## Highlights

- Clinical prevalence of PRGBS is a potential public health concern.
- Most commercially-available MRSA-selective agar plates contain cefoxitin.
- The MICs of cefoxitin for 96.6% of PRGBS isolates were ≥ 8 μg/mL.
- A commercially available MRSA-selective agar detected PRGBS.
- ChromID$^{TM}$ MRSA showed 72.4% sensitivity and 98.4% specificity for PRGBS.

**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.

**Word counts**: Abstract, 46 words; Main text, 988 words.

**Abbreviations**

CLSI, Clinical and Laboratory Standards Institute; CTB, ceftibuten; FOX, cefoxitin; GBS, group B *Streptococcus*; MDR, multi-drug resistant; MIC, minimum inhibitory concentration; MRSA, methicillin-resistant-*Staphylococcus aureus*; OXA, oxacillin; PBP, penicillin-binding protein; PCG, penicillin G; PRGBS, group B streptococci with reduced penicillin susceptibility; PSGBS, penicillin-susceptible group B *Streptococcus*; ZOX, ceftizoxime

**ABSTRACT**

We evaluated the feasibility and efficacy of a commercially available methicillin-resistant *Staphylococcus aureus* (MRSA)-selective agar, chromID$^{TM}$ MRSA, to detect group B streptococci with reduced penicillin susceptibility (PRGBS) in this study. The results showed 72.4% (21/29) sensitivity and 98.4% (60/61) specificity to detect PRGBS using this method.

**Keywords**: *Streptococcus agalactiae*, Group B *Streptococcus*, Group B streptococci with reduced penicillin susceptibility, PRGBS, methicillin-resistant *Staphylococcus aureus* (MRSA)-selective agar plate, commercially available.

**Main text**

Group B *Streptococcus* (GBS; *Streptococcus agalactiae*), the causative agent of neonatal sepsis and meningitis, is an invasive pathogen that also affects the elderly and those with underlying medical conditions (Baker, 2000; Okike et al., 2014; Verani et al., 2010). Clinical isolates of GBS are considered to be uniformly susceptible to β-lactams including penicillins; therefore, penicillins are used as first-line for the prevention and treatment of GBS infections (CLSI, 2013; Verani et al., 2010). However, we previously identified clinical isolates of GBS with reduced penicillin susceptibility (PRGBS) which harbor amino acid substitutions, especially V405A and/or Q557E, near the conserved active site motifs in the penicillin-binding protein (PBP) 2X (Kimura et al., 2006, 2008; Nagano et al., 2008). The minimum inhibitory concentrations (MICs) of penicillin G for PRGBS (0.25 to 1 μg/mL) (Kimura et al., 2006, 2008; Nagano et al., 2008) are above the breakpoint of "susceptible" to penicillin G (≤ 0.12 μg/mL), as set by the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2013). Moreover, the MICs of oxacillin, ceftizoxime, and ceftibuten for PRGBS tend to be higher than those for penicillin-susceptible GBS (PSGBS) (Kimura et al., 2008, 2009). Following our previous report (Kimura et al., 2006), other groups in Japan and North America have also reported clinical PRGBS isolates (Dahesh et al., 2008; Gaudreau et al., 2010;

Longtin et al., 2011; Murayama et al., 2009). We previously reported that PRGBS isolates tend to be non-susceptible to macrolides and fluoroquinolones, in addition to penicillins (Kimura et al., 2013a), and also reported one case of nosocomial spread of such multi-drug resistant (MDR)-PRGBS (Nagano et al., 2012). Moreover, we reported that 14.7% (45/306 GBS isolates) and 10.1% (31/306 GBS isolates) of GBS clinical isolates obtained in Japan between January 2012 and July 2013 were PRGBS and MDR-PRGBS, respectively (Seki et al., 2015). In another previous study, we also detected one clinical isolate of GBS that was highly resistant to ceftizoxime (ZOX) (MIC $\geq$ 256 μg/mL) due to amino acid substitutions in PBP1A, in addition to PBP2X (Kimura et al., 2013c). PRGBS, especially the MDR type, therefore represent a potential, public health concern.

Though we previously reported that an automated susceptibility testing machine erroneously classifies half of PRGBS as PSGBS (Kimura et al., 2013b), we developed 2 practical methods to detect PRGBS: the disk diffusion method (Kimura et al., 2009) and the locally-prepared PRGBS-selective agar containing 128 μg/mL ceftibuten (Kamiya et al., 2015). However, these methods have limitations; namely, the disk diffusion method requires isolated, single colonies, whereas the PRGBS-selective agar plate is not commercially available at present. Therefore, we evaluated the

feasibility of commercially available selective agar plates for detection PRGBS. We previously observed that several PRGBS isolates show growth on methicillin-resistant-*Staphylococcus aureus* (MRSA)-selective agar plates. One such commercially available, MRSA-selective agar plate, chromID$^{TM}$ MRSA agar (SYSMEX bioMérieux Co., Ltd., Tokyo, Japan), seemed the most likely candidate. Although most commercially available MRSA-selective agar plates contain cefoxitin (FOX), the MICs of FOX for PRGBS isolates were unknown. In this study, we used 29 genetically-confirmed PRGBS and 61 PSGBS isolates, including 67 isolates previously reported (Kimura et al., 2008, 2011, 2015; Nagano et al. 2008; Seki et al., 2015). We determine the MICs of FOX for these isolates and analyzed their growth on chromID$^{TM}$ MRSA agar to ascertain the feasibility and efficacy of using this commercially available, MRSA-selective agar plate to detect PRGBS.

The MICs of FOX for the 29 PRGBS and 61 PSGBS isolates were 4–64 and 1–8 μg/mL, respectively, showing that the MICs for PRGBS tended to be higher than those for PSGBS (Table 1). Since chromID$^{TM}$ MRSA agar contains 4 μg/mL FOX, we classified the GBS isolates using an MIC threshold of 4 μg/mL FOX (Table S1). The MICs of FOX for 28 (28/29, 96.6%) PRGBS isolates were ≥ 8 μg/mL, while those for 60 (60/61, 98.4%) PSGBS isolates were ≤ 4 μg/mL. These data indicate that chromID$^{TM}$

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 (μg/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 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 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 |

| | | | | | | |
|---|---|---|---|---|---|---|
| MRY09-13 | 0.03 | 0.5 | 0.12 | 16 | 2 | Negative |
| MRY09-14 | 0.03 | 1 | 0.25 | 32 | 4 | Negative |
| MRY09-15 | 0.03 | 0.5 | 0.12 | 16 | 2 | Negative |
| MRY09-16 | 0.03 | 0.5 | 0.12 | 16 | 4 | Negative |
| MRY09-17 | 0.03 | 0.25 | 0.12 | 16 | 2 | Negative |
| MRY09-18 | 0.03 | 0.5 | 0.12 | 32 | 2 | Negative |
| MRY09-19 | 0.03 | 0.5 | 0.12 | 16 | 2 | Negative |
| MRY09-20 | 0.03 | 0.25 | 0.12 | 16 | 4 | Negative |
| MRY09-21 | 0.03 | 0.25 | 0.25 | 16 | 2 | Negative |
| MRY09-22 | 0.06 | 0.5 | 0.25 | 32 | 4 | Negative |
| MRY09-23 | 0.03 | 0.5 | 0.12 | 32 | 4 | Negative |
| MRY09-24 | 0.03 | 0.5 | 0.12 | 8 | 4 | Negative |
| MRY09-25 | 0.06 | 0.25 | 0.12 | 8 | 2 | Negative |
| MRY09-26 | 0.03 | 0.25 | 0.12 | 8 | 2 | Negative |
| MRY09-27 | 0.03 | 0.5 | 0.12 | 8 | 4 | Negative |
| MRY09-28 | 0.03 | 0.5 | 0.12 | 8 | 4 | Negative |
| MRY09-29 | 0.06 | 0.25 | 0.25 | 8 | 4 | Negative |
| MRY09-30 | 0.03 | 0.25 | 0.12 | 8 | 4 | Negative |
| MRY09-31 | 0.06 | 1 | 8 | 256 | 4 | Negative |
| MRY09-32 | 0.03 | 0.5 | 0.12 | 16 | 4 | Negative |
| MRY09-33 | 0.03 | 0.12 | 0.12 | 8 | 4 | Negative |
| MRY09-34 | 0.06 | 1 | 16 | 256 | 4 | Negative |
| MRY09-35 | 0.03 | 0.5 | 0.12 | 16 | 4 | Negative |
| MRY09-36 | 0.03 | 0.5 | 0.12 | 16 | 4 | Negative |
| MRY09-37 | 0.03 | 0.5 | 0.12 | 16 | 4 | Negative |
| 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$^{TM}$ MRSA agar with 3 streaks each, of 29 PRGBS and 61 PSGBS isolates using inoculating loops. Appearance of round, separate colonies on

the third and/or second streak was designated as “positive” growth, whereas less growth was designated as “negative” (Supplementary text). A total of 21 out of 29 PRGBS isolates showed “positive” results on the MRSA-selective agar, while 60 of the 61 PSGBS isolates showed “negative” results (Table 1, S2). Sensitivity of chromID$^{TM}$ MRSA agar for detecting PRGBS was 72.4% (21/29), while specificity was 98.4% (60/61).

Based on the MIC of FOX, use of the MRSA-selective agar plate to detect PRGBS among GBS seems reasonable. However, although the MICs of FOX for 7 PRGBS isolates were > 4 μg/mL, these were not considered as showing “positive” result on the MRSA-selective agar plate. Thus, although the specificity of this method was high (98.4%), the sensitivity was relatively low (72.4%). Nonetheless, due to high specificity, growth of GBS isolates on the MRSA-selective agar implies that the colonies are most probably PRGBS. These results suggest that MRSA-selective agar can be used to detect PRGBS.

At the present, there is no well-evaluated and/or established method to detect PRGBS in clinical laboratories ; only three publications concerning this topic are currently available, including this report. Moreover, reports concerning the clinical significance of PRGBS are also limited. Therefore, considering that MRSA-selective

agar is commercially available and commonly used in clinical practice, this medium could be useful in the detection of PRGBS, although the sensitivity of this method is low. Moreover, if the MRSA-selective agar is modified to include chromogenic substrates of GBS-specific enzymes, the identification of PRGBS isolates could be simplified via a specific color. Therefore, although further evaluation of this method is required, the use of the MRSA-selective agar to detect PRGBS is a feasible alternative in addition to the two previously reported methods. Thus, the findings in this investigation are precedent to development of new MRSA-selective media to detect PRGBS.

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