

Heterogeneity of penicillin-non-susceptible group B streptococci isolated from a single patient in Germany

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Received 10 September 2019; returned 25 September 2019; revised 10 October 2019; accepted 11 October 2019

Objectives: *Streptococcus agalactiae* [group B streptococci (GBS)] have been considered uniformly susceptible to penicillin. However, increasing reports from Asia and North America are documenting penicillin-non-susceptible GBS (PRGBS) with mutations in *pbp* genes. Here we report, to the best of our knowledge, the first two PRGBS isolates recovered in Europe (AC-13238-1 and AC-13238-2), isolated from the same patient.

Methods: Two different colony morphologies of GBS were noted from a surgical abscess drainage sample. Both were serotyped and antimicrobial susceptibility testing was performed by different methodologies. High-throughput sequencing was done to compare the isolates at the genomic level, to identify their capsular type and ST, to evaluate mutations in the *pbp* genes and to compare the isolates with the genomes of other PRGBS isolates sharing the same serotype and ST.

Results: Isolates AC-13238-1 and AC-13238-2 presented MICs above the EUCAST and CLSI breakpoints for penicillin susceptibility. Both shared the capsular type Ia operon and ST23. Genomic analysis uncovered differences between the two isolates in seven genes, including altered *pbp* genes. Deduced amino acid sequences revealed critical substitutions in PBP2X in both isolates. Comparison with serotype Ia clonal complex 23 PRGBS from the USA reinforced the similarity between AC-13238-1 and AC-13238-2, and their divergence from the US strains.

Conclusions: Our results support the in-host evolution of β -lactam-resistant GBS, with two PRGBS variants being isolated from one patient.

Introduction

Streptococcus agalactiae [group B streptococci (GBS)] are widely recognized pathogens of the neonatal period, increasingly associated with invasive disease in adults, particularly among the elderly and those with underlying conditions.¹ While GBS have been considered universally susceptible to β -lactams, with penicillin being the first-line drug for both prevention and treatment of GBS infections,² penicillin-non-susceptible GBS (PRGBS) clinical isolates have been reported for over a decade. The first description from Japan included isolates recovered as early as 1995 that were non-susceptible to penicillin and cephalosporins through the acquisition of point mutations in *pbp* genes, leading to the production of proteins with low-affinity for β -lactam binding.³ Allelic exchange experiments revealed the critical role

of the acquisition of two substitutions (V405A and/or Q557E) adjacent to the conserved active-site motifs in the transpeptidase domain of PBP2X,³ but numerous substitutions have been found among PRGBS in PBP1A, PBP2A and PBP2B.^{3–11} While multiple studies from Japan^{7,8,12} initially suggested a geographically confined distribution of PRGBS, more recent reports from other countries including Canada,^{4,9} Korea⁵ and the USA⁶ support a wider dissemination of these isolates. In Canada, two studies have demonstrated the acquisition of PBP mutations by β -lactam-susceptible GBS after prolonged administration of penicillin resulting in PRGBS.^{4,9} To date there have been no reported cases of PRGBS in Europe, even though PBP mutations were found among GBS with reduced cephalosporin susceptibility in Italy.¹³

Materials and methods

Serotyping and antimicrobial susceptibility testing

Capsular serotyping was done by slide agglutination with IMMULEX STREP-B Kit (Statens Serum Institut, Copenhagen, Denmark).

Antimicrobial susceptibility testing of both isolates was performed by broth microdilution. Penicillin MIC was also determined with Etest strips (bioMérieux, Marcy-l'Étoile, France), according to the guidelines of CLSI¹⁴ and EUCAST.¹⁵ Additional testing with the automated systems WalkAway (Beckman Coulter, Brea, CA, USA) and VITEK 2 (bioMérieux) was performed. Finally, a test for PRGBS was done by standard disc diffusion with ceftibuten, oxacillin and ceftizoxime discs, as described by Kimura *et al.*¹⁶

High-throughput sequencing

The two GBS isolates (AC-13238-1, ERR3464558 and AC-13238-2, ERR3464559) were sequenced using the NextSeq system (Illumina, San Diego, CA, USA) and analysed by both mapping and *de novo* assembly approaches. The draft genomes and detailed methods can be found at <https://doi.org/10.5281/zenodo.3479195>.

Amino acid sequences were deduced from the *pbp1a*, *pbp1b*, *pbp2a*, *pbp2b* and *pbp2x* genes and compared with the penicillin-susceptible reference strain 2603V/R (GenBank accession number NC_004116) using Geneious software 8.9.1 (Biomatters, Ltd, Auckland, New Zealand).

Results and discussion

A >75-year-old male patient presented at the emergency room with progressive right hip pain. Medical history included a cement-free hip prosthesis on both sides, implanted 20 years ago. The soft parts of the right hip and the right upper leg showed no signs of inflammation besides tenderness on palpation, but blood testing revealed leucocytosis and reduced kidney function. Sonography of the right upper leg showed an echo-poor intramuscular formation in *M. vastus lateralis* and an effusion in the right hip joint. Sonographically directed puncture of the echo-poor formation showed a macroscopically sanious fluid. Surgical drainage of the abscess in the right upper leg was sent for culture. Linezolid treatment was started intra-operatively. Post-operatively the patient went to the ICU because of his critical condition.

After observation of Gram-positive streptococci and reduction of infection parameters, treatment was de-escalated to benzylpenicillin. Following improvement of the general condition of the patient, kidney function and infection parameters, the patient

was transferred to standard care, with termination of antibiotic treatment after 8 days. The patient was discharged from hospital 12 days post-operation, with restored kidney function and no infection parameters.

Two different colony morphologies were evident upon culture, one type being more haemolytic and pigmented. Both were confirmed as GBS and named isolates AC-13238-1 and AC-13238-2.

After agglutination with specific sera, AC-13238-1 was considered non-typeable and AC-13238-2 was serotype Ia. Both isolates were susceptible to erythromycin, clindamycin, levofloxacin, linezolid and vancomycin, and resistant to tetracycline (Table S1, available as [Supplementary data](#) at JAC Online). They presented, however, different MICs of penicillin depending on the method used to test susceptibility (Table 1). AC-13238-1 and AC-13238-2 were non-susceptible to penicillin according to broth microdilution and Etest (presenting MICs of 0.5 and 1 mg/L, respectively), following the CLSI or EUCAST methods and interpretative criteria. Both isolates presented reduced diameters of growth-inhibitory zones with oxacillin, ceftizoxime and ceftibuten discs. Additional antimicrobial susceptibility testing with commercial automated systems revealed lower MICs by up to two dilutions with WalkAway and failed to produce results in VITEK 2. This is in agreement with a study from Japan in which MICs determined by broth microdilution and VITEK 2 were significantly different, with VITEK 2 failing to detect almost half the cases of PRGBS,¹⁷ raising the possibility of missed cases of PRGBS in laboratories in which automated systems are routinely used. MICs of other β -lactams were also elevated (Table S1).

The genomes of AC-13238-1 and AC-13238-2 were 1 970 769 bp and 1 970 815 bp long, respectively. Genomic analysis confirmed that both contained the genes encoding the Eps surface protein, pilus island 2a, the type Ia capsular polysaccharide (CPS) loci and shared the same MLST-based ST23. AC-13238-1 and AC-13238-2 differed from each other in seven genes (Table S2). Among these, a single nucleotide deletion at position 302 of the *neuC* gene of AC-13238-1 resulted in a premature stop codon in the CPS locus. This gene is involved in the synthesis of sialic acid, present in all GBS serotypes. Loss of sialylation was shown to significantly reduce the amount of surface-associated CPS produced by GBS,¹⁸ so this mutation potentially impacted capsule production, explaining the observed non-reactivity of this isolate with the type Ia-specific sera. Additional differences included two non-synonymous mutations in each of the *pbp1a* and *pbp2x* genes.

Table 1. MICs, zone diameters and amino acid substitutions in PBPs found in PRGBS isolates AC-13238-1 and AC-13238-2

Isolate	PEN MIC (mg/L) ^a				Disc diffusion (mm)				Amino acid substitutions ^c		
	microdilution	Etest ^b	WalkAway	VITEK 2	PEN	OXA	CTB	CTX	PBP2X	PBP1A	PBP2B
AC-13238-1	0.5	0.5	0.12	UD	27	7	0	16	I377V, F395L, V405A , H438Y, V510I	A521V, del719–722, N723S, V726A	V80A
AC-13238-2	1	1	0.5	UD	23	0	0	15	I377V, V405A , H438Y, V510I, Q557E	T526I, del719–722, N723S, V726A	V80A

PEN, penicillin; OXA, oxacillin; CTB, ceftibuten; CTX, cefotaxime; UD, undetermined.

^aEach MIC value was obtained consistently across three independent assays.

^bSame PEN MIC determined by Etest according to EUCAST and CLSI guidelines.

^cThe amino acid positions are those of the corresponding genes in strain 2603V/R (GenBank accession number NC_004116). Substitutions strongly associated with PRGBS are shown in bold.

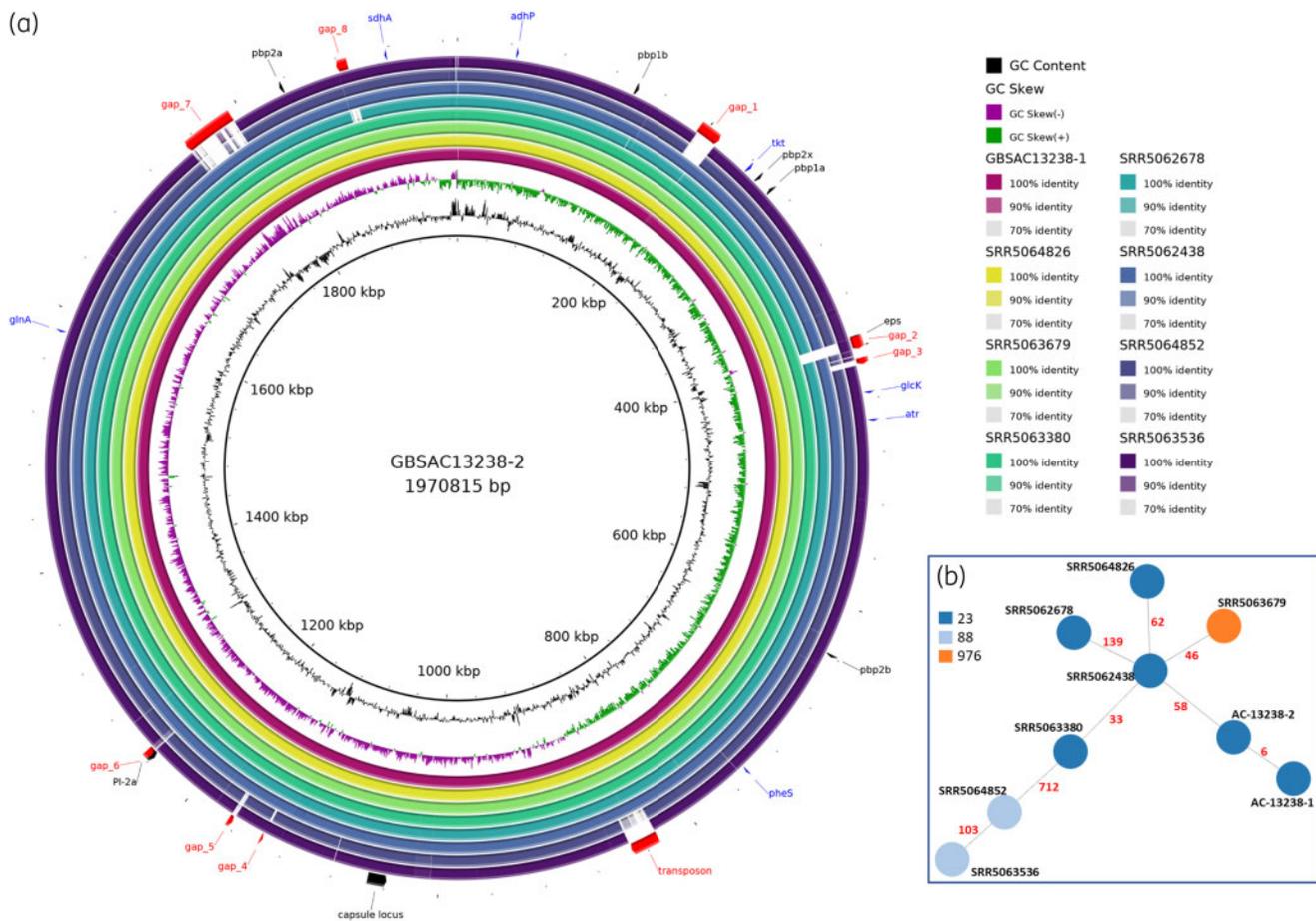


Figure 1. Genomic comparison of AC-13238-1 and AC-13238-2 with the genomes of seven serotype Ia/CC23 isolates identified in the USA carrying PBP mutations associated with decreased β -lactam susceptibility. (a) Whole-genome comparison. The innermost black ring shows the AC-13238-2 genome length (kb), used as the reference. The second and third innermost rings show GC content and GC skew, respectively. The eight outermost rings represent AC-13238-1 and the seven serotype Ia/CC23 isolates, shaded according to their respective percentage nucleotide identity to the query sequence (AC-13238-2), determined by BLAST. Several loci are represented: red, regions present in AC-13238-1 and AC-13238-2, but not in all other Ia/CC23 isolates (gaps); black, regions used to characterize the isolates (PBPs, capsular locus, epsilon surface protein gene and pilus operon); and blue, MLST genes. (b) Core-genome minimum spanning tree of serotype Ia/CC23 PRGBS (1664 genes). Colours were attributed to nodes (strains) according to their ST. Numbers of different alleles between strains are shown in red.

Comparison of the deduced amino acid sequence of the *pbp* genes of AC-13238-1 and AC-13238-2 with those of the susceptible strain 2603V/R revealed, in each isolate, the presence of five amino acid substitutions, four amino acid substitutions and one amino acid substitution in the *pbp2x*, *pbp1a* and *pbp2b* genes, respectively, relative to the reference (Table 1). Of note, both isolates shared the V405A substitution and AC-13238-2 also carried the Q557E substitution in PBP2X. These are considered key substitutions due to their proximity to the transpeptidase domain active site motifs $_{402}SSN_{404}$ and $_{552}KSG_{554}$, which, by allelic exchange experiments, were shown to contribute to a significant increase in β -lactam MIC.³ Other substitutions in PBP2X (I377V, H438Y and V510I in both isolates and F395L in AC-13238-1) were all previously described among both β -lactam-resistant and -susceptible isolates,^{6,8,10,11,13,19} suggesting that these do not contribute to the increased MICs. In PBP1A the isolates shared an N723S (already detected among non-susceptible GBS in Japan),¹¹ a V726A substitution (not previously described) and a deletion of four amino acid residues (719–722), which was shown to be present in various GBS

isolates regardless of penicillin susceptibility.¹¹ Additionally, the isolates each had one different substitution in PBP1A, not previously described: AC-13238-1 and AC-13238-2 carried an A521V and a T526I substitution, respectively, the latter in the same site as the T526A substitution, reported in a PRGBS isolated from a patient with a prosthetic hip joint infection after prolonged low-dose penicillin therapy.⁴ Finally, both isolates carried a V80A substitution in PBP2B, also previously detected in PRGBS isolates from Japan.¹¹

In Japan, most PRGBS isolates share the capsular serotype VI and belong to clonal complex (CC) 1,^{8,20} in agreement with a clonal expansion model of particularly resistant lineages. In the USA, however, decreased β -lactam susceptibility has been found across multiple serotypes and clones,⁶ including serotype Ia/CC23, suggesting that the stepwise accumulation of mutations in the *pbp* genes is occurring independently of the genetic background. We compared our two isolates with the genomes of seven serotype Ia isolates identified in the USA carrying PBP mutations associated with decreased β -lactam susceptibility,⁶ using the genome of AC-13238-2 as the reference (Figure 1). Significant

additional genetic information was found in our isolates relative to some of the Ia/CC23 isolates from the USA (Figure 1a). Comparison of the core genome, consisting of 1664 genes, showed a distance of six alleles between AC-13238-1 and AC-13238-2, and minimum and maximum distances of these to the US serotype Ia/CC23 isolates of 58 and 724 alleles, respectively (Figure 1b). These results indicate that our isolates are very similar to each other, but quite different from the other serotype Ia/CC23 PRGBS described to date. Taken together, genomic analyses support the hypothesis of in-host evolution of PRGBS, with two variants of the same strain being isolated from one surgical sample. Even though the patient recovered completely from the infection, it is not possible to state that the altered PBPs had no effect on the penicillin treatment course since the patient was given linezolid (to which both isolates were susceptible) intra-operatively and the antimicrobial therapy was de-escalated to benzylpenicillin only after isolation of GBS.

PRGBS are reported at an increasing rate, but the clinical significance of *in vitro* penicillin non-susceptibility of PRGBS is still not fully understood. While studies reporting the acquisition of PBP mutations by GBS isolates recovered from patients undergoing β -lactam therapy have not been associated with treatment failure,^{4,7,9} continued surveillance is critical as PRGBS may impact future therapeutic strategies for the prevention and management of GBS infections.

Acknowledgements

We thank Catarina Inês Mendes for technical support.

Funding

This work was partly supported by the ONEIDA project (LISBOA-01-0145-FEDER-016417) co-funded by FEEI-Fundos Europeus Estruturais e de Investimento from Programa Operacional Regional Lisboa 2020 and by national funds from Fundação para a Ciência e a Tecnologia (FCT) and UID/BIM/50005/2019, project funded by Fundação para a Ciência e a Tecnologia/Ministério da Ciência, Tecnologia e Ensino Superior (MCTES) through Fundos do Orçamento de Estado. E.R.M. was supported by Fundação para a Ciência e a Tecnologia (DL 57/2016/CP1451/CT0009).

Transparency declarations

M.v.d.L. has received research grants administered through his university and received honoraria for serving on the speaker's bureaus of Pfizer and Merck Sharp and Dohme. J.M.-C. has received research grants administered through his university and received honoraria for serving on the speaker's bureaus of Pfizer and Merck Sharp and Dohme. M.R. has received honoraria for serving on the speaker's bureau of Pfizer and for consulting for GlaxoSmithKline and Merck Sharp and Dohme. All other authors: none to declare.

Supplementary data

Tables S1 and S2 are available as [Supplementary data](#) at JAC Online.

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