Phenotypic and molecular study of antimicrobial resistance in Neisseria gonorrhoeae collected by a New Caledonian sentinel network

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ABSTRACT

Gonorrhoea, a major sexually transmitted infection, is a major public health concern, notably because of the antimicrobial resistance of its causative agent, Neisseria gonorrhoeae (NG). Decreased penicillin and fluoroquinolone susceptibilities mainly involve stepwise chromosomal mutations. The objective of our study was to correlate the presence of major determinants detected by multiplex real-time PCR assays with phenotypic antimicrobial results in 453 NG isolates and to evaluate the relevance of these genetic markers in antibiotic resistance surveillance. The genotypes obtained have been validated with NG from different countries and correlated with phenotypes. Recently, we established a sentinel network throughout New Caledonia to collect isolates from both public and private medical centres to reinforce surveillance of the emergence of antibiotic resistance in gonococci.

Keywords
Sexually transmitted infection - Neisseria gonorrhoeae- antimicrobial resistance – genotyping - surveillance

1. INTRODUCTION

Neisseria gonorrhoeae (NG) is the causative agent of gonorrhoea, one of the major sexually transmitted infections in many countries. Its remarkable ability to evolve resistance mechanisms threatens the use of most antimicrobial agents so that attention in an effective surveillance to limit the spread of multi-resistant strains has become an absolute necessity [1].

In New Caledonia where N. gonorrhoeae is increasingly isolated, circulating strains are still regarded as penicillin susceptible, a situation that is similar to that of other countries in Oceania [2] or of most rural parts of Australia [3]. Penicillin G is currently the recommended treatment for gonococcal infections in New Caledonia, notably in public health centers, but fluoroquinolones may alternatively be used in patients allergic to beta-lactams or whose private doctors prescribe the antibiotic according to recommendations issued by the French health authorities in France.

Although culture-based methods remain the gold standard for N. gonorrhoeae identification and antimicrobial susceptibility determination, nucleic acid amplification techniques (NAAT) are rapidly replacing culture for diagnosis because of their efficiency and rapidity [4], notably in developed countries. However, most of these techniques are useful only for diagnoses and do not provide any epidemiologically relevant genotypic information. Together with antimicrobial susceptibility patterns, multi-antigen sequence typing (NG-MAST) or analysis by pulsed-field gel electrophoresis (PFGE) are used to characterize circulating strains of Neisseria gonorrhoeae and allow the development of improved control measures.

In most bacteria, penicillin resistance is most commonly a consequence of the production of β-lactamases that inactivate the antibiotic [5], but in N. gonorrhoeae a major mode of resistance is due to the accumulation of genetic variations in endogenous genes and regulatory regions, which results in an increasing resistance to structurally unrelated groups of drugs [6-9]. Chromosomal gene modifications induce a gradual decrease in susceptibility as successive mutations accumulate.

Genetic mechanisms of chromosomally mediated resistance to penicillin are complicated and multifaceted. Stepwise transformation experiments demonstrated the involvement of mutated alleles of at least 4 genes: penA, mtrR, porB1B (penB determinant), and porA [10-18]. Insertion of an aspartic codon (Asp345a in penA results in reduced affinity of β-lactams for penicillin-binding protein 2 (PBP2) [19]. Specific polymorphisms in the promoter or coding region of mtrR, which encodes the major transcriptional repressor of the mtr locus, increase the expression of the MtrC-MtrD-MtrE efflux pump system and consequently confer resistance to many drugs [20]. Juxtaposed substitutions affecting 2 amino acids of the porin PorB1B decrease permeation of hydrophilic antimicrobials across the outer membrane and lastly a single mutation in porA, which encodes an altered PBPs with reduced affinity for penicillin and contributes to high-level of penicillin-resistance in NG [11]. All of these determinants, referred to as penA, mtrR, penB and porA respectively, have been widely observed in clinical isolates displaying decreased antibiotic susceptibility.

Mechanisms of fluoroquinolone resistance in N. gonorrhoeae are not as complicated as those of resistance to beta-lactams and involve mutations in the quinolone resistance determining regions (QRDRs) of gyrA and parC [21-23]. Mutations in the gyrA QRDR are considered as the first and most frequently acquired leading to either a less susceptible or a resistant phenotype, whereas parC changes constitute later steps always leading to complete resistance [24-26].
Using previously described real-time PCR protocols with hybridization probes [27-30], we genotyped all these chromosomal determinants in NG isolated from New Caledonia in Oceania over a 4-year period but also in a panel of NG from Madagascar in Africa and Cambodia in Asia to evaluate these molecular tools over strains from different geographical areas. We then evaluated the correlation between these genetic variations and the susceptibility to the corresponding antibiotics in order to evaluate the predictive value of these molecular tools and their possible use for an active surveillance of antibiotic resistance.

2. MATERIALS AND METHODS

2.1 N. gonorrhoeae isolates
A total of 441 *N. gonorrhoeae* isolates were studied, including 47 from Madagascar and 15 from Cambodia. Most were obtained from persons attending a sexually transmitted disease clinic and collected from cervical or urethral swabs. Identification of the organism was confirmed by Gram staining, oxidase activity and specific characteristics after conventional culture. All isolates collected from different centers were suspended in transport medium and stored at -20°C until DNA extraction. Twelve *N. gonorrhoeae* strains, referenced WHO-C, WHO-G, WHO-L, WHO-M, WHO-O, WHO-P, and 07QA-01 through 07QA-06 from Australian Reference Center and WHO Collaborating Center for STD, were used as controls for antimicrobial susceptibility testing.

2.2 Antimicrobial determination
MICs for penicillin G and ciprofloxacin were determined using the E-test diffusion method according to the manufacturer’s instructions. Breakpoint criteria defined by the Antibiogram Committee of the French Society for Microbiology (CA-SFM) for NG were followed for penicillin: susceptible strain, MIC ≤ 0.06 µg/mL, and resistant strain, MIC of > 1 µg/mL [31]. β-lactamase production was analyzed using nitrocefin discs (BioMérieux SA); strains testing positive were referred to as penicillinase-producing NG (PPNG). For ciprofloxacin, isolates were considered as susceptible (S) when MICs were ≤ 0.06 µg/mL, as intermediate (I) when MICs were 0.125 to 0.5 µg/mL, as resistant (R) when MICs were ≥ 1.0 µg/mL according to CLSI [32].

2.3 Real-time PCR genotyping assay
Genomic DNA was isolated from 0.2 mL of a thawed bacterial suspension in 2-SP using the QIAamp DNA minikit according to the manufacturer’s instructions. Oligonucleotide primers and probes were designed using LightCycler Probe Design software 2.0 (Roche Diagnostics), and synthesized by Sigma-Prolog (Singapore). The additional aspartic acid codon (Asp-345a) in penA, the single-base substitution in ponA, the –A deletion within mtrR promoter and porB1h characterization (penB determinant) were identified using PCR assays previously described [27,29]. Another duplex assay for +T insert and G45 codon genotyping was achieved within a specific fragment including mtrR promoter and coding region (Nandi et al., in prep.). Duplex genotyping of glyA and parC QRDRs was also recently described [30]. Melting curves analyses allowed the identification of different genotypes after a compensation color has been activated as described previously [29,30]. Controls in every experiment included a blank capillary, wild-type DNA and mutated genotype DNA of each known sequence for profile comparisons.

3. RESULTS

3.1 N. gonorrhoeae strains
NG strains were isolated in New Caledonia from 2005 to 2008 from samples collected through a public medical center. Since the beginning of 2008, they have been collected with the participation of private clinical laboratories and medical centers throughout the territory in a NG sentinel network named RESGO and coordinated by the Institut Pasteur of New Caledonia. A total of 379 strains from 191 females and 188 males were examined in this study. 62.8% (238/379) were susceptible to penicillin while 36.7% (139/379) were intermediate with MICs ranging from 0.064 to 0.75 g/mL. Two strains (0.5%) isolated in 2008, documented as imported strains (one from Indonesia and one from New Zealand), were resistant to both penicillin (PPNG) and ciprofloxacin.

The 47 Malagasy isolates were obtained from 42 men and 5 women. Twelve (25.5%) were resistant to penicillin (all of them were PPNG), 27 (57.5%) intermediate and 8 (17%) susceptible. For ciprofloxacin, 1 isolate was resistant, 2 intermediate and 44 (93.6%) were susceptible.

Within a panel of 15 isolates, collected from 12 men and 3 women, from the Institut Pasteur of Cambodia, twelve (80%) were resistant to penicillin (10 were PPNG) and 3 were intermediate with high MIC values (in the range 0.5-1 µg/mL). Almost all isolates (14/15 or 93.3%) were resistant to ciprofloxacin, with the remaining isolate being susceptible.

3.2 Genotyping results
Genotyping assays were successfully performed on all 453 NG strains. Different genotypes involved in chromosomally mediated resistance to penicillin and to fluoroquinolones were observed; some examples are presented in Figure 1. From 379 New Caledonian strains, 184 harboured the Asp-345a codon in *penA* whereas 195 were *penA* wild-type (WT). Analysis of the mtrR promoter revealed 1 strain with the –A deletion and 58 out of 236 tested with a +T insertion. In the coding region of *mtrR*, the G45D mutation was not observed in any strain, but one genotype later determined by sequencing as G45S was identified in 3 strains by virtue of having a distinct Tm. For the *penB* determinant we discriminated different genotypes: 180 were WT (*G101/A102 or GA*), 29 were *G101D (DA)*, 111 were *A102S (GS)*, 1 was *G101K/A102D (KD)* and 1 was non-typable. The 57 remaining strains (15%) contained the porB1a allele as confirmed by a specific amplon size on agarose gel. Concerning the *ponA* determinant, 327 strains were WT, whereas 52 contained the *ponA1* allele with the L421P mutation. Finally, in chromosomal resistance to fluoroquinolones, 2 out of 379 strains presented altered sequences for both *gyrA* and *parC* QRDRs: 1 was *gyrA* double mutated (S91F+D95G), *parC* single mutated (E91G) and 1 was *gyrA* single mutated (D95V) and *parC* single mutated (S87R). It should be noted that these 2 strains are the same as those described above that were resistant to penicillin and originated from importation.

All the 15 Cambodian strains contained mutated *penA* and *ponA* sequences, whereas KD and *G101K/A102N* (KN) genotypes were observed in the *penB* determinant in 5 and 4 isolates, respectively. These 9 strains harboured all the determinants described as involved in chromosomal penicillin resistance (*penA*, *mtrR*, *penB* et *ponA*), 1 strain presented both the –A deletion within the
promoter and a G45D mutation in the coding region of mtrR. The remaining 6 isolates had a porB1a allele with 5 WT and one mutated mtrR (–A deletion). Only one of the 15 isolates was characterized as WT for both QRDRs. Genotype distribution was as follows: (i) for gyrA, 3 distinct Tm groups with double mutations [S91F+D95A (8/14); S91F+D95G (3/14); S91F+D95Y (3/14)], (ii) for parC, 3 strains had a WT QRDR, 9 were single mutated [E91G (4); S87N (2); S87R (3)] and 3 isolates had the double mutation S87N+E91Q in parC.

Out of 47 Malagasy isolates, 37 bore the Asp-345a codon, 9 were penA WT and one was penA non-typable. No mtrR mutation was found. About penB determinant, 22 strains were characterized as WT (GA), 3 GS, 19 presented a porB1a allele. Three strains were penB non-typable including the discrimination between alleles porB1a or porB1b. The penA determinant occurred in 12 isolates, whereas 35 were WT. On the whole, the Malagasy panel had no changes in either gyrA or parC QRDRs.

3.3 Correlations between genotype / phenotype

Out of 238 New Caledonian strains susceptible to penicillin, 21.8% contained the penA determinant, out of which 81% had MIC ≥ 0.023 µg/mL. All of the strains with MIC ≥ 0.047 µg/mL harboured the additional codon. Similarly, all non-PPNG strains among Malagasy and Cambodian panels with the penA determinant had a MIC ≥ 0.032 µg/mL.

For mtrR, the +T insert within mtrR promoter represents the most prevalent sequence feature in New Caledonian isolates and was detected in 46% of intermediate strains but in only 8% of susceptible ones. The 3 strains with a G45S sequence in mtrR coding region had also an intermediate susceptibility to penicillin and the only strain displaying the –A deletion was producing a penicillinase, so it was already highly resistant to penicillin. The +T insert and G45S clearly correlated with a decrease in susceptibility, but not as strong as the one associated with the –A deletion. This latter mutation was absent in any non-PPNG isolate from Madagascar (MICs were < 0.5 µg/mL, except one) but present in all non-PPNG from Cambodia classified resistant to penicillin (penicillin MICs ranging from 0.5 to 2.0 µg/mL).

The penB (porB1b) genotypes GA or DA were predominantly observed in susceptible isolates, whereas the GS genotype, which conferring a moderately decreased susceptibility to penicillin [29], was found in 53.2% of the intermediate strains from New Caledonia with MICs ~ 0.15 µg/mL. The GS genotype, which does not confer a phenotype as strong as KD or KN, was rarely found in Madagascar isolates and was absent in Cambodian isolates. Interestingly, the porB1a allele has a high prevalence (40%) in Malagasy and Cambodian isolates including strains with altered MIC, but is much less prevalent (15%) in New Caledonia. These results suggested that penB genotypes can be indicative of the geographical origin of NG isolates. All non-PPNG from Cambodia classified as resistant to penicillin presented a KD or KN penB genotype, in agreement with their phenotype. Both genotypes appeared to be associated with similarly increased MIC.

Consistent with a previous study [ropp, 2002], the presence of the penA determinant was correlated with an increased MIC only when determinants penA, mtrR (–A deletion) and penB (KD or KN genotypes) were already acquired.

With regards to fluoroquinolone resistance, we observed a good correlation between genotypes and phenotypes with first, variations in gyrA QRDR (here double mutations in all resistant isolates from Cambodia and New Caledonian) and secondarily, mutations in parC QRDR, be they single or double as the MICs increased. Conversely, all susceptible strains presented wild-type QRDR.

4. DISCUSSION

In recent years, the diagnosis of infections caused by Neisseria gonorrhoeae has considerably increased in New Caledonia, and therefore has commanded greater attention, especially regarding the remarkable ability of this pathogen to become resistant to antimicrobials. For 5 years, we have observed a significant number of strains with reduced sensitivity not only to penicillin, which is the current recommended treatment for gonococcal infections in New Caledonia, but also to tetracycline, erythromycin and/or spectinomycin. Conventional isolation with susceptibility determination remains the mainstay of antimicrobial resistance surveillance but the feasibility of molecular methods for the specific detection of genetic alterations known to be involved in chromosomally-mediated resistance offers an alternate and efficient means in clinical resistance prediction. Admittedly, all these genetic markers have not yet been elucidated; nevertheless, acquisition of some variations in structural genes have, singly or cumulatively, an unambiguous and significant role in resistance development to structurally unrelated drugs. In New Caledonia as in other developed countries, NAAT are increasingly used because they can be implemented faster. The considerable advantage of such methods is to affirm the presence of gonococci and to assist the clinician in his therapeutic proposal, including situations where the pathogen is no longer viable, as is frequent with samples collected in remote peripheral health centers.

Our genotyping results were consistent with mechanisms described in the literature for non-PPNG. Only 5/453 (1.10%) strains presented some difficulty with one non-typable determinant per strain, mainly with penB. Out of these 5 NG, 4 were from Madagascar likely to highlight sequence diversity within the target related to their geographical origin. Apart from these few discrepancies, the analysis of these known resistance determinants reveals that their absence is strongly associated with antibiotic susceptibility. The penA allele with a D345a insertion was observed in 97% of the non-PPNG isolates with penicillin-
MIC ≥ 0.047 mg/mL, confirming this variation as the initial and most important mechanism for chromosomally mediated resistance to penicillins [6,11,17]. In contrast, the ponA determinant, considered as a final element, will significantly raise the MIC to high-level penicillin resistance only if all other determinants (penA, mtrR and penB) are present.

The genetic markers mtrR and penB are central determinants in broad spectrum antibiotic resistance, as they act collaboratively in a stepwise and specific order to confer resistance. The –A deletion in mtrR promoter is the main mechanism by which the MtrC-MtrD-MtrE efflux pump is overexpressed, and this mutation is required for the penB determinant to increase resistance by decreasing permeation of many agents. The presence of a +T insert in the mtrR promoter and mutations as G45D or G45S in mtrR coding region may increase the expression of the MtrCDE pump, mainly in strains lacking the dominant -A deletion in the promoter, but these variations alone only induce a moderate decrease in susceptibility to penicillin (Nandi et al., in prep). Regarding penB, only the KD and KN genotypes were associated with high intermediate- or resistant-levels of resistance to penicillin, with GA and DA being clearly present in all susceptible or low-intermediate strains. Interestingly, the genotype GS we recently observed as associated with a moderate decrease of susceptibility [29] is highly prevalent in New Caledonian isolates (29.3% with around 84% of them having a penicillin-MIC ≥ 0.047 mg/mL). Conversely, this GS sequence was rare in our Malagasy and absent in our Cambodian panels.

Out of 453 strains, our duplex genotyping assay developed to detect variations within QRDRs of gyrA and parC provided results that correlated very well with the susceptibility patterns with the exception of 3 discrepancies. Single, then double mutations in gyrA clearly correlated with a gradual increase in MIC sufficient to reach resistant status. parC mutations (single then double) occur only in gyrA mutated isolates and correlated with high-level fluoroquinolone-resistance. All 3 discrepancies affected Malagasy isolates with intermediate level of resistance to ciprofloxacin, though no QRDR mutation was found. Possible unknown variations outside the QRDRs could explain this result. If so, they would illustrate the fact that such assays can only detect mutations within designed targets.

A current limitation of these molecular tools could be the non-screening of any β-lactamase plasmid; however an adapted improvement to geographical environment would be feasible using available techniques [33]. Although successfully performed directly on clinical samples [28] it would be wise to realize genotyping test on DNA from isolated organisms because of the very high conservation of some target genes between bacterial species that could cause misinterpretations of the genotyping data.

All of the results observed through this study have been validated over different geographical areas and allow us to suggest that monitoring of specific mutations reflect phenotypic trends with a very good agreement. At the present time, genotypic analyse cannot be used to accurately predict MICs, but do constitute good predictive tools of clinical resistance. In order to expand our local surveillance program, a multi-center collaboration with all private medical laboratories as well as hospitals from the north of New Caledonia has been proposed and set up: the N. gonorrhoeae RESGO sentinel network. The number of strains collected through this network was limited, but allowed us to target people from different geographical and socio-economic origins, thus offering as complete as possible an overview of strains circulating in New Caledonia. As an illustration of this better collection of isolates, the RESGO network was recently able to collect 2 independent strains resistant both to penicillin and fluoroquinolones that could have gone undetected without this network.

In conclusion this study proved the reliability and usefulness of the genotyping assays to carry out a monitoring as extensive as possible in the continuous local surveillance of N. gonorrhoeae antimicrobial resistance. Such molecular tools could also help clinicians adapt an efficient therapeutic protocol to assure successful treatment of the bacteria. It especially provides valuable resistance trends for health authorities as decision-helping tools as whether and when to update treatment recommendations.

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6. REFERENCES


