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Novel Mutations in a Patient Isolate of *Streptococcus agalactiae* with Reduced Penicillin Susceptibility Emerging after Long-Term Oral Suppressive Therapy▽

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Penicillin nonsusceptibility has been demonstrated in group B streptococci (GBS), but there is limited information regarding mechanisms of resistance. We report a case of GBS with reduced susceptibility to penicillin emerging after long-term suppressive oral penicillin therapy for a prosthetic joint infection. Molecular characterization of the isolate before and after long-term penicillin therapy revealed 5 mutations in the ligand-binding regions of PBP1a, -2a, and -2x not previously reported in GBS.

Streptococcus agalactiae (group B streptococci [GBS]) is generally considered uniformly susceptible to penicillins, making these first-line agents for both prophylaxis and treatment of GBS infections (5, 17). The Clinical and Laboratory Standards Institute (CLSI) defines GBS susceptibility to penicillin as less than or equal to 0.12 μg/ml and does not indicate an interpretative breakpoint for resistance (2). However, GBS strains with reduced penicillin susceptibility (GBSRPS; MICs, 0.25 to 1 μg/ml) have been reported periodically since 1994 and are caused mainly by stepwise accumulation of mutations in PBP1a, PBP2b, and PBP2x (1, 3, 4, 7, 14–16).

A GBS isolate with reduced susceptibility to penicillin was identified during routine clinical work-up from a 69-year-old patient. He underwent right total hip replacement for osteoarthritis in May 2003 and presented with sepsis in September 2003. Penicillin-susceptible GBS (PSGBS) was cultured from blood, urine, and a right hip joint aspirate. A one-stage revision arthroplasty was performed and followed by intravenous penicillin G (4 millions units every 6 h) for 6 weeks. In October 2004, a left total hip replacement was undertaken and was complicated 1 month later with a deep surgical-site infection, also due to penicillin-susceptible GBS. The patient was treated with oral penicillin VK (300 mg 4 times daily), with prompt resolution of the left hip soft tissue infection and reduction in pain in the right hip. The patient then continued on this oral suppressive penicillin regimen, which was intended to be lifelong. However, increasing pain in the right hip resulted in a second revision in August 2009. Debridement and implantation of a gentamicin-containing cement spacer were performed. Right hip acetabular tissue grew GBS with a penicillin MIC of 0.5 μg/ml. The patient completed a 6-week course of intravenous cefazolin (2 g every 8 h) postoperatively and underwent implantation of a new prosthesis to complete a standard "two-step" procedure in October 2009. No signs of infection were noted at the time of reimplantation, and all intraoperative specimens submitted to the microbiology laboratory were culture negative.

Two strains of GBS were available for further analysis: one isolate from September 2003 and the strain from August 2009. Both were identified as Lancefield group B streptococcus (serotype II) and confirmed by sequencing of the 16S rRNA genes. Genetic relatedness of the two isolates was assessed by multilocus sequence typing (MLST), and both were identified as ST2 (11). Antimicrobial susceptibility testing was done by agar dilution according to CLSI standards in triplicate (2). Penicillin MICs were ≤0.06 μg/ml in 2003 and 0.5 μg/ml in 2009, while ceftriaxone MIC increased to 0.5 μg/ml in 2009 (Table 1). These MIC values were confirmed at the Canadian National Centre for Streptococcus (Edmonton, Canada) using standard broth microdilution (2). A nitrocefin test was performed to rule out beta-lactamase activity. To investigate the genetic environment of the nonsusceptible strain, five genes encoding high-molecular-weight penicillin-binding proteins (PBPs) (*PBP1a*, *PBP1b*, *PBP2a*, *PBP2b*, and *PBP2x*) were amplified and sequenced in both the 2003 and 2009 isolates, as

TABLE 1. MICs of the GBS isolates from September 2003 and August 2009 and mutations in their ligand-binding regions of PBP[a]

Isolate	MIC (μg/ml)		Mutation(s) in the ligand-binding regions				
	PCN	CTX	PBP1a	PBP1b	PBP2a	PBP2b	PBP2x
2003	≤0.06	≤0.5					
2009	0.5	0.5	T546P		E636G, S644F, S676F		G371D

[a] CLSI-defined breakpoints for susceptibility are as follows: penicillin (PCN), ≤0.12 μg/ml; ceftriaxone (CTX), ≤0.5 μg/ml.

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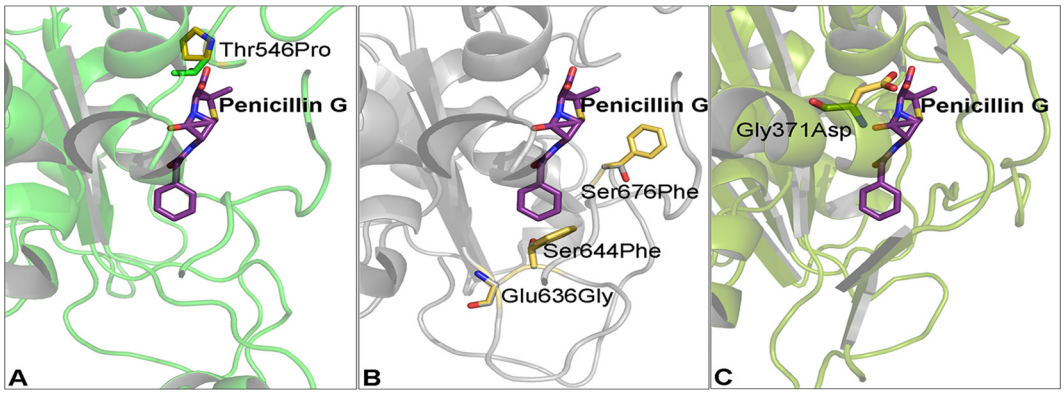
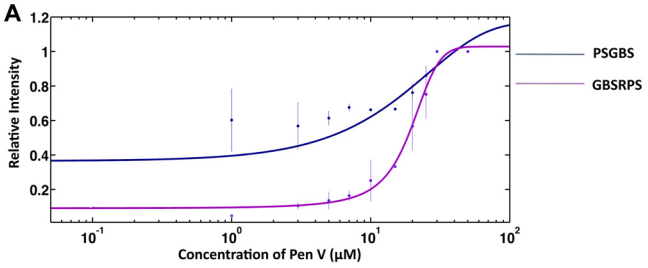


FIG. 1. Computational modeling of the novel mutations that arose in the penicillin-nonsusceptible strain in PBP1a (A), PBP2a (B), and PBP2x (C). (A) The mutation of threonine 546 (green) to proline (yellow) disrupts the hydrogen bonding with the penicillin carboxyl group (red). (B) The three mutations Glu636Gly, Ser644Phe, and Ser676Phe alter the conformation of the penicillin binding pocket. The introduction of the two phenylalanine residues reduces the available space for penicillin binding. (C) The replacement of glycine 371 with aspartate introduces an electronegative carboxylic group that repels the penicillin G carboxyl group.

described elsewhere (16). Sequencing products were compared with the *S. agalactiae* A909 NCBI reference strain. Mutations in the ligand-binding regions (amino acids 350 to 650) of the PBPs are reported in Table 1. No mutations in the penicillin binding sites of PBP1b and PBP2b were found. Substitutions within the penicillin binding domains of PBP1a, PBP2a, and PBP2x were analyzed based on a computational docking simulation as described previously (13). Docking of penicillin was conducted using Coot, while DaliLite was used for structural superimposition (6, 10). Results were visualized using PyMOL (DeLano Scientific, San Carlos, CA). To evaluate the contribution of these mutations to the observed increase in beta-lactam MIC, a fluorescent penicillin (Bocillin FL) binding assay was performed as previously reported (18).

Mutations reported so far in GBSRPS include T546N/T526A in PBP1a and P278L/G613R in PBP2b (3, 7, 14–16). Of interest, T526A and P278L were identified along with N575D in PBP2x in a susceptible GBS strain that became nonsusceptible after prolonged low-dose oral penicillin (7). We did not identify these mutations, but our strain carries T546P in PBP1a. This substitution replaces an electronegative residue and subsequently leads to the disruption of a hydrogen bond with the ligand, likely contributing to an increase in penicillin MIC (Fig. 1A). By comparison, T546N has been reported previously in a strain with a penicillin MIC of 0.12 μg/ml (3). We also identified 3 substitutions in the penicillin binding region of PBP2a that appeared in the nonsusceptible strain (Fig. 1B). E636G substitution affects both the size and the acidic environment of the penicillin binding pocket, potentially resulting in loosening of the bond between protein and ligand. This phenomenon can be further disrupted by the observed mutations S644F and S676F. To our knowledge, there are no published reports of mutations in PBP2a causing increased penicillin MIC in GBS, but such a mutation has been previously reported in *Streptococcus pneumoniae* through lateral transfer events (9). To date, the most frequently reported mutation is Q557E in PBP2x. In the United States, it has been found in four geographically distant ST19 sterile-site isolates (MIC, 0.12 μg/ml) (3). Similarly, in Japan the primary amino acid substitutions implicated in 14 GBSRPS isolated between 1995 and 2005 were Q557E and/or V405A in PBP2x (MICs, 0.25 to 1 μg/ml) (14). These studies suggest that amino acid substitutions adjacent to the conserved SSN and KSG motifs (amino acids 402 to 404 and 552 to 554, respectively) of PBP2x are important to the development of an increased penicillin MIC. Our isolate developed a mutation (G371D) in PBP2x



C

Isolate	Pbp1a/1b K_i (μM)	Pbp2A/2B/2X K_i (μM)
PSGBS	9.2	8.6
GBSRPS	19.5	15.2

FIG. 2. A competitive binding assay using fluorescent penicillin V (Pen V; Bocillin FL) and penicillin G. Relative intensity designates the fluorescence emitted upon PBP binding with fluorescent penicillin for each protein. Error bars represent standard deviations of the means ($n = 2$). (A) Penicillin affinity of the susceptible (dark blue) and GBSRPS (purple) isolate PBP1a/1b. (B) Penicillin affinity of the susceptible (dark blue) and GBSRPS (purple) isolate PBP2a/2b/2x. (C) The equilibrium dissociation constant (K_i) is tabulated for each isolate.

that is further upstream from the SSN motif but can nonetheless bring structural instability to the penicillin binding pocket by changing the propensity for alpha-helix formation based on computational modeling (Fig. 1C). The penicillin affinities of PBP1a/1b and PBP2a/2b/2x in the GBSRPS isolate were 2-fold lower than those in the PSGBS strain (Fig. 2). The equilibrium inhibitor dissociation constant (K_i) is in the micromolar range and comparable to that reported previously (8, 12, 18). These data suggest that both PBP1a/1b and PBP2a/2b/2x mutations are contributing to reduced susceptibility.

The access to type-related strains isolated before and after the emergence of nonsusceptible GBS gave us the confidence that these mutations arose *de novo*. The GBS PBP structures were predicted with 100% expected precision using the PHYRE homology modeling algorithm as predicted by three-dimensional (3D) sequence alignment (13). However, allelic replacement assays would be needed to definitively demonstrate the effect of each mutation on penicillin MIC. In conclusion, beta-lactams are the first-line agents against *Streptococcus agalactiae* and resistance is rarely reported. Our case demonstrates acquisition of mutations with drug selection pressure in PBP1a, -2a, and -2x but not PBP1b or -2b conferring reduced penicillin susceptibility due to diminished drug affinity.

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