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Classification of group B streptococci with reduced β-lactam susceptibility (GBS-RBS) based on the amino acid substitutions in PBPs

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All clinical isolates of group B *Streptococcus* (GBS; *Streptococcus agalactiae*) are considered uniformly susceptible to β-lactams, including penicillins. However, GBS with reduced penicillin susceptibility (PRGBS) were first identified by our group in Japan and have also been reported from North America. PRGBS are non-susceptible to penicillin because of acquisition of amino acid substitutions near the conserved active-site motifs in PBP2X. In particular, V405A and Q557E are considered the key amino acid substitutions responsible for penicillin non-susceptibility. We revealed that in addition to the substitutions in PBP2X, an amino acid substitution in PBP1A confers high-level cephalosporin resistance in GBS. As the number of publications on GBS with reduced β-lactam susceptibility (GBS-RBS), especially PRGBS, and concomitantly the need for a systematic classification of GBS-RBS is increasing, we propose here a classification of GBS-RBS based on the amino acid substitutions in their PBPs.

Keywords: *Streptococcus agalactiae*, non-susceptibility, group B streptococci with reduced penicillin susceptibility, PRGBS

Introduction

Group B *Streptococcus* (GBS; *Streptococcus agalactiae*) is the major cause of neonatal sepsis and meningitis; it also causes invasive infections in the elderly and in people with underlying illness.[1-3] At present, no approved vaccine to prevent GBS infections is available. Clinical isolates of GBS continue to be widely regarded as susceptible to β-lactams, with penicillin commonly being a first-line option for the treatment or prevention of GBS infections. However, we have identified GBS with reduced penicillin susceptibility (PRGBS) in Japan and these have also been reported from North America.[4-16] PRGBS show non-susceptibility to penicillin due to the acquisition of amino acid substitutions near the active-site motifs in the transpeptidase domain of PBP2X.[4-6] In particular, two amino acid substitutions in PBP2X, V405A and/or Q557E, are considered the key substitutions accounting for penicillin non-susceptibility.[4] Moreover, we have shown that in addition to substitutions in PBP2X, an amino acid substitution in PBP1A confers high-level resistance to cephalosporins in GBS.[13] Recently, penicillin-susceptible, but ceftibuten-resistant, GBS with amino acid substitutions in PBP2X were reported.[15] Because the number of publications on GBS with reduced β-lactam susceptibility (GBS-RBS) is increasing, a classification scheme for GBS-RBS, including PRGBS, based on the amino acid substitutions in their PBPs is very important to prevent future confusion in the nomenclature of GBS-RBS, including PRGBS.

Rationale for the classification of GBS-RBS

A substantial body of literature on β-lactam-non-susceptible clinical isolates of GBS is available. These publications are based solely on drug susceptibility tests and lack supportive molecular data on the mechanism of β-lactam non-susceptibility, e.g. sequence analysis data of the PBP genes. As phenotypic drug susceptibility tests are subjective and hence prone to potential measurement errors, we cannot rule out possible inaccuracies in the susceptibility data of β-lactam-non-susceptible clinical isolates described in these publications if PBP sequence data are not available. Moreover, as the reported penicillin MICs for PRGBS (0.25–1 mg/L)[4-6] are close to the 'susceptible' breakpoint (≤0.12 mg/L) as defined by the CLSI, international cooperation on antibiotic resistance research using a systematic approach is essential. Therefore, we propose a classification scheme for GBS-RBS clinical isolates, including PRGBS, for which the sequence data of the PBP genes are available.

Perspective on the classification of GBS-RBS

Our perspective on the classification of GBS-RBS based on amino acid substitutions in PBPs is outlined in Table 1. First, we classified GBS-RBS into classes I–IV based on the distinct PBPs that harbour amino acid substitutions. Class I contains critical amino acid substitutions in PBP2X, but not in PBP1A, 1B, 2A or 2B. Although it is difficult to define a 'critical' amino acid substitution, we considered substitutions near the active-site motifs (within five amino acids) as 'critical' amino acid substitutions, except in PBP2X. Because amino acid substitutions in PBP2X are considered the first step of β-lactam non-susceptibility, we considered all substitutions in the transpeptidase domain of PBP2X as 'critical' amino acid substitutions. Class II contains critical amino acid substitutions in both PBP2X and PBP1A, but not in PBP1B, 2A or 2B. Class

Table 1. Classification of GBS-RBS, including PRGBS, based on amino acid substitutions in their PBPs

Class	Subclass	PBP2X	PBP2B	PBP2A	PBP1B	PBP1A	Strains (reference)
I	Ia	V405A	—	—	—	—	B8[4], B502[4], B503[4], B514[4], B516[4], R1[5], R2[5], R5[5], R6[5], R9[7], no. 1–8[12], M19[14], MRY08-517[14], MRY08-528[14], MRY11-004[16], MRY11-005[16], NUBL-2449[16]
	Ib	Q557E	—	—	—	—	B6[4], B10[4], B12[4], B40[4], B60[4], B68[4], 3789-04[6], 6138-03[6], 7507-03[6], 8607-03[6], R3[5], R4[5], M16[14]
	Ic	V405A, Q557E	—	—	—	—	B513[4], MRY08-527[14], A1[15], A2[15]
	Iz	other	—	—	—	—	B7[4], MRY08-1422[14], one clinical isolate[8], GBS2007[9], B1-6[15]
II	IIa	V405A	—	—	—	yes	
	IIb	Q557E	—	—	—	yes	B1[4,13]
	IIc	V405A, Q557E	—	—	—	yes	
	IIz	other	—	—	—	yes	
III	IIIa	V405A	yes	—	—	—	
	IIIb	Q557E	yes	—	—	—	R7[5], R8[5]
	IIIc	V405A, Q557E	yes	—	—	—	
	IIIz	other	yes	—	—	—	
IV	IVa	V405A	—	yes	—	yes	
	IVb	Q557E	—	yes	—	yes	
	IVc	V405A, Q557E	—	yes	—	yes	
	IVz	other	—	yes	—	yes	2009 isolate[10]

'Yes', the PBP harbours a critical mutation; '—', the PBP does not harbour a critical amino acid substitution; V405A, PBP2X contains the V405A substitution close to the $_{402}$SSN$_{404}$ motif; Q557E, PBP2X contains the Q557E substitution close to the $_{552}$KSG$_{554}$ motif; V405A, Q557E, PBP2X contains both the V405A and the Q557E substitutions; other, PBP2X contains neither the V405A substitution nor the Q557E substitution, but it contains novel potentially critical amino acid substitutions. We considered substitutions near the active-site motifs (within five amino acids) in the transpeptidase domain as 'critical' amino acid substitutions, except in PBP2X. Because amino acid substitutions in PBP2X are considered the first step of β-lactam non-susceptibility, we considered all substitutions in the transpeptidase domain of PBP2X as 'critical' amino acid substitutions.

III harbours critical substitutions in both PBP2X and PBP2B, but not in PBP1A, 1B or 2A. Class IV harbours critical substitutions in PBP2X, PBP2A and 1A, but not in PBP1B or 2B. If other substitutions are reported in the future, new classes can be added, e.g. class V, class VI etc.

In addition, we divided each class into four subclasses, based on the key amino acid substitutions in PBP2X. Subclass 'a' of each class harbours the V405A substitution close to the $_{402}$SSN$_{404}$ motif in PBP2X. Subclass 'b' contains the Q557E substitution close to the $_{552}$KSG$_{554}$ motif. Subclass 'c' harbours both the V405A and Q557E substitutions. Subclass 'z' harbours neither V405A nor Q557E substitutions, but it harbours novel potentially critical amino acid substitutions in PBP2X. If other key amino acid substitutions in PBP2X are reported in the future or if the substitutions in PBP2X now classified under subclass 'z' are experimentally confirmed to be critical, novel subclasses can be added, e.g. subclass 'd', subclass 'e' etc.

Finally, we propose to add an asterisk to each class for which the amino acid substitutions in the PBPs have been proven critical by using molecular genetic techniques. For example, clinical isolate B1 belongs to IIb* because amino acid substitutions in PBP2X and PBP1A were shown to be critical by using allelic exchange experiments.[13]

Prospect of the classification of GBS-RBS

At present, the number of publications on the PBPs of GBS-RBS is increasing, owing to the ongoing emergence and discovery of novel β-lactam-non-susceptible clinical isolates. Concomitantly, the number of reported distinct amino acid substitution patterns in the PBPs will likely continue to increase. Therefore, we propose a classification scheme that is easily expandable with the increasing identifications of critical substitutions in PBPs.

For some β-lactam-non-susceptible clinical isolates, PBP gene sequence data are unfortunately missing. We recommend performing sequence analysis of the PBP genes for all β-lactam-non-susceptible clinical isolates of GBS. Furthermore, when novel putative key amino acid substitutions in the PBPs are found, we recommend molecular genetic analyses including allelic exchange in order to reveal the importance of each amino acid substitution in the development of non-susceptibility to β-lactams.

We hope that this classification scheme for GBS-RBS will be adopted and expanded and that it will aid in predicting their β-lactam susceptibility profiles.

Transparency declarations

We have no conflicts of interest to declare.

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Comparative analysis of penicillin-susceptible and non-susceptible isolates in group B streptococci by multilocus sequence typing

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Short Communication

Comparative Analysis of Penicillin-Susceptible and Non-Susceptible Isolates in Group B Streptococci by Multilocus Sequence Typing

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Keywords

Group B *Streptococcus*, *Streptococcus agalactiae*, multilocus sequence typing, Group B *Streptococcus* with reduced penicillin susceptibility, Penicillin-susceptible GBS

Running title: MLST of PRGBS and PSGBS

SUMMARY: Since Group B *Streptococcus* (GBS, *Streptococcus agalactiae*) clinical isolates were believed to be uniformly susceptible to β-lactams, penicillin G has been the first-line agent for the prevention and treatment of GBS infections. However, Kimura et al. recently reported the existence and characteristics of GBS isolates with reduced penicillin susceptibility (PRGBS) in Japan. Sequence type (ST) 458 is predominant among PRGBS in Japan. However, although most PRGBS isolates in Japan have been recovered from respiratory specimens from adults, no information about genotype is available concerning GBS isolates from such specimens. Therefore, whether ST458 predominates among GBS isolates obtained from such specimens is not known. We characterised the STs of 38 GBS isolates with penicillin susceptibility (PSGBS) recovered from respiratory specimens and compared them to PRGBS STs.

ST458, the predominant ST of PRGBS (10/19, 53%), was not found in PSGBS. Thirty-six PSGBS isolates belonged to the ST1/19/10 group (6 different STs); the remaining 2 isolates belonged to ST23. PRGBS were divided between the ST1 (3 STs) and ST23 groups (2 STs).

ST458 was not predominant among PSGBS recovered from respiratory specimens in Japan and may be specific to PRGBS. The ST distribution of PRGBS does not merely reflect that of PSGBS.

Main Text

Group B *Streptococcus* (GBS, *Streptococcus agalactiae*) is often isolated from the digestive or lower genital tract and is an important pathogen. GBS is the main cause of sepsis and meningitis in neonates. It is also a cause of serious infections in pregnant women, the elderly, and people with underlying disease (1, 2). Since GBS clinical isolates were believed to be uniformly susceptible to β-lactams, penicillin G has been the first-line agent for the prevention and treatment of GBS infections. However, Kimura et al. recently reported the existence and characteristics of GBS isolates with reduced penicillin susceptibility (PRGBS) in Japan (3). PRGBS was later reported in the US and Canada (4-6). We had previously reported that PRGBS tends to be resistant to other drugs, fluoroquinolones and macrolides (7), and that a clinical isolate of PRGBS became highly cephalosporin resistant through the acquisition of amino acid substitutions in PBP1A and PBP2X (8). Therefore, PRGBS may become a significant public health concern.

Multilocus sequence typing (MLST) analyses of PRGBS isolates in Japan and the US have been reported. According to these reports, the 28 strains of PRGBS found in Japan are divided into 7 sequence types. Eleven (39%) belonged to ST458, which was newly identified in that study (9). The ST1 group ("ST1 group" includes ST1 and STs similar to ST1), made up of 5 different STs including ST458, was predominant (23/28, 82%) (9). Four PRGBS isolates recovered in the US belonged to the same ST (ST19) (4). Although PRGBS isolates recovered from a sacral decubitus ulcer have been reported elsewhere (10), most PRGBS isolates in Japan were recovered from adult respiratory specimens (9). However, MLST data for GBS isolates with penicillin susceptibility (PSGBS) isolated from adult respiratory specimens are quite limited (11) and no information is available on GBS isolates from adult respiratory specimens in Japan. Therefore, we determined the STs of 38 PSGBS recovered from independent adult sputum samples in Japan and compared them to the reported STs of PRGBSs to deduce the process of PRGBS development.

We selected 19 PRGBS (patient age: 20-64 years, 4 isolates, ≧65 years, 15 isolates) and 38 PSGBS isolates (patient age: 20-64 years, 12 isolates, ≧65 years, 24 isolates) recovered during

2001–2008 from various Japanese medical institutions. The isolates were recovered mainly from respiratory specimens.

MICs of penicillin G were determined by the agar dilution method using *Streptococcus pneumoniae* ATCC 49619 as the quality control, as recommended by the Clinical and Laboratory Standards Institute (CLSI) (12).

STs of 38 PSGBS were determined as described (9). Chromosomal DNA was extracted using the Wizard genomic DNA purification kit (Promega) with mutanolysin, and MLST was performed as described (13). Seven housekeeping genes (*adhP, pheS, atr, glnA, sdhA, glcK,* and *tkt*) were PCR-amplified with the high fidelity PrimeSTAR HS DNA polymerase (Takara), followed by amplicon purification using the Wizard SV gel and the PCR clean-up system (Promega). The nucleotide sequences were determined using BigDye Terminator V3.1 on the Applied Biosystems 3130xl and 3730xl instruments. One allele type was assigned to each sequence according to the MLST database for *S. agalactiae* (http://pubmlst.org/sagalactiae/). STs were identified by combining 7 allele types, and linkages were analysed by eBURST V3 (http://eburst.mlst.net/).

We selected 19 PRGBS from 28 previously analysed PRGBS (9, 14), in order to match the isolation years to the 38 PSGBS analysed in this study. ST458 was the most common one (10/19, 53%), followed by ST1 (5/19, 26%) (Fig. 1A). All the 38 PSGBS were susceptible to PCG (MIC range: 0.03 to 0.06 μg/mL). The PSGBS STs are shown in Fig. 1B. ST458, the predominant ST in PRGBS, was not found at all in PSGBS tested in the present investigation. ST1 was most common (25/38, 66%). The frequencies of ST19, ST10, and ST23 were 4 (11%), 4 (11%) and 2 (5%), respectively. ST12, ST153, and ST573 were identified only once, and ST573 was newly identified in this study.

According to the eBURST analysis, 6 PSGBS STs (ST1, ST153, ST19, ST10, ST12, and ST573) have genetic connections, as described in Figure 2. Therefore, these STs formed the ST1/19/10 group. ST153 differed from ST1 by a single allele, and ST12 and ST573 differed from ST10 by a single allele. ST23 was one of the STs belonging to the ST23 group. The PRGBS STs were also divided into 2 groups, ST1 group (ST458, ST1, and ST358) and ST23 group (ST23 and ST464).

Figure 1 shows the STs of 38 PSGBS isolates and 19 PRGBS isolates. PRGBS in Japan can be

classified into at least 2 groups: ST1 (16/19, 84%) and ST23 (3/19, 16%). In the present study, the PSGBS STs were divided into the ST1/19/10 (36/38, 95%) and ST23 (2/38, 5%) groups.

This investigation would demonstrate that the ST distribution of PRGBS is not merely reflecting the population of STs among PSGBS isolated from respiratory specimens of adults in Japan. This speculation would also imply that ST458 may be a specific lineage to PRGBS.

There is not sufficient data to conclude the specificity of ST458 with respect to PRGBS. However, in this investigation, we eliminated the possibility that ST458 is predominant among the PSGBS isolated from the respiratory specimens obtained from adults in Japan and the ST distribution of PRGBS merely reflects that of PSGBS. PRGBS tends to be multidrug-resistant (7) and a clinical isolate of GBS became highly resistant to cephalosporin through amino acid substitutions in 2 PBPs (8). The nosocomial spread of multidrug-resistant PRGBSs belonging to ST458 has been reported (15). At the present, All STs of PRGBS belong to ST1/19/10 group or ST23 group and there is no report that PRGBS clinical isolates belong to ST17 group, which are often isolated from neonatal meningitides. Because PRGBS may become future public health concerns, a greater deal of and more advanced attention should be focused on monitoring and researching PRGBS.

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Conflict of interest None to declare

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Figure legends

Fig. 1. Stacked bar graphs of PRGBS and PSGBS STs. Graphs A and B show the STs of PRGBS and PSGBS, respectively.

Fig. 2. eBURST analysis of STs of GBS. Numbers stand for STs, and neighbouring STs connected by a line differ at 1 allele. STs marked with an asterisk were identified previously in PRGBS in Japan. STs surrounded by squares were found in PSGBS in this study. A shows ST1/19/10 group and B shows ST23 group. STs in the figure were picked randomly from all STs.

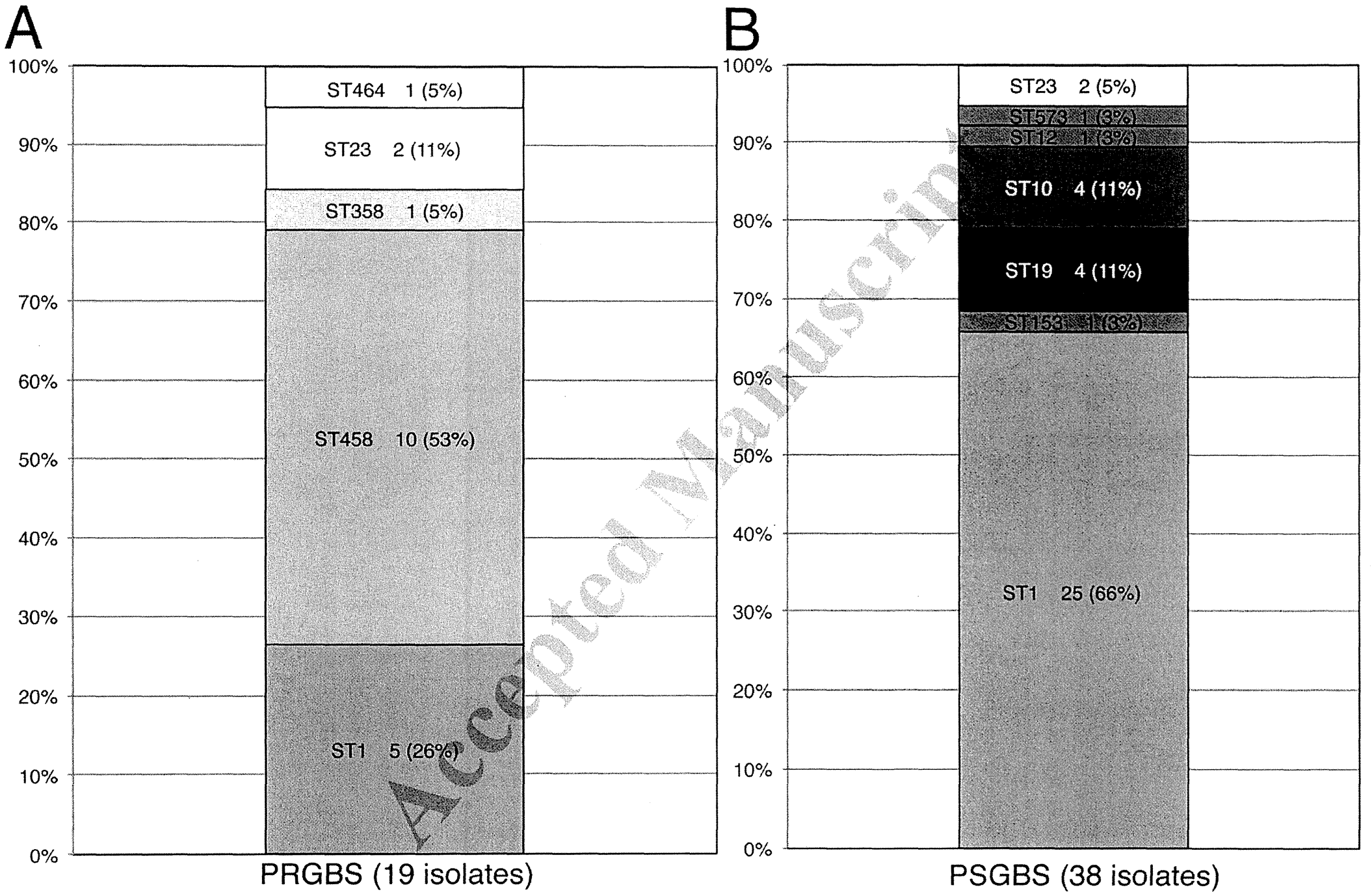

Figure 1

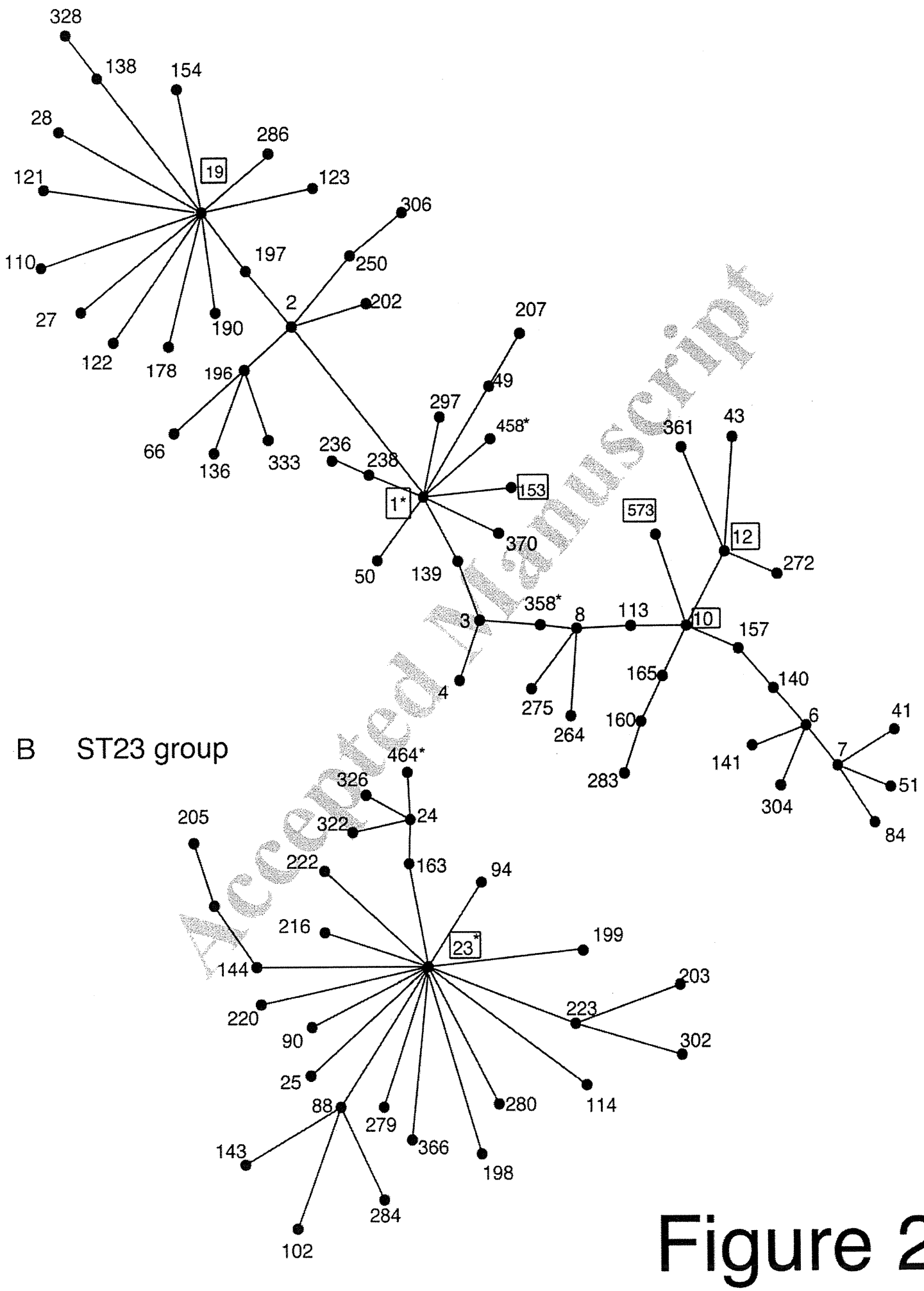

Figure 2

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New plasmid-mediated aminoglycoside 6′-*N*-acetyltransferase, AAC(6′)-Ian, and ESBL, TLA-3, from a *Serratia marcescens* clinical isolate

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Objectives: Enterobacteriaceae clinical isolates showing amikacin resistance (MIC 64 to >256 mg/L) in the absence of 16S rRNA methyltransferase (MTase) genes were found. The aim of this study was to clarify the molecular mechanisms underlying amikacin resistance in Enterobacteriaceae clinical isolates that do not produce 16S rRNA MTases.

Methods: PCR was performed to detect already-known amikacin resistance determinants. Cloning experiments and sequence analyses were performed to characterize unknown amikacin resistance determinants. Transfer of amikacin resistance determinants was performed by conjugation and transformation. The complete nucleotide sequence of the plasmids was determined by next-generation sequencing technology. Amikacin resistance enzymes were purified with a column chromatography system. The enzymatic function of the purified protein was investigated by thin-layer chromatography (TLC) and HPLC.

Results: Among the 14 isolates, 9 were found to carry already-known amikacin resistance determinants such as *aac(6′)-Ia* and *aac(6′)-Ib*. Genetic analyses revealed the presence of a new amikacin acetyltransferase gene, named *aac(6′)-Ian*, located on a 169 829 bp transferable plasmid (p11663) of the *Serratia marcescens* strain NUBL-11663, one of the five strains negative for known *aac(6′)* genes by PCR. Plasmid p11663 also carried a novel ESBL gene, named bla_{TLA-3}. HPLC and TLC analyses demonstrated that AAC(6′)-Ian catalysed the transfer of an acetyl group from acetyl coenzyme A onto an amine at the 6′-position of various aminoglycosides.

Conclusions: We identified *aac(6′)-Ian* as a novel amikacin resistance determinant together with a new ESBL gene, bla_{TLA-3}, on a transferable plasmid of a *S. marcescens* clinical isolate.

Keywords: amikacin resistance, *S. marcescens*, antibiotic resistance genes

Introduction

Aminoglycosides have been widely used for the treatment of bacterial infections caused by Gram-negative and Gram-positive bacteria in combination with β-lactams.[1] However, bacteria are known to acquire various mechanisms of resistance to aminoglycosides.[2] In Enterobacteriaceae, the acquisition of plasmid-mediated 16S rRNA methyltransferase (MTase) genes such as *armA*, *rmtB* and *rmtC* has been reported worldwide and is becoming a major clinical concern because these MTases confer a high level of resistance to clinically important aminoglycosides including amikacin.[3]

We previously reported that 16S rRNA MTase-producing Enterobacteriaceae showing a very high level of amikacin resistance (MIC ≥256 mg/L) have already spread in Japanese clinical settings, although the prevalence is very low.[3,4] This survey led us to realize that the high amikacin resistance (MIC ≥256 mg/L) in Enterobacteriaceae mostly depends on 16S rRNA MTase production and at the same time raises the question of what resistance determinant is involved in amikacin resistance in Enterobacteriaceae strains without 16S rRNA MTase genes.

Aminoglycoside 6′-*N*-acetyltransferases, AAC(6′), which acetylate the amino group at the 6′-position of aminoglycosides, and aminoglycoside 3′-*O*-phosphotransferases, APH(3′), which phosphorylate the hydroxyl group at the 3′-position of aminoglycosides, are known to underlie amikacin resistance by disrupting the ability of aminoglycosides to bind to target 16S rRNA molecules.[5,6] Several new AAC(6′) enzymes, such as AAC(6′)-Iad,[7] AAC(6′)-Iae,[8] AAC(6′)-Iaf,[9] AAC(6′)-Iaj[10] and AAC(6′)-Iag,[11] have been reported exclusively in amikacin-resistant non-fermenting Gram-negative pathogens over the last 10 years in Japan, but it remains unclear whether these AAC(6′) enzymes are involved in amikacin resistance in Enterobacteriaceae. The aim of this study was thus to clarify the molecular mechanism underlying amikacin