ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 2008, p. 3293–3300 Vol. 52, No. 9
0066-4804/08/$08.00+0 doi:10.1128/AAC.00020-08

Genotyping as a Tool for Antibiotic Resistance Surveillance of *Neisseria gonorrhoeae* in New Caledonia: Evidence of a Novel Genotype Associated with Reduced Penicillin Susceptibility▽

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Received 6 January 2008/Returned for modification 4 February 2008/Accepted 11 June 2008

Antibiotic resistance in *Neisseria gonorrhoeae* continues to be a major concern in public health. Resistance of *N. gonorrhoeae* bacteria to penicillin G is widespread in most developed countries, which has necessitated a change to newer drugs for treatment of gonococcal infections. Recent reports indicate that resistance to these newer drugs is increasing, highlighting the need for accurate therapeutic recommendations. In some countries or communities, however, *N. gonorrhoeae* isolates are still susceptible to penicillin, so the use of this antibiotic for single-dose treatments of medically under-resourced patients is beneficial. In order to evaluate the adequacy and sustainability of this treatment approach, we explored the presence and prevalence of chromosomally mediated resistance determinants in *N. gonorrhoeae* isolates collected from 2005 to 2007 in New Caledonia. We developed two new real-time PCR assays targeting the *penB* and *mtrR* determinants, to be used together with a previously described duplex assay targeting the *penA* and *ponA* determinants. The results of this study provided evidence that neither the most-common *mtrR* determinants nor the most-resistance-associated *penB* alleles are currently circulating in New Caledonia, suggesting that penicillin should still be considered a valuable treatment strategy. Additionally, using our genotyping assay, we observed an unexpected *penB* genotype at a relatively high frequency that was associated with a decreased susceptibility to penicillin (average MIC, 0.15 μg/ml). Sequencing revealed that this genotype corresponded to an A102S mutation in the *penB* gene. The molecular tools developed in this study can be used successfully for prospective epidemiological monitoring and surveillance of penicillin susceptibility.

Neisseria gonorrhoeae is an obligate gram-negative human pathogenic bacterium that continues to be a public health problem worldwide (31). It is well documented that *N. gonorrhoeae* bacteria have evolved resistance mechanisms to almost all antimicrobial compounds that have been used to treat infections, an ability that has largely been related to the capacity for natural recombination between strains of the same species within a human host and with other members of the natural human mucosal flora, notably saprophytic and other pathogenic *Neisseria* species (7, 27, 29, 30).

Antimicrobial resistance mechanisms include the production of antibiotic-degrading enzymes, modification of the target site, and decreased influx or increased efflux of the antibiotic (1, 13). Penicillin G was used to treat gonococcal infections up to the mid-1980s, until the emergence and spread of resistance rendered the antibiotic ineffective. These strains utilized either plasmid-mediated or chromosomally mediated mechanisms to become resistant to penicillin G (9, 36). The spread of strains harboring a plasmid-encoded TEM-like β-lactamase has been very rapid and has forced the withdrawal of penicillin for treating gonococcal infections in many areas. Chromosomally mediated resistance to penicillin, tetracycline, and macrolides (25) in *N. gonorrhoeae* bacteria is also recognized as being a major means by which resistance emerges and spreads in various geographic areas (10, 12, 19, 20, 22–24). The results of in vitro transformation experiments have shown that chromosomally mediated high-level penicillin resistance is complex and involves at least five genes, including those encoding altered alleles of the two essential penicillin-binding proteins PBP1 and PBP2 (*ponA* and *penA*, respectively) and porin (*penB*) and those resulting in overexpression of the MtrC-MtrD-MtrE efflux system (*mtrR*). The results of recent studies have demonstrated that alterations in the *penA* gene which create a mosaic PBP2 decrease susceptibility to cefixime and ceftriaxone (16, 17, 21, 40).

The major porin of *N. gonorrhoeae* is encoded by *porB* that has two alleles, *porB1b* and *porB1a* (3). The results of early studies of chromosomally mediated penicillin resistance demonstrated that recombination near the *porB* locus was associated with a decreased susceptibility to antibiotics upon acquisition of the *penB* resistance determinant and that *penB* resistance was correlated with the *porB1b* allele (6, 10). The important sequence modifications of *porB1b* related to the decrease in susceptibility to penicillin have been located in loop 3 at positions 101 and 102 of the mature protein (12, 22).

Transcriptional regulation of the *mtrCDE* operon in *N. gonorrhoeae* bacteria has been shown to be a major determinant in susceptibility to hydrophobic agents (14). The *mtrR* promoter drives the transcription of *mtrR* (a transcriptional repressor) in one direction and overlaps with the promoter for the *mtrCDE* operon, which encodes three proteins that assemble to form the MtrC-MtrD-MtrE efflux pump, in the other direction. The

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▽ Published ahead of print on 30 June 2008.

MtrR repressor binds to a 13-bp inverted repeat upstream of the *mtrCDE* operon and represses transcription. A single-nucleotide deletion (−T) in the inverted repeat, which causes both the loss of transcription of *mtrR* and increased transcription of *mtrCDE*, is the most-prevalent mechanism by which clinical isolates overexpress the MtrC-MtrD-MtrE efflux system (5, 18, 37, 41). Within the coding region of *mtrR*, mutations in the DNA-binding domain have also been shown to be associated with increased expression of the *mtrCDE* operon (26). Recent findings have revealed that *mtrR* might play an additional role in chromosomally mediated resistance to β-lactams in *N. gonorrhoeae* bacteria by contributing to transcriptional regulation of the *ponA* gene encoding PBP1 (11).

Whereas plasmid-encoded antibiotic-degrading enzymes usually confer complete resistance to an antibiotic, chromosomally mediated gene modifications often induce a gradual decrease of susceptibility to antibiotics as successive resistance determinants accumulate. These resistance determinants, which are mutated alleles of normal genes, are acquired in a stepwise manner, and each acquisition incrementally increases resistance until clinical failure occurs. Because of this, the complete loss of efficacy of an antibacterial treatment in chromosomally mediated resistance appears as a final step that might be detected before its emergence and spread. Particular combinations of genes involved in resistance can be regarded as "predetermining" resistance risk factors, with a complete loss of susceptibility occurring when a bacterium acquires the last of several determinants. Identifying these determinants by genotyping therefore offers a unique opportunity to develop epidemiological surveillance approaches for antibiotic resistance, so that antibiotic treatment guidelines adapted to current susceptibility patterns can be provided. Following the genetic acquisition of resistance determinants in a particular geographic region could help to foreshadow the appearance of higher-level resistant strains before their clinical emergence and might provide valuable information to public health decision-makers (2).

Due to the spread of resistance, penicillin G was largely abandoned in favor of fluoroquinolones as a treatment for gonococcal infections in most developed countries. Unfortunately, this switch resulted in the rapid emergence of fluoroquinolone-resistant *N. gonorrhoeae* strains and the recent withdrawal of fluoroquinolones as recommended drugs, leaving only the expanded-spectrum cephalosporins as the main recommended antibiotics (4). However, penicillin G is still recommended in particular areas or communities of developed countries or in areas where circulating strains are still susceptible (32).

In New Caledonia, *N. gonorrhoeae* is frequently isolated and constitutes a serious health concern. Plasmid-encoded β-lactamases have seldom been observed in New Caledonian gonococcal isolates and only in a few isolates from infections acquired overseas. The circulating strains are still regarded as penicillin susceptible, a situation that is very similar to that of other countries in Oceania (32) or in most rural parts of Australia (33). Therefore, penicillin G is the recommended treatment for gonococcal infections in New Caledonia, used, notably, in public health centers.

The aims of our study were to develop genotyping methods for the two major determinants of susceptibility to β-lactams, tetracyclines, and macrolides in *N. gonorrhoeae* bacteria, namely, mutations in the loop 3 region of *porB1b* and either a single-nucleotide deletion in the 13-bp inverted-repeat region in the *mtrR* promoter or mutations of G45 in the DNA-binding region of *mtrR*, using real-time PCR technologies on the LightCycler 2.0 platform and to evaluate the possible emergence and spread of these determinants throughout a collection of New Caledonian *N. gonorrhoeae* strains isolated over nearly a 3-year period.

MATERIALS AND METHODS

***N. gonorrhoeae* isolates.** A total of 208 isolates of *N. gonorrhoeae* isolated from February 2005 through October 2007 were examined in this study. These strains were isolated at the bacteriology laboratory of the Institut Pasteur in New Caledonia from (i) persons attending a sexually transmitted disease clinic; (ii) their partner(s) when possible (even if asymptomatic); and (iii) symptomatic patients consulting private doctors or the hospital. Samples were collected from cervical or urethral swabs from female (111) and male (96) patients, respectively, and included one sample from an eye culture from a newborn baby. None of these strains was knowingly isolated posttreatment or twice from the same patient. The identity of the organism was confirmed by Gram staining, oxidase activity, and specific characteristics on API NH (BioMérieux SA) after culture on chocolate agar supplemented with PolyVitex plus colistin, vancomycin, amphotericin B, and trimethoprim antibiotics (BioMérieux SA, Marcy l'Etoile, France). The isolates were suspended into homemade 2-sucrose phosphate (sucrase, 200 mM; K_2HPO_4, 12 mM; KH_2PO_4, 8 mM) transport medium (supplemented with vancomycin, gentamicin, and fungizone) and stored at −20°C until DNA extraction.

Archival isolates in our collection classified as chromosomally mediated resistant *N. gonorrhoeae* (CMRNG) and 12 *N. gonorrhoeae* reference strains (WHO-C, WHO-G, WHO-L, WHO-M, WHO-O, WHO-P, and 07QA-01 through 07QA-06) kindly provided by John Tapsall, Australian Reference Center and WHO Collaborating Center, were used as controls for antimicrobial susceptibility testing.

Antimicrobial susceptibility testing and penicillin MICs. In vitro susceptibility to conventional antibiotics was tested by using a disk agar diffusion method, and β-lactamase production was analyzed by using nitrocefin discs (BioMérieux SA). MICs (μg/ml) for penicillin G were determined by using the E-test diffusion method (AB Biodisk, Solna, Sweden) on chocolate agar plus PolyVitex and were interpreted by directly reading the intercept of the penicillin gradient strip and the zone of inhibition. The Antibiogram Committee of the French Society for Microbiology (CA-SFM) recommendations for *N. gonorrhoeae* were followed (susceptible strain, MIC of ≤0.06 μg/ml, and resistant strain, MIC of >1 μg/ml) (28). MICs lower than 0.016 μg/ml (the lowest value on the E-test scale) were given a value of 0.004 μg/ml to allow statistical analysis as a continuous variable and, notably, the calculation of average MICs. The data were then analyzed by using StatView software for Windows (SAS Institute, Inc.).

Real-time PCR genotyping assay. Genomic DNA for PCR amplification and genotyping analysis was isolated from 0.2 ml of each thawed *N. gonorrhoeae* isolate by using a QIAamp DNA minikit (Qiagen, Doncaster, Victoria, Australia) according to the manufacturer's instructions. The DNA concentration for each specimen was adjusted to 1 μg/ml.

The additional aspartic acid residue (Asp-345a) in the *penA* gene and the single-base substitution in the *ponA* gene were identified by using a duplex PCR protocol, previously described (39), on a LightCycler 2.0 real-time PCR system (Roche Molecular Biochemicals, Mannheim, Germany). Primers and hybridization probes for genotyping the *penB* and *mtrR* determinants were designed by using LightCycler Probe Design software, version 2.0 (Roche Diagnostics), and were synthesized by Sigma-Proligo (Singapore Pty. Ltd.). The primers for *penB*, the *mtrR* promoter, and the region encompassing G45 in the coding sequence of *mtrR* amplified products of 754, 760, and 638 bp, respectively. The probes were designed to target the −T deletion in the inverted repeat of the *mtrR* promoter; mutations of the G45 codon in the DNA-binding region of *mtrR*; or codons 120 and 121 of the coding region of the *porB1b* gene (*penB* determinant), corresponding to amino acids 101 and 102 of the mature protein, respectively. An unusual system of three hybridization probes targeting the *penB* determinant was designed: (i) one single-anchor probe labeled with fluorescein at its 3′ end; (ii) a sensor probe specific to the sequence G101K/A102D that was labeled with LC Red-705 at its 5′ end; and (iii) a second sensor probe specific to the wild-type sequence of *porB1b* that was labeled with LC Red-640 at its 5′ end. For analysis

TABLE 1. Oligonucleotide sequences used to target *porB1b* and *mtrR* sequence variations in this study

Primer or probe (purpose)	5′ → 3′ sequence
penB-F (forward primer)	AAAGGCCAAGAAGACCTCGGC
penB-R (reverse primer)	GAGAAGTCGTATTCCGCACCG
penB-P1 (anchor probe)	GCAGCCTGAACAGCCCCCTGAAA-fluorescein
penB-P2 (sensor probe 1)	LC Red-640-CACCGGCGCCAACGT-phosphate
penB-P3 (sensor probe 2)	LC Red-705-CACCAAGGACAACGTCAATGCTTG-phosphate
mtrR-F (forward primer)	GCAAAGCAGGTTATACCTGTT
mtrR-R (reverse primer)	TTCAAGGCTTCGGTTTTGG
mtrR-P1 (sensor probe)	ACATACACGATTGCACGGATAAAAAGTCT-fluorescein
mtrR-P2 (anchor probe)	LC Red-670-TTTATAATCCGCCCTCGTCAAACCGACCC-phosphate
mtrRcod-F (forward primer)	CCTCGTCAAACCGACCC
mtrRcod-R (reverse primer)	CCAAGTTGTCCATCATTATCCC
mtrRcod-P1 (sensor probe)	CGCTCAACGAAATCGCCCAAGCCGCCG-fluorescein
mtrRcod-P2 (anchor probe)	LC Red-640-GTAACGCGCGACGCGCT-phosphate

of the −T deletion in the *mtrR* promoter, an anchor probe labeled with LC Red-670 at its 5′ end was combined with a sensor probe labeled with fluorescein at its 3′ end. For *mtrR* G45 codon genotyping, a sensor probe specific for G45D labeled with LC Red-640 at its 5′ end was combined with an anchor probe labeled with fluorescein at its 3′ end. The primer and probe sequences are summarized in Table 1.

Genotyping of both *penB* and the *mtrR* promoter was conducted in a single multiplex amplification run. Each PCR mixture (20 μl) contained 1× buffer from a LightCycler FastStart DNA master HybProbe kit (Roche Diagnostics), 4 mM $MgCl_2$, 0.5 μM of each forward primer, 1.0 μM of each reverse primer, 0.2 μM of each probe, and 2 μl template DNA. The amplification parameters were as follows: an initial enzyme activation step at 95°C for 10 min followed by 50 sequential cycles of denaturation at 95°C for 8 s, primer and probe annealing at 60°C for 8 s (55°C for 10 s for *mtrR* G45 codon mutations), and elongation at 72°C for 30 s. The subsequent melting-curve analysis consisted of heating PCR products up to 95°C for 5 s, cooling them at 40°C for 15 s, and finally, slowly heating them (0.1°C/s) up to 85°C with continuous reading of fluorescence. Controls in every experiment included a blank capillary, wild-type DNA, and mutated genotype DNA of known sequence. This protocol ensures multiplex real-time PCR genotyping after a compensation color has been activated to correct emission spectra overlaps of the dyes.

All genotyping assays were performed after a compensation color had been activated, following LightCycler software instructions, and this correction program was updated with each new batch of probes.

Sequencing of the *porB1b* and *mtrR* coding regions. A panel (n = 10 to 15 when possible) of DNAs from *N. gonorrhoeae* strains representative of each group of samples with identical melting-temperature (T_m) values after melting-curve analysis was amplified as described above, purified using a MinElute PCR purification kit (Qiagen, Australia) according to the manufacturer's instructions, and sequenced at the Waikato University sequencing facilities (Hamilton, New Zealand) using the same primers (Table 1). Multiple-sequence alignments were performed by using ClustalW on BioEdit software, version 7.0.9.0 (15).

Transformation of the novel *porB1b* GS allele. Transformation experiments were carried out essentially as described by Ropp et al. (25). Piliated colonies of the recipient strain, FA19 *penA mtrR* (FA19 previously transformed with the *penA* and *mtrR* alleles from strain FA6140, a CMRNG strain [6]), were passaged on a fresh GC broth (Difco, Detroit, MI) agar plate and grown overnight. The next day, the cells were swabbed from the plate, resuspended in GC broth with supplements I and II and 10 mM $MgCl_2$, and diluted to an optical density at 590 nm of 0.18. Cells were incubated for 5 h with 5 μg of a PCR product encompassing the entire *porB1b* coding sequence and ~500 bp of flanking sequence at both the 5′ and 3′ ends amplified from a strain harboring the GS allele of *porB1b*. Aliquots of the cells were plated on GC broth plates containing penicillin at concentrations just above the MIC of the recipient strain (0.25 μg/ml) and allowed to grow overnight. The sequence of the *porB* gene from resistant transformants was verified by PCR amplification and sequencing, and the MIC of penicillin was determined as described previously (25).

RESULTS

Genotyping assays. The protocols using the fluorescent hybridization probe format were optimized by testing various primer and probe sequences in order to obtain clearly distinct and reproducible melting peaks after melting-curve analyses. These peaks, which reflect the T_m of each hybrid sensor probe-complement strand duplex of the PCR product, were automatically generated by LightCycler software from plotting of the negative derivative of the fluorescence ratio versus temperature. Representative profiles of the melting curves are shown in Fig. 1. According to the selected fluorescence channel, we identified specific T_m values that corresponded to a specific nucleotide sequence as described previously for detection of *penA* and *ponA* mutations (38, 39). For each gene, PCR products from a panel of 10 to 15 isolates classified according to their T_ms obtained with our multiplex PCR assay were sequenced and confirmed to be the genotypes evidenced by our hybridization probe genotyping assay.

Genotyping of the *penB* determinant. Following amplification, fluorescein- and LC Red-705-labeled probes for the *porB1b* alleles (*penB*) produced four distinct peaks, each of which indicated a gene sequence of interest. The amplified fragments containing sequences encoding G101K/A102D (KD) had the highest T_m (67.0 ± 0.5°C [mean ± standard deviation]), which reflects 100% homology between probe and target. Three other distinct peaks at 64.0°C (±0.2°C), 60.0°C (±1.0°C), and 49.0°C (±0.5°C) were shown to correspond to nucleotide sequences encoding G101K/A102N (KN); wild-type (GA) or G101D (DA) (these two had the same T_m value on the 705-nm channel); and A102S (GS), respectively. Variations in T_m values (0.2 to 1.0°C) within each genotype were always much lower than between genotypes. Thus, all melting peaks were easily grouped into specific genotypes, with the exception of the wild type (GA) and G101D (DA), which could only be differentiated with the LC Red-640 sensor probe. With this probe, the wild-type sequence had a T_m of 65.0 ± 1.0°C and was clearly distinct from the G101D sequence, which had a T_m of 57.0 ± 0.5°C. Isolates that did not display fluorescence either during amplification or during the melting-point analysis in the 640- and 705-nm channels were all confirmed to have *porB1a* alleles, based on the difference in size following agarose gel electrophoresis (692 bp instead of 754 bp).

Genotyping of the *mtrR* promoter −T deletion and *mtrR* G45 codon mutations. Using fluorescein and LC Red-670 probes, we observed two distinct melting peaks with reference strains that contained either wild-type sequence or the single-nucleotide −T deletion in the *mtrR* promoter. All isolates with the wild-type sequence produced a discernible peak with a T_m

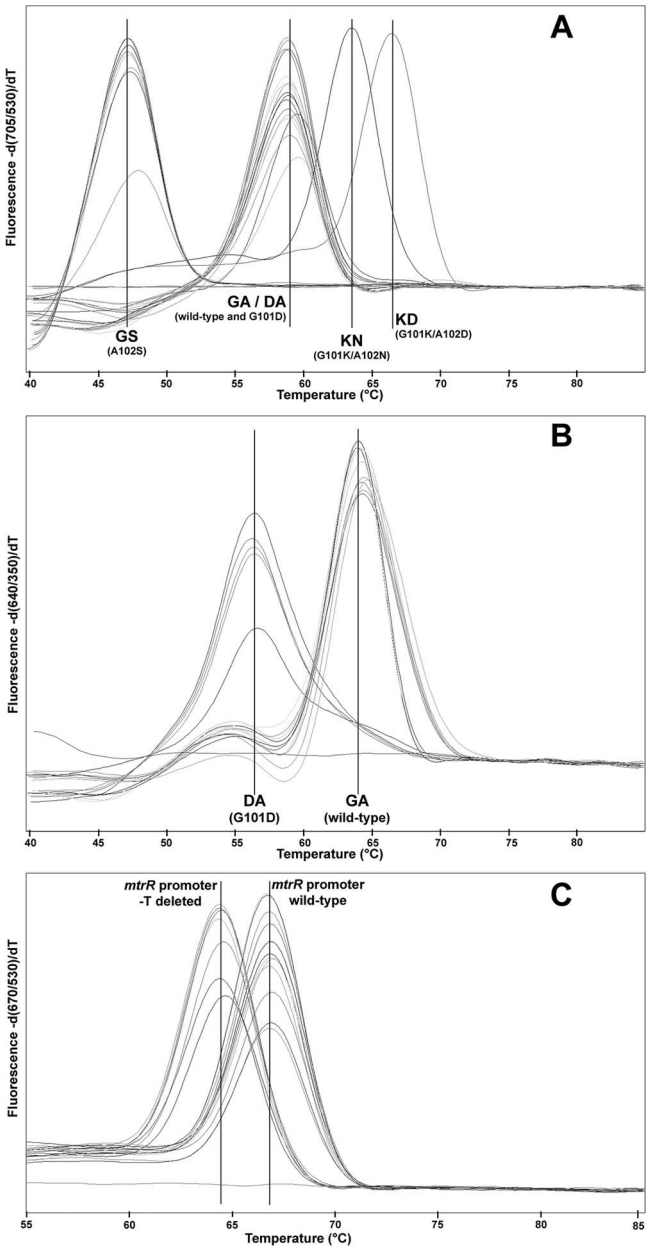

FIG. 1. Melting peaks derived from negative derivatives of fluorescence ratios with respect to temperature of the amplicons obtained for *penB* and *mtrR* promoter genotyping. (A) Readings of wild-type versus mutated genotypes of *porB1b* alleles (705 nm/530 nm). (B) Discrimination between the wild-type (GA) and the G101D mutants (DA) (640 nm/530 nm). Both of these spectra were generated using color compensation as detailed in the text. (C) Discrimination between *mtrR* promoter genotypes (670 nm/530 nm).

value of 67.0 ± 0.5°C, while those that contained the −T deletion produced a peak with a T_m value of 64.5 ± 0.3°C.

The assay for genotyping mutations at the G45 codon within the *mtrR* coding region allowed reproducible typing with clearly distinct melting peaks with T_m values of 61.7 ± 0.5°C for the wild type, 69.4 ± 0.2°C for G45D mutants, and 56.6 ± 0.2°C for G45S mutants (codon change of GGC to AGC demonstrated by sequencing) (data not shown).

Phenotypes and genotypes identified. All isolates from our collection covering an almost 3-year period were non-penicillinase producing and displayed susceptible to intermediate resistance to penicillin, with MICs ranging from <0.016 μg/ml to 0.38 μg/ml (Fig. 2). A total of 125 isolates (60.1%) were penicillin susceptible and inhibited by concentrations lower than 0.064 μg/ml, whereas 83 isolates (39.9%) were classified as intermediate-level resistant, for which the MIC of penicillin was in the range of 0.064 to 0.38 μg/ml. Additionally, all isolates were susceptible to quinolones (i.e., nalidixic acid and ciprofloxacin) and to expanded-spectrum cephalosporins (ceftriaxone).

The genotypes of the 208 strains of *N. gonorrhoeae*, which are summarized in Table 2, led to the identification of 95 isolates harboring the Asp-345a codon insertion in *penA* (45.7%) and 33 isolates with the *ponA* L421P mutation (15.9%). The *porB* genotypes of our collection were as follows: the *porB1a* allele was observed in 30 isolates (14.4%), the wild-type *porB1b* (GA) allele in 100 isolates (48.1%), the single mutation G101D (DA) in 22 isolates (10.6%), and the single mutation A102S (GS) in 56 isolates (26.9%). The G101K/A102N (KN) and G101K/A102D (KD) alleles were not found in any of our isolates. No *mtrR* promoter −T deletion was observed in any isolate. Out of 83 isolates displaying an intermediate resistance to penicillin (MIC ≥ 0.06 μg/ml), none displayed the G45D mutation and only two harbored a G45S mutation. Out of the 208 isolates of our collection, 54 strains (26%) were identical to wild-type sequences in all of the assays carried out. The other genotypes were not randomly distributed over our strain collection; while there is no clear association between the *porB1a* or wild-type *porB1b* (GA) allele and the genotypes of other determinants, the G101D (DA) and A102S (GS) genotypes appear strongly linked with wild-type and Asp-345a *penA* genotypes, respectively ($\chi^2 = 81.993$; $P < 0.001$). Therefore, the effect of these *porB1b* genotypes on penicillin susceptibility cannot be easily evaluated in our strain collection.

Interestingly, out of the 54 strains genotyped as wild type for all four determinants, 52 displayed penicillin-susceptible and 2 displayed intermediate penicillin-resistant phenotypes (MICs of 0.064 and 0.094 μg/ml). Taken together, the average MICs of strains with the *porB1a* allele and the wild-type (GA) and G101D (DA) *porB1b* genotypes were 0.031, 0.037, and 0.016 μg/ml, respectively, whereas the average MIC of strains with the A102S (GS) genotype (0.155 μg/ml) was significantly higher ($F = 52.419$; $P < 0.001$). Similarly, strains harboring the Asp-345a codon insertion in the *penA* gene exhibited a higher penicillin MIC (0.122 versus 0.019 μg/ml; $P < 0.001$; Student's *t* test, 11.694) than those without the insertion, whereas the MICs of strains with the *ponA* mutation were not different from those of the wild type (0.059 versus 0.067 μg/ml; $P = 0.62$; Student's *t* test, −0.488). Thus, a *penA-porB* GS (A102S) genotype predicts a significantly higher penicillin MIC than the other genotypes represented in our strain selection (Table 2).

In contrast to the strains in our collection, all of the archived CMRNG isolates displaying high-level penicillin resistance harbored both the −T deletion in the *mtrR* promoter and/or the G101K/A102N (KN) or G101K/A102D (KD) *porB1b* genotype. Interestingly, all of these archive isolates originated from infections obtained overseas.

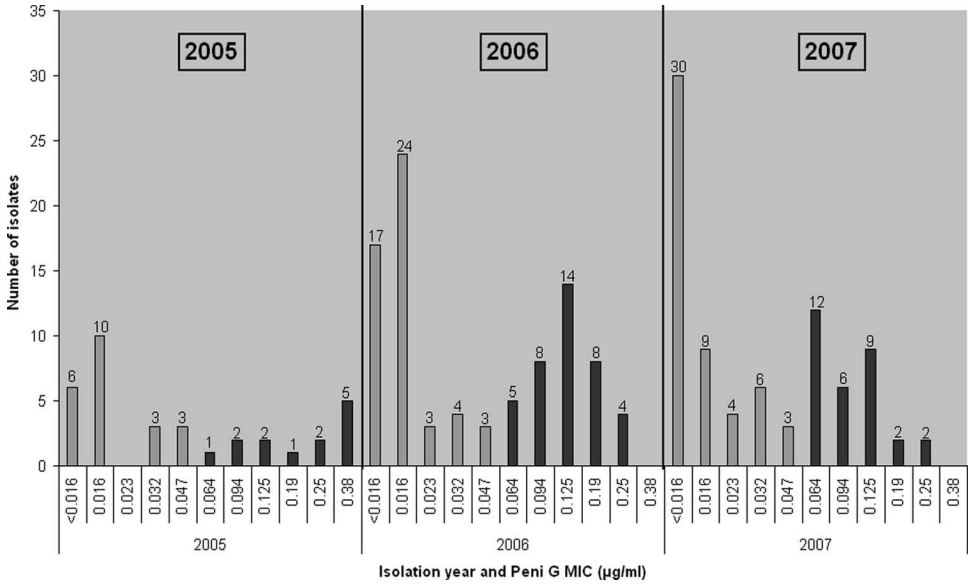

FIG. 2. Penicillin G (Peni G) MICs of the *N. gonorrhoeae* isolates according to their year of isolation. Gray and black bars indicate susceptible and intermediate levels of MIC, respectively. MICs are expressed as μg/ml.

Determination of the resistance phenotype of the *porB1b* GS allele (A102S). Although the *porB1b* GS allele is correlated with significantly higher penicillin MICs than the other genotypes, the A102 mutation has not been previously associated with resistance. To determine directly whether this allele is involved in resistance, we transformed strain FA19 *penA mtrR* with PCR products encompassing the *porB1b* gene from one of the intermediate resistant strains harboring the GS allele and selected for transformants on penicillin concentrations slightly higher than the MIC of the recipient strain (MIC = 0.25 μg/ml). Multiple transformants were obtained with PCR products amplified from the GS strain and from FA6140 (a CMRNG strain with a KD allele), but not from the *porB1b* gene from FA1090 (GA). The GS allele transformants exhibited increased penicillin MICs (0.63 μg/ml), but not to the level of the recipient strain transformed with the KD allele (MIC = 1.0 μg/ml). These data verify that the GS alleles are *penB* determinants, although they do not appear to have as strong a resistance phenotype as the KD allele.

TABLE 2. Genotypes and phenotypes observed in our collection of *N. gonorrhoeae* strains isolated from sexually transmitted infections in New Caledonia[a]

Type of *porB* allele	Type of *penA* allele	No. of strains with indicated type of *ponA* allele		Total no. of strains	MIC for penicillin G[b]
		WT	Mutant		
GA (WT)	WT	54	14	68	0.017*
	Asp-345a	31	1	32	0.081†
	Total	85	15	100	
DA (G101D)	WT	21	1	22	0.016*
	Asp-345a	0	0	0	
	Total	21	1	22	
GS (A102S)	WT	3	0	3	0.133‡
	Asp-345a	46	7	53	0.156‡
	Total	49	7	56	
porB1a	WT	10	10	20	0.011*
	Asp-345a	10	0	10	0.070†
	Total	20	10	30	
Total		175	33	208	

[a] Two hundred eight strains were isolated between February 2005 and October 2007. Results for isolates with wild-type genotypes are underlined. None of the isolates contained the single −T deletion in the *mtrR* promoter, and only two of the isolates with low susceptibility had a G45S mutation in the *mtrR* gene. WT, wild type.

[b] Symbols indicate significantly different average MICs between genotypes (analysis of variance and Fischer's protected least significant difference test: $0.001 < P < 0.013$).

Temporal trend. The mean MICs for penicillin were 0.100 μg/ml, 0.068 μg/ml, and 0.049 μg/ml for 2005 (35 isolates), 2006 (90 isolates), and 2007 (83 isolates), respectively (Fig. 2). Interestingly, there was a significant decrease in MICs between 2005 and 2006 and between 2005 and 2007 (analysis of variance and Fischer's protected least significant difference test, $P = 0.0441$ and $P = 0.0015$), but not between 2006 and 2007 ($P = 0.108$). The increased proportion of strains displaying MICs lower than 0.016 μg/ml confirms this trend, with this category representing 17%, 19%, and 36% of the isolates from 2005, 2006, and 2007, respectively. However, from a therapeutic point of view, there is no difference between the successive years, as the isolates being identified as having intermediate resistance accounted for 37%, 43%, and 37% of the total isolates in 2005, 2006, and 2007, respectively. Regarding genotypes, there is no evidence of a particular temporal evolution when considering either single-gene or multiple-gene genotypes. There is an apparent increased proportion of *porB1a* alleles in the 2007 isolates, reaching 18.1% in 2007, whereas the proportions were 14.3% and 11.1% in 2005 and 2006, respectively, but this difference is also not significant ($\chi^2 = 1.696$; $P = 0.4283$).

DISCUSSION

Real-time PCR technologies facilitate the ability to rapidly identify and quantify microorganisms. Various technologies have been developed that allow the genotyping of sequence variations in target genes (8). Many microbiology and diagnostic labs now have access to real-time PCR platforms, which are used for identification, quantification, and genotyping and, increasingly, for monitoring the emergence of antibiotic resistance in pathogenic *N. gonorrhoeae* (38, 39). In this study, we developed real-time PCR assays that permitted us to simultaneously genotype two major chromosomal determinants of penicillin resistance in an *N. gonorrhoeae* isolate in a single capillary and a third determinant in a separate assay. The design of our assay for the *penB* determinant is original, combining one single-anchor probe with two different sensor probes labeled with distinct fluorochromes. The −T deletion in the *mtrR* promoter could also be clearly observed with an additional hybridization probe pair in which the sensor probe, labeled with a third fluorochrome, targets the deletion region and its corresponding anchor targets a conserved region upstream of the *mtrR* promoter. Testing various primer pairs designed to conserved regions of the genes allowed us to optimize our assay so that both determinants could be studied in a single-capillary reaction using four wavelengths for fluorescence readings together with a color compensation algorithm using LightCycler software. Lastly, another assay clearly identifies *mtrR* G45D or G45S mutants and should also be able to identify mutations in codons 42 to 46 of the *mtrR* gene. When these assays are carried out in concert with another assay that we developed previously (39), we are able to genotype the *penA*, *ponA*, *penB*, and *mtrR* determinants within 4 h using three capillaries during two real-time PCR runs on a LightCycler 2.0 platform. This approach was optimized and validated by using archival and reference strains of various geographical origins, including several CMRNG strains. Once optimized, our assays were applied to either all 208 (*penA*, *ponA*, *penB*, and *mtrR* promoter deletion) or to the 83 intermediate-penicillin-resistant (*mtrR* G45 genotyping) *N. gonorrhoeae* strains isolated by our medical bacteriology lab over a 33-month period (February 2005 to October 2007). Interestingly, none of these strains produced a β-lactamase, indicating that all of the strains showing increased resistance did so through alterations of chromosomal genes.

The phenotypes and genotypes of the isolates with wild-type or low-level resistance were in good accordance. For example, out of 54 isolates genotyped as wild type with our assays, 52 were penicillin susceptible (MICs of <0.06 μg/ml) and the other 2 showed only very low levels of resistance (MICs of 0.064 and 0.094 μg/ml). Similarly, the Asp codon insert in *penA* was observed in 95 isolates, of which 89 have a penicillin MIC of ≥ 0.032 μg/ml, which is in good accordance with published results (34). The accordance between phenotypes and genotypes within a large strain collection demonstrates the relevancy of these particular determinants to susceptibility to β-lactams in CMRNG.

In the 208 isolates of our study period, the Asp-345a codon insert in *penA* sequences was found to be widespread, being found in almost half of the isolates. The *ponA* mutation (L421P, resulting from a T to C substitution) was observed in ~16% of the isolates. In contrast, and perhaps rather surprisingly, neither the *mtrR* promoter −T deletion nor the *penB* G101K/A102N (KN) or G101K/A102D (KD) genotypes were observed in any of our isolates. Instead, we observed a rare *mtrR* mutation genotype, G45S, to our knowledge yet unreported in the literature. Whether this mutation alters the DNA binding of MtrR was not evaluated in our study and remains to be explored. We also identified a particular *porB1b* mutation genotype, A102S (GS), that to our knowledge has not yet been reported. This GS genotype was observed frequently, with 56 out of our 208 isolates (ca. 27%) having these two amino acids at positions 101 and 102 in $PorB_{IB}$. Most (53 out of 56) of the isolates containing this *porB1b* A102S allele also carry the Asp-345a codon insertion in *penA*. Whether these isolates originate from a common strain circulating in New Caledonian communities remains unknown but can be explored in future studies; however, the variability of MICs within these isolates (in the range of 0.032 to 0.125 μg/ml) seems to indicate a nonclonal origin.

We were quite surprised to find that strains with the GS genotype had a significantly higher MIC than other isolates, suggesting that this *porB* allele might confer resistance to penicillin. Indeed, the results of transformation experiments confirmed that the *porB* GS allele reduces penicillin susceptibility in a strain harboring both *penA* and *mtrR*, although not to the same extent as a *porB* KD allele. The lower levels of resistance conferred by the GS allele correlate with the observation that mutations at G101 are more strongly associated with resistance than those at A102 (22). Besides the A102S mutation, these GS alleles have additional mutations relative to *porB1b* from FA1090 (i.e., the wild type), and additional studies will be necessary to identify which of these changes are most critical for conferring increased resistance to penicillin. It has been reported that other mutated *penB* alleles, such as G101K/A102D, are phenotypically silent in the absence of an *mtrR* determinant (23), but none of the isolates with the GS allele harbored either the *mtrR* promoter −T deletion or an *mtrR* G45D or G45S mutation. These results suggest that either the changes in the GS allele disrupt antibiotic permeation in the absence of an *mtrR* determinant or that these strains contain an *mtrR* determinant that is distinct from those examined in this study. Given the somewhat weaker phenotype of the GS allele in a model strain (FA19 *penA mtrR*), the latter seems the most likely, and current studies are focused on this possibility.

Similarly, 13 out of 16 isolates (including archival isolates) identified as harboring a *porB1a* allele together with wild-type genotypes of the other three determinants were highly susceptible to penicillin (MIC of 0.016 μg/ml or lower), but 3 isolates showed higher resistance, with MICs of 0.032 or 0.064 μg/ml, and these could also be further investigated. Whether sequence modification of *porB1a* alleles is responsible for decreased susceptibility to penicillin remains largely unknown and has seldom been studied experimentally (22). Isolates containing the *porB1a* allele represented 14.4% of the isolates from our 3-year study period, a percentage similar to the results of other studies in developed countries. Though it was not significant during our survey, we observed an increased frequency of isolation of *porB1a* strains between 2005 and 2007. Because these are considered to be responsible for most serious and invasive infections caused by *N. gonorrhoeae*, a longer-

term survey should pay attention to the evolution of this isolation frequency. An evaluation of whether some particular genotypes of this allele increase resistance to penicillin would also deserve consideration.

The order of acquisition of penicillin resistance determinants, which has been described in detail from laboratory transformation experiments (22, 23, 25), is *penA*→*mtr*→*penB*→*ponA*. Interestingly, this order of acquisition does not appear to be followed in our isolates. For example, 25 of 113 isolates that do not possess the Asp-345a insertion in *penA* harbor a mutated *ponA* allele. Indeed, these strains display lower penicillin MICs (from <0.016 to 0.064 μg/ml) than those usually observed when both the *mtrR* and *penB* determinants are present, confirming that the *ponA* mutation alone is unable to significantly decrease penicillin susceptibility (25). Our results within a large collection of strains highlight the importance of the order of stepwise acquisition of multiple resistance genes to conferring an incremental increase in resistance. However, isolates that have randomly acquired later-stage determinants can be regarded as "predisposed" to chromosomally mediated resistance should they acquire the other determinants.

Taken together, our genotyping and phenotypic data indicate that β-lactams, such as penicillin, can still be considered a useful option for curing *N. gonorrhoeae* infections in New Caledonia. A few archival penicillin-resistant isolates from 2003 and 2004 were found to contain both the *mtrR* promoter −T deletion and the G101K/A102N (KN) or G101K/A102D (KD) *porB1b* genotypes, and another isolate displayed high-level penicillin resistance due to a plasmid-encoded β-lactamase. However, all of these isolates resulted from infections obtained overseas and, because these determinants were not observed again in the following 3 years, they most likely did not get established in New Caledonia. These archival isolates nevertheless clearly illustrate that travelers constitute the major risk for importing penicillin-resistant isolates and changing antibiotic susceptibility patterns (35).

The results of our study highlight the utility of having a surveillance scheme aimed at evaluating the presence and incidence of penicillin resistance determinants in *N. gonorrhoeae* bacteria. Considering the high prevalence of both the Asp-345a codon insertion in *penA* and the *ponA* mutation and the probable absence of the *mtrR* promoter deletion, *mtrR* G45D/S mutations, and G101K/A102N (KN) or G101K/A102D (KD) *porB1b* genotypes, this surveillance could specifically focus on these latter determinants. The genotyping assays developed and reported in this study would constitute adequate tools for this purpose.

ACKNOWLEDGMENTS

Thanks are due to the staff of the bacteriology lab of the Institut Pasteur in New Caledonia under the authority of R. Goursaud and to C. Lethezer for providing the New Caledonian isolates of the study and MIC data. We are grateful to John Tapsall for sharing WHO reference isolates as part of the WHO-GASP.

This work was supported by grants from the International Network of Pasteur Institutes (General Delegation, Institute Pasteur, Paris, France).

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