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

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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. 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, 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, 2003a; von Eiff et al., 1997; Spanu et al., 2005; Seifert et al., 2003), and they form characteristics such as 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, 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., 1987; Proctor et al., 2006; von Eiff et al., 2004). The mutants are deficient in thymidylate synthetase, which catalyzes the conversion of deoxyuridine monophosphate to deoxythymidine monophosphate (Besier et al., 2007; Chatterjee et al., 2008). Thus, they are SXT-resistant (Gilligan et al., 1987; Proctor et al., 2006). TD-SCVs fail to grow on commercially prepared Mueller–Hinton agar because of the low thymidine content of this agar (Gilligan et al., 1987).

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

Abbreviation: TD-SCVs, thymidine-dependent small-colony variants.

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

Strain	Patient	Identification (16S rRNA)[a]	*mecA*[b]	Auxotrophism[c]		
				Thymidine	Hemin	Menadione
SH1	A	*S. aureus*	−	+	−	−
SH2		*S. aureus*	+	+	−	−
SH3	B	*S. aureus*	+	+	−	−
SH4	C	*S. aureus*	+	+	−	−
SH5	D	*S. aureus*	+	+	−	−
SH6	E	*S. aureus*	−	+	−	−
SH7	F	*S. aureus*	−	+	−	−
SH8	G	*S. aureus*	−	+	−	−
SH9	H	*S. aureus*	−	+	−	−
SH10	I	*S. aureus*	−	+	−	−

[a] Identification by 16S rRNA gene analysis.
[b] +: positive for *mecA*, −: negative for *mecA*
[c] +: growth only around the filter paper disks, −: no growth.

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 h 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 stains 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 before examination. We cultured 10 TD-SCVs of *S. aureus* strains and *S. aureus* ATCC 29213 on trypticase soy agar with 5% sheep blood 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 (OD_{600}) 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 OD_{600} 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 trypticase soy agar with 5% sheep blood 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 trypticase soy agar with 5% sheep blood and Mueller–Hinton agar as controls. We also examined with modified media (five MRSA screening media which 200 μL 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 μg of thymidine in each plate, to produce a final thymidine concentration of approximately 10 μg/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).