

Highlights

- Clinical prevalence of PRGBS is a potential public health concern.
- Most commercially-available MRSA-selective agar plates contain ceftiofloxacin.
- The MICs of ceftiofloxacin for 96.6% of PRGBS isolates were $\geq 8 \mu\text{g/mL}$.
- A commercially available MRSA-selective agar detected PRGBS.
- ChromIDTM MRSA showed 72.4% sensitivity and 98.4% specificity for PRGBS.

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Note

Effectual detection of group B streptococci with reduced penicillin susceptibility
(PRGBS) by commercially available methicillin-resistant-*Staphylococcus aureus*
(MRSA)-selective agar

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Running title: Commercial MRSA-selective agar detects PRGBS.

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3 **Abbreviations**
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6 CLSI, Clinical and Laboratory Standards Institute; CTB, ceftibuten; FOX, cefoxitin;
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10 GBS, group B *Streptococcus*; MDR, multi-drug resistant; MIC, minimum inhibitory
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12 concentration; MRSA, methicillin-resistant-*Staphylococcus aureus*; OXA, oxacillin; PBP,
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14 penicillin-binding protein; PCG, penicillin G; PRGBS, group B streptococci with
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16 reduced penicillin susceptibility; PSGBS, penicillin-susceptible group B *Streptococcus*;
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3 **ABSTRACT**
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6 We evaluated the feasibility and efficacy of a commercially available
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9 methicillin-resistant *Staphylococcus aureus* (MRSA)-selective agar, chromID™ MRSA,
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12 to detect group B streptococci with reduced penicillin susceptibility (PRGBS) in this
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15 study. The results showed 72.4% (21/29) sensitivity and 98.4% (60/61) specificity to
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18 detect PRGBS using this method.
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25 **Keywords:** *Streptococcus agalactiae*, Group B *Streptococcus*, Group B streptococci with
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28 reduced penicillin susceptibility, PRGBS, methicillin-resistant *Staphylococcus aureus*
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31 (MRSA)-selective agar plate, commercially available.
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3 **Main text**
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6 Group B *Streptococcus* (GBS; *Streptococcus agalactiae*), the causative agent of
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8 neonatal sepsis and meningitis, is an invasive pathogen that also affects the elderly and
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10 those with underlying medical conditions (Baker, 2000; Okike et al., 2014; Verani et al.,
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12 2010). Clinical isolates of GBS are considered to be uniformly susceptible to β -lactams
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14 including penicillins; therefore, penicillins are used as first-line for the prevention and
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16 treatment of GBS infections (CLSI, 2013; Verani et al., 2010). However, we previously
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18 identified clinical isolates of GBS with reduced penicillin susceptibility (PRGBS) which
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20 harbor amino acid substitutions, especially V405A and/or Q557E, near the conserved
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22 active site motifs in the penicillin-binding protein (PBP) 2X (Kimura et al., 2006, 2008;
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24 Nagano et al., 2008). The minimum inhibitory concentrations (MICs) of penicillin G for
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26 PRGBS (0.25 to 1 $\mu\text{g}/\text{mL}$) (Kimura et al., 2006, 2008; Nagano et al., 2008) are above
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28 the breakpoint of “susceptible” to penicillin G ($\leq 0.12 \mu\text{g}/\text{mL}$), as set by the Clinical and
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30 Laboratory Standards Institute (CLSI) (CLSI, 2013). Moreover, the MICs of oxacillin,
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32 ceftizoxime, and ceftibuten for PRGBS tend to be higher than those for
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34 penicillin-susceptible GBS (PSGBS) (Kimura et al., 2008, 2009). Following our
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36 previous report (Kimura et al., 2006), other groups in Japan and North America have
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38 also reported clinical PRGBS isolates (Dahesh et al., 2008; Gaudreau et al., 2010;
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3 Longtin et al., 2011; Murayama et al., 2009). We previously reported that PRGBS
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6 isolates tend to be non-susceptible to macrolides and fluoroquinolones, in addition to
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9 penicillins (Kimura et al., 2013a), and also reported one case of nosocomial spread of
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12 such multi-drug resistant (MDR)-PRGBS (Nagano et al., 2012). Moreover, we reported
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15 that 14.7% (45/306 GBS isolates) and 10.1% (31/306 GBS isolates) of GBS clinical
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18 isolates obtained in Japan between January 2012 and July 2013 were PRGBS and
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21 MDR-PRGBS, respectively (Seki et al., 2015). In another previous study, we also
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24 detected one clinical isolate of GBS that was highly resistant to ceftizoxime (ZOX)
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27 (MIC \geq 256 μ g/mL) due to amino acid substitutions in PBP1A, in addition to PBP2X
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30 (Kimura et al., 2013c). PRGBS, especially the MDR type, therefore represent a
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33 potential, public health concern.
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38 Though we previously reported that an automated susceptibility testing
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41 machine erroneously classifies half of PRGBS as PSGBS (Kimura et al., 2013b), we
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44 developed 2 practical methods to detect PRGBS: the disk diffusion method (Kimura et
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47 al., 2009) and the locally-prepared PRGBS-selective agar containing 128 μ g/mL
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50 ceftibuten (Kamiya et al., 2015). However, these methods have limitations; namely, the
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53 disk diffusion method requires isolated, single colonies, whereas the PRGBS-selective
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56 agar plate is not commercially available at present. Therefore, we evaluated the
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3 feasibility of commercially available selective agar plates for detection PRGBS. We
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6 previously observed that several PRGBS isolates show growth on
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9 methicillin-resistant-*Staphylococcus aureus* (MRSA)-selective agar plates. One such
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12 commercially available, MRSA-selective agar plate, chromID™ MRSA agar
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15 (SYSMEX bioMérieux Co., Ltd., Tokyo, Japan), seemed the most likely candidate.
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18 Although most commercially available MRSA-selective agar plates contain ceftiofuran
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21 (FOX), the MICs of FOX for PRGBS isolates were unknown. In this study, we used 29
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24 genetically-confirmed PRGBS and 61 PSGBS isolates, including 67 isolates previously
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27 reported (Kimura et al., 2008, 2011, 2015; Nagano et al. 2008; Seki et al., 2015). We
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30 determine the MICs of FOX for these isolates and analyzed their growth on chromID™
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33 MRSA agar to ascertain the feasibility and efficacy of using this commercially available,
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36 MRSA-selective agar plate to detect PRGBS.
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41 The MICs of FOX for the 29 PRGBS and 61 PSGBS isolates were 4–64 and
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44 1–8 µg/mL, respectively, showing that the MICs for PRGBS tended to be higher than
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47 those for PSGBS (Table 1). Since chromID™ MRSA agar contains 4 µg/mL FOX, we
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50 classified the GBS isolates using an MIC threshold of 4 µg/mL FOX (Table S1). The
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53 MICs of FOX for 28 (28/29, 96.6%) PRGBS isolates were ≥ 8 µg/mL, while those for
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56 60 (60/61, 98.4%) PSGBS isolates were ≤ 4 µg/mL. These data indicate that chromID™
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MRSA agar is feasible for detection of PRGBS among GBS isolates.

Table 1. MICs of PCG, OXA, ZOX, CTB, and FOX and growth on the MRSA-selective agar

Category	Clinical isolates	MIC ($\mu\text{g/mL}$)					Growth on the MRSA-selective agar
		PCG	OXA	ZOX	CTB	FOX	
PRGBS	NUBL7912	0.25	1	1	32	16	Positive
	NUBL7916	1	8	32	> 256	16	Negative
	NUBL7917	0.5	8	32	> 256	16	Positive
	NUBL7918	0.5	8	32	> 256	16	Negative
	NUBL7919	0.5	8	32	> 256	32	Positive
	NUBL7920	0.5	8	32	> 256	16	Positive
	NUBL7921	1	16	64	> 256	32	Positive
	NUBL7922	1	16	64	> 256	8	Negative
	NUBL7923	1	8	32	> 256	8	Positive
	NUBL7924	1	8	3	> 256	64	Positive
	B502	0.25	2	32	256	8	Positive
	B503	0.25	1	16	256	4	Negative
	B513	0.25	8	64	256	16	Negative
	B514	0.25	2	32	256	16	Positive
	B516	0.25	2	32	256	16	Negative
	M16	0.25	2	2	64	8	Negative
	M19	0.25	4	16	256	16	Positive
	MRY08-517	0.25	4	16	256	8	Positive
	MRY08-527	0.5	8	32	256	16	Positive
	MRY08-528	0.25	2	128	256	8	Positive
	MRY08-1422	0.25	2	2	256	16	Negative
	R1	0.25	2	16	256	8	Positive
	R2	0.25	2	16	256	16	Positive
	R3	0.25	2	2	256	16	Positive
R4	0.25	2	1	64	8	Positive	
R5	0.25	2	8	256	8	Positive	
R6	0.25	2	64	256	8	Positive	
R7	0.25	4	32	256	64	Positive	
R8	0.5	8	8	256	64	Positive	

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PSGBS	NUBL7892	0.06	0.5	0.12	16	2	Negative
	NUBL7893	0.06	0.5	0.25	16	4	Negative
	NUBL7894	0.06	0.5	0.12	16	4	Negative
	NUBL7895	0.06	0.5	0.25	16	2	Negative
	NUBL7896	0.06	0.5	0.25	16	2	Negative
	NUBL7897	0.06	1	0.25	16	4	Negative
	NUBL7898	0.06	1	0.25	32	4	Negative
	NUBL7899	0.06	0.5	0.25	16	4	Negative
	NUBL7900	0.06	0.5	0.12	8	2	Negative
	NUBL7901	0.03	0.5	0.12	<4	1	Negative
	NUBL7902	0.06	0.5	0.12	16	4	Negative
	NUBL7903	0.06	0.5	0.12	16	4	Negative
	NUBL7904	0.06	0.5	0.12	16	2	Negative
	NUBL7905	0.06	0.5	0.12	16	2	Negative
	NUBL7906	0.06	0.5	0.12	8	2	Negative
	NUBL7907	0.06	0.5	0.12	16	4	Negative
	NUBL7908	0.06	0.5	0.12	16	2	Negative
	NUBL7909	0.06	0.5	0.12	16	2	Negative
	NUBL7910	0.06	0.5	0.25	16	4	Negative
	NUBL7911	0.06	0.5	0.12	16	8	Positive
	NUBL7913	0.06	0.5	0.25	16	4	Negative
	NUBL7914	0.06	0.5	0.25	16	4	Negative
	NUBL7915	0.06	0.5	0.25	16	4	Negative
	MRY09-01	0.03	0.25	0.12	16	2	Negative
	MRY09-02	0.03	0.25	0.12	16	2	Negative
	MRY09-03	0.03	0.25	0.12	16	2	Negative
	MRY09-04	0.03	0.25	0.12	16	2	Negative
	MRY09-05	0.03	0.5	0.25	32	4	Negative
	MRY09-06	0.03	0.25	0.25	16	4	Negative
	MRY09-07	0.03	0.25	0.12	16	4	Negative
	MRY09-08	0.03	0.5	0.25	32	4	Negative
	MRY09-09	0.03	0.5	0.25	16	4	Negative
	MRY09-10	0.03	0.5	0.25	32	2	Negative
	MRY09-11	0.03	0.25	0.12	16	4	Negative
	MRY09-12	0.03	0.25	0.12	8	2	Negative

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3		MRY09-13	0.03	0.5	0.12	16	2	Negative
4		MRY09-14	0.03	1	0.25	32	4	Negative
5		MRY09-15	0.03	0.5	0.12	16	2	Negative
6		MRY09-16	0.03	0.5	0.12	16	4	Negative
7		MRY09-17	0.03	0.25	0.12	16	2	Negative
8		MRY09-18	0.03	0.5	0.12	32	2	Negative
9		MRY09-19	0.03	0.5	0.12	16	2	Negative
10		MRY09-20	0.03	0.25	0.12	16	4	Negative
11		MRY09-21	0.03	0.25	0.25	16	2	Negative
12		MRY09-22	0.06	0.5	0.25	32	4	Negative
13		MRY09-23	0.03	0.5	0.12	32	4	Negative
14		MRY09-24	0.03	0.5	0.12	8	4	Negative
15		MRY09-25	0.06	0.25	0.12	8	2	Negative
16		MRY09-26	0.03	0.25	0.12	8	2	Negative
17		MRY09-27	0.03	0.5	0.12	8	4	Negative
18		MRY09-28	0.03	0.5	0.12	8	4	Negative
19		MRY09-29	0.06	0.25	0.25	8	4	Negative
20		MRY09-30	0.03	0.25	0.12	8	4	Negative
21		MRY09-31	0.06	1	8	256	4	Negative
22		MRY09-32	0.03	0.5	0.12	16	4	Negative
23		MRY09-33	0.03	0.12	0.12	8	4	Negative
24		MRY09-34	0.06	1	16	256	4	Negative
25		MRY09-35	0.03	0.5	0.12	16	4	Negative
26		MRY09-36	0.03	0.5	0.12	16	4	Negative
27		MRY09-37	0.03	0.5	0.12	16	4	Negative
28		MRY09-38	0.03	0.5	0.12	16	4	Negative

Data concerning the MICs of PCG, OXA, ZOX, and CTB for several clinical isolates have been previously reported (Kamiya et al., 2015; Seki et al., 2015).

Abbreviations: MIC, minimum inhibitory concentration; PCG, penicillin G; OXA, oxacillin; ZOX, ceftizoxime; CTB, ceftibuten; FOX, cefoxitin; **MRSA, methicillin-resistant-*Staphylococcus aureus***; PRGBS, group B streptococci with reduced penicillin susceptibility; PSGBS, penicillin susceptible group B streptococci.

We inoculated chromID™ MRSA agar with 3 streaks each, of 29 PRGBS and 61 PSGBS isolates using inoculating loops. Appearance of round, separate colonies on

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3 the third and/or second streak was designated as “positive” growth, whereas less growth
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6 was designated as “negative” (Supplementary text). A total of 21 out of 29 PRGBS
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9 isolates showed “positive” results on the MRSA-selective agar, while 60 of the 61
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12 PSGBS isolates showed “negative” results (Table 1, S2). Sensitivity of chromID™
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15 MRSA agar for detecting PRGBS was 72.4% (21/29), while specificity was 98.4%
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19 (60/61).
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22 Based on the MIC of FOX, use of the MRSA-selective agar plate to detect
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25 PRGBS among GBS seems reasonable. However, although the MICs of FOX for 7
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28 PRGBS isolates were > 4 µg/mL, these were not considered as showing “positive”
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31 result on the MRSA-selective agar plate. Thus, although the specificity of this method
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34 was high (98.4%), the sensitivity was relatively low (72.4%). Nonetheless, due to high
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37 specificity, growth of GBS isolates on the MRSA-selective agar implies that the
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40 colonies are most probably PRGBS. These results suggest that MRSA-selective agar
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43 can be used to detect PRGBS.
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47 At the present, there is no well-evaluated and/or established method to detect
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50 PRGBS in clinical laboratories ; only three publications concerning this topic are
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53 currently available, including this report. Moreover, reports concerning the clinical
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56 significance of PRGBS are also limited. Therefore, considering that MRSA-selective
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3 agar is commercially available and commonly used in clinical practice, this medium
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6 could be useful in the detection of PRGBS, although the sensitivity of this method is
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9 low. Moreover, if the MRSA-selective agar is modified to include chromogenic
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12 substrates of GBS-specific enzymes, the identification of PRGBS isolates could be
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15 simplified via a specific color. Therefore, although further evaluation of this method is
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18 required, the use of the MRSA-selective agar to detect PRGBS is a feasible alternative
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21 in addition to the two previously reported methods. Thus, the findings in this
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24 investigation are precedent to development of new MRSA-selective media to detect
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28 PRGBS.
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66 Kimura K, Nagano N, Nagano Y, Suzuki S, Wachino J, Shibayama K, et al. High
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68 frequency of fluoroquinolone- and macrolide-resistant streptococci among clinically
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