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Research Paper

Molecular basis of high-level ciprofloxacin resistance in *Neisseria gonorrhoeae* strains from Shandong Province, China

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Abstract

In the study, the ciprofloxacin resistance rate was 100%. High-level ciprofloxacin resistance rate was 63.55%. Sixteen different mutation patterns involved in the formation of ciprofloxacin resistance were identified. The most prevalent were patterns P7 (25.2%), P8 (15.0%), P9 (11.2%), P1 (10.3%), and P5 (10.3%). All of the 107 *NG* isolates analyzed for mutations in the study have demonstrated a change of Ser-91 → Phe in the *gyrA* gene, and all except one have demonstrated a change in position 95 of the amino acid sequence. All of the 68 high-level QRNG isolates had double mutations in *gyrA* gene combined with a single or two mutations in *parC* gene. It is most important that a new mutation site of Ile-97 → Met in *gyrA* and a new mutation of *Leu-106* → Ile in *parC* were found in the study, both leading to high-level ciprofloxacin resistance (MIC values, 8 μg/mL, 32 μg/mL, respectively). Therefore, we confim that *gyrA* mutations are necessary for the fluoroquinolone resistance phenotype and *parC* mutations are correlated intimately with high-level fluoroquinolone resistance. In China fluoroquinolone resistance in *Neisseria gonorrhoeae* strains is very serious and the new mutation sites in the fluoroquinolone resistance-determining regions emerge more and more quickly. Hence, in China fluoroquinolones, which are used to treat *gonorrhoea presently, should be substituted by a new antibiotics.*

Key words: *Neisseria gonorrhoeae,* ciprofloxacin resistance, high-level, molecular analysis.

Introduction

Neisseria gonorrhoeae (*N. gonorrhoeae*) is the pathogen of gonorrhoea, one of the most common sexually transmitted diseases worldwide. In China, gonorrhea is the second one of the sexually transmitted diseases, with a cumulative total of 119, 824 reported cases in 2009 (http://www.chinacdc.cn/en/, 2010). *Neisseria gonorrhoeae* urogenital infection leads to pelvic inflammatory disease and infertility. Therefore, it is very important for early detection and appropriate treatment of *N. gonorrhoeae* infection. In the face of widespread penicillin resistance, fluoroquinolones have been used as an effective antimicrobial agent for treatment of *N. gonorrhoeae* infection. However, presently fluoroquinolone-resistance in *N. gonorrhoeae* (QRNG) has emerged and increased rapidly in the United States, Western Europe, and Asia (Fiorito *et al.*, 2001; Bala *et al.*, 2003; Vereshchagin *et al.*, 2004; Yong *et al.*, 2004; http://www.who.int/en/, 2004; Xie *et al.*, 2006; Allen *et al.*, 2011; Angeliki *et al.*, 2011;). In China, the range of the rate of fluoroquinolone resistance is about 30% to 80%, depending on the regions studied (Su *et al.*, 2004; Liu *et al.*, 2006; Tu *et al.*, 2006).

Fluoroquinolone resistance in *N. gonorrhoeae* has been shown to be due to point mutations at Ser-91 and Asp-95 in the *gyrA* gene (Fiorito *et al.*, 2001). Besides this, *parC* mutation has also been involved in high-level fluoroquinolone resistance of *N. gonorrhoeae (*Lindback *et al.*, 2002), but *parC* mutations in isolation do not appear to be associated with antimicrobial resistance (Zhou *et al.*, 2004). Zafar Sultan shows that double alterations at Ser-91 and Asp-95 in *gyrA* plus a single or double *parC* alteration(s) play an important role in the development of high-level fluoroquinolone resistance in *N. gonorrhoeae (*Sultan *et al.*, 2004). Isolates with only *gyrA* mutations has lower MICs than those with mutations in both *gyrA* and *parC* gene (Wang *et al.*, 2006; Yang *et al.*, 2006).

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In this study we investigated the occurrence of ciprofloxacin resistance among 107 isolates of *N. gonorrhoeae* collected from Shandong Province from 2007 to 2011, and analyzed the molecular basis of ciprofloxacin resistance in the *Neisseria gonorrhoeae* isolates from Shandong Province, China.

Materials and Methods

Bacterial strains

107 non-duplicate isolates of *N. gonorrhoeae* were collected from patients who attended sexually transmitted disease (STD) clinics in Tai'an between June 2007 and June 2011. The age range of the patients was from 18 to 51 years (mean, 29.6 years), and the ratio of male to female patients was 1.2:1. The swab specimens were isolated initially on Thayer-Martin agar plates, then subcultured onto chocolate agar plates in a 5% CO_2 incubator at 35 °C for 24 to 48 h, and then identified by colony morphology, Gram stain and oxidase reaction, followed by carbohydrate-utilization studies of glucose, maltose, fructose and sucrose (API NH, bioMérieux). Colonies collected from plates and supplemented with 8% skimmed milk and 10% fetal calf serum were stored at -70 °C.

Antimicrobial susceptibility testing

MICs of ciprofloxacin (Bayer AG, Germany) were determined by an agar dilution method with GC agar base (bioMérieux, France) supplemented with PolyViteX (bioMérieux, France) according to the Clinical and Laboratory Standards Institute (National Committee for Clinical Laboratory Standards, 2006). *N. gonorrhoeae* ATCC 49226 (American Type Culture Collection, USA) was used as a control. All tests were performed in triplicate. The MIC (μg/mL) was defined as the concentration at which no colony was detected after agar plates were incubated at 35 °C in 5% CO_2 for 24 h. The criteria for ciprofloxacin resistance in *N. gonorrhoeae* isolates was MIC values ≥ 1 μg/mL (National Committee for Clinical Laboratory Standards, 2006). The criteria for high-level ciprofloxacin resistance in *N. gonorrhoeae* isolates was MIC values ≥ 4 μg/mL(Allen *et al.*, 2011).

$QRDR_s$ amplification and sequencing

The fluoroquinolone resistance-determining regions (QRDRs) were amplified with the primers designed by Primer Premier 5.0 (Premier, Canada). The forward primer for the *gyrA* gene was 5'-GAT GTA TCA ATC CGC CAC G-3' and the reverse was 5'-AGC AGT TGA CGA GCA GTG TG-3', leading to a product of 529 bp from position 7 to 535. The PCR mixtures were composed of 0.5 μL of each of primers (25 μM, each), 0.5 μL of *Ex-Taq* (5 U/μL) (TaKaRa Biotech., Dalian, China), 2.5 μL of 10*LA* PCR Buffer II (Mg^{2+} Free), 2.5 μL of $MgCl_2$ (25 mM), 4 μL of dNTP mixtures (2.5 mM, each), 5 μL of template DNA, and 9.5 μL of the distilled water added to a final volume of 25 μL. The PCR for the *gyrA* gene was carried out as follows: 93 °C denaturation for 2 min, followed by 35 cycles of denaturation at 93 °C for 45 s, annealing at 54 °C for 55 s and extension at 72 °C for 60 s, with final extension at 72 °C for 3 min. The forward primer for amplification of the *parC* gene was 5'-CGC TTC CCA TAC TGA TTC CAA C-3' and the reverse was 5'-TTG TGC TAC GCA ATC TCG GT-3', leading to a product of 552 bp from position 18 to 569. The PCR mixtures were composed of 0.5 μL of each of primers (25 μM, each), 1.0 μL of *Ex-Taq* (5 U/μL) (TaKaRa Biotech., Dalian, China), 2.5 μL of 10*LA* PCR Buffer II (Mg^{2+} Free), 2.5 μL of $MgCl_2$ (25 mM), 4 μL of dNTP mixtures (2.5 mM, each), 5 μL of template DNA, and 9.0 μL of the distilled water added to a final volume of 25 μL. The PCR for the *parC* gene was carried out as follows: 95 °C denaturation for 2 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 60 s and extension at 72 °C for 60 s, with final extension at 72 °C for 2 min. The amplified fragments was purified by *Genomic DNA Purification Kit* (Promega Wizard, USA), and the PCR products were sent to Invitrogen Biotech.(Shanghai, China) for sequence analysis on an ABI373 automatic sequencer (Applied Biosystems, USA), and data were aligned with QRDR sequences for amino acids (aa) 91 to 95 of *gyrA* (GenBank accession number U08810) and aa 86 to 91 of *parC* (GenBank accession number U08907).

Results

Susceptibilities to ciprofloxacin

The ciprofloxacin susceptibilities of 107 Chinese gonococcal isolates were shown in Table 1. All of the 107 isolates were resistant to ciprofloxacin (MICs, ≥ 1 μg/mL). Of the 107 isolates tested, 68 isolates demonstrated high-level resistance to ciprofloxacin (MICs, ≥ 4 μg/mL).

Mutation patterns in the *gyrA* and *parC* genes

Table 2 showed the mutation patterns found in *gyrA* and *parC* genes in 107 ciprofloxacin-resistant isolates. Six-

Table 1 - Ciprofloxacin MICs for 107 Chinese *N. gonorrhoeae* isolates.

Agent	No. of strains with MIC (μg/mL) of:													No. of highly resistant strains (%)	MIC50	MIC90
	0.015	0.03	0.06	0.125	0.5	1	2	4	8	16	32	64	128			
Ciprofloxacin						17	22	30	22	8	6	1	1	68(63.55%)	4	16

Table 2 - Alterations in *gyrA* and *parC* genes of 107 Chinese QRNG isolates.

Mutation Pattern	Phe.	Amino acid substitution in:									No. of strains (%)	Cip MIC range (μg/mL)
		GyrA					ParC					
		Ser-91 (TCC)	Ala-92 (GCA)	Asp-95 (GAC)	Ile-97 (ATC)	Gln-102 (CAA)	Asp-86 (GAC)	Ser-87 (AGT)	Ser-88 (TCC)	Glu-91 (GAG)	Leu-106 (TTA)	
P1	Cip^r	Phe (TTC)		Asn (AAC)				Asn (AAT)				11 (10.3%)	1-2
P2	Cip^r	Phe (TTC)		Asn (AAC)						Lys (AAG)		5 (4.7%)	1-2
P3	Cip^r	Phe (TTC)		Asn (AAC)					Pro (CCC)			3 (2.8%)	1-2
P4	Cip^r	Phe (TTC)		Ala (GCC)						Gly (GGG)		5 (4.7%)	1-2
P5	Cip^r	Phe (TTC)		Ala (GCC)			Asn (AAC)					11 (10.3%)	1-2
P6	Cip^h	Phe (TTC)		Asn (AAC)				Ile (ATT)				2 (1.8%)	4-16
P7	Cip^h	Phe (TTC)		Gly (GGC)				Arg (CGT)				27 (25.2%)	4-32
P8	Cip^h	Phe (TTC)		Ala (GCC)				Arg (CGT)				16 (15.0%)	4-32
P9	Cip^h	Phe (TTC)		Ala (GCC)			Asn (AAC)					12 (11.2%)	4-32
P10	Cip^h	Phe (TTC)		Gly (GGC)			Asn (AAC)					8 (7.5%)	4-32
P11	Cip^h	Phe (TTC)		Ala (GCC)				Arg (CGT)		Ala (GCG)		1 (0.9%)	64
P12	Cip^r	Phe (TTC)	Pro (CCA)									1 (0.9%)	12
P13	Cip^h	Phe (TTC)	Pro (CCA)	Gly (GGC)				Arg (CGT)				1 (0.9%)	32
P14	Cip^h	Phe (TTC)		Ala (GCC)	Met (ATG)				Pro (CCC)			1 (0.9%)	8
P15	Cip^h	Phe (TTC)		Ala (GCC)		His (CAC)	Asn (AAC)					1 (0.9%)	16
P16	Cip^h	Phe (TTC)		Ala (GCC)				Arg (CGT)			Ile (ATA)	1 (0.9%)	32

Abbreviations: Cip, ciprofloxacin; r, resistant; h, high-level resistant; Phe, Phenotype.

teen different mutation patterns were characterized. Seven mutation sites were found on the *gyrA* gene, leading to seven amino acid changes. Twelve mutation sites were found on the *parC* gene, leading to nine amino acid changes. Three silent mutations in codons 104 (TAT to TAC), 129 (GCG to GCA), and 131 (CTC to CTG) of *parC* genes were detected. These silent mutations occurred in 60 QRNG strains (56.1%).

Discussion

Nowadays, in China, fluoroquinolones have been continually used to treat infections caused by *N. gonorrhoeae* despite widespread resistance. The continued use of fluoroquinolone antibiotics results in a substantial of fluoroquinolones resistant *N. gonorrhoeae* strains and raises the possibility that horizontal gene transfer is an important mechanism for the acquisition of resistance (Loubna *et al.*, 2010).

In this study the ciprofloxacin resistance rate was 100%. High-level resistance to ciprofloxacin (MICs, ≥ 4 μg/mL) was 63.55% (Table 1). Therefore, this antibiotic should not be recommended for treatment of gonococcal infections in the Shandong Province, China. In this study sixteen different mutation patterns involved in the formation of ciprofloxacin resistance were identified. The most prevalent were patterns P7 (25.2%), P8 (15.0%), P9 (11.2%), P1 (10.3%), and P5 (10.3%), which differed from those reported in other countries and regions (Vereshchagin *et al.*, 2004; Yong *et al.*, 2004; Angeliki *et al.*, 2011;). All of the 107 *NG* isolates analyzed for mutations in the study have demonstrated a change of Ser-91 → Phe in the *gyrA* gene, and all except one have demonstrated a change in position 95 of the amino acid sequence. All of the 68 high-level QRNG isolates in the study had double mutations in position 91 and 95 in *gyrA* gene combined with a single or two mutations in *parC* gene (Table 2). It is most important that a new mutation site of Ile-97 → Met in *gyrA* and a new mutation of *Leu-106* → Ile in *parC* were found in this study, both leading to high-level ciprofloxacin resistance (MIC values, 8 μg/mL, 32 μg/mL, respectively) (Table 2). It shows that in China fluoroquinolone resistance in *Neisseria gonorrhoeae* strains is very serious and that the new mutation sites in the fluoroquinolone resistance-determining regions emerge more and more quickly. Therefore, in China fluoroquinolones, which are used to treat gonorrhoea presently, should be substituted by a new antibiotics.

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