 1.0, 10,

and 100 µg/mL. The strains were then incubated at 35°C under aerobic conditions with shaking, and the OD₆₀₀ values were measured every 2 h for 12 h. The measurements were performed one time.

2.6 Antibiotic susceptibility test using the modified medium

To investigate the effect of thymidine on bacterial susceptibility to oxacillin and cefoxitin, the MICs of 10 isolates each of wild-type MRSA and MSSA were examined. MSSA and MRSA strains were determined by the agar dilution method, according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (Clinical and Laboratory Standards Institute, 2009). Control strains recommended by the CLSI (*S. aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, and *Escherichia coli* ATCC 25922) were also examined. Agar dilution susceptibility tests were performed using Mueller–Hinton agar (supplemented with 2% sodium chloride for testing oxacillin susceptibility) (Clinical and Laboratory Standards Institute, 2009), with or without 10 µg/mL of thymidine. Antibiotics were serially diluted two-fold in Mueller–Hinton agar medium to obtain final concentrations ranging from 0.25 µg/mL to 128 µg/mL. To determine the MICs of oxacillin and cefoxitin, the strains were cultured

at 35°C under aerobic conditions for 24 and 20 h, respectively. MICs of the TD-SCVs of *S. aureus* were then determined using the same modified Mueller–Hinton agar. MIC determination was performed one time for each method.

2.7 Detection of TD-SCVs of MRSA by using MRSA screening media and the modified medium

We used the following five MRSA screening media, which are selective and differential chromogenic media: ChromID™ MRSA (SYSMEX bioMérieux, Tokyo, Japan), MS-CFX (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan), BBL™ CHROMagar™ MRSA II (Becton Dickinson), CHROMagar™ MRSA (Kanto Chemical, Tokyo, Japan), and MDRS-II (Kyokuto Pharmaceutical Industrial Co. Ltd., Tokyo, Japan) to screen for TD-SCVs of MRSA. Colonies of TD-SCVs of *S. aureus*, which were cultured on TSA II at 35°C under aerobic conditions for 48 h, were suspended in saline and adjusted to 0.5 McFarland turbidity standard. These suspensions were then diluted tenfold. Subsequently, 2 µL of each suspension was inoculated on the five media and incubated for 48 h. The suspensions were also inoculated on TSA II agar and Mueller–Hinton agar as controls. We also examined with modified media (five MRSA screening media

which 200 μ L of 1.0 mg/mL thymidine had been added to the surface of the plate, then spread and dried). The volume of an agar plate is approximately 20 mL; therefore, we used a total of 200 μ g of thymidine in each plate, to produce a final thymidine concentration of approximately 10 μ g/mL. The TD-SCVs of MRSA were diagnosed using the methods employed in routine laboratory tests (green colonies on ChromID™ MRSA, reddish violet colonies on BBL™ CHROMagar™ MRSA II and CHROMagar MRSA, and positive results of egg yolk reaction and/or mannitol fermentation on MS-CFX and MDRS-II).

3 Results

Data regarding molecular identification, the presence or absence of *mecA*, and auxotrophisms in the 10 TD-SCVs are summarized in Table 1. All the strains were identified as *S. aureus* and showed thymidine auxotrophism. Further, four of them possessed *mecA*. We found that TD-SVCs showed insufficient growth on modified medium that contained < 10 μ g/mL of thymidine; however, their growth

137 Table 1 Identification, *mecA* possession, and auxotrophisms of small-colony variants of
 138 *Staphylococcus aureus*

Strain	Patient	Identification (16S rRNA) ^a	<i>mecA</i> ^b	Auxotrophism ^c		
				thymidine	hemin	menadione
SH1	A	<i>S. aureus</i>	—	+	—	—
SH2		<i>S. aureus</i>	+	+	—	—
SH3	B	<i>S. aureus</i>	+	+	—	—
SH4	C	<i>S. aureus</i>	+	+	—	—
SH5	D	<i>S. aureus</i>	+	+	—	—
SH6	E	<i>S. aureus</i>	—	+	—	—
SH7	F	<i>S. aureus</i>	—	+	—	—
SH8	G	<i>S. aureus</i>	—	+	—	—
SH9	H	<i>S. aureus</i>	—	+	—	—
SH10	I	<i>S. aureus</i>	—	+	—	—

139 ^a Identification by 16S rRNA gene analysis

140 ^b + : positive for *mecA*, — : negative for *mecA*

141 ^c + : growth only around the filter paper disks, — : no growth

remained constant when the concentration of thymidine was increased beyond 10 $\mu\text{g/mL}$. The growth of *S. aureus* ATCC 29213 was not affected by thymidine concentration (Figure 1).

MICs of oxacillin and cefoxitin for MSSA and MRSA, as determined on the modified medium containing 10 $\mu\text{g/mL}$ of thymidine and on the control medium are summarized in Table 2. We observed that the difference in the MICs of cefoxitin and oxacillin between the modified and control medium was not more than two-fold and that the susceptibility categories did not change even in strains that showed one-fold differences in MICs. The MICs of TD-SCVs are shown in Table 3. According to the CLSI guidelines, the MICs of oxacillin and cefoxitin determined using an agar dilution method or a broth micro-dilution method for determination of MRSA were $\geq 4 \mu\text{g/mL}$ and $\geq 8 \mu\text{g/mL}$, respectively. All four strains of TD-SCVs of *S. aureus* that possessed *mecA* showed MIC of $\geq 4 \mu\text{g/mL}$ for oxacillin and $\geq 8 \mu\text{g/mL}$ for cefoxitin. On the other hand, six TD-SCVs that did not possess *mecA* demonstrated MIC of $\leq 2 \mu\text{g/mL}$ for oxacillin and $\leq 4 \mu\text{g/mL}$ for cefoxitin.

The results of five MRSA screening media are summarized in Table 4. When thymidine was not added to the media, all four strains of TD-SCVs that possessed *mecA*

159 grew only on ChromID™ MRSA after 48 h of incubation and did not grow on the
160 remaining four media. However, the same strains grew on all MRSA screening media
161 when thymidine was added. All colonies appeared green on ChromID™ MRSA and
162 reddish violet on BBL™ CHROMagar™ MRSA II and CHROMagar™ MRSA, and
163 showed positive egg yolk reaction and/or mannitol fermentation on MS-CFX and
164 MDRS-II.

168 Figure 1. Growth curves of thymidine-dependent small-colony variants of
169 *Staphylococcus aureus* using Mueller–Hinton broth in the presence of various
170 concentrations of thymidine (0, 0.1, 1.0, 10, and 100 µg/mL)

Table 2 Antibiotic susceptibility test of wild-type methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* and quality control strains to oxacillin and ceftioxin with or without 10 µg/mL thymidine

Strain	Ceftioxin (µg/mL)		Oxacillin (µg/mL)	
	Thymidine ^a		Thymidine ^a	
MSSA	1	2	≤0.25	≤0.25
	2	2	≤0.25	≤0.25
	3	4	≤0.25	≤0.25
	4	2	≤0.25	≤0.25
	5	4	≤0.25	≤0.25
	6	4	≤0.25	≤0.25
	7	4	≤0.25	≤0.25
	8	4	≤0.25	≤0.25
	9	4	≤0.25	≤0.25
	10	4	≤0.25	≤0.25
MRSA	1	32	128	128
	2	128	>128	128
	3	128	>128	>128
	4	32	32	64
	5	32	32	32
	6	>128	>128	>128
	7	>128	>128	>128
	8	>128	>128	>128
	9	>128	>128	>128
	10	16	32	32
<i>S. aureus</i> ATCC29213	2 ^b	2 ^b	≤0.25 ^c	≤0.25 ^c
<i>E. faecalis</i> ATCC29212	NT ^f	NT ^f	8 ^d	8 ^d
<i>E. coli</i> ATCC25922	2 ^e	2 ^e	NT ^f	NT ^f

^a 10 µg/mL of thymidine added

^b Acceptable range : 1–4 µg/mL

^c Acceptable range : 0.12–0.5 µg/mL

^d Acceptable range : 8–32 µg/mL

^e Acceptable range : 2–8 µg/mL

^f Not tested

Table 3 Antibiotic susceptibility test of thymidine-dependent small-colony variants of *Staphylococcus aureus* to oxacillin and ceftioxin on Mueller–Hinton agar containing 10 µg/mL of thymidine

Strains	<i>mecA</i> ^a	Ceftioxin (µg/mL)	Oxacillin (µg/mL)
SH1	—	2	≤0.25
SH2	+	16	64
SH3	+	64	128
SH4	+	8	32
SH5	+	>128	>128
SH6	—	4	≤0.25
SH7	—	4	≤0.25
SH8	—	4	≤0.25
SH9	—	2	≤0.25
SH10	—	4	≤0.25
<i>S. aureus</i> ATCC29213		2 ^b	≤0.25 ^c
<i>E. faecalis</i> ATCC29212		NT ^f	8 ^d
<i>E. coli</i> ATCC25922		2 ^e	NT ^f

^a + : positive for *mecA*, — : negative for *mecA*

^b Acceptable range : 1–4 µg/mL

^c Acceptable range : 0.12–0.5 µg/mL

^d Acceptable range : 8–32 µg/mL

^e Acceptable range : 2–8 µg/mL

^f Not tested

Table 4 Comparison of five MRSA screening media and the modified media with added thymidine for detection of thymidine-dependent small-colony variants of *Staphylococcus aureus*

Strain	<i>mecA</i> ^a	ChromID MRSA ^b		MS-CFX ^c		BBL CHROMagar MRSA II ^d		CHROMagar MRSA ^d		MDRS-II ^e	
		Thymidine ^e		Thymidine ^e		Thymidine ^e		Thymidine ^e		Thymidine ^e	
SH1	—	—	—	—	—	—	—	—	—	—	—
SH2	+	+	—	+	—	—	+	+	—	+	+
SH3	+	+	—	+	—	—	+	+	—	+	+
SH4	+	+	—	+	—	—	+	+	—	+	+
SH5	+	+	—	+	—	—	+	+	—	+	+
SH6	—	—	—	—	—	—	—	—	—	—	—
SH7	—	—	—	—	—	—	—	—	—	—	—
SH8	—	—	—	—	—	—	—	—	—	—	—
SH9	—	—	—	—	—	—	—	—	—	—	—
SH10	—	—	—	—	—	—	—	—	—	—	—

^a + : positive for *mecA*, — : negative for *mecA*

^b + : growth and formed green colonies, — : no growth

^c + : growth and egg yolk reaction and/or mannitol fermentation positive, — : no growth

^d + : growth and formed reddish-violet colonies, — : no growth

^e With 200 µg/plate of thymidine added

4 Discussion

TD-SCVs of *S. aureus* can grow in humans because they uptake external thymidine (Chatterjee et al., 2007; Kriegeskorte et al., 2014); however, it is difficult to examine their antibiotic susceptibility by the methods that are routinely employed in a clinical bacteriological laboratory. Therefore, the accurate detection of TD-SCVs of MRSA is difficult, and they could go undetected while testing clinical isolates. SCVs are often misidentified using biochemical tests due to their low biological activity; therefore, we used analysis of sequences of 16S ribosomal RNA genes to correctly identify them in this study. Four of the 10 clinical TD-SCVs of *S. aureus* possessed *mecA* and did not grow on the Mueller–Hinton medium during the susceptibility tests. They also failed to grow on the four commercial MRSA screening media, except for ChromID™ MRSA medium.

Kipp et al. (2004) reported that PCR for the detection of *mecA* and MRSA-Screen latex agglutination test are useful for rapid and reliable detection of SCVs of MRSA; however, these tests are not usually carried out in a clinical bacteriological laboratory. Therefore, MRSA usually can be detected by antibiotic susceptibility tests or by using screening media. Several types of MRSA screening

media are commercially available; however, almost none of the MRSA screening media used in the current study, including Mueller–Hinton medium, contain sufficient thymidine to support the growth of TD-SCVs; hence, TD-SCVs failed to grow on them. Thymidine was removed from the Mueller–Hinton medium, as its presence in the medium could increase susceptibility to SXT (Koch and Burchall, 1971; Stokes and Lacey, 1978). Incorporation of 10 µg/mL thymidine in a medium was sufficient for the growth of TD-SCVs of *S. aureus*; TD-SCVs of *S. aureus* grew insufficiently until a concentration of 10 µg/mL. Therefore, we recommend the use of the modified medium containing 10 µg/mL thymidine for detecting TD-SCVs of MRSA.

Addition of 10 µg/mL thymidine to the culture medium did not affect the susceptibilities of the clinical isolates of MSSA and MRSA to cefoxitin and oxacillin. When the modified medium containing 10 µg/mL thymidine was used, MICs of cefoxitin and oxacillin for all four TD-SCVs of *S. aureus* that possessed *mecA* were ≥ 8 µg/mL and ≥ 4 µg/mL, respectively, and those for the other TD-SCVs of *S. aureus* that did not possess *mecA* were ≤ 4 µg/mL and ≤ 2 µg/mL, respectively. Therefore, the modified medium containing 10 µg/mL thymidine could distinguish *S. aureus*, including TD-SCVs, with *mecA* from those without *mecA*, in accordance with the CLSI criteria.

In our experiment, TD-SCVs of MRSA could be cultured only on the MRSA screening media ChromID™ MRSA, which consists of animal- and plant-derived peptones that might include thymidine. The four other MRSA screening media might be devoid of or have an insufficient concentration of thymidine. We observed that the TD-SCVs of MRSA rather than those of MSSA grew on all four MRSA screening media after the incorporation of thymidine. Therefore, we recommend the addition of thymidine to the MRSA screening media for accurate examination of TD-SCVs of MRSA. To strengthen the robustness of these findings, it will be necessary to investigate a large number of *S. aureus* isolates, including TD-SCVs of MRSA, and to confirm that TD-SCVs of MRSA can be detected in a bacterial mixture. Furthermore, it may be necessary to examine it using a laboratory-generated thymidine-requiring mutant.

In conclusion, we recommend the addition of thymidine to both the culture media and MRSA screening media that are routinely used in clinical bacteriological laboratories. This would lessen the chance of TD-SCVs going undetected, especially the TD-SCVs of MRSA, during a routine clinical examination.

249 **Conflict of interest:** None to declare.

250

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