Highlights (85 characters, 3 to 5 bullet points)

- PRGBS have a multidrug-resistance tendency and tend to be misclassified as PSGBS.
- We improved the disk diffusion method for detecting PRGBS.
- Using 73 PRGBS and 81 PSGBS, we determined more rational cutoff values.
- Oxacillin, ceftizoxime, and ceftibuten disks showed high sensitivity and specificity.
- This method can be performed without other special and/or expensive equipment.

Improved disk diffusion method for simple detection of group B streptococci with reduced penicillin susceptibility (PRGBS)

Rikuko Goto[a], Wanchun Jin[a, 1], Jun-ichi Wachino[a, 2], Yoshichika Arakawa[a, 2], Kouji Kimura[a*]

[a]Department of Bacteriology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan

[1]Present address: College of Pharmacy, Kinjo Gakuin University, 2-1723 Omori, Moriyama-ku, Nagoya, Aichi 463-8521, Japan

[2]Present address: Laboratory of Microbiology, Department of Medical Technology, Faculty of Medical Sciences, Shubun University, 6 Nikkou-cho, Ichinomiya, Aichi 491-0938, Japan.

Running title: Improved PRGBS detection method

Abstract: 50 words, Main text: 991 words.

[*]Address correspondence to Kouji Kimura, koujikim@med.nagoya-u.ac.jp, Department of Bacteriology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan, TEL: +81-52-744-2106, FAX: +81-52-744-2107.

ABSTRACT (50 words)

We used 73 group B *Streptococcus* with reduced penicillin susceptibility (PRGBS) isolates and determined more rational cutoff values of previously developed disk diffusion method for detecting PRGBS using oxacillin, ceftizoxime, and ceftibuten disks. Using the novel cutoff values, the three disks showed high sensitivity and specificity, which were above 90.0%.

KEYWORDS *Streptococcus agalactiae*, reduced penicillin susceptibility, detection method, disk diffusion method, multidrug resistance

Streptococcus agalactiae (Group B *Streptococcus*, GBS) is the leading cause of neonatal sepsis and bacterial meningitis, and is a causative pathogen of invasive diseases in the elderly and those suffering from underlying medical conditions, such as diabetes [1–5]. β-Lactams are the first-line agents for the intrapartum antibiotic prophylaxis and treatment of GBS infections; all clinical isolates of GBS were previously considered to be uniformly susceptible to β-lactams [5]. However, we reported clinical isolates of GBS with reduced penicillin susceptibility (PRGBS) harboring amino acid substitutions, including V405A and/or Q557E, in penicillin-binding protein (PBP) 2X [6, 7]. After our first report in 2008 (6), clinical isolates of PRGBS that were confirmed to harbor amino acid substitutions in PBPs were also reported from other groups in Japan [8], USA [9, 10], Canada [11, 12], Germany [13], Mozambique [14], China [15], Hong Kong [16], and South Korea [17]. Besides reduced penicillin susceptibility, PRGBS clinical isolates tend to be non-susceptible/resistant to fluoroquinolones and macrolides, and exhibit multidrug-resistance tendency [18]. Therefore, drug choice against infections caused by such multidrug-resistant PRGBS is narrower than that caused by penicillin-susceptible GBS (PSGBS), although clinical outcomes of high dose penicillins administration against PRGBS infections are unknown. Consequently, it is necessary to detect such multidrug-resistant PRGBS accurately and rapidly to support better

47 drug choice against the infections.

48 The MICs of penicillin G for PRGBS (0.25-2 μg/ml) are near the “susceptible” breakpoints
49 (≤0.12 μg/ml) set by the Clinical and Laboratory Standards Institute (CLSI) [19], therefore,
50 misclassifications of PRGBS to PSGBS tend to happen [20]. To overcome this, we had
51 previously developed a simple disk diffusion method for detecting PRGBS using oxacillin,
52 ceftizoxime, and ceftibuten disks [21]. However, at that time, it was not long since we first
53 reported PRGBS, the number of PRGBS isolates used in that study was limited (16 strains of
54 PRGBS and 34 strains of PSGBS), and the cutoff values between PRGBS and PSGBS were
55 proposed tentatively. In this study, the number of PRGBS isolates we collected is larger than
56 the number of PRGBS isolates we possessed at that time. It was reported that the MICs of
57 three antibiotics, cefoxitin [22], cefaclor [23], and cefotiam [8], for GBS with reduced
58 β-lactam susceptibility (GBS-RBS) including PRGBS tended to be elevated; therefore, we
59 examined whether or not the three antibiotics disks: cefoxitin, cefaclor, and cefotiam, are
60 useful for detecting PRGBS in addition to oxacillin, ceftizoxime, and ceftibuten disks, and
61 tried to determine more rational cutoff values for each disk using a larger number of PRGBS
62 isolates.

63 We defined strains with the MICs of penicillin G ≥ 0.25 μg/ml as PRGBS and strains with

the MICs of penicillin G ≤ 0.12 μg/ml as PSGBS, as determined by the agar dilution method that was set by the CLSI [19]. In accordance with this definition, we used 73 PRGBS and 81 PSGBS isolates. All 73 PRGBS isolates harbored amino acid substitutions in the PBPs.

Disk diffusion methods standardized by the CLSI were performed using 10U penicillin G, 1 μg oxacillin, 30 μg ceftizoxime, 30 μg ceftibuten, 30 μg cefoxitin, 30 μg cefaclor, and 30 μg cefotiam disks (Eiken Chemical Co. Ltd., Tokyo, Japan). We compared the MICs of penicillin G determined by the agar dilution method standardized by the CLSI and the diameters of the growth inhibitory zones around each disk of the disk diffusion method. We performed receiver operating characteristic (ROC) curve analysis and determined the cutoff value for each disk using the statistical analysis software Easy R (EZR; https://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmedEN.html [accessed 5 August 2022]). We calculated the sensitivity and specificity for the determined cutoff values.

Comparison between the diameters of the growth inhibitory zones around each disk identified from the disk diffusion method and the MICs of penicillin G determined by the agar dilution method standardized by the CLSI are shown in Fig. 1. The selected cutoff values, sensitivity, and specificity of each disk diffusion method are listed in Table 1 (also see Fig. S1 and S2). The cutoff values of penicillin G, oxacillin, ceftizoxime, ceftibuten, cefoxitin, cefaclor, and

cefotiam, were 31 mm, 18 mm (previous tentative cutoff: 16 mm), 29 mm (previous tentative cutoff: 28 mm), 19 mm (previous tentative cutoff: 19 mm), 24 mm, 29 mm, and 27 mm, respectively. Among the seven disks investigated in this study, penicillin G, cefoxitin, cefaclor, and cefotiam disks exhibited relatively lower sensitivity and specificity (82.2% and 86.4%, 78.1% and 87.7%, 64.4% and 87.7%, and 84.9% and 67.9%, respectively); therefore, these disks are not very useful for detecting PRGBS using disk diffusion method. However, oxacillin, ceftizoxime, and ceftibuten disks exhibited higher sensitivity and specificity (93.2% and 98.8%, 90.4% and 95.1%, and 94.5% and 95.1%, respectively); consequently, the disk diffusion method using these disks are very useful for detecting PRGBS. Moreover, in the case of that we judge as PRGBS when the diameters around oxacillin or ceftibuten disks are below or equal to cutoff values, the sensitivity and specificity were 97.3% and 95.1%, respectively.

The fact that all clinical isolates used in this study are recovered from only Japan is one of the limitations of this study.

Although most clinical isolates of PRGBS were recovered from respiratory specimens of adults, especially the elderly, PRGBS clinical isolates from neonatal invasive GBS diseases which were confirmed to harbor amino acid substitutions in PBP2X have been already

98 reported from Mozambique [14]. Therefore, active screening for PRGBS among clinical
99 isolates, including clinical isolates recovered from neonatal invasive GBS infections, using
100 this method for detecting PRGBS, is required to support the determination of better drug
101 choice against GBS infections and to facilitate the accumulation of clinical information as
102 well as promoting researches concerning PRGBS through efficient detection of PRGBS.

103 In conclusion, using the novel cutoff values, oxacillin, ceftizoxime, and ceftibuten disks
104 showed high sensitivity and specificity. This improvement to our previous disk diffusion
105 method for detecting PRGBS could serve as a promising method for detecting PRGBS at
106 bacterial laboratories in medical institutes worldwide, without need of other special and/or
107 expensive equipment.
108

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Competing interests statement

The authors declare no competing interests.

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Author contributions statement

Rikuko Goto: Data curation, Formal analysis, Investigation, Validation, Writing – original draft, Writing – review & editing

Wanchun Jin: Data curation, Formal analysis, Investigation, Validation, Writing – review & editing

Jun-ichi Wachino: Data curation, Formal analysis, Investigation, Validation, Writing – review & editing

Yoshichika Arakawa: Conceptualization, Funding acquisition, Supervision, Writing – review & editing

Kouji Kimura: Conceptualization, Data curation, Formal analysis, Investigation, Validation, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing

221

222

223 **Figure Legends**

224 **Fig. 1 Comparison between MICs of penicillin G determined by the agar dilution**
225 **methods and the diameters of growth inhibitory zones around each disk**

226 The vertical axis is MICs of penicillin G determined by the agar dilution methods [μg/ml].
227 The horizontal axis is diameters [mm] of growth inhibitory zones around penicillin G disk
228 (A), oxacillin disk (B), ceftizoxime disk (C), ceftibuten disk (D), cefoxitin disk (E), cefaclor
229 disk (F), and cefotiam disk (G). Numbers in the intersection are the numbers of isolates. We
230 used 73 PRGBS (MICs of penicillin G ≥0.25 μg/ml) and 81 PSGBS (MICs of penicillin G
231 ≤0.12 μg/ml). Penicillin G MICs ≤0.12 μg/ml is the "susceptible" breakpoint set by the CLSI.
232 The horizontal line shows this breakpoint. The vertical line shows the cutoff values
233 determined by the receiver operating characteristic (ROC) curve analysis.

234

235 **Fig. S1 Receiver operating characteristic (ROC) curve analysis**

236 ROC curve concerning comparison between MICs of penicillin G determined by the agar
237 dilution methods and the diameters of growth inhibitory zones around each disk in Figure 1.
238 Each block among (A)-(D) is the disk diffusion method using penicillin G disk (A), oxacillin
239 disk (B), ceftizoxime disk (C), and ceftibuten disk (D). The vertical axis is the sensitivity and

240 the horizontal axis is the specificity. The three numbers in each blocks show cutoff values
241 (specificity, sensitivity).

242 **Fig. S2 Receiver operating characteristic (ROC) curve analysis**

243 ROC curve concerning comparison between MICs of penicillin G determined by the agar
244 dilution method and the diameters of growth inhibitory zones around each disk in Figure 1.
245 Each block among (A)-(C) is the disk diffusion method using cefoxitin disk (A), cefaclor disk
246 (B), and cefotiam disk (C). The vertical axis is the sensitivity and the horizontal axis is the
247 specificity. The three numbers in each blocks show the cutoff value (specificity, sensitivity).
248

249 **Table 1. Cutoff values, sensitivity, and specificity of the seven disks identified from the**
250 **disk diffusion method**
251

Disk	Cutoff [Previous tentative cutoff] (mm)	Sensitivity (%)	Specificity (%)
Penicillin G	31	82.2	86.4
Oxacillin	18 [16]	93.2	98.8
Ceftizoxime	29 [28]	90.4	95.1
Ceftibuten	19 [19]	94.5	95.1
Cefoxitin	24	78.1	87.7
Cefaclor	29	64.4	87.7
Cefotiam	27	84.9	67.9
Combination (Oxacillin or Ceftibuten)	-	97.3	95.1

252

253

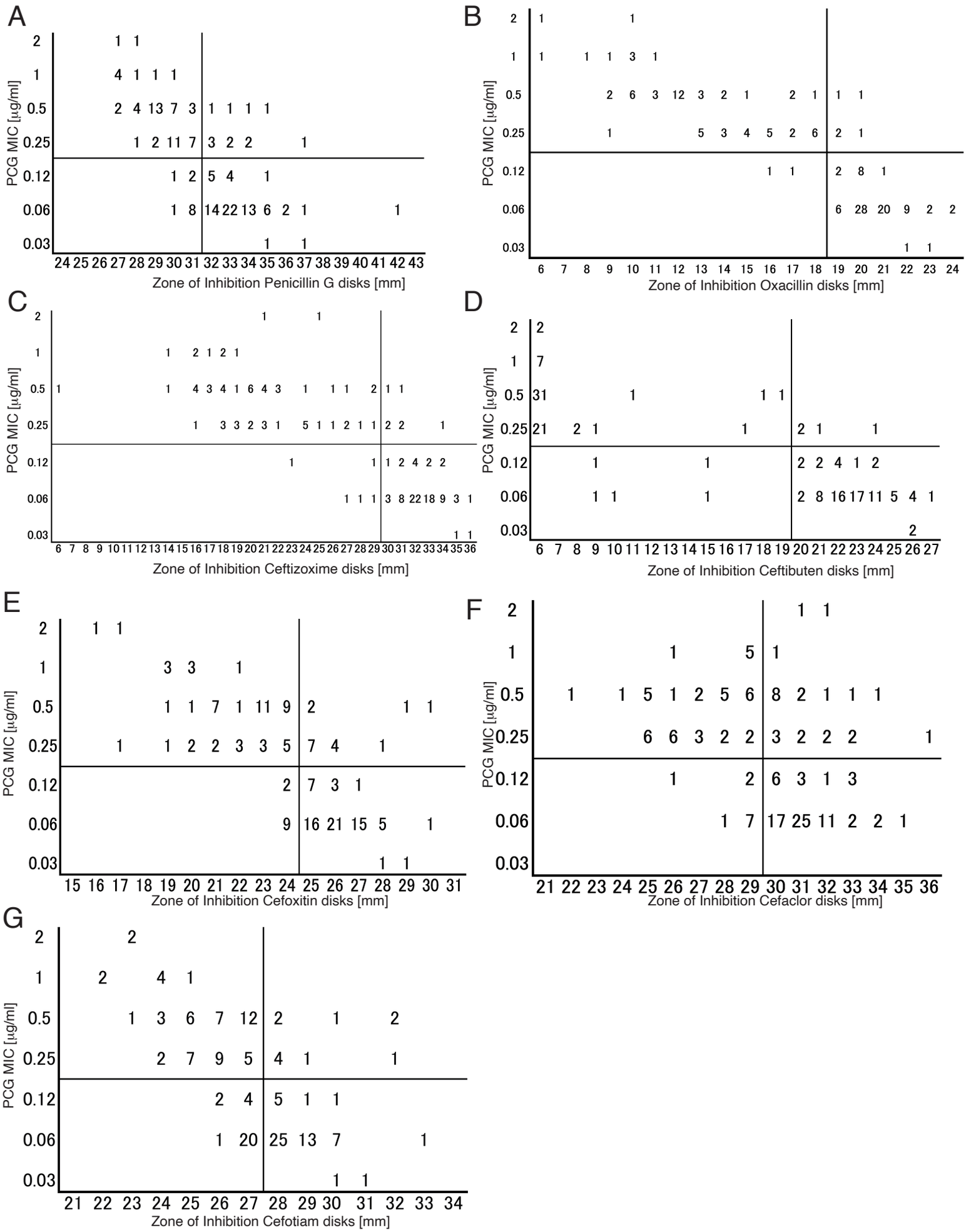

A
PCG MIC [μg/ml]
Zone of Inhibition Penicillin G disks [mm]
B
PCG MIC [μg/ml]
Zone of Inhibition Oxacillin disks [mm]
C
PCG MIC [μg/ml]
Zone of Inhibition Ceftizoxime disks [mm]
D
PCG MIC [μg/ml]
Zone of Inhibition Ceftibuten disks [mm]
E
PCG MIC [μg/ml]
Zone of Inhibition Cefoxitin disks [mm]
F
PCG MIC [μg/ml]
Zone of Inhibition Cefaclor disks [mm]
G
PCG MIC [μg/ml]
Zone of Inhibition Cefotiam disks [mm]

Figure 1

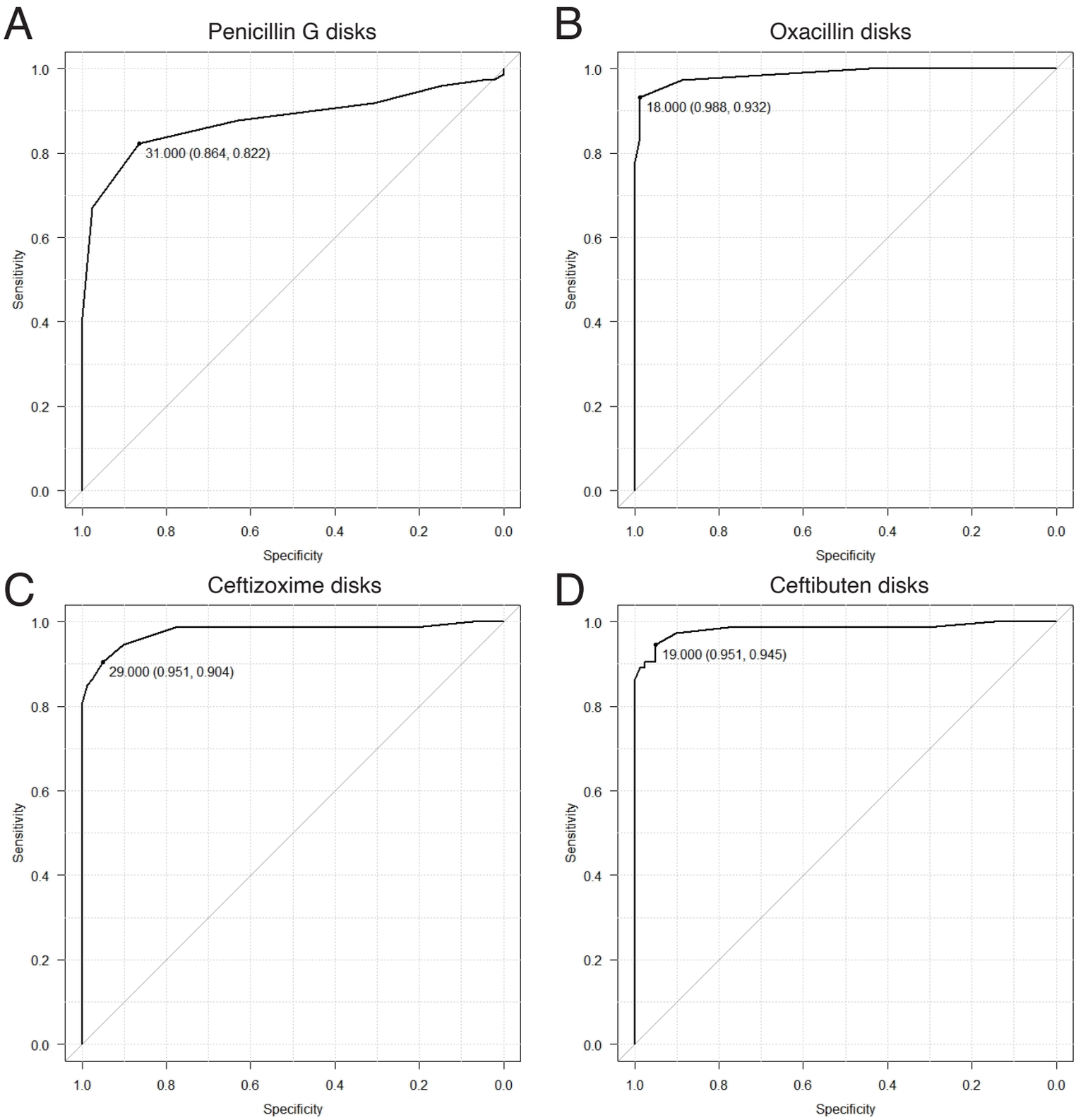
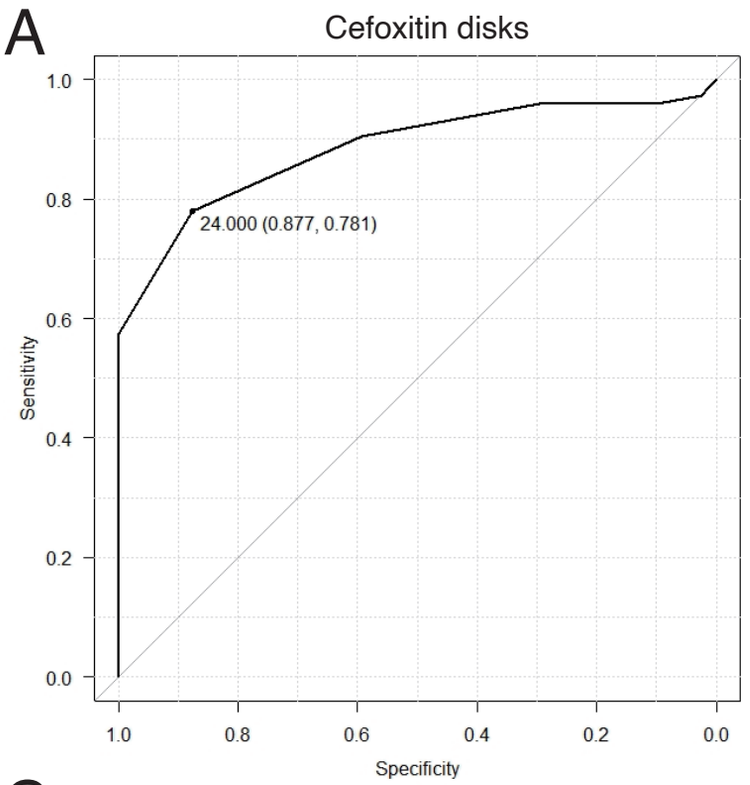
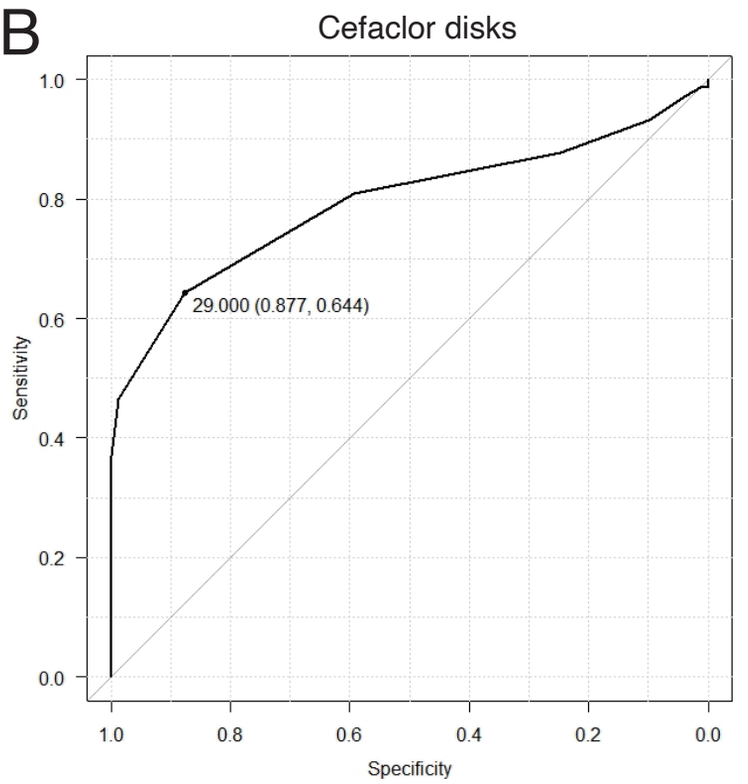

A
Penicillin G disks
31.000 (0.864, 0.822)
Sensitivity
Specificity
B
Oxacillin disks
18.000 (0.988, 0.932)
Sensitivity
Specificity
C
Ceftizoxime disks
29.000 (0.951, 0.904)
Sensitivity
Specificity
D
Ceftibuten disks
19.000 (0.951, 0.945)
Sensitivity
Specificity

Figure S1


A
Cefoxitin disks
24.000 (0.877, 0.781)
Sensitivity
Specificity
1.0
0.8
0.6
0.4
0.2
0.0


B
Cefaclor disks
29.000 (0.877, 0.644)
Sensitivity
Specificity
1.0
0.8
0.6
0.4
0.2
0.0

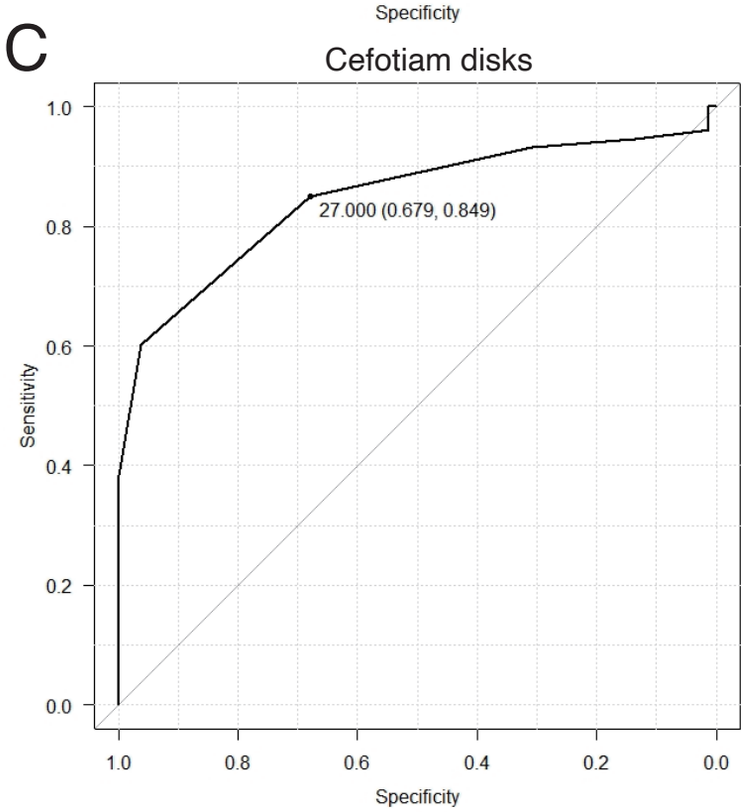

C
Cefotiam disks
27.000 (0.679, 0.849)
Sensitivity
Specificity
1.0
0.8
0.6
0.4
0.2
0.0

Figure S2