Practical Disk Diffusion Test for Detecting Group B Streptococcus with Reduced Penicillin Susceptibility[▽]

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Although group B streptococcus (GBS) has been considered to be uniformly susceptible to β -lactams, the presence of GBS with reduced penicillin susceptibility (PRGBS) was recently confirmed genetically. We developed a feasible and reliable method for screening PRGBS in clinical microbiology laboratories using a combination of ceftibuten, oxacillin, and ceftizoxime disks.

Streptococcus agalactiae (group B streptococcus [GBS]) is a leading cause of neonatal sepsis and meningitis and is also an important pathogen for elderly people and those suffering from underlying medical disorders (1, 5, 7, 11). GBS results in the highest mortality and morbidity if it causes invasive infections in neonates, including very-low-birth-weight infants (6, 10, 12). About 5% of GBS-infected infants die, and if they survive, they often suffer from severe neurological sequelae, such as mental retardation and vision and/or auditory disabilities (2), but development of GBS vaccines is still under investigation (8). Penicillins are the first-line agents in the treatment of GBS infections because all clinical GBS isolates have been considered to be uniformly susceptible to β -lactams, including penicillins (2, 3). However, we have recently identified and molecularly characterized several clinical GBS isolates demonstrating reduced penicillin susceptibility (PRGBS) through acquisition of multiple mutations in the penicillin-binding protein 2X (*pbp2x*) gene (9), and similar PRGBS isolates were recently reported in the United States (4). PRGBS isolates were indeed confirmed to be nonsusceptible to penicillin G (PCG) by the agar dilution method, but this PCG nonsusceptibility was not apparent even if the PCG disk diffusion method was performed in accordance with the recommendations of the CLSI (Clinical and Laboratory Standards Institute) (3). Here we developed, therefore, a feasible and practical new disk test method for discriminating PRGBS from the clinically isolated GBS using three disks containing ceftibuten, oxacillin, and ceftizoxime, respectively.

Forty-eight clinical isolates were identified as GBS using a streptococcus grouping kit (Slidex Strepto [bioMérieux, Marcy l'Etoile, France] and Streptex [Mitsubishi Chemical Medience Corporation, Tokyo, Japan]). *Streptococcus agalactiae* ATCC BAA-611 and ATCC 12403 were used as the reference strains in bacteriological identification. The MICs of PCG for two ATCC standard strains and clinical isolates were determined by the agar dilution method recommended by the CLSI. *Strep-*

tococcus pneumoniae 49619 was used for validation of the MIC measurements. The disk diffusion method was performed as recommended by the CLSI in evaluation of the applicability of each β -lactam disk for discrimination of PRGBS strains from penicillin-susceptible strains.

At first, the MICs of PCG for 2 ATCC strains and 48 clinical GBS isolates were measured by the CLSI standard agar dilution methods, and 34 strains, including 2 reference strains, proved susceptible to PCG (MIC, ≤ 0.12 μ g/ml), whereas the remaining 16 clinical isolates were nonsusceptible to PCG (MIC, >0.12 μ g/ml) (Fig. 1A and Table 1). The highest PCG MIC for such PCG-nonsusceptible GBS strains was 1 μ g/ml.

In the next step, the nucleotide sequences of the PBP2X genes of all GBS strains used in this investigation were determined, and all 34 penicillin-susceptible GBS (PSGBS) strains were found to harbor neither V405A nor Q557E substitutions in PBP2X, which are conserved among PBP2Xs of almost all PRGBS strains. Among the 16 PRGBS isolates, 15 isolates harbored the PRGBS-specific mutations in PBP2X genes that cause V405A and/or Q557E substitutions in PBP2X, but no such substitution was found in one PRGBS strain, B7, which harbored multiple substitutions in PBP2X other than V405A and Q557E (Table 1), as reported previously (9).

To validate the PCG disk for screening PRGBS, we applied the standard disk diffusion method for GBS with PCG disks in accordance with the CLSI recommendation for streptococci other than pneumococci. For each isolate, the PCG MIC determined by the agar dilution method and the diameter of the growth-inhibitory zone measured by the standard disk diffusion method were plotted on a scatter diagram (Fig. 1A). In all isolates tested, the growth-inhibitory zones around the PCG disk were >24 mm (CLSI susceptibility criteria). Thus, it seemed very difficult to exactly discriminate all of the PRGBS from PSGBS by the CLSI standard disk diffusion method using only a PCG disk.

We previously reported that PRGBS showed reduced susceptibility not only to PCG but also to oxacillin and ceftizoxime (9). Thus, we examined the applicability of 44 β -lactam disks commercially available in Japan for detection of PRGBS (Table 2) and found that the ceftibuten disk was also applicable for screening PRGBS.

To evaluate the sensitivity and specificity of the disk diffu-

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FIG. 1. Scatter diagram of MICs and the sizes of the growth-inhibitory zone. PCG MICs determined by the CLSI agar dilution method and diameters of the growth-inhibitory zone measured by CLSI-recommended standard disk diffusion method are plotted using a PCG disk (A). MICs determined by the agar dilution method and the diameters of growth inhibitory zone measured by the CLSI-recommended standard disk diffusion method are plotted using an oxacillin disk (B), ceftizoxime disk (C), and ceftibuten disk (D). The numbers in the scatter diagram indicate the number of clinical isolates in each intersection.

sion method, we performed the standard disk diffusion method of CLSI using oxacillin, ceftizoxime, and ceftibuten disks (Fig. 1B, C, and D). Because the CLSI has not determined the cutoff values for susceptible criteria in these three disks, we set the

provisional cutoff values for “reduced susceptible” criteria in the three disks using the smallest-diameter values of susceptible strains: e.g., oxacillin, <17 mm; ceftizoxime, <29 mm; and ceftibuten, <20 mm. Under this condition, the sensitivities of

TABLE 1. MICs of PCG for 16 strains of PRGBS, amino acid substitutions in PBP2X of PRGBS, and the diameters of growth-inhibitory zones around oxacillin, ceftizoxime, and ceftibuten disks

Strain	MIC (µg/ml) of PCG	Amino acid substitutions in PBP2X	Diam (mm) of growth-inhibitory zone with:		
			Oxacillin	Ceftizoxime	Ceftibuten
B1	0.5	M349I, I377V, F399I, P445S, T555S, Q557E	13	7	6
B6	0.25	E411K, T555S, Q557E	12	15	6
B7	0.25	I377V, T394A, G398A	16	22	6
B8	0.25	I377V, F395L, P396T, V405A, R433H, H438Y, G648A	16	17	6
B10	0.5	G526R, Q557E, S726L	15	16	7
B12	0.25	G526R, Q557E, S726L	14	19	6
B40	0.5	P396S, G526R, Q557E, S726L	11	20	6
B60	0.25	I377V, T394A, G398A, Q557E	16	22	6
B68	0.5	A514V, Q557E	18	28	10
B502	0.5	I377V, F395L, V405A, R433H, H438Y, G648A	12	18	6
B503	0.25	I377V, F395L, V405A, R433H, H438Y, G648A	20	24	6
B513	1	A400V, V405A, Q557E	7	17	6
B514	0.25	I377V, F395L, V405A, R433H, H438Y, G648A	12	19	6
B516	0.25	I377V, F395L, V405A, R433H, H438Y, G648A	17	18	6
M16	0.5	A514V, Q557E	12	31	19
M19	0.25	I377V, F395L, V405A, R433H, H438Y, G648A	12	20	6

TABLE 2. Antibiotic concentrations of 44 Kirby-Bauer disks used in the applicability check for detecting PRGBS

Antibiotic	Concn
PCG.....	10 U
Oxacillin.....	1 µg
Ampicillin.....	10 µg
Amoxicillin.....	25 µg
Aspoxicillin.....	100 µg
Piperacillin.....	100 µg
Cephalothin.....	30 µg
Cefazolin.....	30 µg
Cefamandole.....	30 µg
Cefotiam.....	30 µg
Cefoperazone.....	75 µg
Cefuroxime.....	30 µg
Cefotaxime.....	30 µg
Ceftizoxime.....	30 µg
Cefmenoxime.....	30 µg
Cefpiramide.....	75 µg
Ceftazidime.....	30 µg
Ceftriaxone.....	30 µg
Cefodizime.....	30 µg
Cefpirome.....	30 µg
Cefepime.....	30 µg
Cefozopran.....	30 µg
Cefsulodin.....	30 µg
Cefoxitin.....	30 µg
Cefmetazole.....	30 µg
Cefotetan.....	30 µg
Cefbuperazone.....	75 µg
Cefminox.....	30 µg
Cephalexin.....	30 µg
Cefaclor.....	30 µg
Cefixime.....	5 µg
Ceftibuten.....	30 µg
Cefdinir.....	5 µg
Cefpodoxime.....	10 µg
Cefteram.....	10 µg
Cefcapene.....	5 µg
Cefditoren.....	5 µg
Moxalactam.....	30 µg
Flomoxef.....	30 µg
Imipenem.....	10 µg
Panipenem.....	10 µg
Meropenem.....	10 µg
Aztreonam.....	30 µg
Carumonam.....	30 µg

these disks were 13/16 (81%), 15/16 (94%), and 16/16 (100%), respectively, and the respective specificities were 34/37 (92%), 34/35 (97%), and 34/34 (100%). Thus, the sensitivity and specificity of the CLSI standard disk method using oxacillin, ceftizoxime, and ceftibuten disks were confirmed to be fully applicable for discrimination of PRGBS from the clinical GBS isolates. Moreover, all of the clinical PRGBS isolates were detected by at least one of the three disks. Therefore, the combination of the three disks could be expected to successfully distinguish all of the PRGBS from PSGBS (Table 3) in the routine work of clinical microbiology laboratories.

Indeed, one PRGBS isolate showed a 19-mm growth-inhibitory zone around the ceftibuten disk (Fig. 1D and Table 1), and this strain might well be misclassified as PSGBS. However, the results from the other disk containing oxacillin would complement exact detection of PRGBS. The PRGBS detection method developed in this investigation using ceftibuten, as well

TABLE 3. Specificity and sensitivity of each β -lactam disk used in the disk diffusion methods

Disk	No. of isolates/total (%) ^a	
	Specificity	Sensitivity
Penicillin	34/50 (68)	0/16 (0)
Oxacillin	34/37 (92)	13/16 (81)
Ceftizoxime	34/35 (97)	15/16 (94)
Ceftibuten	34/34 (100)	16/16 (100)
Combination	34/34 (100)	16/16 (100)

^a Denominators of specificity values are the numbers of isolates with a growth-inhibitory zone diameter around each disk above the tentative cutoff value. Denominators of sensitivity values are the numbers of PRGBS isolates determined by PCG MICs.

as oxacillin and ceftizoxime disks, would promise high specificity and sensitivity without necessitating any expensive or special equipment. This method, therefore, could come into wide use for detection of PRGBS in the daily antimicrobial susceptibility testing of GBS after its validation by multiple reference laboratories. Due to the lack of exact detection test methods for PRGBS, no confirmed case of penicillin treatment failure has been reported in GBS infections to date, and no clinical significance of PRGBS has so far been evaluated. Indeed, the PRGBS isolates were identified by measurement of MICs using the agar dilution method without regard for penicillin treatment failure. Moreover, it seems very difficult without case-controlled analyses to conclude whether the antibiotic treatment failure in the GBS infection cases is mainly due to bacterial penicillin nonsusceptibility or depends on a deteriorated ability to defend against microbial infections of the host. The new test method reported here offers a promising, easy, and reliable way to detect PRGBS and thus promote case analyses of infections with PRGBS in the future.

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