

Mutation patterns in *gyrA* and *parC* genes of ciprofloxacin resistant isolates of *Neisseria gonorrhoeae* from India

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Aim: To analyse mutations in the *gyrA* and *parC* genes leading to possible increase in ciprofloxacin resistance (high MIC values for ciprofloxacin) in clinical isolates of *Neisseria gonorrhoeae* in Delhi, India.

Method: MIC of ciprofloxacin for 63 clinical isolates of *N gonorrhoeae* were examined by the Etest method. Subsequently, *gyrA* and *parC* genes of these isolates were amplified and sequenced for possible mutations.

Results: Out of the 63 clinical isolates tested, only five (8%) isolates were found to be susceptible to ciprofloxacin (MIC <0.06 µg/ml). DNA sequence analysis of the *gyrA* and the *parC* genes of all these isolates (n = 63) revealed that all isolates which were not susceptible to ciprofloxacin (n=58) had mutation(s) in *gyrA* and *parC* genes. 12 isolates (19%) exhibited high resistance with an MIC for ciprofloxacin of 32 µg/ml. Two out of these 12 isolates (UD62 and UD63), harboured triple mutations (Ser-91 to Phe, Asp-95 to Asn and Val-120 to Leu) in the *gyrA* gene. The third mutation of Val-120 to Leu, lies downstream of the quinolone resistance determining region (QRDR) of the *gyrA* and has not been described before in gonococcus. In addition, both these isolates had a Phe-100 to Tyr substitution in the *parC*, a hitherto unknown mutation.

Conclusions: Emergence of ciprofloxacin resistance with high levels of MIC values (up to 32 µg/ml) in India is alarming. Double and triple mutations in *gyrA* alone or together in *gyrA* and *parC* could be responsible for such a high resistance.

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Neisseria gonorrhoeae, a common causative agent of sexually transmitted diseases (STD), is still prevalent in high numbers in developing countries.^{1,2} Owing to the emergence of penicillin and tetracycline resistant strains of *N gonorrhoeae*, CDC and WHO recommended third generation cephalosporins and fluoroquinolones for the treatment of gonococcal infections worldwide, including India.^{3,4} However, the decreased susceptibilities of *N gonorrhoeae* to fluoroquinolones has recently been reported from a large number of countries.^{5–12} Ciprofloxacin, a fluoroquinolone, inhibits DNA replication by targeting DNA gyrase and topoisomerase IV.^{13,14} In laboratory mutants of *N gonorrhoeae*, mutations in the *gyrA* and *gyrB* genes of DNA gyrase and in the *parC* gene of topoisomerase IV, associated with decreased susceptibilities to quinolones, have been identified.^{15–18} An amino acid change in the GyrB tends to confer a low level resistance to nalidixic acid.¹⁶ Alterations in the ParC have been identified only in strains having GyrA mutations and had a complementary role in further decreasing susceptibility of *N gonorrhoeae* to fluoroquinolones.^{11,15,17,18}

The present study was performed to examine the susceptibility to ciprofloxacin of *N gonorrhoeae* isolated from clinical samples (n = 63) collected from patients visiting Safdarjung Hospital, Delhi, India, from January 2000 to March 2001. The studies were further extended to understand the sharp increase in ciprofloxacin resistance, observed in Delhi, and to find out a correlation between mutation patterns in *gyrA* and the *parC* genes and the level of ciprofloxacin resistance.

MATERIALS AND METHODS

Gonococcal isolates

A total of 78 male patients with signs and symptoms of acute gonococcal urethritis attending the STD Teaching and

Research Centre of Safdarjung Hospital in New Delhi, India, from January 2000 to March 2001 were investigated for the presence of Gram negative intracellular diplococci in their urethral discharge. A total of 63 samples were smear positive. Specimens from these smear positive patients were inoculated directly onto Thayer-Martin selective agar (Hi Media, India), and incubated for 24–48 hours at 35°C in a humid atmosphere containing 5–10% CO₂ (candle extinction jar).¹⁹ The culture positive isolates were further confirmed as *N gonorrhoeae* by colony morphology, Gram staining, oxidase test, and carbohydrate degradation test.¹⁹ Post-treatment isolates or repeat isolates from the same patients were excluded in this study.

Susceptibility testing

The MIC of ciprofloxacin was determined by the Etest method in all the culture positive isolates (63 cultures) using the steps suggested by the manufacturer (AB Biodisks, Solna, Sweden). An inoculum of 10⁷ cfu/ml was allowed to grow on chocolate agar medium for 24 hours in a candle extinction jar.¹⁹ Based on MIC values, the strains were labelled as susceptible (MIC <0.06 µg/ml), intermediate resistant (MIC 0.06 µg/ml to 0.75 µg/ml), and resistant (MIC ≥1 µg/ml).²⁰ WHO A-E, H, and J strains were used as controls.²¹

Molecular study

The *gyrA* and *parC* genes of each of the gonococcal isolate were amplified using oligonucleotide primers as reported by Tanaka *et al.*¹¹ These primers amplified *gyrA* gene from nucleotides 160–438 (278 bp product), corresponding to the amino acids 54–146. The 255 bp amplified product of *parC* gene represented the nucleotides 166–420, which corresponds to amino acids 56–140.¹⁵ Chromosomal DNA was extracted by standard methods²² and the PCR amplification was performed in 50 µl of a reaction mixture which contained 5 µl of 10x Taq

Table 1 Susceptibility to ciprofloxacin of *Neisseria gonorrhoeae* isolated in India and the number of GyrA and ParC alterations

Name of isolates	No of isolates	% isolates	MIC* ($\mu\text{g/ml}$) of ciprofloxacin	No of mutations in	
				<i>gyrA</i>	<i>parC</i>
Clinical isolates					
UD1–UD5	5	8%	<0.06 (S)	No mutation	No mutation
UD6	1	1%	0.1 (IR)	One	No mutation
UD7–UD9	3	5%	0.25 (IR)	Two	One (silent)
UD10–UD13	4	6%	0.38 (IR)	Two	One (silent)
UD14–UD21	8	13%	0.50 (IR)	Two	One (silent)
UD22–UD28	7	11%	0.75 (IR)	Two	Two (silent)
UD29–UD32	4	6%	1.0 (R)	Two	One
UD33–UD40	8	13%	1.5 (R)	Two	One
UD41–UD44	4	6%	2.0 (R)	Two	One
UD45, UD46	2	3%	3.0 (R)	Two	One
UD47–UD50	4	6%	4.0 (R)	Two	One
UD51	1	1%	12.0 (R)	Two	Two (one silent)
UD52–UD61	10	16%	32.0 (R)	Two	Two (one silent)
UD62, UD63	2	3%	32.0 (R)	Three	Three (two silent)
WHO reference strains:					
WHO strain A	1	–	0.016 (S)	No mutation	No mutation
WHO strain H	1	–	2.0 (R)	Two	One
WHO strain J	1	–	8.0 (R)	Two	One

*Based on MICs (minimum inhibitory concentrations), isolates were labelled as susceptible (S), intermediate resistant (IR), and resistant (R).²⁰

polymerase buffer (500 mM KCl, 100 mM TRIS-HCl (pH 8.3), 15 mM MgCl₂, 0.1% gelatin), 25 pmol of each of the two primers (reverse and forward), 200 μM of each of the four deoxynucleoside triphosphates (New England Biolabs, Inc, USA), 1.5 U of Taq DNA polymerase (BangaloreGenei India Pvt Ltd, India), and 100 ng of template DNA. Thirty five cycles were performed for each reaction. Each cycle consisted of 30 seconds at 93°C, 1 minute at 52°C, and 1 minute at 72°C. The amplified PCR product was separated by electrophoresis on agarose gel and eluted from agarose by using a DNA isolation kit from Biological Industries, Israel, as in the manufacturer's instructions. The purified DNA was air dried and suspended in 12–15 μl of sterile water. Gel purified PCR amplified product (100 ng) of *gyrA* gene was digested with *HinfI* (1 unit) in a final volume of 10 μl for 2 hours at 37°C. The digests were subjected to electrophoresis on 1.5% agarose gel and visualised by ethidium bromide staining. Purified PCR amplified product of *gyrA* (corresponding to 160–438 nucleotides) and *parC* (corresponding to 166–420 nucleotides) genes of all the isolates as well as WHO reference strains A, H, and J, were subjected to DNA sequencing using PCR primers (forward primer) with a Taq-Dye Terminator cycle sequencing kit and a Model 377A autosequencer (Applied Biosystems) as in the manufacturer's instructions. The region sequenced includes the quinolone resistance determining region (QRDR) of the GyrA protein (amino acids 55 to 110) and the ParC protein (amino acids 66–119). DNA sequences were analysed using the BLAST program.

RESULTS

Susceptibilities of clinical isolates to ciprofloxacin

Table 1 shows the MIC values of ciprofloxacin for the 63 gonococcal isolates. The susceptibility pattern of *N gonorrhoeae* isolates was based on criteria proposed by Knapp *et al.*²⁰ Only five isolates (8%) were found to be susceptible to ciprofloxacin with an MIC <0.06 $\mu\text{g/ml}$. Out of the 58 quinolone resistant *N gonorrhoeae* (QRNG) isolates, 23 (36%) isolates were exhibiting intermediate resistance towards ciprofloxacin (MIC values ranging from 0.06–0.75 $\mu\text{g/ml}$), while 35 isolates (56%) were resistant to ciprofloxacin with a wide range of MIC (1–32 $\mu\text{g/ml}$). Among these 35 resistant isolates, 12 isolates (35%) exhibited high levels of resistance with an MIC for ciprofloxacin of 32 $\mu\text{g/ml}$.

HinfI digestion to screen mutations in *gyrA* gene

Quick screening of all the isolates, for the mutation at the Ser-91 codon in *gyrA* gene, was done by *HinfI* restriction endonuclease digestion. PCR amplified *gyrA* gene product (278 bp) of all the isolates, which were susceptible to ciprofloxacin, when digested with the *HinfI* restriction endonuclease enzyme gave two bands of 165 bp and 113 bp on the agarose gel (fig 1). However, PCR products of *gyrA* gene, amplified from all other isolates, were not digested with *HinfI*. The amplified products of the *gyrA* gene of WHO reference strains H and J (resistant to ciprofloxacin) were also resistant to digestion with the *HinfI* enzyme while that of WHO strain A (sensitive to ciprofloxacin) gave two bands of 165 bp and 113 bp (fig 1). Thus, all the gonococcal isolates, resistant to ciprofloxacin (n=58), were found to be mutated at Ser-91 position within QRDR of GyrA protein.

Novel mutations in *gyrA* and *parC* genes in isolates showing high resistance to ciprofloxacin

DNA sequencing of the PCR amplified region of the *gyrA* and the *parC* genes from the ciprofloxacin susceptible isolates (UD1–UD5), revealed no alteration in either of the genes (table 2). However, multiple mutations were observed in the PCR amplified region of *gyrA* and the *parC* genes from the QRNG isolates (n = 58). All these isolates had mutation in the codon 91 of the *gyrA* leading to Ser-91 to Phe substitution. Single mutation, at position 91 of GyrA, was observed in only one isolate, exhibiting intermediate resistance towards ciprofloxacin with an MIC of 0.1 $\mu\text{g/ml}$ (table 2). All other QRNG isolates (n = 57) had at least two mutations in the *gyrA* gene along with *parC* mutation(s). The most frequent mutations (in 81% of QRNG isolates) are the Ser-91 to Phe and Asp-95 to Asn substitutions in the GyrA protein. Among the 12 isolates with MIC for ciprofloxacin of 32 $\mu\text{g/ml}$, 10 isolates (UD52 to UD61) had an Asp-95 to Gly substitution along with the Ser-91 to Phe in the *gyrA* gene. The other two isolates, UD62 and UD63, had three point mutations in the *gyrA* gene resulting in three amino acid substitutions (Ser-91 to Phe, Asp-95 to Asn, and Val-120 to Leu) in the GyrA (table 2). Interestingly this third mutation (Val-120 to Leu) lies outside the so far recognised QRDR (amino acids 55 to 110 of *N gonorrhoeae* GyrA protein).¹⁵ WHO strain A, which is sensitive to ciprofloxacin (MIC of 0.016 $\mu\text{g/ml}$), had no mutation in the *gyrA* gene while WHO strain H (MIC of 2.0 $\mu\text{g/ml}$ for ciprofloxacin) and WHO

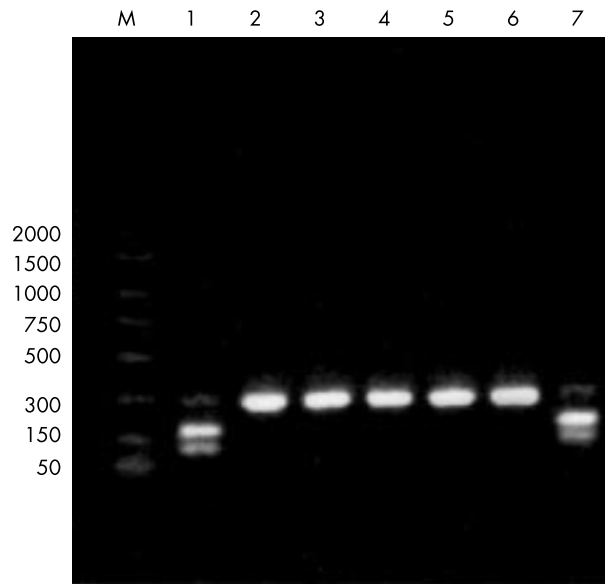


Figure 1 *Hinf*I restriction fragment length polymorphism. Region spanning QRDR of *gyrA* gene of *Neisseria gonorrhoeae* was amplified and subjected to *Hinf*I restriction enzyme digestion. Ciprofloxacin susceptible isolates (lane 1; susceptible clinical isolate, lane 7; WHO reference strain A) with no mutation produced two fragments of 165 bp and 113 bp on *Hinf*I digestion. The ciprofloxacin resistant isolates (lanes 2–4; QRNG clinical isolates, lane 5; WHO reference strain H, lane 6; WHO reference strain J) remain undigested as *Hinf*I cleavage site is lost because of the C to T transition at nucleotide position 272 [Ser-91 to Phe]. Lane M; 100 bp molecular weight standard.

strain J (MIC of 8 µg/ml for ciprofloxacin), had two mutations leading to Ser-91 to Phe and Asp-95 to Gly substitutions.

Sequence analysis of the *parC* gene demonstrated a variety of mutations at codons 91, 100, 104, 109, and 131 (table 2). The isolates (UD7–UD28), exhibiting intermediate resistance to ciprofloxacin (MIC from 0.06–0.75 µg/ml), had only silent mutations at the *parC* locus. The most common substitution of Glu-91 to Gly in the *parC* gene was found in 33 QRNG isolates (57%). The two isolates (UD62, UD63), having a novel mutation in the *gyrA* gene, also possessed a hitherto undescribed mutation at the *parC*, resulting in the substitution of Phe-100 with Tyr. Along with this mutation, two silent mutations at codon 104 (Tyr) and 109 (Gly) were also observed in the *parC* gene of these two isolates. The silent mutation at the codon 109 has also not been described before. WHO strain A (MIC of 0.016 µg/ml) had no mutation in the *parC* gene while WHO Strain H (MIC of 2.0 µg/ml for ciprofloxacin) and WHO strain J (MIC of 8 µg/ml for ciprofloxacin) possessed a single mutation at the codon 88 (Ser-88 to Pro) and 86 (Asp-86 to Asn) respectively.

DISCUSSION

During the past few years, an overall increase in resistance to various antimicrobial agents have been observed in *N gonorrhoeae*.^{6 10 23 24} Despite a high prevalence of gonorrhoea in India, a systematic study regarding resistance to various antimicrobial agents is limited.^{7 10 23 25} In the present study, an increase in the number of QRNG isolates (92%) as well as the level of resistance to ciprofloxacin (MIC up to 32 µg/ml) was observed in *N gonorrhoeae* isolated from patients visiting STD Centre, Safdarjung Hospital, India. A significant proportion of high level QRNG (63%) has also been reported from Philippines.² Almost 90% of isolates in China and Hong Kong were QRNG and about half of these isolates possessed high level of resistance.² In Japan, although the overall percentage

Table 2 Mutation patterns in the *GyrA* and *ParC* proteins of *Neisseria gonorrhoeae* isolates

Name of isolates	Sequence change(s) in <i>GyrA</i> *					Sequence change(s) in <i>ParC</i> *					
	Ser-91 (TCC)	Asp-95 (GAC)	Val-120 (GTG)	Glu-91 (GAG)	Phe-100 (TTT)	Tyr-104 (TAT)	Gly-109 (GGC)	Leu-131 (CTC)	Tyr-104 (TAC)	Gly-109 (GGG)	Leu-131 (CTG)
UD1–UD5†	Ser-91 (TCC)	Asp-95 (GAC)	Val-120 (GTG)	Glu-91 (GAG)	Phe-100 (TTT)	Tyr-104 (TAT)	Gly-109 (GGC)	Leu-131 (CTC)	Tyr-104 (TAC)	Gly-109 (GGG)	Leu-131 (CTG)
UD6	Phe-91 (TTC)	–	–	–	–	–	–	–	–	–	–
UD7–UD9	Phe-91 (TTC)	Asn-95 (AAC)	–	–	–	–	–	–	–	–	–
UD10–UD21	Phe-91 (TTC)	Asn-95 (AAC)	–	–	–	–	–	–	–	–	–
UD22–UD28	Phe-91 (TTC)	Asn-95 (AAC)	–	–	–	–	–	–	–	–	–
UD29–UD50	Phe-91 (TTC)	Asn-95 (AAC)	–	–	–	–	–	–	–	–	–
UD51	Phe-91 (TTC)	Asn-95 (AAC)	–	Gly-91 (GGG)	–	–	–	–	–	–	–
UD52–UD61	Phe-91 (TTC)	Gly-95 (GGC)	–	Gly-91 (GGG)	–	–	–	–	–	–	–
UD62, UD63	Phe-91 (TTC)	Asn-95 (AAC)	Leu-120 (TTG)	Gly-91 (GGG)	Tyr-100 (TAT)	Tyr-104 (TAC)	Gly-109 (GGG)	–	–	–	–

*Associated codon is indicated in the parenthesis; †and –, identical to wild type.

of resistant strains has not changed appreciably in past 2 years, there was a gradual increase in level of resistance (MIC >1.0 µg/ml).¹¹ Decreased susceptibility to ciprofloxacin and ofloxacin has also been reported from Bangladesh (11.7% were resistant and 26.6% had reduced susceptibility).⁹ This widespread emergence of ciprofloxacin resistance in South East Asia is rather alarming.

Mutations in the *gyrA* and the *parC* genes leading to amino acid changes in GyrA (especially at Ser-91 and Asp-95) and ParC proteins are significantly associated with ciprofloxacin resistance.^{5 11 15 17 18 26–28} Mutation at codon 91 of the *gyrA* gene results in the loss of natural *HinfI* site. Direct *HinfI* digestion of PCR amplified *gyrA* gene has been used to screen *gyrA* mutations in *N gonorrhoeae*.^{27 29} Another restriction endonuclease site (*AlwI*) spans the novel mutation (Val-120 to Leu), identified by us. Double digestion with *HinfI* and *AlwI* would result in release of a fragment from the wild type but not mutated *gyrA* gene and may be used to check for mutations at these two loci.

Since QRNG isolates (intermediate and resistant) exhibit a wide range of MIC for ciprofloxacin (0.1–32 µg/ml), mutation pattern in *gyrA* and *parC* genes was checked in all the isolates. Double and triple mutations in the QRDR region of *gyrA* and the *parC* genes appear to be the major contributors to the high level of ciprofloxacin resistance. It is significant to note that the most common *gyrA* mutations (in 75% of the isolates) found by us were Ser-91 to Phe and Asp-95 to Asn, while only 16% isolates showed change of Asp-95 to Gly along with Ser-91 to Phe. None of the isolates sequenced in this study had tyrosine at position 91 in the *gyrA* gene.^{11 12 28} We also did not observe any isolate carrying mutation at Ala-67, Ala-75, and Ala-84 in the *gyrA*, as reported in some of the isolates from Japan.¹¹ Isolates (UD7-UD28), with low MIC for ciprofloxacin (0.25–0.75 µg/ml), had silent mutations at the *parC* locus, while all other isolates (MIC of 1–32 µg/ml), except UD62 and UD63, had Glu-91 to Gly substitution. This supports the earlier observations that mutations in *parC* have a complementary role in decreasing susceptibility to ciprofloxacin.^{11 15 17 18 26 27} Alterations at the Gly-85, Asp-86, Ser-87, Ser-88, or Arg 116 residues, in *parC*, as reported in the earlier studies,^{11 17 27} have not been found in any of the clinical isolates in the present study. Instead, two isolates (UD62 and UD63) with high MIC for ciprofloxacin had a Phe-100 to Tyr substitution along with the triple mutations in the QRDR of the GyrA protein (Ser-91 to Phe, Asp-95 to Asn, and Val-120 to Leu). The third mutation observed in these two isolates has not been reported so far. Though close to QRDR in the GyrA subunit, this novel mutation is present downstream of the identified QRDR. Additional mutations in the *gyrA* gene, about 20–30 amino acids away from Ser-83 in *Escherichia coli* and Thr-83 in *Pseudomonas aeruginosa*, leading to quinolone resistance, have also been reported in *E coli* (Gln-106 to Arg, Gln-106 to His) and *P aeruginosa* (Asn-116 to Tyr) respectively.^{30 31} Acknowledging that transformation studies need to be performed for confirmation, these results suggest that triple mutations in *gyrA* along with the Phe-100 to Tyr substitution in the *parC* may have some bearing on the action of ciprofloxacin in *N gonorrhoeae*.

The results of our study clearly indicate that resistance to ciprofloxacin, which was introduced as first line therapy only in 1990, has increased rapidly with an alarming high level of MIC values and new mutational patterns in *N gonorrhoeae*. Unless continued monitoring of antimicrobial sensitivity patterns of gonococcal strains circulating in a community is carried out, treatment failures may occur and lead to prolonged infection and spread of multiple drug resistant strains of *N gonorrhoeae*.

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Key messages

- Emergence of ciprofloxacin resistant *Neisseria gonorrhoeae*
- High percentage of isolates have high level of MIC for ciprofloxacin
- Characterisation of novel mutations in the QRDR region of *gyrA* and *parC* genes of *Neisseria gonorrhoeae*
- Rapid screening of ciprofloxacin resistant isolates by *HinfI* digestion of the *gyrA* amplified product.

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CONTRIBUTORS

KR (head, STD laboratory) and MB collected the clinical samples and conducted the drug sensitivity for *Neisseria gonorrhoeae*; UC carried out PCR and analysis of mutations in the QRDR region of *gyrA* and *parC* genes; DS instigated the project, designed the study, and analysed the data; DS and UC wrote the manuscript.

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ECHO

Multiple sclerosis may be sexually acquired



Please visit the Sexually Transmitted Infections website [www.sextransinf.com] for link to this full article.

MS research may change direction if researchers take up the challenge of testing the hypothesis that a recent review proposes—namely, that MS is a sexually transmitted infection (STI). The author emphasises that this may be just one way in which the disease occurs but maintains that there is sufficient evidence to suggest that it is spread sexually, manifesting itself in those who are genetically susceptible.

MS is generally assumed to have a genetic or immune basis, but it shares parallels with STIs—not least tropical spastic paraplegia—an STI whose similarity to primary progressive MS is striking.

Historically, clusters or epidemics of MS recorded after an influx of troops in communities with a previously low incidence of the disease—for example, Allied troops sent to Iceland, Shetland, and the Faroes in World War II—are consistent with the spread of an STI. MS is initially more prevalent in females, and is passed on more efficiently from men to women. Migration at a young age from low risk to high risk areas for MS rapidly increases risk status. Prevalence mirrors social and cultural norms: MS is most prevalent in permissive societies and least so in Moslem Arab countries, in ethnic populations living apart from white populations—Aborigines, Maoris—and in closed white communities with strict moral attitudes. It also mirrors changing social norms: the Danish MS Registry, with records from 1949, reported a significant increase in MS in women when oral contraceptives became available and use of barrier methods offering more protection against STIs declined.

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