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Pyrosequencing of the DNA gyrase gene in *Neisseria* species: effective indicator of ciprofloxacin resistance in *Neisseria gonorrhoeae*

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Abstract

The quinolone resistance determining region (QRDR) of the *gyrA* gene in ciprofloxacin-susceptible strains (n=53) and strains of *Neisseria* spp. with reduced susceptibility (n=70) was determined by the pyrosequencing method. Results showed that the QRDR of the *gyrA* gene is an effective molecular indicator of resistance to ciprofloxacin in *Neisseria gonorrhoeae*, and presumably in *Neisseria meningitidis*, but not in all other *Neisseria* spp. This sequence was not unique for *N. gonorrhoeae* and seems unsuitable for species verification of *N. gonorrhoeae*. However, whether it is also possible to use this region for verification depends on the specificity of the primary screening method used.

Keywords

Neisseria gonorrhoeae; *Neisseria* species; ciprofloxacin; *gyrA*; species verification

Several commercial kits for molecular diagnosis of *Neisseria gonorrhoeae* in a duplex PCR with *Chlamydia trachomatis* are available. Many laboratories, as well as clinicians, are interested in these techniques, since they provide fast negative results, are easy to perform, and also urine samples can be analyzed (1-7).

However, most of the commercial kits suffer from low specificity (1-7), and therefore require a confirmatory PCR (1,2,6,7). The AMPLICOR *N. gonorrhoeae* PCR (Roche Diagnostics, Indianapolis, IN, USA) targets the putative cytosine DNA methyltransferase gene. A PCR targeting the 16S rRNA gene has been recommended for confirmation (8), but we found it suboptimal for this purpose (6). Our previous results showed that the QRDR of the *gyrA* could be used as an indicator of ciprofloxacin resistance in *N. gonorrhoeae* and possibly for species verification of *N. gonorrhoeae* (6,8,9). In addition, the QRDR of the *gyrA* seems to be an effective indicator of ciprofloxacin resistance in *N. meningitidis* (10-12).

The aims of this study were to investigate if the molecular method indicating resistance to ciprofloxacin in *N. gonorrhoeae*, based on pyrosequencing of the QRDR of the *gyrA* gene, can

be used as an indicator of resistance to ciprofloxacin in most *Neisseria* spp. and if this QRDR sequence is suitable also for species confirmation of *N. gonorrhoeae*.

MATERIALS AND METHODS

In the present study, *N. gonorrhoeae* reference strains (CCUG 37534 and ATCC 43069) and clinical isolates (n=83), as well as reference strains (*N. lactamica* ATCC 23970 and CCUG 17628, *N. meningitidis* ATCC 13090 and ATCC 13077, *N. subflava* CCUG 30607 and CCUG 32186, *N. flavescens* CCUG 26476, CCUG 17913 and CCUG 806, *N. cinerea* CCUG 25879 and CCUG 27178, *N. mucosa* ATCC 9913, and *N. sicca* CCUG 26877) and clinical isolates (n= 27) of nine other *Neisseria* spp. were examined.

The species identification of the isolates was based on growth ability in different incubational conditions, colony morphology and pigmentation on different culture media, reduction of nitrate, sugar oxidation tests (glucose, maltose, fructose, and lactose) (13), RapID NH (Remel, Lenexa, USA) and/or API NH v2.0 (BioMérieux, Lyon, France), and sequencing of the 16S rRNA gene (Unemo et al. personal communication). All isolates were cultured as previously described (14). Minimum inhibitory concentration (MIC) of ciprofloxacin was determined by Etest (AB Biodisk, Solna, Sweden).

PCR was performed with the previously described primers gyrA2-1 and gyrA2-2 (9), followed by sequencing using the pyrosequencing technique with the primer gyrA2-3, as previously described (9).

According to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (<http://www.srga.org/eucastwt/MICTAB/index.html>, access date: 2006-04-21) as well as the Swedish Reference Group for Antibiotics (SRGA) (<http://www.srga.org/MICTAB/MICTAB2.htm>, access date: 2006-04-21) *N. gonorrhoeae* and *N. meningitidis* ciprofloxacin-susceptible strains have MIC $\leq$ 0.03 mg/L and resistant strains have MIC $>$ 0.06 mg/L. In the present study, susceptibility of all *Neisseria* spp. was interpreted according to these breakpoints. Of the *Neisseria* isolates that gave a sequence (n=113), 46 were susceptible to ciprofloxacin, 65 were resistant, and two isolates were intermediate (Table 1). All sequenced reference strains were susceptible to ciprofloxacin.

RESULTS AND DISCUSSION

The pyrosequencing method gave sequences for all strains of *N. gonorrhoeae* (Table 1). The *gyrA* sequences encoding amino acids 91–95 of GyrA revealed the wild-type sequence in all ciprofloxacin-susceptible strains and six different patterns of non-synonymous substitutions in the ciprofloxacin-resistant strains.

We found the ciprofloxacin-resistant strain comprising a mutation that changed Ser91 to Thr and also a synonymous substitution in Ala92 especially interesting, since this sequence was a unique QRDR of *gyrA* variant in our large collections of *N. gonorrhoeae*, and also it was identical to the sequences of *N. subflava* and *N. flavescens* ciprofloxacin-susceptible strains. To our knowledge this sequence has not been published (Table 1).

Most isolates of the species *N. cinerea*, *N. elongata*, *N. mucosa*, and *N. sicca* showed a sequence identical to one of the *N. gonorrhoeae* ciprofloxacin-resistant sequences (Table 1). The same *gyrA* sequence was found in both ciprofloxacin-susceptible as well as in intermediate strains of *N. cinerea*. The susceptible strain of *N. sicca* showed the same sequence as one of the *N. gonorrhoeae* ciprofloxacin-resistant *gyrA* sequences; in contrast, the two ciprofloxacin-resistant strains of *N. sicca* showed the *N. gonorrhoeae* ciprofloxacin-susceptible wild-type

pattern (Table 1). *N. subflava* and *N. flavescens* showed the same sequence as that of one of the *N. gonorrhoeae* ciprofloxacin-resistant strains (Table 1).

Regarding *N. gonorrhoeae* subsp. *kochii*, both ciprofloxacin-susceptible as well as ciprofloxacin-resistant strains showed an identical *gyrA* sequence, and this sequence was also the same as for ciprofloxacin-susceptible strains of *N. meningitidis* and *N. lactamica* (Table 1).

The following 12 strains did not give a PCR product and were excluded from further analysis: one clinical isolate of *N. mucosa* and ATCC 9913, one clinical isolate of *N. cinerea* and CCUG 27178, CCUG 25879, one clinical isolate of *N. sicca* and CCUG 26877, three clinical isolates of *N. subflava* and two clinical isolates of *N. meningitidis*. Of these 12 strains, 7 were susceptible, 2 were intermediate, and 3 were resistant to ciprofloxacin.

The results presented in Table 1 implicate that, in *Neisseria* spp., also other mechanisms of resistance to ciprofloxacin than *gyrA* mutations at position Ser91 and Asp95 are involved. The sequence variation in the QRDR in the *gyrA* of *N. gonorrhoeae* may be due to genetic exchange among *Neisseria* spp.

Additional strains need to be examined in order to draw conclusions respecting those *Neisseria* spp. only represented by a few ciprofloxacin-susceptible strains in this study, but it seems that the correlation between ciprofloxacin susceptibility and mutations in the QRDR of the *gyrA* is unique for *N. gonorrhoeae* and probably also for *N. meningitidis*. Unfortunately, no *N. meningitidis* strain with reduced susceptibility to ciprofloxacin was available to us. According to previous studies, sequencing of the QRDR in the *gyrA* will presumably also identify the rare strains of *N. meningitidis* with reduced susceptibility to ciprofloxacin (10-12). Meanwhile, since two clinical isolates of *N. meningitidis* were not amplified with the primers that we used, our method needs to be further optimised for this purpose.

Despite the fact that a few ciprofloxacin-resistant *N. gonorrhoeae* strains without alterations in the QRDR of the *gyrA* have been reported (15-17), the present study supports previous studies (6-9) of pyrosequencing of the QRDR in the *gyrA* gene as an effective molecular method to indicate resistance to ciprofloxacin in *N. gonorrhoeae*. However, the method is useful for *N. gonorrhoeae* and presumably for *N. meningitidis*, but not for other *Neisseria* spp.

Neisseria spp. are highly recombinogenic and naturally transformable (18-19). Clinical methods to distinguish between *Neisseria* spp. are designed mainly to discriminate *N. gonorrhoeae* and *N. meningitidis* from other *Neisseria* spp. *Neisseria* multilocus sequence typing (MLST) showed that in *Neisseria* spp. the definition of a species will not be clear cut, especially among those species that colonize the same body site, such as *N. meningitidis* and *N. lactamica*. Thus, both these species colonize the human pharynx and there are also mosaic overlaps due to recombination (19). Also *N. gonorrhoeae* can colonize the human pharynx and thus recombination between species probably occurs. This might explain the atypical *gyrA* sequence of one *N. gonorrhoeae* strain, the same sequence as that of *N. subflava* and *N. flavescens*. Even if future commercial kits are further optimised for *N. gonorrhoeae* diagnostics the results should be evaluated bearing in mind the high recombination frequency.

The clinical application of this method as a molecular indicator of ciprofloxacin resistance adds valuable information to any commercial molecular *N. gonorrhoeae* diagnostic test. So far, all *N. gonorrhoeae* samples have been amplified using this method, but some strains of other *Neisseria* spp. gave no PCR products. Consequently, samples that give no PCR product can be verified as non-*N. gonorrhoeae*. In a previous study Airell et al. (6) showed that out of 24 *N. gonorrhoeae* culture-negative pharynx samples, 16 were positive in AMPLICOR *N. gonorrhoeae* PCR (Roche Diagnostics). *N. gonorrhoeae* species verification using the 16S

rRNA gene was negative in 14 of these samples and in all 16 with verification with the *gyrA* gene. With further optimisations of the PCR protocol, it is possible that *Neisseria* spp. other than *N. gonorrhoeae* can be excluded with acceptable accuracy.

In conclusion, the examined QRDR of the *gyrA* gene may not seem suitable for species verification of *N. gonorrhoeae*, since this sequence only discriminates *N. gonorrhoeae* from *N. meningitidis*, *N. gonorrhoeae* subsp. *kochii* and *N. lactamica* (Table 1). However, whether it is possible to use this region for species verification still depends on the specificity of the primary screening method used. In addition, this sequence is an effective indicator of ciprofloxacin resistance in *N. gonorrhoeae* and probably in *N. meningitidis*, but not all other *Neisseria* spp.

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Sequence of the wild-type *gyrA* gene encoding amino acid positions 91–95 of *GyrA* in *N. gonorrhoeae*, identified divergences from *N. gonorrhoeae* wild-type *gyrA* in isolates of *N. gonorrhoeae* and other *Neisseria* spp., and ciprofloxacin susceptibility of all the isolates

TABLE 1

Species	MIC ciprofloxacin (mg/L)		Number of strains		Amino acid position in <i>GyrA</i>				
	S≤0.03	I (MIC)	R>0.06 (MIC)		91	92	93	94	95
<i>N. gonorrhoeae</i>	24				S	A	V	Y	D
					TCC	GCA	GTT	TAC	GAC
			4		F				
					TTC				
			5						N
<i>N. lactamica</i>	6		7		F				AAC
					TTC				A
			33		F				GCC
					TTC				G
			11		F				GCC
<i>N. meningitidis</i>	4				TTC				N
			1		T	A			AAC
					ACA	GCC			
			2 (4)		T	A	V		
					ACC	GCC	GTA		
<i>N. subflava</i>	3				T	A	V		
					ACC	GCC	GTA		
					T	A	V		
					ACC	GCC	GTA		
					T	A	V		
<i>N. flavescens</i>	3				ACA	GCC			
					T	A			
					ACA	GCC			
					T	A			
					ACA	GCC			
<i>N. cinerea</i>	1		2 (0.06)		F				G
					TTC				GCC
					F				G

Species	MIC ciprofloxacin (mg/L) Number of strains			Amino acid position in GyrA				
	S≤0.03	I (MIC)	R>0.06 (MIC)	91	92	93	94	95
<i>N. mucosa</i>	1			TTC				GGC
				F				G
				TTC				GGC
<i>N. sicca</i>	1			F				G
				TTC				GGC
<i>N. sicca</i>			2 (0.5;1.0)	Sequence identical to <i>N. gonorrhoeae</i> wild-type <i>gyrA</i> (TCC GCA GTT TAC GAC)				

The 12 strains of *Neisseria* spp. that gave no sequence are included in Table 1.