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Effect of Variants of Penicillin-Binding Protein 2 on Cephalosporin and Carbapenem Susceptibilities in *Neisseria gonorrhoeae*

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To characterize the relationship between penicillin-binding protein 2 (PBP2/*penA*) and susceptibility to extended-spectrum cephalosporins (ESCs) and carbapenem antibiotics, we compared 17 PBP2 variants in *Neisseria gonorrhoeae*. Nonmosaic and mosaic variants of PBP2 caused decreased susceptibility to ESCs and, to a lesser extent, to carbapenems. An A501P substitution in mosaic XXXIV_A501P conferred decreased susceptibility to ESCs but restored carbapenem susceptibility to wild-type levels. These results could aid the molecular surveillance of antimicrobial resistance to these agents.

The World Health Organization and the U.S. Centers for Disease Control and Prevention have named antimicrobial resistant gonococcus (AMR-GC) as a top concern to human health (1, 2). The recommended treatment for gonorrhea is an extended-spectrum cephalosporin (ESC), preferably ceftriaxone or, alternatively, cefixime, in combination with the macrolide azithromycin (3–5); however, treatment failures have been reported in many countries (6–10). Ertapenem has recently been explored as a potential treatment for gonorrhea (11–14). Resistance to β-lactam antibiotics, including ESCs and carbapenems, is primarily caused by changes in their cellular target, penicillin-binding protein 2 (PBP2), which is encoded by the *penA* gene in *Neisseria gonorrhoeae*. Nonmosaic variants of PBP2 contain an aspartic acid insertion after position 345 (termed D345a), while mosaic variants contain many segments from commensal *Neisseria* species that are less susceptible to ESCs (15, 16).

Surveillance of AMR-GC is important for predicting potential treatment failure due to empirical therapy; however, diagnosis of gonococcal infection is now done by nucleic acid amplification testing (NAAT) so fewer cultures are available for surveillance of resistance (17). In the absence of live cultures, a better understanding of the genetic markers of ESC resistance in *N. gonorrhoeae* might help to make molecular surveillance a viable alternative. To elucidate the link between susceptibility to ESC and carbapenem antibiotics and changes in their cellular target, we have conducted a systematic study of 17 variants of PBP2 in an isogenic background.

Characterization of PBP2 variants in clinical isolates. Seventeen alleles of *penA* were identified from whole-genome sequences of 169 clinical isolates collected across Canada between 1989 and 2013 (18) and from the F89 strain from France (10). Alleles were extracted from whole-genome sequence data by BLAST using the sequence of wild-type *penA* (GenBank accession no. M32091). Five alleles were novel, while 12 were known (19). GenBank accession nos. of the novel alleles are KP721215 (nonmosaic I H168Y), KP721216 (nonmosaic II A501V), KP721217 (nonmosaic II V78I), KP721218 (nonmosaic V MNP), and KP721219 (nonmosaic XXXIV E538G). Figure 1A highlights the amino acid positions that were altered in the PBP2 variants of this study and that of a high MIC H041 variant from Japan (19). Three allele pairs differed only by variation at A501: nonmosaic II and nonmosaic II_A501V; nonmosaic XII and nonmosaic XIII (which contains A501V); and mosaic XXXIV and mosaic XXXIV_A501P. In the dendrogram of PBP2 sequences, variants that were found here to be associated with elevated cephalosporin MICs are highlighted (Fig. 1B).

Transformation of *penA* alleles into a susceptible recipient strain. Wild-type *penA* was replaced by non-wild-type alleles in *N. gonorrhoeae* isolate NML 22890, which was chosen because it lacked the markers of ESC resistance (no mutations in *penA*, *mtrR* −35A deletion, MtrR A39T, MtrR G45D, PonA L421P, or PorB G120) except for PorB A121G. NML 22890 was highly susceptible to both cefixime (MIC of 0.001 mg/liter) and ceftriaxone (MIC of 0.00025 mg/liter). The *penA* gene (1,749 bp) along with 1,150 bp of the upstream sequence (containing a natural DNA uptake sequence) and 800 bp of the downstream sequence was amplified with primers 1150-F and 800-R (Table 1). Transformation was carried out by the method outlined by Dillard (20) by spotting 0.5 μg *penA* PCR product onto supplemented GC agar medium and selection on 0.0005 mg/liter ceftriaxone (2× MIC). Spontaneous resistance did not occur at a detectable frequency. The sequences of the *penA* alleles in the transformants were verified with primers P0-FS, P1-RCS, P2-RCS, P3-RCS, and either P4-RCS-nonmosaic or P4-RCS-mosaic (Table 1).

Effects of PBP2 variants on cephalosporin susceptibility. MICs were determined by agar dilution according to the standard method of the Clinical and Laboratory Standards Institute (CLSI) (21). Nonmosaic alleles I, I_H168Y, II, II_V78I, IV, V, V_MNP, IX, XII, and XXII caused 2-fold to 5-fold increases in cefixime MICs and 5-fold to 11-fold increases in ceftriaxone MICs with additional 2-fold to 3-fold increases contributed by the A501V substitutions in nonmosaic II A501V and nonmosaic XIII (Table 2). Variations in the C-terminal transpeptidase domain after res-

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A.

	Variable positions in amino acid sequence of *PBP2*
	3477111111222222222223333333333333333333333333334444444444444444444445555555555555555555 5108004667000013678891111222222233333444445777788800011134456666667788800111133444555556775 0140831234402958112634678901251235634678369147023848823569031462513673823602367756
WT (M32091)	MCAVKDDVHNYGEDQQAADRRAIVAGTDLNERLQPSPR.SRGAEFEITLNRRPAVLQIFESRENPTTAFANVAAEHGGAPPKII.A
H041	.....E.A.SHAGEE..VEKQVMPS.V.TTDTFLSATQ.TMTPK.DVSV.K..VEVKVIA.KKEASI.LVY....N.ST.VQVVNV
X	.....E.A.SHAGEE..VEKQ.MTS.V.ATDTFLSATQ.TMTPK.DV..S.QKVEVKVIA.KKEA...LVY....N.ST.VQVVNV
XXXIV	.....E.A.SHAGEE..VEKQ.MTS.V.ATDTFLSATQ.TMTPK.DV..S.QKVEVKVIA.KKEA...LVY....N.S.....
XXXIV E538G	.....E.A.SHAGEE..VEKQ.MTS.V.ATDTFLSATQ.TMTPK.DV..S.QKVEVKVIA.KKEA...LVY...**G**N.S.....
XXXIV A501P	.....E.A.SHAGEE..VEKQ.MTS.V.ATDTFLSATQ.TMTPK.DV..S.QKVEVKVIA.KKEA..PLVY....N.S.....
I	.....D.....
I H168Y	.....**Y**.....D.....
II	.....D.....LV..G.....
II A501V	.....D.....**V**LV..G.....
II V78I	...**I**.....D.....LV..G.....
IV	.....D.....LV..G...S.....
V	.....D.....LV..G...S.....VNV
V MNP*	.....**HAGEE**.....D.....LV..G...S.....VNV
IX	.....D.....LV..G.....L.....
XII	.....D.....LV..G.....S.....
XIII	.....D.....VLV..G.....S.....
XXII	.....D.....LV..G..N....VQVVNV

B.

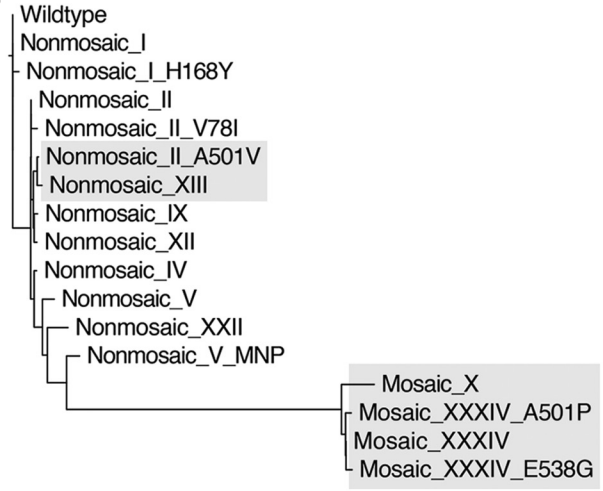

FIG 1 Sequence variations and phylogenetic relationship between PBP2 variants of this study. (A) The altered positions in the amino sequences of wild-type PBP2 (M32091), H041 PBP2 (AB546858) (19), and the variants of this study are shown. The residue numbers of wild-type and mosaic variants were shifted by +1 to maintain alignment with the nonmosaic alleles, which contain an Asp insertion after position 345; thus A501P and A501V substitutions are shown at position 502. Dots indicate consensus with the wild-type sequence, while bold text indicates novel variants and substitutions. The last 12 alleles (I to XXII) are nonmosaic alleles. The variant named V MNP* was encoded by an allele containing multiple nucleotide polymorphisms (MNP) compared to variant V. (B) A dendrogram based on the amino acid sequences of the PBP2 variants of this study was created with ClustalX by the nearest neighbor-joining method. Highlighted branches contain either mosaic alleles or nonmosaic alleles with A501V substitutions.

TABLE 1 Primers used in this study

Primer[a]	Sequence (5′ to 3′)	Position (bp)
1150-F	ACCCTGCGGTTTGATTTCCT	1,150 upstream
800-R	TGGTGAAGAGCGGTTTAGCC	800 downstream
P0-FS	GGGTAATGGCGTTTTAATTC	490
P1-RCS	CGAGCTTGTCGATGTGCCGGT	345
P2-RCS	GCGGTCGAATACCATCAGGCA	760
P3-RCS	TTGGATGTGCGCGGCATTATG	1,060
P4-RCS-nonmosaic	TGATGGTTTCCGTAACCGA	1,420
P4-RCS-mosaic	TGATGGTTTCCGTTACTGA	1,420

[a] Primers were designed using the sequence of strain FA1090 (GenBank accession no. NC_002946.2).

idue 500 or in the N-terminal domain did not have a significant effect on ESC susceptibility in this study.

Mosaic alleles X, XXXIV, and XXXIV_E538G conferred 27-fold to 53-fold increases in cefixime MICs and 16-fold to 21-fold increases in ceftriaxone MICs, with additional 16-fold to 30-fold increases conferred by the A501P substitution in mosaic XXXIV A501P. The larger effect of A501P than A501V may be due to the allelic context, or perhaps the proline causes significant chain bending in PBP2 (10). Mosaic XXXIV_A501P raised the MICs to 1 mg/liter for cefixime and 0.25 mg/liter for ceftriaxone, which were above the breakpoints for decreased susceptibility for both

TABLE 2 MICs of cephalosporin and carbapenem antibiotics against variants of PBP2 in isogenic strains of *N. gonorrhoeae*

PBP2 variant	MIC (mg/liter)[a]			
	Cefixime	Ceftriaxone	Ertapenem	Meropenem
Wild type	0.0023 ± 0.0015	0.0005 ± 0	0.016 ± 0	0.016 ± 0
Nonmosaic I	0.008 ± 0	0.004 ± 0	0.0267 ± 0.0092	0.016 ± 0
Nonmosaic I H168Y	0.0053 ± 0.0023	0.0033 ± 0.0012	0.0267 ± 0.0092	0.016 ± 0
Nonmosaic II	0.0067 ± 0.0023	0.0033 ± 0.0012	0.0267 ± 0.0092	0.016 ± 0
Nonmosaic II A501V	0.0213 ± 0.0092	0.008 ± 0	0.0267 ± 0.0092	0.016 ± 0
Nonmosaic II V78I	0.0067 ± 0.0023	0.004 ± 0	0.0267 ± 0.0092	0.016 ± 0
Nonmosaic_IV	0.008 ± 0	0.004 ± 0	0.0267 ± 0.0092	0.016 ± 0
Nonmosaic_V	0.008 ± 0	0.004 ± 0	0.032 ± 0	0.016 ± 0
Nonmosaic V MNP*	0.008 ± 0	0.004 ± 0	0.0213 ± 0.0092	0.016 ± 0
Nonmosaic IX	0.008 ± 0	0.004 ± 0	0.032 ± 0	0.0213 ± 0.0092
Nonmosaic XII	0.0107 ± 0.0046	0.0053 ± 0.0023	0.032 ± 0	0.0213 ± 0.0092
Nonmosaic XIII	0.032 ± 0	0.008 ± 0	0.032 ± 0	0.016 ± 0
Nonmosaic XXII	0.004 ± 0	0.0027 ± 0.0012	0.0213 ± 0.0092	0.016 ± 0
Mosaic X	0.125 ± 0	0.0107 ± 0.0046	0.125 ± 0	0.125 ± 0
Mosaic XXXIV	0.0637 ± 0.0006	0.008 ± 0	0.125 ± 0	0.0843 ± 0.0352
Mosaic XXXIV E538G	0.0637 ± 0.0006	0.008 ± 0	0.1047 ± 0.0352	0.0843 ± 0.0352
Mosaic XXXIV A501P	1 ± 0	0.25 ± 0	0.016 ± 0	0.016 ± 0
ATCC 49226	0.0267 ± 0.0092	0.0133 ± 0.0046	0.125 ± 0	0.1047 ± 0.0352
WHO F	0.0033 ± 0.0012	0.001 ± 0	0.016 ± 0	0.0133 ± 0.0046
F89	≥4 ± 0	2 ± 0	0.1047 ± 0.0352	0.1047 ± 0.0352

[a] MICs were measured in triplicate by the agar dilution method and averages ± SD are shown.

antibiotics according to WHO guidelines (2). The magnitudes of the changes conferred by these mosaic alleles were similar to those reported for similar alleles (10, 19, 22–25).

Effects of PBP2 variants on carbapenem susceptibility. Ertapenem has been investigated as a potential future treatment for gonorrhea infections (11–14). Nonmosaic alleles, regardless of the presence of A501V, caused 2-fold decreased susceptibility to ertapenem, raising the MICs from 0.016 mg/liter to 0.032 mg/liter, but had no effect on meropenem MICs. Mosaic alleles caused 5-fold to 8-fold decreased susceptibility to both ertapenem and meropenem, increasing the MIC to ≥0.125 mg/liter. Resistance breakpoints for carbapenems against *N. gonorrhoeae* have not been established. Interestingly, susceptibilities to ertapenem and meropenem were restored by the A501P mutation in mosaic XXXIV_A501P, suggesting that this substitution might block the binding or inactivation of carbapenems by PBP2. Thus, carbapenem MICs were less affected than ESC MICs by PBP2 variants, and susceptibility to carbapenems was restored by the A501P substitution suggesting that carbapenem antibiotics may be an effective treatment for infections that are associated with mosaic XXXIV_A501P mutations.

Effects of PBP2 variants on susceptibility to other antibiotics. All PBP2 variants had the same MICs as the recipient strain for gentamicin (MIC of 4 mg/liter), azithromycin (MIC of 0.063 mg/liter), spectinomycin (MIC of 16 mg/liter), erythromycin (MIC of 0.25 mg/liter), tetracycline (MIC of 1 mg/liter), and ciprofloxacin (MIC of 0.125 mg/liter). The average increases in penicillin MICs were 1.6-fold and 2.1-fold for the nonmosaic and mosaic alleles, respectively.

Elucidating the relationship between the genetic markers of resistance and antimicrobial susceptibility can help in interpretation of molecular surveillance data. In this comparative study of 17 PBP2 variants, we found that nonmosaic alleles caused small elevations in ESC MICs, mosaic alleles caused larger elevations in ESC MICs, and further contributions were made by substitutions at A501. Carbapenem antibiotics were less affected than ESCs, and the A501P substitution restored carbapenem susceptibility to wild-type levels, suggesting that carbapenem antibiotics may be effective against cases of ESC treatment failures that are associated with mosaic XXXIV_A501P mutations. The results of this study will strengthen the link between the genotype and phenotype for PBP2 variants and susceptibility to the last approved treatment for gonorrhea infections.

Nucleotide sequence accession numbers. The novel alleles were deposited in GenBank under accession numbers of KP721215 (nonmosaic I H168Y), KP721216 (nonmosaic II A501V), KP721217 (nonmosaic II V78I), KP721218 (nonmosaic V MNP), and KP721219 (nonmosaic XXXIV E538G).

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