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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.

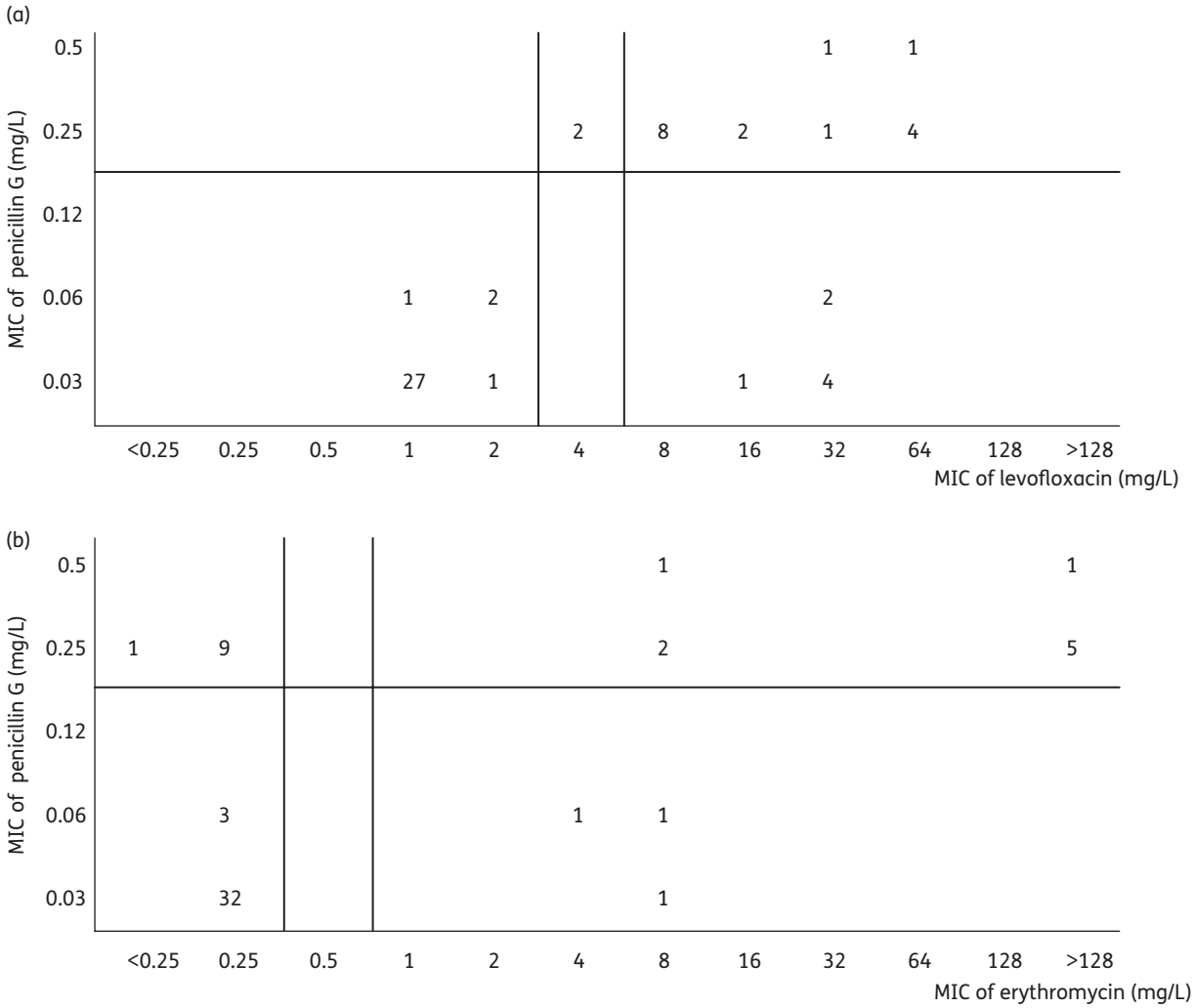

Figure 1. Scatter diagram depicting the relationship between reduced penicillin susceptibility and levofloxacin (FQ) non-susceptibility (a) and erythromycin (macrolide) resistance (b). The numbers at each intersection indicate the number of isolates. The vertical and horizontal lines in the scatter diagrams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