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Neisseria gonorrhoeae Isolates with Reduced Susceptibility to Cefixime and Ceftriaxone: Association with Genetic Polymorphisms in *penA*, *mtrR*, *porB1b*, and *ponA*▽

Robert Lindberg,[1] Hans Fredlund,[1] Robert Nicholas,[2] and Magnus Unemo[1]*

National Reference Laboratory for Pathogenic Neisseria, Department of Clinical Microbiology, Örebro University Hospital, Örebro, Sweden,[1] and Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina[2]

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The recent emergence and transmission of *Neisseria gonorrhoeae* isolates with reduced susceptibility to expanded-spectrum cephalosporins such as cefixime and ceftriaxone have been reported. The aim of this study was to determine the correlation of different polymorphisms in the *penA*, *mtrR*, *porB1b* (*penB*), and *ponA* genes of *N. gonorrhoeae* with reduced susceptibility to cefixime and ceftriaxone. Eighteen gonococcal isolates with reduced cefixime and ceftriaxone susceptibility (Cef^{i}) and two susceptible isolates were characterized using serovar determination, antibiograms, *N. gonorrhoeae* multiantigen sequence typing (NG-MAST), and sequencing of *penA*, *mtrR*, *porB1b*, and *ponA* alleles. For the Cef^{i} isolates ($n = 18$), the MICs of cefixime and ceftriaxone ranged between 0.032 to 0.38 μg/ml and 0.064 to 0.125 μg/ml, respectively. These isolates were assigned five different serovars and six divergent NG-MAST sequence types. Eleven isolates (61%) with higher MICs of cefixime and ceftriaxone contained a nearly identical *penA* mosaic allele and previously described polymorphisms in *mtrR* (a single nucleotide [A] deletion in the promoter), *penB* (mutations in *porB1b* encoding loop 3 of PorB1b), and *ponA* (*ponA1* polymorphism). The remaining seven Cef^{i} isolates (39%), which had somewhat lower MICs of cefixime and ceftriaxone, contained an aspartic acid insertion (Asp-345a) in PBP 2 in conjunction with alterations of 4 to 10 amino acid residues in the C-terminal region of the transpeptidase domain of *penA*. In conclusion, an unambiguous association between *penA* mosaic alleles, in conjunction with genetic polymorphisms in *mtrR*, *porB1b*, and *ponA*, and greater reduced susceptibility to cefixime and ceftriaxone was identified.

Since the mid to late 1990s, the number of infections caused by *Neisseria gonorrhoeae*, the etiologic agent of the sexually transmitted infection gonorrhea, has increased in many West European countries (11, 33). Resistance of *N. gonorrhoeae* to traditional antimicrobial agents, e.g., penicillin and tetracycline, in the 1980s led to discontinuation of their use in treating gonococcal infections, and the more recent emergence of strains resistant to currently prescribed antibiotics, such as fluoroquinolones, azithromycin, and expanded-spectrum cephalosporins, is now a serious concern worldwide (2, 5, 7, 14, 18, 24, 26). Presently, the recommended first-line treatments for gonorrhea in most countries include antibiotics such as cefixime, ceftriaxone, spectinomycin, and in some cases azithromycin or ciprofloxacin. However, resistance to fluoroquinolones in many countries is high (e.g., 31% in Western Europe in 2004), resistance to azithromycin is rapidly increasing (18), and resistance to spectinomycin was already reported in the 1980s (4). Recently and most disquieting, the emergence of *N. gonorrhoeae* isolates with reduced susceptibility or resistance in vitro to broad-spectrum oral cephalosporins such as cefixime and parenteral cephalosporins such as ceftriaxone has been described (1, 2, 16, 18, 24, 25, 30). In occasional cases, treatment failures when using cefixime have been reported (6, 30).

Thorough antimicrobial susceptibility testing requires culturing of *N. gonorrhoeae*. However, in some geographic areas with high-prevalence populations, nucleic acid amplification tests are rapidly replacing culture for diagnosis of gonorrhea. Accordingly, comprehensive knowledge regarding the genetic basis of reduced susceptibility and resistance to many antimicrobials and, subsequently, development of fast and objective genetic assays for screening of resistance are crucial.

The genetic mechanisms of chromosomally mediated high-level resistance to penicillin are complicated and multifaceted. Stepwise transformation experiments using DNA from a resistant strain to increase the resistance of a susceptible strain demonstrate the involvement of mutated alleles of at least four genes: *penA*, *mtrR*, *porB1b* (*penB*), and *ponA*. Moreover, these polymorphisms are acquired in a particular order and often increase resistance only when other mutated alleles are present. Insertion of an aspartic acid codon (Asp-345a) in the *penA* gene together with downstream mutations causes a reduced affinity of penicillins for penicillin-binding protein 2 (PBP 2) (8, 9). Specific mutations in the promoter or coding segments of *mtrR*, which encodes the major transcriptional repressor of the *mtr* locus, increase transcription of three tandemly linked genes in the *mtrCDE* operon encoding the MtrC-MtrD-MtrE efflux pump (34). Increased levels of the efflux pump confer resistance to multiple hydrophobic agents (i.e., crystal violet, Triton X-100, and erythromycin) and some hydrophilic antibiotics such as the penicillins (15). Nonsynonymous substitutions at two positions within the constriction loop

* Corresponding author. Mailing address: Department of Clinical Microbiology, Örebro University Hospital, SE-701 85 Örebro, Sweden. Phone: 46 19 6021520. Fax: 46 19 127416. E-mail: magnus.unemo@orebroll.se.

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TABLE 1. Country of origin, serovar, antibiogram, NG-MAST, *porB* allele, and the polymorphisms in *penA, mtrR, porB1b,* and *ponA* alleles of *N. gonorrhoeae* isolates with reduced susceptibility to cefixime and ceftriaxone

Isolate (no./yr)	Origin	Serovar	*porB* allele	NG-MAST	MIC[a] (μg/ml)			Polymorphisms in:				
								penA				
					CFM	CRO	PEN	Mosaic allele	Insertion (GAC)	*mtrR*[e]	*porB1b*[f]	*ponA*[g]
119/04	Sweden	IB-2	3	ST5	0.032	0.064	2.0	No	D^{345a}	Deletion of A	K^{101}, D^{102}	P^{421}
158/04	Sweden	IB-3	4	ST1724[b]	0.047	0.094	1.5	No	D^{345a}	Deletion of A	K^{101}, D^{102}	WT
188/03	United Kingdom	IB-16	5	ST1619	0.064	0.064	1.0	No	D^{345a}	WT[c,d]	K^{101}, D^{102}	P^{421}
201/03	United Kingdom	IB-16	5	ST1619	0.064	0.064	1.0	No	D^{345a}	WT[d]	K^{101}, D^{102}	P^{421}
253/04	Sweden	IB-4	6	ST1723[b]	0.064	0.064	1.5	No	D^{345a}	WT[d]	D^{101}, WT	P^{421}
273/04	Sweden	IB-4	6	ST1723[b]	0.064	0.094	1.5	No	D^{345a}	WT[d]	D^{101}, WT	P^{421}
196/03	United Kingdom	IB-16	5	ST1619	0.094	0.094	1.0	No	D^{345a}	WT[d]	K^{101}, D^{102}	P^{421}
66/02	Sweden	IB-1	7	ST326	0.19	0.094	2.0	Yes		Deletion of A[d]	K^{101}, D^{102}	P^{421}
30/02	Sweden	IB-1	7	ST326	0.25	0.094	3.0	Yes		Deletion of A[d]	K^{101}, D^{102}	P^{421}
64/02	Sweden	IB-1	7	ST326	0.25	0.094	2.0	Yes		Deletion of A[d]	K^{101}, D^{102}	P^{421}
65/02	Sweden	IB-1	7	ST326	0.25	0.094	1.5	Yes		Deletion of A[d]	K^{101}, D^{102}	P^{421}
67/02	Sweden	IB-1	7	ST326	0.25	0.094	2.0	Yes		Deletion of A[d]	K^{101}, D^{102}	P^{421}
59/03	United States	IB-1	8	ST925	0.25	0.094	6.0	Yes		Deletion of A	K^{101}, D^{102}	P^{421}
66/03	United States	IB-1	8	ST925	0.25	0.125	4.0	Yes		Deletion of A	K^{101}, D^{102}	P^{421}
35/02	Sweden	IB-1	7	ST326	0.38	0.094	2.0	Yes		Deletion of A[d]	K^{101}, D^{102}	P^{421}
82/03	United States	IB-1	8	ST925	0.38	0.125	6.0	Yes		Deletion of A	K^{101}, D^{102}	P^{421}
90/03	United States	IB-1	8	ST925	0.38	0.125	6.0	Yes		Deletion of A	K^{101}, D^{102}	P^{421}
91/03	United States	IB-1	8	ST925	0.38	0.125	6.0	Yes		Deletion of A	K^{101}, D^{102}	P^{421}
119/05[h]	Sweden	IB-23	1	ST1722[b]	<0.016	<0.002	0.012	WT		WT	WT, WT	WT
128/05[h]	Sweden	IB-1	2	ST1580	<0.016	<0.002	0.008	WT		WT	WT, WT	WT

[a] CFM, cefixime; CRO, ceftriaxone; PEN, penicillin G.
[b] STs not previously identified.
[c] WT, wild type.
[d] Contained the G^{45}→D amino acid replacement in the DNA-binding motif of MtrR, which also may increase the expression of the MtrCDE efflux pump (34), mainly in strains lacking the dominant deletion of A in the promoter.
[e] The features of the 13-bp repeat in the promoter are indicated.
[f] Wild type: G^{101}, A^{102}.
[g] Wild type: L^{421}.
[h] Isolates susceptible to cefixime and ceftriaxone.

of *porB1b*, i.e., *penB* mutations, decrease the permeability of the outer membrane porin PorB1b for hydrophilic antimicrobials (13, 20, 21). Surprisingly, these mutations do not increase resistance in the absence of an *mtrR* mutation, suggesting that the MtrC-MtrD-MtrE efflux pump and PorB1b work together to increase penicillin resistance by limiting the concentration of the antibiotic in the periplasm (21). Additionally, a single substitution in *ponA* (the *ponA1* allele), which encodes an altered PBP 1 with a reduced affinity for penicillin, contributes to high-level penicillin resistance in *N. gonorrhoeae* (22). Finally, *pilQ2* mutations (previously named *penC*) or a *pilQ* deletion increase penicillin resistance if the *penA*, *mtrR*, and *penB* resistance determinants are present, presumably because PilQ forms an outer membrane pore through which antimicrobials diffuse into the periplasm (22, 35). However, the role of *pilQ* mutations in clinical resistance to antimicrobials has not yet been established.

The reduced susceptibility of *N. gonorrhoeae* strains to broad-spectrum cephalosporins such as cefixime and ceftriaxone has been proposed to be associated with polymorphisms in several of these genes and especially with certain *penA* mosaic alleles (1, 16, 24, 25, 31, 32). However, thorough knowledge regarding these molecular mechanisms is still lacking. For example, all these genes need to be systematically sequenced in more numerous and evidently diverse clinical *N. gonorrhoeae* strains with reduced susceptibility to broad-spectrum cephalosporins, the cooperation of the genetic polymorphisms mentioned above needs to be comprehensively examined, and it is crucial to investigate the contribution of specific polymorphisms in different regions of these genes as well as other genetic loci to the MICs of divergent broad-spectrum cephalosporins. Thus, the aim of this study was to correlate different polymorphisms in *penA*, *mtrR*, *porB1b* (*penB*), and *ponA* of *N. gonorrhoeae* with reduced susceptibility to cefixime and ceftriaxone.

MATERIALS AND METHODS

***N. gonorrhoeae* isolates.** A total of 18 *N. gonorrhoeae* isolates with reduced susceptibility to cefixime and/or ceftriaxone (referred to hereafter as Cef[i]) and, for comparison, two additional clinical isolates susceptible to these cephalosporins were examined (Table 1). In Sweden, the breakpoints used for cefixime and ceftriaxone are MICs of ≤0.064 μg/ml (susceptible) and >0.5 μg/ml (resistant). All isolates were received at the National Reference Laboratory for Pathogenic Neisseria, Örebro University Hospital, Örebro, Sweden, from February 2002 through May 2005. The Cef[i] isolates included clinical isolates (n = 10) from six different gonorrhea patients in Sweden and clinical isolates provided in 2003 by Catherine Ison, Health Protection Agency, United Kingdom (n = 3), and Joan Knapp, Centers for Disease Control and Prevention (n = 5), for antimicrobial susceptibility testing.

Phenotypic and genotypic characterization. β-Lactamase production was analyzed using nitrocefin discs, and the antibiotic susceptibility profiles (expressed as MICs in μg/ml) to cefixime, ceftriaxone, penicillin G, ciprofloxacin, azithromycin, and spectinomycin were analyzed using the Etest method (AB Biodisk, Solna, Sweden) as previously described (3).

Cultivation, serovar determination, isolation of genomic DNA, *porB1b* gene sequencing, and *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) were performed as previously described (28, 29). The promoter and coding regions of

mtrR and *ponA* were amplified in a LightCycler real-time PCR system (Roche Molecular Biochemicals, Mannheim, Germany) using previously described *mtrR* (19) and *ponA* primers (22). Briefly, each PCR mixture (20 μl) contained 2 μl LightCycler-FastStart DNA Master SYBR green I (Roche Diagnostics GmbH, Mannheim, Germany), 3 mM $MgCl_2$, 0.5 μM of each primer, and 2 μl of DNA template. The parameters of the amplifications were as follows: an enzyme activation step at 95°C for 10 min, followed by 40 sequential cycles of heating up to 95°C, 49°C (*mtrR*) or 68°C (*ponA*) for 10 s, and 72°C for 37 s (*mtrR*) or 51 s (*ponA*). The parameters of the subsequent melting curve analyses were as follows: heating the PCR products up to 95°C, cooling at 63°C (*mtrR*) or 74°C (*ponA*) for 45 s, and finally slowly heating (0.1°C/s) up to 95°C.

Using two previously described primer pairs, PA2/B1 or B1/C2 (25), the *penA* genes from all of the different isolates were amplified by PCR in a PTC-100 instrument (MJ Research, Watertown, MA). The PCR mixtures (50 μl) contained 1.25 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), 1× PCR Gold buffer (Applied Biosystems), 2.5 mM $MgCl_2$, 0.8 mM deoxynucleoside triphosphates, 1 μM of each primer, and 1 μl of DNA template. The following PCR amplification parameters were used: an enzyme activation step at 94°C for 10 min, followed by 30 sequential cycles of 94°C for 1 min, 54°C for 1 min, and 72°C for 2 min. At the end of the final cycle, an extension phase of 72°C for 7 min was included. A positive control (DNA of *N. gonorrhoeae* reference strain CCUG 15821) and a negative control (distilled water) were included in each PCR run. The products were analyzed by gel electrophoresis as previously described (28). All PCR products were stored at 4°C prior to purification.

The PCR products were purified using a High Pure PCR product purification kit (Roche Diagnostics GmbH, Mannheim, Germany) and sequenced using the PCR primers mentioned above as previously described (28, 29). However, for sequencing of the entire *penA* genes of divergent isolates, the primers B2 (25), Gcup2, Fo, Ro, AA-1, and PenA-R2 (27) were also utilized. Multiple-sequence alignments of nucleotide and amino acid sequences and phylogenetic analysis using BioEdit (version 5.0.9) software and TREECON (version 1.3b) software, respectively, were performed as previously described (29).

RESULTS

The origins of the isolates, results of serovar determinations and genotyping, and antibiotic resistance profiles are summarized in Table 1.

Phenotypic characterization. The Cef^i isolates ($n = 18$) were assigned five different serovars. The ranges of MICs of cefixime (0.032 to 0.38 μg/ml), ceftriaxone (0.064 to 0.125 μg/ml), and penicillin G (1.0 to 6.0 μg/ml) of all the Cef^i isolates were markedly higher than the MICs of the susceptible strains, 119/05 and 128/05 (Table 1). None of the isolates produced a β-lactamase.

Genotypic characterization. The Cef^i isolates ($n = 18$) comprised six divergent *porB1b* sequences and were assigned six different NG-MAST sequence types (STs) (Table 1). The 18 Cef^i isolates also comprised 11 divergent *penA* sequences. However, 11 (61%) of these isolates displayed identical ($n = 4$) or highly similar (≥99.6% identity; $n = 7$) *penA* alleles, which comprised mosaic patterns from nucleotide 294 to the end of the gene (Table 1). These *penA* mosaic alleles encoded three slightly divergent amino acid sequences (Fig. 1). All of these isolates also contained the three nonsynonymous nucleotide substitutions, resulting in amino acid alterations G^{545}→S, I^{312}→M, and V^{316}→T, which have been proposed to be important for reduced susceptibility to cefixime according to data from site-directed mutagenesis (24). These isolates also displayed the highest MICs of cefixime (range: 0.19 to 0.38 μg/ml) and ceftriaxone (range: 0.094 to 0.125 μg/ml). The *penA* genes from the remaining seven (39%) Cef^i isolates harbored an insertion of a single codon (GAC) encoding an aspartic acid residue (D^{345a}) in PBP 2 in conjunction with alterations of 4 to 10 amino acid residues in the C-terminal region of the transpeptidase domain of *penA*. These isolates contained five slightly divergent amino acid sequences (Fig. 1) and displayed lower MICs to cefixime (range: 0.032 to 0.094 μg/ml) and ceftriaxone (range: 0.064 to 0.094 μg/ml) (Table 1).

Seven (39%) of the Cef^i isolates contained only a single nucleotide (A) deletion in the 13-bp inverted repeat located between the −10 and −35 sequences of the *mtrR* promoter, six (33%) contained this A deletion in the promoter and in addition a G^{45}→D amino acid replacement in the DNA-binding motif of MtrR, and five (28%) displayed only the G^{45}→D amino acid replacement. Furthermore, 16 (89%) of the Cef^i isolates contained identical nonsynonymous nucleotide substitutions resulting in alterations of both the amino acid residues of PorB1b, G^{101}→K and A^{102}→D, which are known to be important for phenotypic expression of *penB* resistance (13, 20, 21), while the two remaining Cef^i isolates contained only a G→D alteration in residue 101. Finally, 17 (94%) of the Cef^i isolates contained the *ponA1* polymorphism, i.e., a single nucleotide transition (T→C) causing one amino acid alteration (L^{421}→P) (Table 1). The *penA*, *mtrR*, *porB1b*, and *ponA* alleles of the two ceftriaxone-susceptible isolates, which were included for comparison, all comprised wild-type sequences (Table 1).

DISCUSSION

In the present study, an unambiguous association between *penA* mosaic alleles, but also genetic polymorphisms in *mtrR*, *porB1b (penB)*, and *ponA*, and reduced susceptibility to cefixime and ceftriaxone was identified. This reduced susceptibility is not due to β-lactamase-encoding plasmids (present study; see also references 1 and 25). In the present study, the *penA* alleles displayed mosaic segments or other polymorphisms, such as a single codon insertion encoding D^{345a}, in all of the Cef^i isolates. In addition, the main variant of the *penA* mosaic alleles (61% of the Cef^i isolates) was identical or nearly identical to the *penA* mosaic alleles identified in *N. gonorrhoeae* isolates with reduced susceptibility to cefixime and ceftriaxone in Japan (1, 16, 24) and to ceftriaxone in Australia (31, 32). According to the serovar determination, *porB1b* sequencing, and NG-MAST, many of the Cef^i isolates were indistinguishable or closely related, which has also been reported in previous studies using pulsed-field gel electrophoresis (16). In the present study, all the Cef^i isolates with *penA* mosaic alleles were serovar IB-1 and ST326 or ST925, which are STs that have previously been associated with *penA* mosaic alleles and reduced susceptibility to ceftriaxone in Australia (31). Consequently, these Cef^i isolates may have emerged from a limited number of strains and been subsequently disseminated in many countries worldwide. However, more comprehensive data are needed to support this idea, especially given the observation that *penA* mosaic alleles were identified in isolates assigned also five other STs in the Australian study (31), which instead may suggest horizontal genetic exchange of the *penA* mosaic sequences.

In concordance with previous studies, the main variant of the *penA* mosaic alleles from the present study comprised segments, especially in the transpeptidase domain, that were identical or highly similar to the corresponding regions of *penA* in commensal or other pathogenic *Neisseria* species such as *N.*

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M32091    MLIKSEYKPR MLPKEEQVKK PMTSNGRISF VLMAMAVLFA CLIARGLYLQ TVTYNFLKEQ GDNRIVRTQA LPATRGTVSD RNGAVLALSA PTESLFAVPK  100
AB071984  .......... .......... .......... .......... .......... .......... .......... .......... ..V....... ..........  100
30/02     .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  100
59/03     .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  100
35/02     .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  100
158/04    .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  100
19/04     .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  100
188/03    .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  100
201/03    .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  100
273/04    .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  100

M32091    DMKEMPSAAQ LERLSELVDV PVDVLRNKLE QKGKSFIWIK RQLDPKVAEE VKALGLENFV FEKELKRHYP MGNLFAHVIG FTDIDGKGQE GLELSLEDSL  200
AB071984  E......... .......... .......... .......... .......... .........A .......... ..S....... .......... ..........  200
30/02     E......... .......... .......... .......... ...A...... .........A .......... ..S....... .......... ..........  200
59/03     E......... .......... .......... .......... .......... .........A .......... ..S....... .......... ..........  200
35/02     E......... .......... .......... .......... .......... .........A .......... .......... .......... ..........  200
158/04    .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  200
19/04     .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  200
188/03    .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  200
201/03    .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  200
273/04    .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  200

M32091    YGEDGAEVVL RDRQGNIVDS LDSPRNKAPQ NGKDIILSLD QRIQTLAYEE LNKAVEYHQA KAGTVVVLDA RTGEILALAN TPAYDPNRPG RADSEQRRNR  300
AB071984  HAGE...... ...E...... .......... .......... .......... .......... .......... ........V. ....E..K.. Q.........  300
30/02     HAGE...... ...E...... .......... .......... .......... .......... .......... ........V. ....E..K.. Q.........  300
59/03     HAGE...... ...E...... .......... .......... .......... .......... .......... ........V. ....E..K.. Q.........  300
35/02     HAGE...... ...E...... .......... .......... .......... .......... .......... ........V. ....E..K.. Q.........  300
158/04    .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  300
19/04     .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  300
188/03    .......... .......... .......... .......... .......... .......... .T........ .......... .......... ..........  300
201/03    .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  300
273/04    .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  300

M32091    AVTDMIEPGS AIKPFVIAKA LDAGKTDLNE RLNTQPYKIG PSPVR-DTHV YPSLDVRGIM QKSSNVGTSK LSARFGAEEM YDFYHELGIG VRMHSGFPGE  399
AB071984  .......... .M...T.... ..S..V.ATD TF..L..... SAT.Q..... ..T....... .......... ...M.TPK.. .....D..V. ..........  399
30/02     .......... .M...T.... ..S..V.ATD TF..L..... SAT.Q..... ..T....... .......... ...M.TPK.. .....D..V. ..........  399
59/03     .......... .M...T.... ..S..V.ATD TF..L..... SAT.Q..... ..T....... .......... ...M.TPK.. .....D..V. ..........  399
35/02     .......... .M...T.... ..S..V.ATD TF..L..... SAT.Q..... ..T....... .......... ...M.TPK.. .....D..V. ..........  399
158/04    .......... .......... .......... .......... .....D.... .......... .......... .......... .......... ..........  400
19/04     .......... .......... .......... .......... .....D.... .......... .......... .......... .......... ..........  400
188/03    .......... .......... .......... .......... .....D.... .......... .......... .......... .......... ..........  400
201/03    .......... .......... .......... .......... .....D.... .......... .......... .......... .......... ..........  400
273/04    .......... .......... .......... .......... .....D.... .......... .......... .......... .......... ..........  400

M32091    TAGLLRNWRR WRPIEQATMS FGYGLQLSLL QLARAYTALT HDGVLLPLSF EKQAVAPQGK RIFKESTARE VRNLMVSVTE PGGTGTAGAV DGFDVGAKTG  499
AB071984  ......S... .QK....... .......... .......V.. ...E...V.. .......K.. .VI.A...KK ..E....... A.........  ..........  499
30/02     ......S... .QK....... .......... .......V.. ...E...V.. .......K.. .VI.A...KK ..E....... A.........  ..........  499
59/03     ......S... .QK....... .......... .......V.. ...E...V.. .......K.. .VI.A...KK ..E....... A.........  ..........  499
35/02     ......S... .QK....... .......... .......V.. ...E...V.. .......K.. .VI.A...KK ..E....... A.........  ..........  499
158/04    .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  500
19/04     .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  500
188/03    .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  500
201/03    .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  500
273/04    .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  500

M32091    TARKFVNGRY ADNKHVATFI GFAPAKNPRV IVAVTIDEPT AHGYYGGVVA GPPFKKIMGG SLNILGISPT KPLTA-AAVK TPS* 582
AB071984  ....L..... V.Y....... .......... .......... .N...S...T ..V..QV... ......V... ....NV.... ...* 583
30/02     ....L..... V.Y....... .......... .......... .N...S...T ..V..QV... ......V... ....NV.... ...* 583
59/03     ....L..... V.Y....... .......... .......... .N...S...T ..V..QV... ......V... ....NV.... ...* 583
35/02     ....L..... V.Y....... .......... .......... .N...S...T ..V..QV... ......V... ....NV.... ...* 583
158/04    ....L..... V.....G... .......... .......... .N........ ..V..QV... ......V... ....NV.... ...* 584
19/04     ....L..... V.....G... .......... .......... .......... .L........ .......... .......... ...* 583
188/03    .V..L..... V.....G... .......... .......... ..S....... .......... .......... .......... ...* 583
201/03    .V..L..... V.....G... .......... .......... ..S....... .......... .......... .......... ...* 583
273/04    .V..L..... V.....G... .......... .......... .......... .S........ .......... .......... ...* 583
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FIG. 1. Multiple-sequence alignment of the amino acid sequences of PBP 2 in Cef^{i} strains of *N. gonorrhoeae*. One representative of each distinct sequence variant associated with reduced susceptibility to cefixime and ceftriaxone in the present study is displayed ($n = 8$). Dots denote identity with the wild-type PBP 2 amino acid sequence of the *N. gonorrhoeae* strain LM306 (GenBank accession no. M32091), and dashes represent alignment gaps due to insertions/deletions. The mosaic PBP 2 sequence of the *N. gonorrhoeae* strain NG-3 (GenBank accession no. AB071984), which previously has been associated with reduced cefixime susceptibility (1), is also shown.

perflava, *N. sicca*, *N. cinerea*, *N. flavescens*, and *N. meningitidis* (data not shown) (1, 24, 25). Accordingly, this *penA* mosaic allele may have evolved in vivo due to interspecies recombination of partial *penA* sequences from other *Neisseria* species. In previous studies (1, 24), the importance of *penA* mosaic alleles for reduced susceptibility to cefixime and ceftriaxone was suggested by transformation in vitro of *penA* mosaic alleles from donor isolates with reduced susceptibility to fully susceptible recipients. However, although the MICs of the recipient isolates were significantly increased, they still were substantially lower than those of the donor isolates. In addition, in the study by Takahata et al. (24), isolates with *penA* mosaic alleles

showed only a fourfold increase in the MIC of ceftriaxone, compared to a 16-fold increase in the MIC of cefixime. This difference was suggested to be due to the long side chain at the C-3 position of the cephem skeleton of ceftriaxone, which might increase the affinity for the altered PBP 2. Overall, this clearly indicates that polymorphisms in other genetic loci, e.g., *mtrR*, *penB*, and *ponA*, influence susceptibility to cefixime and especially ceftriaxone. In the present study, all Cef^i isolates containing the main variant of *penA* mosaic alleles also contained the previously described polymorphisms in *mtrR*, *penB*, and *ponA*, and these strains were the ones with the highest MICs of cephalosporins. These data provide further evidence of a link between polymorphic *penA* alleles and the *mtrR*, *penB*, and *ponA* alleles (see below) in the development of intermediate resistance to cefixime and ceftriaxone.

Of the 18 Cef^i isolates, 72% contained the previously described single nucleotide deletion in the *mtrR* promoter, which results in the loss of expression of MtrR and, consequently, enhanced expression of the MtrC-MtrD-MtrE efflux pump and increased resistance to antibiotics, including β-lactams, and multiple hydrophobic agents. In addition, the remaining five Cef^i isolates contained a $G^{45}\rightarrow D$ amino acid replacement in the DNA-binding motif of MtrR, which may also increase the expression of the MtrCDE efflux pump (34), mainly in strains lacking the dominant deletion of A in the promoter. These genetic polymorphisms may explain why all of these Cef^i isolates also displayed an increased MIC of azithromycin (range: 0.125 to 0.5 μg/ml) (34). Sixteen (89%) of the Cef^i isolates also contained alterations in amino acid residues 101 and 102 in putative loop 3 of PorB1b (i.e., *penB* mutations), which reduce the permeability of PorB1b to penicillin, cephalosporins, and ciprofloxacin (17, 22). Interestingly, two relatively low-level resistant isolates, 253/04 and 273/04, had only a $G^{101}\rightarrow D$ mutation in loop 3. The presence of a single aspartic acid at position 101 was shown to provide only a partial increase in penicillin and tetracycline resistance compared to that for a $G^{101}\rightarrow D/A^{102}\rightarrow D$ double mutation (13, 20, 21), and this may explain in part the lower level of resistance in these strains. Finally, all except one (94%) of the Cef^i isolates contained the *ponA1* polymorphism that results in one amino acid alteration ($L^{421}\rightarrow P$) in PBP 1, resulting in a decreased affinity for penicillin that even in normal cases is approximately 10-fold lower than that for PBP 2. This genetic polymorphism contributes to high-level penicillin resistance by decreasing the acylation rate of β-lactam antimicrobials in PBP 1 by three- to fourfold (22).

It is important to point out that the polymorphic alleles examined here (*penA*, *mtrR*, *penB*, and *ponA*) are necessary but not sufficient for high-level resistance to penicillin (22). That is, genetic studies have shown that transformation of a susceptible strain to the same level of resistance as a high-level penicillin-resistant strain with DNA from the resistant strain is exceedingly difficult, if even possible, to achieve in vitro (8, 10, 22). In conjunction with *penA*, *mtrR*, *penB*, and *ponA*, the *pilQ2* mutation was shown recently to confer the same level of penicillin resistance (MIC = 4 μg/ml) in laboratory strains as in high-level clinical isolates. However, it is unlikely that *pilQ2*, which interferes with type IV pilus formation that is critical for pathogenesis, is present in clinical isolates. Indeed, mutations in *pilQ* arise spontaneously and have not been observed in any clinical isolates examined thus far. Taken together, these data indicate that there remains at least one unidentified resistance determinant present in clinical isolates that is not transferable and, along with *penA*, *mtrR*, *penB*, and *ponA*, helps strains achieve high-level penicillin resistance.

In contrast to penicillin resistance, the roles of polymorphic *penA*, *mtrR*, *penB*, and *ponA* alleles in increased MICs of expanded-spectrum cephalosporins are mainly unknown. It is clear that the mosaic *penA* alleles are critical for increased cephalosporin resistance, but it has not yet been determined which of the other polymorphic alleles are important and whether an unidentified allele similar to the one responsible for high-level penicillin resistance is involved. It is also important to note that as yet no high-level cefixime- or ceftriaxone-resistant strain (MIC $\geq$ 1 μg/ml) has been reported. Consequently, the maximum levels of MICs of these cephalosporins that can be attained by solely divergent polymorphisms in these genes, single locus and in cooperation, are unclear at the present time. However, given the proclivity of the gonococcus to become resistant to all previously prescribed antibiotics, it may be more a matter of when and not if strains emerge that are resistant to the currently prescribed cephalosporins.

A serious concern emerging from our study is that many of the Cef^i isolates comprise a multiantimicrobial-resistant phenotype (1, 19, 25). For example, 13 of the isolates (72%) showed increased MICs of azithromycin (range: 0.125 to 0.5 μg/ml), 17 (94%) were resistant to ciprofloxacin (range: 0.25 to >32 μg/ml), and all 18 displayed a highly reduced susceptibility to penicillin G (range: 1.0 to 6.0 μg/ml). In contrast, all the isolates were susceptible to spectinomycin (MIC range: 6 to 16 μg/ml). Although resistance to spectinomycin is rare today, as early as the 1980s widespread use of spectinomycin was shown to rapidly increase the prevalence of resistance to this antimicrobial (4), possibly due to mutations in the 16S rRNA gene (12).

In recent times, ciprofloxacin was the recommended first-line treatment for gonorrhea in Sweden; however, resistance to this antimicrobial has rapidly increased and ciprofloxacin is no longer the first-line antibiotic of choice (23, 29). Presently, ceftriaxone (250-mg parenteral administration), cefixime (400-mg oral administration), spectinomycin (2-g parenteral administration), or in some more rare cases azithromycin (2-g oral administration; e.g., for patients with concurrent chlamydial infection) are the recommended first-line treatments when the results of antimicrobial susceptibility testing are not yet known. Even though the emergence of Cef^i isolates is currently not a major clinical problem, as treatment failures using ceftriaxone caused by resistance have not yet been reported, it is critical to monitor the increasing MICs to expanded-spectrum cephalosporins. Moreover, it will be important to elucidate the genetic mechanisms that are responsible for decreased susceptibility and future resistance. Overall, the present study highlights the importance of continuous local and national surveillance of *N. gonorrhoeae* antimicrobial resistance in order to reveal the emergence of new resistant strains, to monitor the changing patterns of resistance, and to be able to update treatment recommendations, including dose regimens, on a regular basis to assure successful eradication of the bacteria and, consequently, treatment of gonococcal infections.

In conclusion, we report an unambiguous association between particularly *penA* mosaic alleles but also genetic poly-

morphisms in *mtrR*, *penB*, and *ponA* and reduced susceptibility to cefixime and ceftriaxone. Further studies are needed to unambiguously elucidate associations between polymorphisms in these genes, and perhaps other genes, and reduced susceptibility to newer, broad-spectrum cephalosporins.

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