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High frequency of fluoroquinolone- and macrolide-resistant streptococci among clinically isolated group B streptococci with reduced penicillin susceptibility

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Objectives: Recently several clinical isolates of *Streptococcus agalactiae* [also known as group B *Streptococcus* (GBS)] that have acquired reduced penicillin susceptibility (PRGBS) by amino acid substitutions in the penicillin-binding protein 2X have emerged. The frequency of fluoroquinolone (FQ)- and macrolide-resistant streptococci among PRGBS is not yet known.

Methods: Fifty-seven GBS [19 PRGBS and 38 penicillin-susceptible GBS (PSGBS)], isolated from different medical institutions in Japan, were studied. For GBS, the MICs of penicillin G, levofloxacin and erythromycin were determined using the agar dilution method. Nineteen PRGBS were previously confirmed as genetically diverse streptococci by PFGE. Further, the mechanisms underlying penicillin, FQ and macrolide non-susceptibility/resistance were analysed.

Results: The frequency of non-susceptibility to FQs among PSGBS was 18.4% (7/38), whereas that among PRGBS was 100% (19/19). The frequency of resistance to erythromycin among PSGBS was 7.9% (3/38), while that among PRGBS was 47.4% (9/19). Statistical significance was determined using Fisher's exact test between reduced penicillin susceptibility and FQ non-susceptibility ($P \leq 0.0001$) and macrolide resistance ($P = 0.0012$). The resistance/non-susceptibility mechanisms among PRGBS were diverse, suggesting that the PRGBS examined were not clonal.

Conclusions: PRGBS isolates tend to show resistance to FQs and/or macrolides. Because the drug choice for treating these multidrug-resistant GBS is more limited than that for usual GBS, these strains may present future public health challenges.

Keywords: GBS, PRGBS, levofloxacin, erythromycin

Introduction

Streptococcus agalactiae [group B *Streptococcus* (GBS)] is the primary cause of neonatal (from birth to 4 weeks of age) invasive infections such as sepsis and meningitis and is an important pathogen in elderly people and those with underlying medical disorders.[1–6] Because GBS is consistently susceptible to β-lactams, including penicillins and cephems,[1,6] the β-lactam resistance breakpoints have not yet been set by the CLSI.[7] β-Lactams are prescribed as first-line drugs, without susceptibility testing, to treat GBS infections. Moreover, the US CDC has recommended β-lactams to prevent neonatal invasive infections in pregnant women who harbour GBS in their gestational and/or enteric tracts.[6] However, several GBS clinical isolates with reduced penicillin susceptibility (PRGBS) have been identified by molecular methods in both Japan[8–12] and North America.[13–15] The MICs of penicillin, oxacillin and ceftizoxime for PRGBS isolates are usually above 'susceptible' levels for the *Streptococcus* spp. of the β-haemolytic group.[7]

EUCAST (http://www.eucast.org/clinical_breakpoints/) has defined a clinical penicillin MIC breakpoint for *Streptococcus* groups A, B, C and G, together with the penicillin MIC resistance breakpoint (>0.25 mg/L).[16] Both EUCAST and CLSI state that penicillin-non-susceptible isolates are extremely rare in GBS.

High MICs of fluoroquinolones (FQs) for the PRGBS isolates were usually found in our preliminary investigations. After our first report on PRGBS,[8] almost all newly identified PRGBS clinical isolates in Japan were found to have FQ resistance. However, the frequency of FQ-non-susceptible and macrolide-resistant streptococci among PRGBS has not yet been determined.

We conducted this study to ascertain the frequency of FQ-non-susceptible and macrolide-resistant streptococci among PRGBS isolated in Japan using 57 GBS, including 19 PRGBS and 38 penicillin-susceptible GBS (PSGBS).

Materials and methods

Bacterial isolates

Fifty-seven GBS (19 PRGBS and 38 PSGBS) were isolated from blood or respiratory specimens in Japan between 2001 and 2008. PRGBS were confirmed by penicillin-binding protein 2X (PBP2X) gene sequencing analysis and disc diffusion methods using ceftibuten discs.[17] Among the well-characterized PRGBS, we excluded PRGBS isolated between 1995 and 1998 because we could not obtain PSGBS between 1995 and 1998 retrospectively. We obtained PSGBS between 2001 and 2008 and analysed 19 PRGBS and 38 PSGBS during this period. For statistical analysis, the number of PSGBS analysed was twice that of PRGBS.

MIC determinations

The MICs of penicillin G, levofloxacin (FQ) and erythromycin (macrolide) for 57 GBS were determined using the agar dilution method recommended by the CLSI[7] using *Streptococcus pneumoniae* ATCC 49619 as the quality-control strain.

Molecular biological methods

To analyse the sequences of PBP2X genes and quinolone resistance-determining regions (QRDRs) of *gyrA*, *gyrB*, *parC* and *parE* genes, we amplified each full-length and/or partial gene using the total DNA isolated from GBS as templates and PrimeSTAR HS DNA Polymerase (Takara Bio Inc., Otsu, Shiga, Japan). The primers used for PCR amplification and sequencing have been described previously.[8,12]

To identify the macrolide-resistance genes *erm*(TR), *erm*(B) and *mef*(A/E), we performed PCR amplification of these partial genes using primers described previously.[18,19]

Statistical analysis

Fisher's exact test was performed using GraphPad Prism 4 software (GraphPad Software, La Jolla, CA, USA). Significance was set at $P<0.05$.

Results

The MICs for the 57 GBS are presented in Table S1 (available as Supplementary data at *JAC* Online). The MICs of penicillin G for all 19 PRGBS were above the susceptible limit defined by the CLSI (>0.12 mg/L); however, the MICs of penicillin G for 38 PSGBS were below the susceptible limit. Figure 1 shows the relationship of the MICs of penicillin G with those of levofloxacin (Figure 1a) and erythromycin (Figure 1b). Because PRGBS seem to show higher levofloxacin and erythromycin MICs than PSGBS, we classified the 57 GBS into two groups—susceptible and not susceptible/resistant to levofloxacin and erythromycin—according to CLSI criteria (Table 1). Among the 38 PSGBS, 7/38 (18.4%) were not susceptible to levofloxacin and 3/38 (7.9%) were resistant to erythromycin. In addition, none of the 19 PRGBS isolates (100%, 19/19) were susceptible to levofloxacin and 9/19 isolates (47.4%) were resistant to erythromycin. The statistical significance of reduced penicillin susceptibility and FQ non-susceptibility was determined using Fisher's exact test ($P\leq0.0001$) (Table 1). A similar statistical significance ($P=0.0012$) was obtained between reduced penicillin susceptibility and macrolide resistance among the GBS tested (Table 1). PRGBS tend to display multiple-antimicrobial resistance that may predict FQ and/or macrolide treatment failure.

The PRGBS isolates were found to have several amino acid substitutions in PBP2X (Table S2; available as Supplementary data at *JAC* Online), as found in our previous studies,[8,10] together with several amino acid substitutions in the QRDR of the Gyr and Par enzymes (Table S3; available as Supplementary data at *JAC* Online). In addition, diverse macrolide resistance genes *erm*(TR), *erm*(B) and *mef*(A/E) were detected (Table S4; available as Supplementary data at *JAC* Online). No pair of isolates was identical in terms of amino acid substitutions in PBP2X and QRDR regions of Gyr and Par enzymes, and macrolide resistance gene types. The data show that the 19 PRGBS tested in this study were not clonal, which is consistent with a previous PFGE study.[8,10]

Discussion

This study revealed that PRGBS tend to be FQ non-susceptible and macrolide and multidrug resistant. Only 19 PRGBS were used in this study because the isolation rate in Japan is only 2.3% of all GBS clinical isolates.[10] It is difficult to obtain more isolates, and, as far as we know, this study analysed the largest number of PRGBS compared with other PRGBS studies worldwide. Moreover, our results revealed a statistically significant association between reduced penicillin susceptibility and FQ non-susceptibility and macrolide resistance. Therefore we conclude that PRGBS tend to be multidrug resistant.

Although we did not select the analysed PRGBS from FQ-resistant GBS, all PRGBS were non-susceptible to levofloxacin. The obvious selection bias is unlikely. Therefore, although PRGBS clonal expansion must be considered, we can rule out this possibility according to previous PFGE findings,[8,10] the amino acid substitutions in PBP2X and Gyr and Par enzymes, and the macrolide resistance gene types.

Because an adequate amount of PRGBS isolates for epidemiological study has not yet been recovered from GBS infectious cases and clinical samples often comprise sputa or upper respiratory tract swabs, the clinical significance of PRGBS has not yet been confirmed. Fortunately, no neonatal case of invasive PRGBS infection has been reported in Japan. However, PRGBS tend to demonstrate multidrug resistance. Therefore we must examine PRGBS prevalence from this point onward. To validate the accuracy of GBS susceptibility tests, it is important to determine the resistance criteria of β-lactams for GBS. Additionally, GBS isolates should be subjected to aggressive susceptibility testing against penicillin and cephalosporins such as ceftizoxime to ensure early and accurate identification of PRGBS demonstrating multidrug resistance.

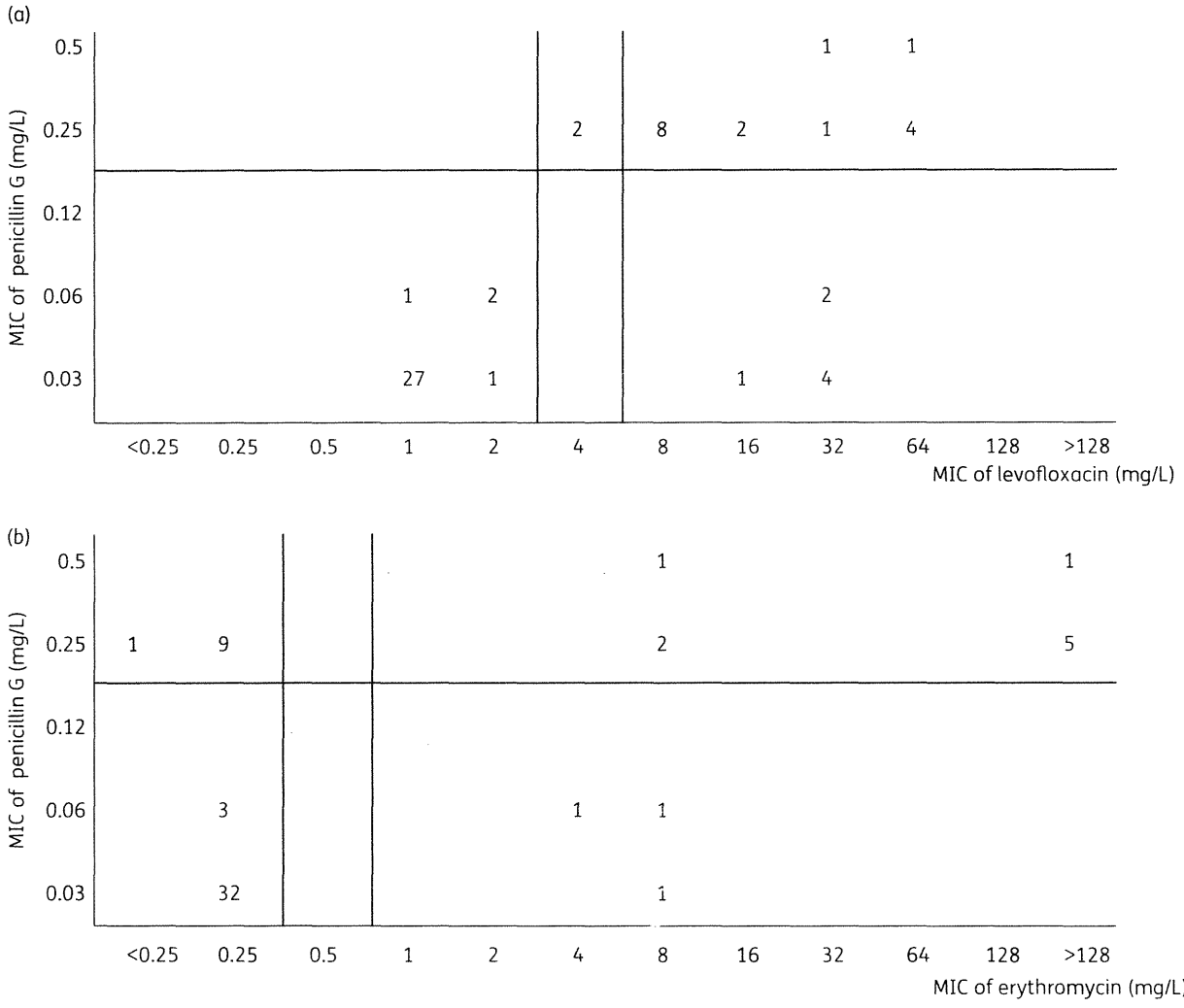

Figure 1. Scatter diagram depicting the relationship between reduced penicillin susceptibility and levofloxacin (FQ) non-susceptibility (a) and erythromycin (macrolide) resistance (b). The numbers at each intersection indicate the number of isolates. The vertical and horizontal lines in the scatter diagrams indicate susceptible, intermediate and resistant breakpoints established by the CLSI.

Table 1. Comparison of levofloxacin non-susceptibility and erythromycin resistance in PRGBS and PSGBS

	PRGBS ($n=19$) (penicillin G MIC >0.12 mg/L)	PSGBS ($n=38$) (penicillin G MIC ≤0.12 mg/L)	P^a
Non-susceptibility to levofloxacin (R or I; MIC ≥4 mg/L)	19 (100%)	7 (18.4%)	≤0.0001
Resistant to erythromycin (R; MIC ≥1 mg/L)	9 (47.4%)	3 (7.9%)	0.0012

I, intermediate; R, resistant. MICs were determined by the agar dilution method according to the recommendations of the CLSI.
[a]Calculated using Fisher's exact test.

In our previous study, sequence type (ST) 458 and serotype VI were predominant among PRGBS isolated in Japan.[9] Because ST458 is a novel GBS ST and is specific to PRGBS found to date, the characteristics and clinical properties of strains with ST458 are largely unknown. However, a case of nosocomial spread of multidrug-resistant PRGBS with ST458 and serotype VI was recently reported.[20] Therefore there is a need to analyse and characterize PRGBS with ST458 and serotype VI.

The MIC of penicillin G for PRGBS is 0.25–1 mg/L, and reduced penicillin susceptibility mechanisms of PRGBS include the accumulation of amino acid substitutions, including Q557E and/or V405A, in PBP2X. Because the MICs of penicillin G for PRGBS are not so high, penicillin G might be effective against PRGBS infections, except meningitis, if PRGBS cause infections. However, the MICs of penicillin G for PRGBS may be elevated by the acquisition of amino acid substitutions in PBPs other than PBP2X because the MICs of penicillins for penicillin-resistant *S. pneumoniae* have been increasing by similar penicillin

resistance mechanisms. As a result, it may be more difficult to achieve a positive outcome with the use of penicillins against PRGBS infection at various sites. Moreover, as this study shows, PRGBS tend to be non-susceptible to FQs and resistant to macrolides, and the available drug selection to treat multidrug-resistant PRGBS infections is more limited than the selection to treat common drug-susceptible GBS infections. If the MICs of penicillins and cephalosporins for PRGBS continue to rise in the future, the available drug selection to treat multidrug-resistant PRGBS infections at various sites will be quite limited. Therefore PRGBS pose future clinical concern and further PRGBS-related research is a necessity from this point forward.

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Transparency declarations

The authors have no conflicts of interest to declare. The manuscript has been edited by Editage, a language-editing company.

Supplementary data

Tables S1–S4 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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Ability of the VITEK® 2 system to detect group B streptococci with reduced penicillin susceptibility (PRGBS)

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Keywords: group B *Streptococcus*, GBS, penicillin G

Sir,

Group B *Streptococcus* (*Streptococcus agalactiae*, GBS) is the leading cause of neonatal sepsis and meningitis and an important pathogen among elderly people and those suffering from underlying medical disorders.[1,2] The highest GBS mortality and morbidity result from invasive infections in neonates.[3] Approximately 5% of GBS-infected infants die and survivors often suffer from severe neurological sequelae.[4] Intrapartum antibiotic prophylaxis has been recommended by the CDC[5] and is prescribed for pregnant women who have GBS isolated from vaginal specimens. Since the introduction of prophylaxis, the rate of GBS infection during the first post-natal week has decreased. Penicillins are the first-line agents in the prophylaxis and treatment of GBS infections because all clinical GBS isolates have been considered to be uniformly susceptible to β-lactams, including penicillins.[6,7] However, we identified and characterized several GBS isolates demonstrating reduced penicillin susceptibility (PRGBS) through acquisition of multiple mutations in the penicillin-binding protein 2X (*pbp2x*) gene,[8] and similar isolates were reported in the USA,[9] Canada[6,7] and Japan.[2] After our research was published, EUCAST (http://www.eucast.org/clinical_breakpoints/) defined a clinical penicillin MIC breakpoint for *Streptococcus* groups A, B, C and G, together with the penicillin MIC resistance breakpoint (>0.25 mg/L). The EUCAST breakpoint is higher than the breakpoint for penicillin susceptibility set by the CLSI (≤0.12 mg/L). Until recently, PRGBS were isolated from respiratory specimens, blood, decubitus ulcers and adult hip-joint fluid,[6,7,9] with no report of PRGBS isolated from neonates or vaginal specimens of pregnant women. The isolation rate of PRGBS from various sources is approximately 2.3% in Japan.[2] The MICs of penicillin G for PRGBS (0.25–1 mg/L) are near the breakpoint set by the CLSI (≤0.12 mg/L). Therefore it is unclear whether automated susceptibility testing machines such as VITEK® 2 can detect PRGBS accurately. Because the VITEK® 2 system is widely used in clinical laboratories in Japan, we used this system as an example in order to evaluate the ability of automated susceptibility testing machines to detect PRGBS.

The MICs of penicillin G were determined for 28 PRGBS using the agar dilution method as per CLSI recommendations.[10] *Streptococcus pneumoniae* ATCC 49619 was used as a quality control for MIC measurements. It was confirmed that these PRGBS harboured the amino acid substitutions in *pbp2x* genes, as described previously.[6,7] We performed the determination of the MICs of penicillin G for 28 PRGBS three times using the VITEK® 2 compact system with AST-P546 cards (bioMérieux Clinical Diagnostics, Marcy l'Étoile, France) in accordance with the manufacturer's instructions.

The results of the comparison between the MICs of penicillin G for 28 PRGBS, as determined by agar dilution and the VITEK® 2 system, are shown in Table 1. Although the MICs determined by the agar dilution method were 0.25–1 mg/L [above the breakpoint (≤0.12 mg/L) set by the CLSI], the MICs determined by the VITEK® 2 system were ≤0.12–1 mg/L. The MICs determined by the VITEK® 2 system were ≤0.12 mg/L in 38 instances (38/84, 45.2%; 84 instances = 28 strains × 3 times). The number of strains for which the MICs determined by the VITEK® 2 system were ≤0.12 mg/L at least two of three times was 13 (13/28, 46.4%).

In this study, we investigated the ability of the VITEK® 2 system to detect PRGBS. It detected only half of the PRGBS in this study. Automated susceptibility testing machines such as VITEK® 2 are used in clinical settings worldwide, and these results suggest that many PRGBS may be misclassified as 'susceptible' to penicillin G. We recently revealed that PRGBS tends to be resistant to fluoroquinolones and macrolides, in addition to having reduced penicillin susceptibility,[8] indicating that the classification of susceptibility to penicillin G is very important. The worldwide misclassification of PRGBS as 'susceptible' to penicillin G is undesirable and hinders attempts to clarify the clinical significance of reduced susceptibility to penicillin G.

The MICs of penicillin G for PRGBS (0.25–1 mg/L) are near the 'susceptible' breakpoint (≤0.12 mg/L) set by the CLSI, while the MICs of oxacillin (2–8 mg/L) and ceftizoxime (4–128 mg/L) for PRGBS are higher than those of penicillin-susceptible GBS.[6,7] However, the VITEK® 2 system AST-P546 cards for *S. agalactiae* do not include MIC determinations of oxacillin or ceftizoxime. We believe that inclusion of these MICs would enable more accurate detection of PRGBS by automated susceptibility testing machines. Moreover, it would be better for these machines to contain systems to alert operators to PRGBS-suspicious isolates when the MICs of penicillin G indicate a range near the susceptibility breakpoint, e.g. at 0.12 mg/L.

Table 1. Comparison between MICs of penicillin G for 28 PRGBS determined by agar dilution and by VITEK® 2

Strain	MIC (mg/L) of penicillin G by agar dilution	MIC (mg/L) of penicillin G by VITEK® 2		
		1st	2nd	3rd
B1	0.5	0.25	0.5	0.5
B6	0.25	0.25	0.25	0.5
B7	0.25	≤0.12	≤0.12	≤0.12
B8	0.25	≤0.12	ND	≤0.12
B10	0.5	0.25	0.25	0.25
B12	0.25	ND	0.5	0.25
B40	0.5	0.5	0.5	0.5
B60	0.25	0.25	0.25	0.25
B68	0.5	0.5	0.25	0.5
B502	0.5	≤0.12	≤0.12	≤0.12
B503	0.25	≤0.12	≤0.12	≤0.12
B513	1	0.25	0.25	0.25
B514	0.25	≤0.12	≤0.12	≤0.12
B516	0.25	≤0.12	≤0.12	≤0.12
MRY06-238	0.5	≤0.12	0.25	0.25
MRY06-241	0.25	≤0.12	≤0.12	≤0.12
MRY08-517	0.25	0.25	0.25	0.25
MRY08-527	0.5	0.25	0.25	0.25
MRY08-528	0.25	≤0.12	≤0.12	≤0.12
MRY08-1422	0.25	≤0.12	≤0.12	≤0.12
R1	0.25	≤0.12	ND	≤0.12
R2	0.25	≤0.12	≤0.12	≤0.12
R3	0.25	0.25	0.25	0.25
R4	0.25	≤0.12	0.25	0.25
R5	0.25	≤0.12	≤0.12	≤0.12
R6	0.25	0.25	≤0.12	≤0.12
R7	0.25	0.5	1	0.25
R8	0.5	ND	ND	ND

ND, not determined.

Previously we reported that disc diffusion methods using oxacillin, ceftizoxime and ceftibuten were useful for detecting PRGBS.[10] The disc diffusion method for detecting PRGBS does not require expensive or specialized equipment. Therefore, prior to any improvements in automated susceptibility testing machines, the disc diffusion method for detecting PRGBS will be useful for clinical microbiological laboratories worldwide.

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Transparency declarations

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Laboratory and Epidemiology Communications

First Report of OXA-48 Carbapenemase-Producing *Klebsiella pneumoniae* and *Escherichia coli* in Japan from a Patient Returned from Southeast Asia

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Various carbapenemases such as NDM-types, KPC-types, and OXA-types have recently emerged and spread worldwide (1). Among these carbapenemases, OXA-types have been identified mainly in *Acinetobacter* spp., particularly in *Acinetobacter baumannii* (2). OXA-type carbapenemases in *A. baumannii* were shown to be OXA-51-like, OXA-23-like, OXA-24/40-like, and OXA-58-like. However, OXA-48 was first identified from *Klebsiella pneumoniae* clinically isolated in Turkey in 2001 (3). After 2009, OXA-48-producing *K. pneumoniae* spread rapidly among European countries and caused several outbreaks in hospital settings (4–6). The first cases in the United States were recently identified (7), although OXA-48-producing Gram-negative bacteria belonging to the family *Enterobacteriaceae* have been reported in several developing countries or regions (8,9).

In November 2012, a man in his 60s was admitted to a general hospital in Kanto. This patient had a history of hospitalization in a Southeast Asian country for the treatment of cerebral infarction before admission to the hospital. Three types of antimicrobial-resistant microbes belonging to the family *Enterobacteriaceae*, namely, multidrug-resistant *K. pneumoniae* and *Escherichia coli*, as well as piperacillin-tazobactam-resistant *K. pneumoniae*, were isolated from the sputa and/or feces of this patient. The minimum inhibitory concentrations (MICs) of imipenem (IPM) and meropenem

Table 1. MICs of antimicrobials for OXA-48-producing *K. pneumoniae* and *E. coli*

Antimicrobial	MIC (μg/ml) for			Antimicrobial	MIC (μg/ml) for		
	Kp1[1)]	Kp2[2)]	Ec[2)]		Kp1	Kp2	Ec
ampicillin	>16	>16	>16	cefpirome	≤8	>16	>16
amoxicillin/clavulanate	>16	>16	>16	cefepime	≤1	>32	>32
piperacillin	>64	>64	>64	cefoxitin	4	4	>32
piperacillin/tazobactam	>64	>64	>64	cefmetazole	2	2	16
cefazolin	≤4	>16	>16	cefotetan	≤1	2	16
cefaclor	>16	>16	>16	imipenem	2	2	2
cefotiam	≤8	>16	>16	meropenem	2	2	2
cefotaxime	1	>128	>128	flomoxef	≤8	≤8	≤8
cefotaxime/clavulanate	0.25/4	>32/4	>32/4	gentamicin	≤1	≤1	>8
ceftazidime	≤0.5	>128	>128	amikacin	≤4	≤4	≤4
ceftazidime/clavulanate	≤0.12/4	4/4	16/4	tobramycin	≤1	4	>8
ceftriaxone	≤0.5	>64	>64	minocycline	2	8	>8
cefpodoxime	1	>64	>64	levofloxacin	≤0.5	1	>4
cefozopran	≤2	>16	>16	ciprofloxacin	≤0.25	2	>2
cefoperazone/sulbactam	≤16	>32	>32	fosfomycin	>16	16	≤4
aztreonam	≤0.5	>64	>64	sulfamethoxazole/trimethoprim	≤2	>2	>2

1): OXA-48 (+), ESBL (−).
2): OXA-48 (+), CTX-M-1-group ESBL (+).

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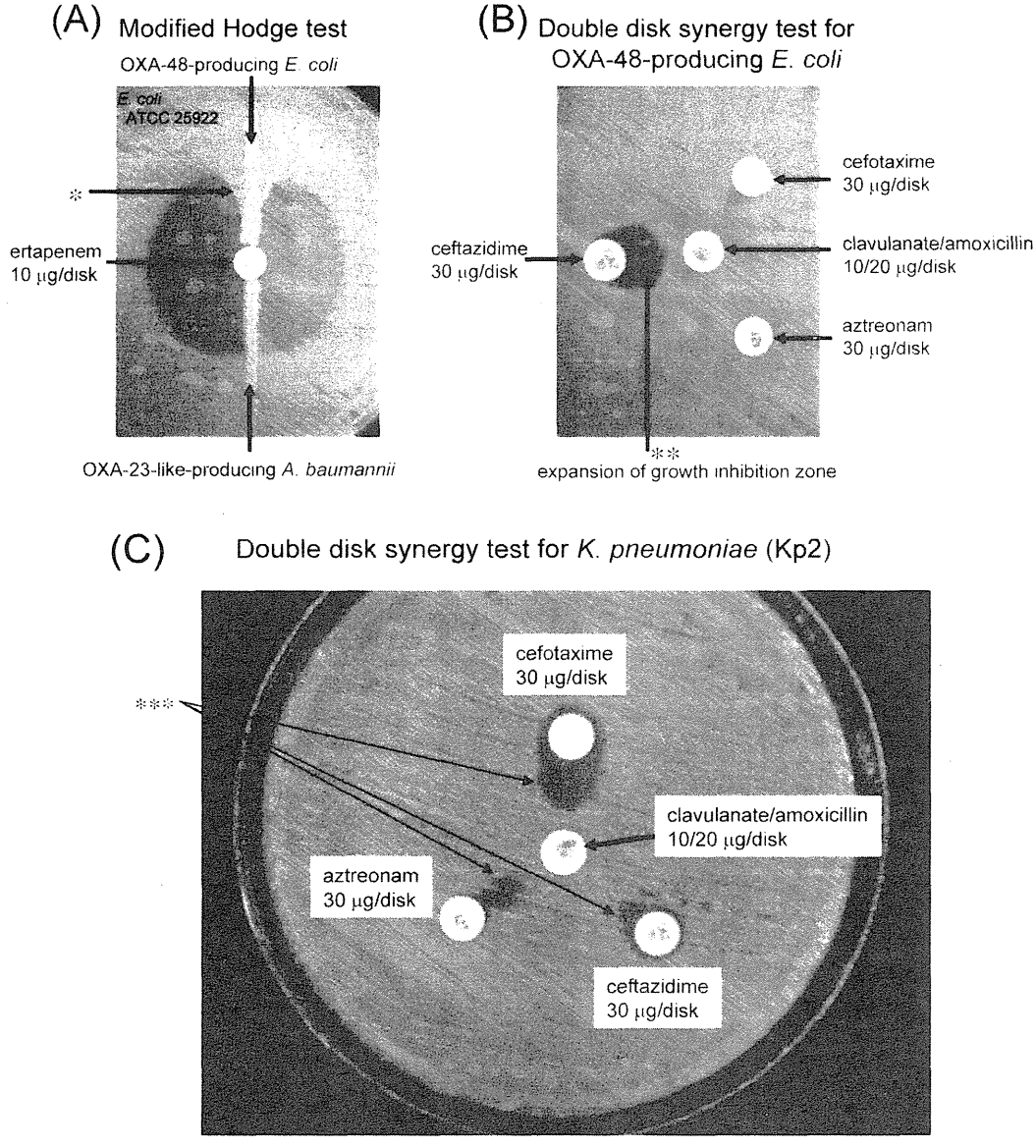

*Typical expansion of growth area of *E. coli* ATCC25922 toward the ertapenem disk along the streak of OXA-48-producing *E. coli* was observed, suggesting production of carbapenemase.
**This phenomenon suggested probable production of class A enzymes such as ESBL.
***In each antimicrobial, atypical slight expansion of growth inhibition zone was observed toward the center disk containing clavulanate/amoxicillin.

Fig. 1. (A) Results of modified Hodge test. Growth of *E. coli* ATCC25922, a carbapenem-susceptible reference strain, expands to the ertapenem disk along the streak of the *E. coli* strain Ec, shown in Table 1, suggesting the production of carbapenemase in the Ec. (B) and (C) Double disk synergy tests for *E. coli* strain Ec and *K. pneumoniae* strain Kp2. Since these strains produce both OXA-48 and CTX-M-type ESBL, the patterns of the growth inhibition zone around the disks containing ceftazidime (CAZ), cefotaxime, or aztreonam with clavulanic acid became atypical. In clinical isolates demonstrating atypical growth inhibition profiles with clavulanic acid and CAZ or cefotaxime increased, production of multiple and different types of β-lactamases should be considered.

(MEPM) for these isolates were below the breakpoint for "resistant," but two multidrug-resistant isolates, Kp2 and Ec (Table 1), consistently showed resistance to various broad-spectrum β-lactams, including cefotaxime, ceftazidime (CAZ), cefpirome, and cefepime, together with aminoglycosides and fluoroquinolones. In contrast, a piperacillin-tazobactam-resistant *K. pneumonia* isolate, Kp1 (Table 1), was found to be susceptible to many β-lactams. Inhibition tests for discriminating between β-lactamases produced in the isolates using sodium mercaptoacetate (SMA), EDTA, and 3-aminophenylboronic acid (APB) were all negative, but a modified Hodge test using the ertapenem disk indicated production of carbapenemases in these isolates (Fig. 1A). Polymerase chain reaction (PCR) analyses of carbapemase genes (10,11) such as bla_{NDM}, bla_{IMP}, bla_{VIM}, bla_{KPC}, and bla_{OXA} including $bla_{OXA\text{-}51\text{-}like}$, $bla_{OXA\text{-}23\text{-}like}$, $bla_{OXA\text{-}24/40\text{-}like}$, $bla_{OXA\text{-}58\text{-}like}$, and $bla_{OXA\text{-}48\text{-}like}$ showed the presence of the $bla_{OXA\text{-}48\text{-}like}$ gene in these isolates. PCR analysis showed that two

multidrug-resistant isolates, Kp2 and Ec, as shown in Table 1, were also found to harbor a gene for the CTX-M-1-group ESBL. The MICs of CAZ for these isolates declined from >128 μg/ml to 4 or 16 μg/ml in the presence of 4 μg/ml clavulanate, suggesting the role of the ESBL production in CAZ resistance. Since these isolates co-produced the CTX-M-1-group ESBL and OXA-48, a double disk synergy test using a clavulanate/ampicillin disk with disks containing CAZ, cefotaxime, or aztreonam showed atypical inhibition profiles (Figs. 1B and 1C). Interestingly, the *E. coli* isolate was not agglutinated with anti-sera specific for *E. coli* serotype O25, although ESBL-producing and fluoroquinolone resistant *E. coli* clinical isolates are usually O25:H4-ST131, a global epidemic lineage. Moreover, an *A. baumannii* that harbors genes for acquired OXA-23-like carbapenemase and ArmA 16S rRNA methyltransferase was co-isolated from this patient.

It has been reported that some clinical isolates were "susceptible" to carbapenems despite harboring carbapenemase genes such as bla_{NDM}, bla_{KPC}, and bla_{OXA}. Therefore, clinical isolates suspected to be ESBL producers due to demonstrating consistent resistance to various cephalosporins and reduced susceptibility (MIC, 2–4 μg/ml) to carbapenems would worth being subjected to PCR analyses using PCR primer sets for detecting known carbapenemase genes after screening using modified Hodge test (12). Furthermore, even in cases in which the clinical isolates show susceptibility to broad-spectrum β-lactams as found in the piperacillin-tazobactam-resistant *K. pneumoniae* (Kp1 in Table 1), the resistance profile to piperacillin-tazobactam may be a good marker for detecting KPC- or OXA-type-carbapenemase producers in addition to the modified Hodge test. Two types of new carbapenemases, the NDM-1 and KPC-2, have already been identified in Gram-negative microbes in Japan, and OXA-48-like carbapenemase was newly identified in *K. pneumoniae* and *E. coli* in this study. Hence, early detection of invasion of carbapenemase-producing Gram-negative microbes and their containment in clinical settings is currently very important in Japan, where NDM, KPC, and OXA producers remain very rare. Furthermore, intensive surveillance of OXA-type carbapenemase-producing Gram-negative microbes should be conducted in Asian countries, as well as enhanced surveillance of NDM and/or KPC producers because OXA-181, a variant of OXA-48, has already spread in India (13). Positive results in the modified Hodge test using an ertapenem disk (14), as well as multidrug-resistance profiles, including piperacillin-tazobactam, are useful in the early detection of OXA-48-like carbapenemase-producing Gram-negative microbes.

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Conflict of interest None to declare.

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Nosocomial spread of multidrug-resistant group B streptococci with reduced penicillin susceptibility belonging to clonal complex 1

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Background: Multiple group B *Streptococcus* (GBS) isolates with reduced penicillin susceptibility (PRGBS) were recovered from several patients, hence a probable nosocomial transmission of PRGBS in a hospital setting was suspected.

Methods: Ten PRGBS recovered from eight patients in a general hospital were characterized. Sequence analysis of genes for penicillin-binding proteins (PBPs) and quinolone resistance-determining regions (QRDRs) of *gyrA*, *gyrB* and *parC* was performed, and the macrolide resistance genes were detected by PCR. Genetic relatedness among the isolates was examined by PFGE and multilocus sequence typing.

Results: All the PRGBS had the key amino acid substitution V405A, together with F395L, R433H, H438Y and G648A in PBP 2X and T567I in PBP 2B. A 23S rRNA methylase gene, *erm*(B), was also found in all 10 PRGBS strains. PFGE analysis revealed considerable genetic relatedness among the isolates. Isolates of pulsotype I were obtained from four patients in ward A and one patient in ward B, while isolates of pulsotypes II and III were obtained from two patients in ward B and one patient in ward C, respectively. Isolates of pulsotype I were resistant to levofloxacin (MIC >8 mg/L) and had the following amino acid substitutions in the QRDRs: S81L in GyrA, E476K in GyrB and S79Y in ParC. However, pulsotype II strains resistant to levofloxacin (MIC 8 mg/L) had no change in GyrA, but changes in GyrB (E476K) and ParC (S79Y). All 10 PRGBS strains belonged to serotype VI and ST458 (where ST stands for sequence type).

Conclusions: This is the first description of the nosocomial spread of multidrug-resistant PRGBS strains belonging to the genetic lineage ST458.

Keywords: horizontal transmission, β-lactams, macrolides, fluoroquinolones, group B *Streptococcus*

Introduction

Group B *Streptococcus* (GBS) is one of the most important causes of serious neonatal infections. In particular, for early onset neonatal diseases, rectal or vaginal GBS colonization found in about 25% of pregnant women is the primary risk factor. GBS also causes invasive infections in adults, including pregnant women, elderly individuals and immunocompromised patients. Penicillin is the first-line antibiotic for treatment of GBS infection, as well as for intrapartum antibiotic prophylaxis to prevent early onset infection, because resistance to this agent has not been reported so far among GBS clinical isolates. However, in 2008 we reported GBS clinical isolates with reduced penicillin susceptibility (PRGBS),[1,2] in which an increase was noted in the MICs of β-lactam antibiotics including penicillin (MICs of 0.25–1 mg/L). Since no typical phenotypic changes are observed between GBS and PRGBS, it is very difficult to distinguish them in routine microbiology tests. However, elevation of ceftizoxime and ceftibuten MICs would be a good marker for screening for PRGBS,[1,3] although these agents are not included in the list of drugs for antimicrobial susceptibility testing. Amino acid substitutions V405A and Q557E in penicillin-binding protein (PBP) 2X, which are shared by most PRGBS strains, have been demonstrated as a major mechanism involved in reduction of GBS penicillin

susceptibility.[1] Besides these two key substitutions that have been identified in PBP 2X, multiple amino acid substitutions were also found in PBPs 2X, 2B and 1A among PRGBS strains depending on their penicillin MIC levels. However, it has also been noted that one of those PRGBS strains had no such key substitution in PBP 2X.[1]

After our aforementioned study, GBS strains isolated in the USA, showing elevated MICs, but which were still susceptible to β-lactam antibiotics, shared amino acid substitution Q557E in PBP 2X,[4] whereas GBS strains with penicillin MICs of 0.25 or 0.5 mg/L from two Canadian studies had amino acid substitutions in several PBPs, but no key substitutions—V405A or Q557E—were found in PBP 2X.[5,6] Interestingly, the amino acid substitutions identified in PBPs of these Canadian PRGBS strains have not been found so far among the strains tested in our studies. Thus characterization of molecular mechanisms underlying the reduced penicillin susceptibility profile in PRGBS is still in progress.

Clindamycin or erythromycin is traditionally used for GBS intrapartum prophylaxis for penicillin-allergic women at high risk of anaphylaxis. However, increasing resistance of GBS to clindamycin or erythromycin has been reported worldwide. In the USA, the rates of resistance among invasive GBS isolates were 13%–15% for clindamycin and 26%–32% for erythromycin.[7,8] The Japan Nosocomial Infections Surveillance (JANIS) of the Ministry of Health, Labour and Welfare showed that the prevalence of resistance was 22% and 28% for clindamycin and erythromycin, respectively, among GBS from various clinical sources in 2010.[9] In GBS isolates, macrolide resistance has been mediated mainly by two classes of resistance genes: the *erm* genes, including *erm*(B), *erm*(TR)/*erm*(A) and *erm*(C), which mediate ribosomal methylation; and the *mef* genes, such as *mef*(A) and *mef*(E), which are involved with efflux of macrolides as they encode membrane-associated transporters. The *erm* genes are associated with the macrolide-lincosamide-streptogramin B resistance phenotype, which usually show cross-resistance to clindamycin, while *mef* genes are the resistance determinants specific for 14- and 15-membered macrolides.

Fluoroquinolone resistance in GBS has recently emerged in several countries including Japan.[10–14] Mutations in the quinolone resistance-determining regions (QRDRs) of the genes encoding topoisomerase IV ParC and DNA gyrase GyrA have mainly been associated with fluoroquinolone resistance of this organism.[10,11,13,15] The presence of GyrA-ParC-ParE triple mutations has recently been reported in Taiwan isolates.[14] High rates of tetracycline resistance have also been noted among GBS isolates with the most common resistance determinant, *tet*(M), which encodes the ribosomal protection protein.[16,17]

Our phylogenetic comparative analyses have shown genetic diversity of *pbp* genes among PRGBS strains, while those genes of the penicillin-susceptible strains were highly conserved, irrespective of their isolation dates.[2] Furthermore, a phylogenic tree showed three distinct genetic lineages of PRGBS strains, implying that those lineages have been independently emerging through the accumulation of different genetic mutations in their *pbp* genes during evolution. PRGBS has been found to be capable of surviving persistently at the site of infection for >3 weeks.[18]

In the present study we investigated the molecular basis of resistance determinants and the clonal relationship of 10 PRGBS isolates showing multidrug resistance to macrolides, lincosamides, fluoroquinolones and tetracyclines detected from eight patients during a 5 month period to characterize the genetic background of the isolates.

Materials and methods

Bacterial strains

Ten GBS clinical strains isolated from eight patients admitted to a general hospital located in Tokyo, Japan, during March–August 2007 were analysed. These strains included two strains, strain numbers 2-1 and 2-2, isolated from different specimens obtained at approximately the same time from one patient and two strains, strain numbers 4-1 and 4-2, isolated at 2 month intervals from another patient. The source of strains and the clinical backgrounds of the patients are shown in Table 1.

GBS strains were grown overnight in Todd–Hewitt broth (BD Diagnostics) and then stored in glycerol at −80 C until use.

Serotyping was performed using antisera (Denka Seiken, Tokyo, Japan) to the type-specific capsular polysaccharides Ia, Ib, II, III, IV, V, VI and VIII.

Antimicrobial susceptibility testing

MICs were determined using a broth microdilution method with a MicroScan MICroFAST panel type 5J system (Dade Behring Inc., Tokyo, Japan) by following the guidelines recommended by the CLSI.[19] For susceptibility categories we referred to the CLSI criteria.[20] Additionally, MICs of penicillin and ceftizoxime were determined by Etest according to the manufacturer's instructions (AB Biodisk, Solna, Sweden).

MIC determinations were repeated independently three times for each strain to ensure the reproducibility of the MICs by using quality control strain *Streptococcus pneumoniae* ATCC 49619.

Susceptibility testing with a ceftibuten disc was also performed by Kirby–Bauer's disc-diffusion method.[21] β-Lactamase activity was detected by a nitrocefin-based disc procedure (BD Diagnostics).

Analysis of pbp gene sequences

PCR amplification and sequencing analysis of each *pbp* gene were performed as previously described, with minor modifications.[4] Briefly, the full-length coding regions of *pbp1a*, *pbp2b* and *pbp2x* genes were amplified from genomic DNA extract using the primer pairs f1 and r1, as listed in Table 2. PCRs were carried out using PrimeSTAR HS DNA polymerase (Takara Shuzo Co., Kyoto, Japan) with reaction conditions of 30 cycles of 98 C for 10 s, 55 C for 5 s and 72 C for 2.5 min.

Sequencing analyses of both strands of purified PCR products were performed using several internal forward and reverse sequencing primers (Table 2), a BigDye Terminator (version 3.1) cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and an ABI 3730x/DNA analyser (Applied Biosystems).

The nucleotide sequences obtained were assembled into contigs with BioEdit (version 5.0.9) software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), then aligned with ClustalW software.[22] *Streptococcus agalactiae* strains 2603 V/R (ATCC BAA-611; GenBank accession number NC004116) and NEM316 (ATCC 12403; GenBank accession number NC004368) were used as reference strains for comparative analysis.

Analysis of macrolide and fluoroquinolone resistance

PCR detection of *erm*(B), *erm*(TR) and *mef*(A/E), conferring resistance to macrolides or lincosamides, was performed with specific primers shown in Table 2 as described previously.[22,23] For three representative strains, the PCR products were subjected to sequence analyses to confirm the identity of amplification products.

Table 1. Clinical associations and microbiological profiles of PRGBS isolates

					Patient					Bacterial isolate		MIC (mg/L)														
Strain no.	Date of admission	Date of isolation	Ward	Department	age (years)	sex	underlying diseases	prior therapy (within 3 months)	specimens	pulsotype	serotype	PEN (≤0.12)[a]	AMP (≤0.25)	CTM	CTX (≤0.5)	CRO (≤0.5)	CDN	FEP (≤0.5)	CFM	ZOX[b]	MEM (≤0.5)	ERY (≤0.25)	CLR (≤0.25)	CLI (≤0.25)	LVX (≤2)	TET (≤2)	VAN (≤1)
1	8 December 2006	17 April 2007	A	NS	86	F	multiple aneurysm	MEM, CIP	PHA	I	VI	0.25	0.5	2	0.25	0.25	0.25	≤0.5	>1	>32	0.25	>1	>1	>1	>8	>4	0.5
2-1	20 March 2007	15 April 2007	A	NS	70	M	cerebral infarction	CAZ, SAM, ABK, CIP	pus	I	VI	0.25	0.5	2	0.25	0.25	0.25	≤0.5	>1	>32	0.25	>1	>1	>1	>8	>4	0.5
2-2		21 April 2007							PHA	I	VI	0.25	0.5	2	0.25	0.25	0.25	≤0.5	>1	>32	0.25	>1	>1	>1	>8	>4	0.5
3	25 March 2007	24 April 2007	B	IM	87	F	acute pneumonia	SAM	PHA	II	VI	0.25	0.5	2	0.25	0.25	0.25	≤0.5	>1	>32	0.25	>1	>1	>1	8	>4	0.5
4-1	5 February 2007	13 March 2007	A	NS	78	F	hydrocephalus	CAZ, ABK, VAN	PHA	I	VI	0.25[b]	0.25	2	0.25	0.25	0.12	≤0.5	>1	>32	0.25	>1	>1	>1	>8	>4	0.5
4-2		7 May 2007							TTA	I	VI	0.25	0.5	2	0.25	0.25	0.25	≤0.5	>1	>32	0.25	>1	>1	>1	>8	>4	0.5
5	24 March 2007	25 April 2007	A	NS	52	M	pancreas cancer	CFZ, SAM	TTA	I	VI	0.25	0.5	2	0.25	0.25	0.25	≤0.5	>1	>32	0.25	>1	>1	>1	>8	>4	0.5
6	18 July 2007	30 July 2007	B	IM	84	M	brain-stem infarction	SAM	TTA	II	VI	0.25	0.5	2	0.25	0.25	0.25	≤0.5	>1	>32	0.25	>1	>1	>1	8	>4	0.5
7	22 February 2007	25 April 2007	B	IM	82	F	cerebral infarction	SAM, CIP, CTM, CFP/SUL	PHA	I	VI	0.25	0.5	2	0.25	0.25	0.25	≤0.5	>1	>32	0.25	>1	>1	>1	>8	>4	0.5
8	31 August 2007	31 August 2007	C	IM	82	F	cerebral infarction sequelae	unknown	TTA	III	VI	0.25[b]	0.25	2	0.25	0.25	0.12	≤0.5	>1	>32	0.25	>1	>1	>1	>8	>4	0.5

NS, neurosurgery; IM, internal medicine; F, female; M, male; PHA, pharyngeal swab; TTA, transtracheal aspirate; MEM, meropenem; CIP, ciprofloxacin; CAZ, ceftazidime; SAM, ampicillin/sulbactam; ABK, arbekacin; VAN, vancomycin; CFZ, cefazolin; CTM, cefotiam; CFP/SUL, cefoperazone/sulbactam; PEN, penicillin; AMP, ampicillin; CTX, cefotaxime; CRO, ceftriaxone; CDN, cefditoren; FEP, cefepime; CFM, cefixime; ZOX, ceftizoxime; ERY, erythromycin; CLR, clarithromycin; CLI, clindamycin; LVX, levofloxacin; TET, tetracycline.

[a]The CLSI's susceptible MICs of each antimicrobial for GBS.

[b]MIC results of the Etest.

Table 2. Oligonucleotide primers

Target gene	Usage			Sequence[a]	Amplicon size (bp)	Reference
pbp1a	PCR primers	forward	f1	5′-CGGAATTCATGGGATTTATTATCTTAGCTA-3′	2209	1
		reverse	r1	5′-ACGTCGACTTAATTACCGTTAGGTACTGTA-3′		
	sequencing primers	forward	f1b	5′-ACACCAAAGAAGAAATTCTTAC-3′		
			f2	5′-TAAAGCAAAAATCTACTTATCC-3′		
			f2b	5′-GTAGTGAGAAAATGGCAGCGGC-3′		
			f3	5′-GCCTACATGATGACGGATATGC-3′		
			f3b	5′-CAAAATTCTGGACAGTCAAGTC-3′		
		reverse	r2	5′-TCCAATCTGCACTGTATCCGCC-3′		
			r3	5′-TAGCTGCTTTAGTACCAGTACC-3′		
			r4	5′-CAGCGGCTTCAAGTGCTCTGAC-3′		
			r5	5′-TGACTTTACCATTAGTCGCATC-3′		
			r6	5′-TTTTATCTTGATACATCTGCTG-3′		
pbp2b	PCR primers	forward	f1	5′-CGGAATTCATGTTGAATCGTAAAAAAAGGT-3′	2062	
		reverse	r1	5′-ACGTCGACTTATTGTCCTGTGAACTGTGAA-3′		
	sequencing primers	forward	f1b	5′-TTCATCTCAGTCTATCAAAGAG-3′		
			f2	5′-CTATTTCTACAGAAAAGGCAGG-3′		
			f2b	5′-AGAAAGTATCTTGAAACAATAC-3′		
			f3	5′-CAACTCTAATGGAATCGTTCGG-3′		
			f3b	5′-TGGACAAACAGTTTCTACCTAC-3′		
		reverse	r2	5′-CTATCTTATTTAGTGTTTTAGG-3′		
			r3	5′-GATAGCCTCGATCAGTTAAAGC-3′		
			r4	5′-CATGATCATTTTTCAGACCAGC-3′		
			r5	5′-CTCGGTCATTCAGTGAATAGCC-3′		
			r6	5′-TAGCGCTCACTGGAACTGCAGC-3′		
pbp2x	PCR primers	forward	f1	5′-CGGAATTCGTGACTTTTTTTAAAAAGCTAA-3′	2275	
		reverse	r1	5′-ACGTCGACTTAATCTCCTATTGTAATTTTG-3′		
	sequencing primers	forward	f1b	5′-AACTATACGACAGCTACAGGTC-3′		
			f2	5′-GTAGTGGGAATGTTCTTTTAGG-3′		
			f2b	5′-TCTAAGCATTTTAACTCTACTG-3′		
			f3	5′-AAGAAGCAGCTAGTAAAACACG-3′		
			f3b	5′-GAAAATCCAGGTCATGTAGCGG-3′		
		reverse	r2	5′-GAACCAGATTACGACGTAATTC-3′		
			r3	5′-CAGATTTTACTGCAACTGATTG-3′		
			r4	5′-ATGAGCTCATAGCGATAGTTAC-3′		
			r5	5′-TTGCAGAGGCTAGAGTCATTAC-3′		
			r6	5′-CCGCCCTACGTTCTGTTGTTGC-3′		
			r7	5′-AAGACAATCCTGAACCTGAACTTCC-3′		
			r8	5′-TATCTGTACCAACGATGATGAC-3′		
erm(B)	PCR primers	forward	f1	5′-ATTGGAACAGGTAAAGGGC-3′	442	23
		reverse	r1	5′-GAACATCTGTGGTATGGCG-3′		
erm(TR)	PCR primers	forward	f1	5′-GAAGTTTAGCTTTCCTAA-3′	395	22
		reverse	r1	5′-GCTTCAGCACCTGTCTTAATTGAT-3′		
mef(A/E)	PCR primers	forward	f1	5′-AGTATCATTAATCACTAGTGC-3′	346	
		reverse	r1	5′-TTCTTCTGGTACTAAAAGTGG-3′		
gyrA	PCR primers	forward	f1	5′-GCCATGAGTGTCATTGTTGC-3′	599	in this study
		reverse	r1	5′-ATCACCAAGGCACCAGTAGG-3′		
gyrB	PCR primers	forward	f1	5′-TTTCGTACTGCCTTGACACG-3′	650	
		reverse	r1	5′-TCAACATCGGCATCAGTCAT-3′		
parC	PCR primers	forward	f1	5′-CGTTTTGGGCGCTATTCTAA-3′	607	
		reverse	r1	5′-TAGCGCCAGTTGGAAAATCT-3′		

[a]Restriction sites are underlined.

PCR amplification and DNA sequencing of the *gyrA*, *gyrB* and *parC* genes, which include the QRDRs responsible for the fluoroquinolone resistance phenotype, were performed using primers specifically designed from known DNA sequences of strain 2603 V/R as shown in Table 2. PCR and sequence analysis were carried out as described above except for an extension time of 1 min.

PFGE

PFGE of SmaI (Takara)-digested chromosomal DNAs was performed as previously described by Nagano *et al.*[2] Lambda DNA ladder (48.5 kb–1 Mb; Takara) was used as a molecular size marker. PFGE results were interpreted according to the Tenover's criteria.[24]

Multilocus sequence typing (MLST)

MLST was performed as described previously.[25] Amplification of seven housekeeping genes—*adhP*, *pheS*, *atr*, *glnA*, *sdhA*, *glcK* and *tkt*—by PCR was carried out using amplification primers and PrimeSTAR HS DNA polymerase (Takara) with reaction conditions of 1 cycle of 98 C for 1 min, followed by 30 cycles of 98 C for 10 s, 55 C for 15 s and 72 C for 1 min, and finally 1 cycle of 72 C for 7 min. PCR products were purified and sequenced using sequencing primers. Allelic profile assignment and sequence type (ST) determinations were made using the GBS MLST databases (http://pubmlst.org/sagalactiae). eBURST analysis was performed to define clonal complexes (CCs) within the isolates by using the eBURST program (http://eburst.mlst.net/v3/enter_data/single/).

Results

Origin of GBS strains

GBS strains were obtained from eight inpatients (five females and three males), aged 52–86 years (mean age 77.6 ± 11 years) (Table 1). Four patients were admitted to neurosurgery in ward A and the remaining four patients were admitted to internal medicine, among whom three were in ward B and one was in ward C. β-Lactams had been administered in all patients except one, whose history of prior therapy was unknown, and ciprofloxacin had been prescribed for three patients within 3 months of isolation of GBS. All GBS clinical strains showed no growth inhibitory zone around the ceftibuten disc, suggesting PRGBS.[3] Of those 10 isolates, 5 (strains 1, 2-2, 3, 4-1 and 7) were recovered from pharyngeal swab samples, 4 (strains 4-2, 5, 6 and 8) were from transtracheal aspirate samples and 1 (strain 2-1) was from pus obtained from the gastrostomy site. All strains were serotyped as type VI.

MICs

The MICs of several antimicrobial agents for *S. pneumoniae* ATCC 49619 were all within the quality control ranges defined by the CLSI,[20] and reproducible MIC results were obtained for GBS strains tested against all antimicrobials (Table 1).

The broth microdilution method showed that the MICs of penicillin and ampicillin were 0.25 mg/L and 0.5 mg/L, respectively, for eight strains, against 0.12 mg/L and 0.25 mg/L, respectively, for strains 4-1 and 8. However, reproducible results of a penicillin MIC of 0.25 mg/L were obtained by the Etest for these two strains. Thus all 10 strains were confirmed to be PRGBS. The MICs of cefotaxime, ceftriaxone and meropenem for PRGBS strains were all still within the susceptible range (≤0.5 mg/L), as shown in Table 1, but were higher than those for penicillin-susceptible strains possessing no amino acid substitutions in PBPs 2X, 2B or 1A.[2] The MICs of erythromycin, clarithromycin and clindamycin were >1 mg/L for all isolates tested, which fell into the resistant category. The 10 strains were resistant to levofloxacin, including MICs >8 mg/L for eight strains and 8 mg/L for strains 3 and 6. All strains were resistant to tetracycline (MIC >4 mg/L), but susceptible to vancomycin. β-Lactamase activity was not detected in any of the PRGBS isolates.

Table 3. Deduced amino acid substitutions in PBPs 2X, 2B and 1A

	Amino acid substitutions in	
	PRGBS	both PRGBS and PSGBS[a]
PBP2X	F395L, V405A, R433H, H438Y, G648A	I377V, V510I
PBP2B	T567I	—
PBP1A	—[b]	—

[a]Refers to Nagano *et al.*[2]
[b]No detected substitution.

Nucleotide sequences and amino acid substitutions in PBPs 2X, 2B and 1A

DNA sequencing of the *pbp* genes revealed that *pbp2x*, *pbp2b* and *pbp1a* genes of 10 PRGBS strains were identical. Those strains shared 10 nucleotide mutations, including five non-synonymous substitutions in *pbp2x* genes when compared with the corresponding genes of strains 2603 V/R and NEM316. The PBP 2X amino acid substitutions included a key substitution, V405A, and four additional substitutions—F395L, R433H, H438Y and G648A—that had been unique to PRGBS in our previous studies.[1,2] In PBP 2B, one amino acid substitution, T567I, which had been unique to PRGBS, was found. No amino acid substitutions were observed in PBP 1A (Table 3).

Macrolide and fluoroquinolone resistance determinants

Amplification of DNA from 10 PRGBS strains yielded PCR products of the expected sizes (442 bp) with *erm*(B)-specific primers. Sequence analyses of the PCR products from three representative strains—1, 3 and 8—selected on the basis of PFGE types revealed that the sequences of those amplified products were identical to the *erm*(B) gene sequence in GenBank accession number EF422361. All strains were negative for the *erm*(TR) and *mef*(A/E) genes.

Table 4 summarizes the QRDR amino acid substitutions in GyrA, GyrB and ParC of the PRGBS strains. Eight strains—1, 2-1, 2-2, 4-1, 4-2, 5, 7 and 8—for which levofloxacin MICs were >8 mg/L had an E476K substitution in GyrB in addition to S81L in GyrA and S79Y substitutions in ParC. The remaining strains—3 and 6—for which levofloxacin MICs were 8 mg/L, also had E476K in GyrB and S79Y in ParC, but had no substitutions in GyrA.

PFGE and MLST analyses

Figure 1 shows the PFGE results of SmaI-digested chromosomal DNAs from 10 PRGBS strains, representing three different PFGE

Table 4. Amino acid substitution in QRDRs of GyrA, GyrB and ParC, and MICs of levofloxacin

Strain	Pulsotype	MIC of levofloxacin (mg/L)	Amino acid substitution		
			GyrA	GyrB	ParC
1, 2-1, 2-2, 4-1, 4-2, 5, 7, 8	I, III	>8	S81L	E476K	S79Y
3, 6	II	8	—[a]	E476K	S79Y

[a]No detected mutation.

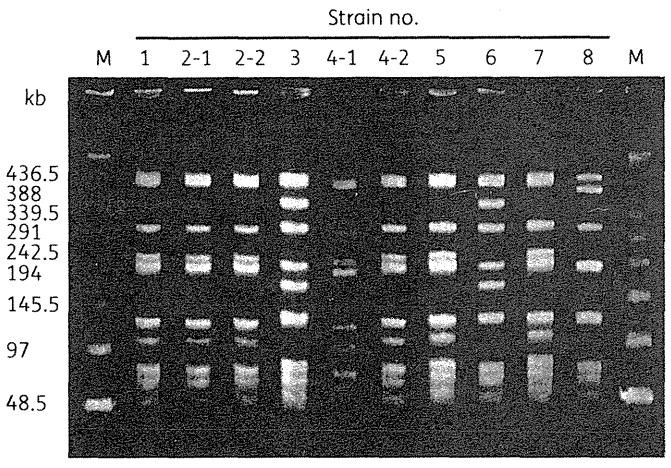

Figure 1. PFGE profiles of genomic DNA of multidrug-resistant PRGBS isolates digested with SmaI. M, bacteriophage lambda DNA ladder as molecular size markers.

patterns. The predominant PFGE type I included seven strains—1, 2-1, 2-2, 4-1, 4-2, 5 and 7—all of which were derived from ward A, except strain 7 from ward B. PFGE type II included strains 3 and 6 derived from ward B, and type III included strain 8 from ward C. PFGE types II and III differed from PFGE type I by four and three bands, respectively, and those three types were therefore considered to have a similar genetic background.

PFGE type I strains had the QRDR amino acid substitutions in GyrA, GyrB and ParC, but PFGE type II strains had those in only GyrB and ParC (Table 4).

All 10 PRGBS with the allelic profile 1, 1, 2, 1, 1, 2 and 3, in the order *adhP*, *pheS*, *atr*, *glnA*, *sdhA*, *glcK* and *tkt*, were assigned to ST458, which is a single-locus variant of ST1 within CC1.

Discussion

This study describes a probable nosocomial spread of PRGBS caused by genetically very similar PRGBS isolates that acquired multidrug resistance to macrolides, lincosamides, fluoroquinolones and tetracyclines, as well as several oral cephalosporins such as ceftizoxime.[1,3]

Among 10 PRGBS isolates derived from eight patients, a close genetic relationship was strongly suggested by PFGE analysis. Four patients in ward A and one patient in ward B had strains with the predominant PFGE type (pulsotype) I, and these strains were isolated in the same period, indicating probable bacterial transmission between these wards. Transmission of PRGBS strains was also noted in ward B, where two patients were found to share the same strains with pulsotype II. A strain with pulsotype III was isolated from a patient in ward C on the day of her admission, so it was suggested that the strain might have been introduced into the hospital from the community or other medical settings. The isolation of PRGBS strains with pulsotype II was preceded by those with pulsotype I, which, initially, might lead one to assume that pulsotype II strains are derivatives of pulsotype I strains. However, pulsotype I strains had the QRDR amino acid substitutions in GyrA, GyrB and ParC, whereas pulsotype II strains had those only in GyrB and ParC, which contradicted our speculation. Thus it may well be that genetically related strains of pulsotypes I and II spread separately on a ward or between two wards. PRGBS strains 4-1 and 4-2 sharing pulsotype I were detected from a patient over a 2 month interval, suggesting those strains might colonize persistently, as has been observed previously.[18]

In eight PRGBS strains of pulsotypes I and III with levofloxacin MICs >8 mg/L, E476K in GyrB was newly detected, together with two substitutions S81L in GyrA and S79Y in ParC, which have been found to be involved with high-level fluoroquinolone resistance. The remaining two strains of pulsotype II for which levofloxacin MICs were 8 mg/L also had E476K in GyrB and S79Y in ParC, but had no substitutions in GyrA. To the best of our knowledge, although amino acid substitutions in GyrB have not been reported in GBS, E476K, which corresponds to the E474K substitution in *S. pneumoniae*, may possibly contribute to fluoroquinolone resistance, as has been suggested for *S. pneumoniae*.[26]

Clindamycin can be used as intrapartum GBS prophylaxis for penicillin-allergic GBS carriers, as has been endorsed by the CDC.[27] All PRGBS strains in this study showing consistent resistance to macrolides and lincosamides harboured *erm*(B) genes that confer high resistance levels to these agents. This finding enhances the need for monitoring of GBS strains multiresistant to macrolides and lincosamides. Those strains were also resistant to tetracycline, and erythromycin resistance genes have sometimes been found on the mobile genetic elements encoding tetracycline resistance genes.[28,29]

The nucleotide sequences of the coding regions of *pbp2x*, *pbp2b* and *pbp1a* genes were completely identical in all 10 PRGBS, although the strains were divided into three genetically related PFGE types. Five amino acid substitutions, including a key substitution (V405A) identified in PBP 2X, have been found to be unique to PRGBS.[1,2] In PBP 2B, one amino acid substitution that is also unique to PRGBS was found. It is of interest that those sequences found in *pbp2x*, *pbp2b* and *pbp1a* genes were 100% identical to the corresponding sequences of the PRGBS strain R5, which has been reported previously.[2] Moreover, the deduced amino acid sequences of PBPs 2X, 2B and 1A were completely identical to the corresponding sequences of the previously described PRGBS strains R1, R2, R5 and R6, which has one additional substitution in PBP 2X. Ten PRGBS strains characterized in the present study and PRGBS strains R1, R2, R5 and R6, which have been found to form one of the three distinct lineages of the *pbp* genes in our previous study,[2] were all serotype VI and had closely related PFGE profiles. MLST analysis

showed that all 10 PRGBS were ST458, which is a single-locus variant of ST1 belonging to CC1. Interestingly, PRGBS strains R1, R2, R5 and R6 were serotype VI and were also assigned to ST458, which has been reported to be the predominant ST among PRGBS in Japan.[30] Thus the spread of a clonal serotype VI PRGBS population of ST458 has occurred in geographically separate areas, while acquiring resistance to macrolides, lincosamides and fluoroquinolones over time. ST458, which has been assigned as a new ST to our PRGBS strains, has not been detected among PRGBS strains from other countries.[4,6] The serotype VI PRGBS with ST458 may well be defined as the Japan clone.

EUCAST (http://www.eucast.org/clinical_breakpoints/) has established a clinical breakpoint for penicillin and *Streptococcus* groups A, B, C and G, including the resistance criteria of penicillin MIC >0.25 mg/L. However, EUCAST notes that the strains with MIC values above the epidemiological cut-off value (0.25 mg/L) are very rare or not yet reported, and they should be reported as 'resistant' until there is evidence regarding clinical response for confirmed isolates with MIC above the current resistant breakpoint. According to CLSI M100-S21,[20] only susceptible interpretive criteria are available for penicillin and β-haemolytic streptococci, with several comments, e.g. non-susceptible isolates with penicillin MICs >0.12 mg/L and ampicillin MICs >0.25 mg/L are extremely rare in β-haemolytic streptococci and have not been reported for group A *Streptococcus*. CLSI also states that susceptibility testing of penicillins and other β-lactams for the treatment of β-haemolytic streptococcal infections need not be performed routinely. However, in Canada, the development of penicillin non-susceptibility in GBS has been described *in vivo* in adult patients by acquiring amino acid substitutions in PBPs during prolonged administration of penicillin V.[5,6] These findings underscore the necessity of routinely monitoring the levels of penicillin MICs, especially when penicillins or cephalosporins are prescribed to a patient over a long period. Moreover, in the present study we have demonstrated probable horizontal transmission of PRGBS strains among patients, leading to their nosocomial spread for at least 5 months. The clinical significance of PRGBS isolates in antimicrobial chemotherapy and also in intrapartum prophylaxis remains unclear. Thus investigation and discussion should be encouraged to predict the therapeutic effect of penicillin therapy, especially for more invasive GBS infections such as meningitis due to PRGBS.

Our findings extend the knowledge about PRGBS with regard to more serious therapeutic and prophylactic problems posed by the possible future prevalence of multidrug-resistant genotypes of PRGBS, together with their ability to spread and survive in hospital environments. The emergence of multidrug-resistant PRGBS is a concern regarding future global spread, as we have experienced with multidrug-resistant *S. pneumoniae*,[31,32] and again emphasizes the need for careful epidemiological monitoring of GBS strains to assess the current prevalence status of PRGBS as well as their multidrug-resistant genotypes.

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Transparency declarations

None to declare.

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Exogenously acquired 16S rRNA methyltransferases found in aminoglycoside-resistant pathogenic Gram-negative bacteria: An update

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ABSTRACT

Exogenously acquired 16S rRNA methyltransferase (16S-RMTase) genes responsible for a very high level of resistance against various aminoglycosides have been widely distributed among *Enterobacteriaceae* and glucose-nonfermentative microbes recovered from human and animal. The 16S-RMTases are classified into two subgroups, N7-G1405 16S-RMTases and N1-A1408 16S-RMTases, based on the mode of modification of 16S rRNA. Both MTases add the methyl group of *S*-adenosyl-L-methionine (SAM) to the specific nucleotides at the A-site of 16S rRNA, which interferes with aminoglycoside binding to the target. The genetic determinants responsible for 16S-RMTase production are often mediated by mobile genetic elements like transposons and further embedded into transferable plasmids or chromosome. This genetic apparatus may thus contribute to the rapid worldwide dissemination of the resistance mechanism among pathogenic microbes. More worrisome is the fact that 16S-RMTase genes are frequently associated with other antimicrobial resistance mechanisms such as NDM-1 metallo-β-lactamase and CTX-M-type ESBLs, and some highly pathogenic microbes including *Salmonella* spp. have already acquired these genes. Thus far, 16S-RMTases have been reported from at least 30 countries or regions. The worldwide dissemination of 16S-RMTases is becoming a serious global concern and this implies the necessity to continue investigations on the trend of 16S-RMTases to restrict their further worldwide dissemination.

1. Outline of aminoglycosides and related issues

Aminoglycoside antibiotics such as amikacin and gentamicin, often along with β-lactams, have been used for the treatment of serious infections caused by Gram-negative and Gram-positive pathogenic bacteria in clinical settings. In livestock-farming settings, aminoglycosides play a crucial role not only in the treatment of bacterial infections, but also in growth promotion. Aminoglycosides are classified into several groups as 4,6-disubstituted 2-deoxystreptamine (DOS), 4,5-disubstituted DOS, and monosubstituted DOS, on the basis of the difference in their chemical structures (Fig. 1). Most aminoglycosides bind to the decoding aminoacyl-tRNA recognition site (A-site) of the 16S rRNA that composes bacterial 30S ribosome, and subsequently interfere with bacterial growth through blocking of protein synthesis (Fig. 2A and B) (Magnet and Blanchard, 2005). On the other hand, bacteria have been furnished with various resistance mechanisms to cope with aminoglycosides (Poole, 2005). Like other antimicrobial resistance mechanisms, the modes of aminoglycoside resistance in bacteria are divided into (i) enzymatic modification/inactivation of aminoglycosides, (ii) mutation or modification of aminoglycoside-binding site in target molecule, (iii) decreased permeability of aminoglycosides across bacterial membranes, and (iv) augmented efflux of aminoglycosides from cytosol to outside. Among them, the most prevalent and clinically relevant mechanism of aminoglycoside resistance in both Gram-negative and Gram-positive bacteria is inactivation of the agents by aminoglycoside-modifying enzyme (Ramirez and Tolmasky, 2010). Based on their molecular mechanisms, the aminoglycoside-modifying enzymes are further divided into 3 groups, acetyltransferases, nucleotidyltransferases, and phosphotransferases (Ramirez and Tolmasky, 2010). Pathogenic bacteria have acquired these aminoglycoside-modifying enzymes via transferable plasmids carrying bacteria-specific DNA recombination systems like transposons and integrons (Partridge et al., 2009; Tolmasky and Crosa, 1987).

Actinomycetes such as *Streptomyces* spp. and *Micromonospora* spp. are the natural producers of aminoglycosides. These aminoglycoside-producing *actinomycetes* are inherently resistant to aminoglycosides, because they harbor intrinsic 16S rRNA methyltransferase (16S-RMTase) genes, that can confer aminoglycoside resistance to bacteria by modifying specific nucleotide residues in the aminoglycoside binding site of 16S rRNA

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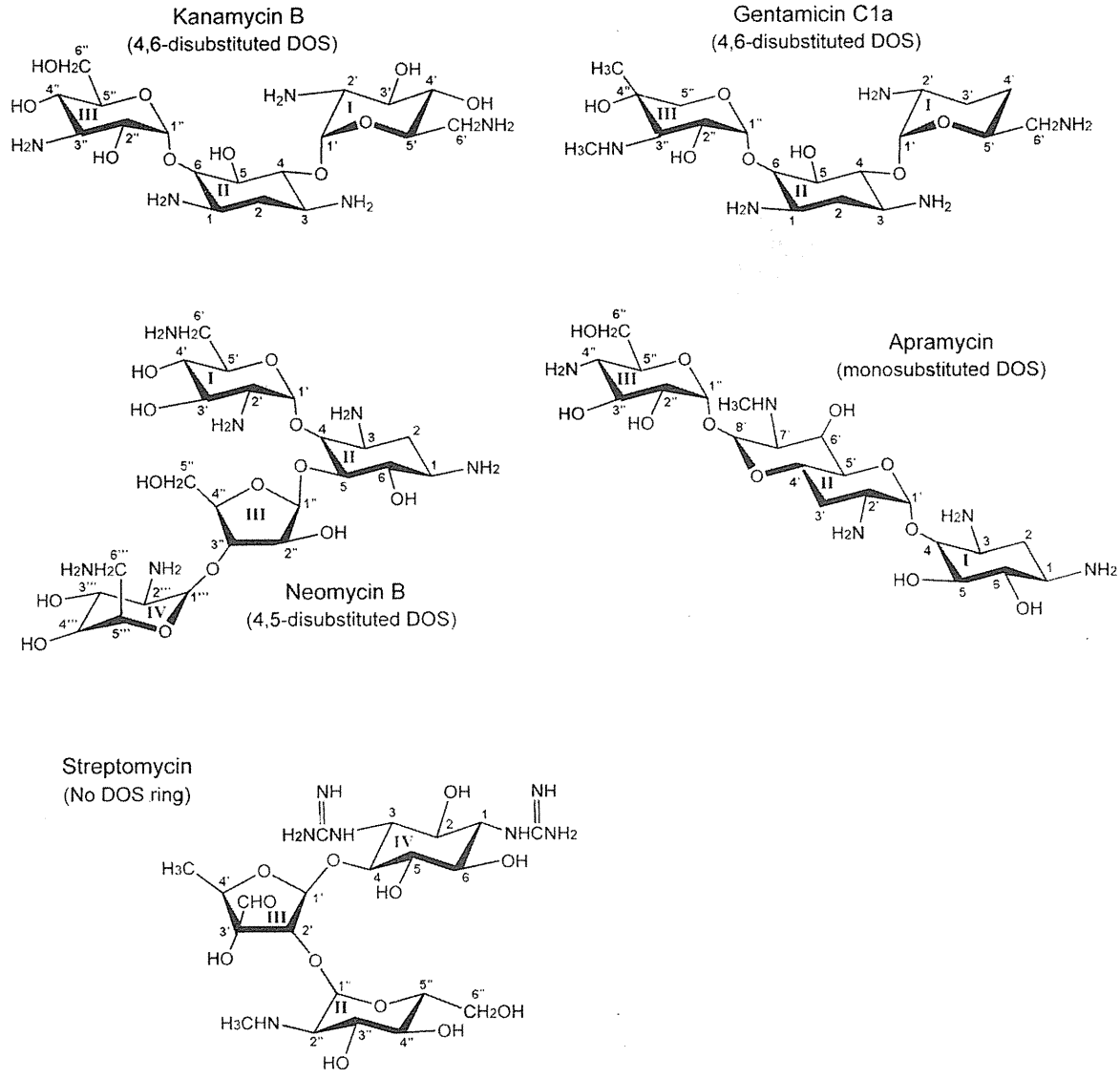

Fig. 1. Chemical structures of major aminoglycosides. Many clinically important aminoglycosides usually belong to the group of 4,6-disubstituted 2-deoxystreptamine (DOS). Among the 4,6-disubstituted DOS, kanamycin, amikacin, tobramycin, and arbekacin are classified into kanamycin group, and gentamicin, sisomicin, and isepamicin are the members of gentamicin group.

(Fig. 2B) (Cundliffe, 1989). Although the aminoglycoside resistance mechanisms through 16S rRNA protection are inherent in aminoglycoside-producing *actinomycetes*, this trait has not been found among the pathogenic bacterial species that cause infectious diseases in human or animal.

However, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* clinical isolates that show high-level resistance to clinically useful aminoglycosides through the production of acquired 16S-RMTases were identified in France and Japan, respectively, in 2003 (Galimand et al., 2003; Yokoyama et al., 2003). These 16S-RMTase genes were mostly located on transferable plasmids, and could be easily transferred to other bacterial species. After these reports, the number of 16S-RMTase-producing Gram-negative bacteria with virulence potential isolated from human and livestock have gradually increased (Doi and Arakawa, 2007). In recent years, the global spread of 16S-RMTase producers has been a concern in association with the rapid worldwide dissemination of the members of *Enterobacteriaceae* that produce NDM-1 metallo-β-lactamase (MBL), because these two enzymes are often coproduced (Mushtaq et al., 2011; Poirel et al., 2011b). Now, global dissemination of the pathogenic microorganisms exhibiting a multidrug-resistant nature by coproduction of NDM-1 MBL and 16S-RMTase, is becoming a serious threat to human health. This review describes the genetic and biochemical characteristics of the 16S-RMTases responsible for aminoglycoside resistance, with focus on those found in pathogenic Gram-negative bacteria, their epidemiology, as well as the practical screening method for early identification of 16S-RMTase producers.

2. Intrinsic 16S-RMTases of aminoglycoside-producing *actinomycetes*

2.1. Aminoglycoside-producing actinomycetes

In the 1980s, a number of aminoglycoside-producing *actinomycetes* like *Streptomyces* and *Micromonospora* species were found to harbor specific 16S-RMTase genes to protect themselves from their own intrinsic aminoglycosides (Fig. 3) (Cundliffe, 1989). Thereby, these aminoglycoside-producing *actinomycetes*