Antimicrobial susceptibility of *Neisseria gonorrhoeae* isolated in Jiangsu Province, China, with a focus on fluoroquinolone resistance

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In this study, the phenotypic and genotypic resistance to fluoroquinolones in *Neisseria gonorrhoeae* isolated in Jiangsu Province, China, was analysed. *In vitro* susceptibility testing of eight antimicrobial agents, including ciprofloxacin and levofloxacin, against 95 clinical isolates was carried out. Detection of mutations in the *gyrA* and *parC* genes was performed by sequence analysis. The clinical isolates demonstrated 100 % resistance to ciprofloxacin and 98.9 % non-susceptibility to levofloxacin. All of the isolates were susceptible to cefotaxime and ceftriaxone. For cefepime, spectinomycin and tetracycline, 98.9, 94.7 and 1.1 % of the isolates were susceptible, respectively. None of the isolates was susceptible to penicillin. Five types based on *gyrA* mutations could be categorized among 54 isolates with seven different mutation sites found on their *parC* gene. Analysis of sequence results showed that the *gyrA* mutation Asp-95→Ala and the *parC* mutations Ser-87→Arg and Ser-87→Asn made a significant contribution to the resistance to fluoroquinolones, in addition to double mutations found in each gene. Therefore, the use of fluoroquinolones in the treatment of *N. gonorrhoeae* infections in Jiangsu Province is not recommended, while the use of third- and fourth-generation cephalosporins and spectinomycin is recommended.

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INTRODUCTION

Neisseria gonorrhoeae is a major pathogen of sexually transmitted diseases in China, with a cumulative total of 185 319 notified cases in 2003 (Chinese Centre for Disease Control and Prevention, 2004). Nowadays, treatment of gonorrhoea using chloramphenicol, sulphonamide, penicillin and tetracycline has become ineffective. The fluoroquinolones were introduced and widely used clinically in the 1980s because of their effectiveness and convenience in single-dose oral administration, together with lower cost. After 10 years of clinical application, an increasing trend of fluoroquinolone resistance in *N. gonorrhoeae* has been reported in Japan, Korea, south-east Asia, Canada and China (Krishna *et al.*, 2005; Ng *et al.*, 2002; Tanaka *et al.*, 2004; World Health Organization, 2003; Yoo *et al.*, 2004). In China, the rate of fluoroquinolone resistance is about 40–80 %, depending on the region studied (Gu *et al.*, 2004; Ye, 2001). However, in China, quinolones are still recommended to treat gonorrhoea (single dose 500 mg for ciprofloxacin) (Cai *et al.*, 2003; Su, 2002; Su *et al.*, 2004; World Health Organization, 2003; Zhu & Zhao, 2003). Accordingly, it is necessary to investigate the resistance rate and

mechanism in *N. gonorrhoeae*. It has been reported that the development of mutations in DNA gyrase subunit A (GyrA) encoded by the *gyrA* gene and mutations in topoisomerase IV encoded by the *parC* gene account for most ciprofloxacin resistance (Belland *et al.*, 1994). We collected 95 isolates of *N. gonorrhoeae* from three regions of Jiangsu Province during 2003 to study their susceptibilities to eight antimicrobial agents and assess the correlation between fluoroquinolone MICs and amino acid changes in GyrA and ParC.

METHODS

Bacterial isolates and culture. Ninety-five non-duplicate isolates of *N. gonorrhoeae* were collected from patients who attended sexually transmitted disease (STD) clinics in the Nanjing, Xuzhou and Wuxi regions between May and December 2003. The organisms were cultured and isolated on NG-Thayer-Martin agar (bioMérieux), and identified by Gram stain and oxidase reaction, followed by carbohydrate-utilization studies of glucose, maltose, fructose and sucrose (API NH, bioMérieux). The bacterial isolates were kept on chocolate agar slants (bioMérieux) for short-term storage. Pure cultures supplemented with 8 % skimmed milk and 10 % fetal calf serum were used for long-term storage at –70 °C.

In vitro antimicrobial susceptibility testing. Material of known potency of the following antimicrobial agents was used for MIC determination: ciprofloxacin (Bayer), levofloxacin (Daiichi), spectinomycin (Pharmacia and Upjohn), penicillin (Sigma), tetracycline (Sigma), cefotaxime (Sigma), ceftipime (Bristol-Myers-Squibb) and ceftriaxone (Sigma). MIC values were determined by an agar dilution method with GC agar base (Oxoid) plus supplements according to the National Committee for Clinical Laboratory Standards (2002). A multipoint inoculator (MIC-2000 System, Dynatech) was used to deliver 10^4 c.f.u. per spot onto GC agar base supplemented with 1% Vitox (Oxoid) and 5% lysed horse blood. *N. gonorrhoeae* ATCC 49226 was included as the control strain for the antimicrobial susceptibility test. The MIC (mg l^{-1}) was defined as the concentration at which no colony was detected after agar plates were incubated at 37 °C in 5% CO_2 for 24 h. Resistance breakpoints for the antimicrobial agents penicillin, cefotaxime, ceftriaxone, ceftipime, tetracycline, ciprofloxacin and spectinomycin were interpreted according to the National Committee for Clinical Laboratory Standards (2002). For levofloxacin, MIC values of $\geq 1 \text{ mg l}^{-1}$ were considered to be resistant (Shigemura *et al.*, 2004; Centers for Disease Control and Prevention, 2005).

Detection of mutations in *gyrA* and *parC* genes. Bacterial isolates were selected from every ciprofloxacin MIC level by the use of a stratified random-sampling method. Bacterial cells were harvested and suspended in Tris/EDTA buffer. Cell suspension (100 μl) was centrifuged at 10 000 *g* at 4 °C for 5 min, the pellet was resuspended in lysing buffer (Tris/EDTA containing 200 ng proteinase K ml^{-1}) at 55 °C for 1 h, and then incubated at 95 °C for 5 min, centrifuged at 10 000 *g* for 5 min, and the supernatant collected and used as template. Primers for the *gyrA* gene were: forward 5'-GTA CTG TAC GCG ATG CAC GA-3' and reverse 5'-CGA GCC GTT GAC GAG CAG T-3', leading to a product of 372 bp. The amplification kit was obtained from the Wuxi Clon-Gen Technique Institute with the following protocol: 94 °C denaturation for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 60 s, with final extension at 72 °C for 2 min. Primers for amplification of the *parC* gene were: forward 5'-GCA CGC TTC CCA TAC CGA-3' and reverse 5'-TCC ACC GTC CCC TGA TTG-3', leading to a product of 410 bp. The amplification protocol for the *parC* gene was identical to that for the *gyrA* gene, with the exception of denaturation at 93 °C. The primers for *gyrA* and *parC* were designed according to primer design principles and compared with sequences of homologous genes in GenBank. The amplified fragment of *gyrA* is from position 175–546, that of *parC* from position 15–443. The PCR products were sent to Shanghai BioAsia Biotechnology for sequence analysis on an ABI377 automatic sequencer (Applied Biosystems). The PCR product sequences were aligned, analysed and compared with the sequence of a wild-type strain of *N. gonorrhoeae* (GenBank accession nos U08817 and U08907) by Bioedit software (Ibis Therapeutics).

Data analysis. SAS 8.1 software (SAS Institute) was used for ANOVA analysis and multiple regression analysis of correlation between mutation sites and MIC results.

RESULTS AND DISCUSSION

The 95 non-duplicate *N. gonorrhoeae* isolates were collected from 78 males and 17 females, with a mean age of 33.96 years (SD 10.49 years).

The antimicrobial MIC distribution for 95 isolates of *N. gonorrhoeae* is shown in Table 1. All of the isolates were susceptible to cefotaxime and ceftriaxone. One isolate was susceptible to tetracycline. None of the isolates was

Table 1. MIC summary and susceptibility for 95 *N. gonorrhoeae* isolates

Abbreviations: CFT, ceftriaxone; CIP, ciprofloxacin; CPE, ceftipime; CTX, cefotaxime; LEV, levofloxacin; PEN, penicillin; SPC, spectinomycin; TET, tetracycline; S, sensitive; I, intermediate; R, resistant.

Antimicrobial agent	Number of isolates with MIC (mg l^{-1}) of:															MIC ₅₀	MIC ₉₀	S (%)	I (%)	R (%)
	≤ 0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	> 128					
CTX	24	22	22	22	3	2										0.06	0.12	100	0	0
CFT	48	30	15	0	2											0.03	0.06	100	0	0
CPE	1	7	15	36	19	16	0	1								0.12	0.5	98.9	0	1.1
PEN				3	0	8	19	13	0	0	2	14	36			32	64	0	31.6	68.4
SPC					1	7	12	35	12	2	6	82	4	0	1	32	32	94.7	4.2	1.1
TET							10	24	16	18	21	5	1	9		2	64	1.1	20	78.9
CIP						1	13	27	17	32	5					4	16	0	0	100
LEV																4	8	0	1.1	98.9

susceptible to penicillin. Spectinomycin resistance was very low: only one isolate showed an MIC $> 128 \text{ mg l}^{-1}$. One isolate was resistant to cefepime with MIC = 2 mg l^{-1} . All isolates were considered resistant to ciprofloxacin and non-susceptible to levofloxacin (Table 1).

Of the 95 isolates, 60 were selected according to the ciprofloxacin MIC distribution. However, two of these could not be amplified and four could not be sequenced. As a result, 54 isolates were selected for PCR of amino acid changes in the GyrA and ParC subunits. Five types of *gyrA* mutations could be categorized, with amino acid changes mainly found in positions 91 and 95. Eleven mutation sites were found on the *parC* gene, leading to seven amino acid changes (Table 2). Four silent mutations were also detected (bases 312T→C, 387G→A, 393C→G and 414G→A) that gave no amino acid change.

Statistical analysis of the relationships between different mutations in the *gyrA* and *parC* genes, and levels of fluoroquinolone resistance, revealed significant correlations. It was noted that when more mutations were found in the two genes of a particular isolate, the organism became more resistant, with a higher MIC, as found with other bacterial species (Hawkey, 2003). Isolates with only *gyrA* mutations had lower MICs than those with mutations in both *gyrA* and *parC* ($P < 0.05$ for ciprofloxacin; Table 3). In addition, analysis also revealed that mutations in *gyrA* resulting in Asp-95→Ala and in *parC* resulting in Ser-87→Arg and Ser-87→Asn had significant influences on the elevation of MIC values ($P < 0.05$).

Fluoroquinolones have been widely used to treat infections caused by *N. gonorrhoeae* since the 1980s because of their strong antibacterial activities, comparatively low costs and convenience of oral administration. However, resistance to these drugs emerged and spread quickly in the 1990s (Gu *et al.*, 2004; Ye, 2001). Resistance to ciprofloxacin from *N. gonorrhoeae* isolated in Nanjing has increased from 2.89% in 1994 to 97.17% in 2002 (Su *et al.*, 2004). Our results in this study indicated that the resistance rate of *N. gonorrhoeae* isolates from Nanjing, Xuzhou and Wuxi against ciprofloxacin has reached 100% (Table 1). It is indeed alarming to note such an increase in drug resistance of over 30-fold within 10 years, and this antibiotic should not be recommended for treatment of gonococcal infections in China, especially in the Jiangsu Province. Other antibiotics, such as third- or fourth-generation cephalosporins or spectinomycin, should be considered.

It has been reported that earlier fluoroquinolone-resistant *N. gonorrhoeae* have mutations in the quinolone-resistant determining region (QRDR) (Belland *et al.*, 1994). While susceptible bacteria have no mutation found in either *gyrA* or *parC* (Shultz *et al.*, 2001; Tanaka *et al.*, 2000), when a single mutation in *gyrA* is found in an isolate, there is an elevation of the fluoroquinolone MIC. In earlier studies by Belland *et al.* (1994) and Shultz *et al.* (2001), it has been demonstrated that resistance levels are correlated with

sequential changes in *gyrA* and *parC* mutations. Higher MICs were obtained when isolates had double *gyrA* mutations or co-existing *parC* mutations. Our present study revealed that nearly all the *N. gonorrhoeae* isolates were fluoroquinolone resistant (Table 1), and among the 54 isolates analysed for mutations, all demonstrated a change of Ser-91→Phe in their *gyrA* gene, and all except one demonstrated a change in position 95 of the amino acid sequence (Table 2).

It was also demonstrated that most of the isolates with *gyrA* mutations that had co-existing *parC* mutations were more resistant than those without *parC* mutations ($P < 0.05$; Table 3). Mutations in the *parC* gene were found to be mainly caused by amino acid changes in position 86 to 91, with 18 types (Table 2), excluding silent mutations. Previous studies of GyrA and ParC in *N. gonorrhoeae* have reported 12 amino acid changes in GyrA, including Ala-67→Ser, Ala-75→Ser, Ala-84→Pro, His-88→Gln, Ser-91→Phe, Ser-91→Tyr, Ser-91→Cys, Asp-95→Gly, Asp-95→Ala, Asp-95→Asn, Asn-103→Ile and Val-120→Leu; and 16 amino acid changes in ParC, including Gly-85→Cys, Asp-86→Asn, Ser-87→Ile, Ser-87→Asn, Ser-87→Arg, Ser-88→Arg, Ser-88→Pro, Ser-88→Thr, Glu-91→Lys, Glu-91→Gly, Glu-91→Gln, Ala-92→Gly, Phe-100→Leu, Phe-100→Tyr, Arg-116→His and Arg-116→Leu (Chaudhry *et al.*, 2002; Shultz *et al.*, 2001; Su & Lind, 2001; Tanaka *et al.*, 1998, 2000; Trees *et al.*, 1999; Yoo *et al.*, 2004). Our results concurred with most of the findings reported in the literature; in addition, we have found two new mutation sites of Ala-92→Pro and Gln-102→His in *gyrA*, and a new mutation of Ala-29→Pro in *parC* (Table 2). We would speculate that there might be geographical differences among resistant isolates of *N. gonorrhoeae* (Shultz *et al.*, 2001; Yong *et al.*, 2004).

We further analysed the correlation between the number of mutation sites, their position, and the MIC results of the two fluoroquinolones. Through multi-regression analysis, it was revealed that the positions of mutations in the *gyrA* and *parC* genes significantly influenced resistance levels against fluoroquinolones, especially the amino acid changes Asp-95→Ala in GyrA, and Ser-87→Arg and Ser-87→Asn in ParC ($P < 0.05$). Since the amino acid change Ser-91→Phe in GyrA was found in all isolates, its effect on fluoroquinolones was not measurable through multi-regression analysis due to the lack of a control group. However, correlation of such a mutation to the MIC for fluoroquinolones has previously been demonstrated to be significant (Chaudhry *et al.*, 2002; Shultz *et al.*, 2001; Su & Lind, 2001; Tanaka *et al.*, 1998, 2000; Trees *et al.*, 1999; Yoo *et al.*, 2004). Although the detection of mutations in *gyrB* and *parE* was not performed in our study, an increase in the number of mutations would result in higher MICs for ciprofloxacin.

Our findings also revealed variations between mutations and levels of fluoroquinolone resistance in *N. gonorrhoeae*. Isolates with the same mutations demonstrated different MICs, and isolates with different mutations could give

Table 2. Distribution of *gyrA* and *parC* mutations in relation to fluoroquinolone MICs

<i>gyrA</i> mutation types (amino acid change)	<i>parC</i> mutations with amino acid change							Number of isolates (<i>n</i> = 54)	MIC range (mg l ⁻¹)	
	Ala-29	Asp-86	Ser-87	Ser-87	Ser-87	Ser-88	Glu-91		Ciprofloxacin	Levofloxacin
A (<i>n</i> = 19) (Ser-91→Phe, Asp-95→Gly)								2	1–4	1–4
		Asn						2	1–8	2–8
							Gly	2	8–16	4–8
			Arg					13	4–32	2–8
B (<i>n</i> = 24) (Ser-91→Phe, Asp-95→Ala)		Asn						1	1	1
						Pro		1	2	2
							Gly	1	2	2
	Pro			Asn				1	2	2
				Asn				9	2–16*	2–8*
			Arg					5	2–8	1–8
C (<i>n</i> = 9) (Ser-91→Phe, Asp-95→Asn)								5	2–16	1–8
		Asn				Ile		1	8	4
								1	2	4
		Asn						3	4–16	4–8
			Arg					3	16–32	8–16
D (<i>n</i> = 1) (Ser-91→Phe, Ala-92→Pro)		Asn				Ile		2	32†	16†
E (<i>n</i> = 1) (Ser-91→Phe, Asp-95→Asn, Gln-102→His)			Arg					1	16	8

*Only one isolate exhibited a MIC of 16 mg l⁻¹ for ciprofloxacin and 8 mg l⁻¹ for levofloxacin; the MIC range of the other eight isolates ranged from 2 to 4 mg l⁻¹.

†The two isolates exhibited the same MIC values for ciprofloxacin and levofloxacin.

Table 3. Number of *parC* mutations in relation to MICs for fluoroquinolones

Antibiotic	Number of <i>parC</i> mutations	Number of isolates	Mean of ln(MIC)	SD	ANOVA analysis results	
					F value	P value
Ciprofloxacin	0	13	0.96	0.76	5.67	0.006
	1	37	1.93	1.02		
	2	4	2.43	1.33		
Levofloxacin	0	13	1.07	0.61	1.89	0.160
	1	37	1.41	0.82		
	2	4	1.91	1.04		

identical MIC results. It is highly likely that fluoroquinolone resistance involves mechanism(s) other than mutations in the *gyrA* and/or *parC* genes, as found with other bacterial species, which would require further investigation.

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