



Exploring quinolone resistance-determining region in *Neisseria gonorrhoeae* isolates from across India

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Received May 11, 2015

Background & objectives: Antimicrobial resistance in *Neisseria gonorrhoeae*, the causative agent of gonorrhoea, is a subject of worldwide attention. The present study was undertaken to examine the rates of ciprofloxacin resistance, to correlate mutations in *gyrA* and *parC* genes with the level of resistance and to look for a variation in mutation pattern, if any, in isolates from across the country.

Methods: A total of 113 isolates of *N. gonorrhoeae* collected from sexually transmitted infection patients in six centres during November 2010 to October 2013 were investigated. Minimum inhibitory concentration (MIC) determination was done by E-test and results interpreted as per Calibrated Dichotomous Sensitivity criteria. DNA sequence analysis of *gyrA* and *parC* genes was done.

Results: Of the 113 isolates, only three (2.6%) were susceptible whereas eight (7.07%) were less susceptible, 32 [28.3%, 95% confidence interval (CI): 20.4-37.6%] resistant (MIC 1-3 µg/ml) and 70 (61.9%, 95% CI: 52.2-70.7%) exhibited high-level resistance (HLR) (MIC ≥4 µg/ml) to ciprofloxacin. A S91F substitution in *gyrA* gene was demonstrated in all ciprofloxacin non-susceptible isolates. All resistant and HLR isolates had a double mutation in *gyrA* gene. However, only 5.7 per cent of HLR isolates showed double mutations in *parC* gene. One isolate (MIC 32 µg/ml) had a previously undescribed G85D substitution in the *parC* gene.

Interpretation & conclusions: A S91F substitution in *gyrA* gene was seen in all non-susceptible isolates of *N. gonorrhoeae*. It may be used as a marker for ciprofloxacin resistance for molecular surveillance approaches to complement the culture-based methods.

Key words Antimicrobial resistance - fluoroquinolones - *gyrA* gene - *Neisseria gonorrhoeae* - *parC* gene - quinolone resistance-determining region

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Antimicrobial resistance (AMR) in *Neisseria gonorrhoeae*, the causative agent of gonorrhoea, is a subject of worldwide attention. The introduction of fluoroquinolones as first-line therapy for gonorrhoea was soon followed by reports of resistance first in Asia¹⁻³ and then other parts of the world⁴⁻⁷. Unlike its predecessor, penicillin, which was introduced in the 1940s and continued to be efficacious till the 1980s, development of resistance to fluoroquinolones was rapid. Emergence of this resistance was a significant moment in the history of gonococcus as it marked the end of inexpensive, oral treatment for gonorrhoea. The Centres for Disease Control and Prevention discontinued its use as first-line therapy in the year 2007⁸. However, it was withdrawn from our country in early 2000. Now, despite over a decade of being discontinued, the rates of resistance are sustained.

Amongst the mechanisms of fluoroquinolone resistance, alterations in the quinolone resistance-determining region (QRDR) of the *gyrA* and *parC* genes which encodes for enzymes DNA gyrase and topoisomerase IV, respectively, play a pivotal role⁹. These can be used as genetic markers of AMR in molecular surveillance protocols to supplement the culture-based methods for surveillance. There is a need for such an approach because world over there is a decline in the number of gonococcal isolates. The resource-rich settings have shifted to nucleic acid amplification tests while the resource-limited settings like ours have been focussing on syndrome case management for the treatment of sexually transmitted infections. Although molecular surveillance of AMR as a concept is still in infancy, its widespread use is anticipated in the long run. It is important because of the looming problem of increased AMR in *N. gonorrhoeae* and limitations of phenotypic methods.

The previous study from India investigating the role of *gyrA* and *parC* genes in ciprofloxacin-resistant *N. gonorrhoeae* was performed in 2000-2001 in isolates from New Delhi alone¹⁰. We collected *N. gonorrhoeae* isolates from different parts of the country and not only examined the rates of ciprofloxacin resistance after over a decade of being excluded from the treatment of gonorrhoea but also investigated the specific genetic events in *gyrA* and *parC* genes. Further, we studied the variation in mutation pattern, if any, in different regions of the country. In addition, a correlation between the number of mutations and the level of ciprofloxacin resistance was also studied. The aim was to identify a genetic target that is fundamental to quinolone resistance in gonococcal isolates across India.

Material & Methods

From November 2010 to October 2013, a total of 670 urethral swabs from male patients and 3500 endocervical swabs from female patients were collected and processed as a part of a multicentric study for the surveillance of AMR in *N. gonorrhoeae*. The six centres included (no. of isolates) All India Institute of Medical Sciences (AIIMS), New Delhi (n=43); Apex Regional STD Teaching, Training & Research Centre, VMMC and Sajdarjung Hospital, New Delhi (n=39); Government Medical College, Srinagar (n=1); Institute of Microbiology, Madurai Medical College, Madurai (n=6); Grant Medical College and Sir JJ Group of Hospitals, Mumbai (n=6) and Regional Institute of Medical Sciences, Imphal (n=9). Dr. Ram Manohar Lohia Hospital and Lal Bahadur Shastri Hospital (LBSH) served as additional sample collection sites (n=9) for AIIMS, New Delhi. LBSH was included only in the third year of the study to obtain data from NCR East region as well as border area of Uttar Pradesh. AIIMS, New Delhi, coordinated the network and also conducted the molecular studies. Culture and identification of *N. gonorrhoeae* was done as per standard protocol¹¹. The study was approved by the Institutional Ethics Committee, AIIMS, New Delhi.

Antimicrobial susceptibility testing: Antimicrobial susceptibility testing was done using disc diffusion method as per Calibrated Dichotomous Sensitivity (CDS) technique¹² against the following antibiotics - penicillin (0.5 IU), tetracycline (10 µg), nalidixic acid (30 µg), ciprofloxacin (1 µg), spectinomycin (100 µg), ceftriaxone (0.5 µg) and cefpodoxime (10 µg) (Oxoid Limited, UK). Azithromycin (15 µg) was initially tested as per Clinical and Laboratory Standards Institute guidelines¹³ but from August 2011 as per CDS technique. The minimum inhibitory concentration (MIC) of ciprofloxacin was determined for all isolates using *E*-test strips of ciprofloxacin as per the manufacturer's instruction (bioMerieux, France). Based on the MIC values, the isolates were categorized as susceptible (MIC ≤0.03 µg/ml), less susceptible (MIC 0.06-0.5 µg/ml) and resistant (MIC 1-3 µg/ml). High-level resistance (HLR) was defined by MIC ≥4 µg/ml. World Health Organization (WHO) 2008 *N. gonorrhoeae* reference strains such as WHO F (ciprofloxacin-S), WHO M (ciprofloxacin-R) and WHO K (ciprofloxacin-HLR) were used as controls.

Mutation studies: The molecular studies were carried out in all ciprofloxacin susceptible and less susceptible *N. gonorrhoeae* isolates and in about 50 per cent of the resistant and high-level resistant isolates. Genomic DNA

was extracted by boiling method¹⁴. DNA extraction was followed by amplification of *gyrA* and *parC* gene¹⁵. The *gyrA* PCR was performed in 50 µl of reaction mixture containing reaction buffer (10 mM KCl & 10 mM HCl), 1.5 mM MgCl₂, 200 µM of dNTP mix, 2 unit Taq DNA polymerase, 12 picomoles of each of the two primers (forward and reverse) and 8 µl of DNA. Reaction conditions were as follows: initial incubation at 93°C for five minutes, followed by 35 cycles of 93°C for 30 sec, 56°C for one minute and 72°C for one minute and finally a hold at 72°C for five minutes. These primers amplified *gyrA* gene from nucleotides 160-438. The amplified PCR product (278 bp) was separated by electrophoresis on 1.5 per cent agarose gel. Similar reaction mixture and reaction conditions except primers were used for amplification of *parC* gene. The primers amplified *parC* gene from nucleotides 166-420. The amplified PCR product (255 bp) was separated by electrophoresis on 1.5 per cent agarose gel and visualized by ethidium bromide staining⁹.

Gel extraction was performed by Qiagen MinElute Gel Extraction kit (Qiagen, The Netherlands), according to the manufacturer's procedural directives. Purified PCR products of *gyrA* and *parC* genes of all the isolates and WHO reference strains F, M and K were subjected to DNA sequencing by the dideoxy chain termination method, using the BigDye Terminator v3.1 cycle sequencing kit and model 3130XL Genetic Analyzer (Applied Biosystems, USA). Sequence analysis was performed by online available bioinformatics tools BLASTX (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastx&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) and LALIGN (www.ch.embnet.org/software/LALIGN_form.html).

Statistical analysis: Fisher's exact test was used to evaluate the variation between mutation pattern in *gyrA*

and *parC* genes in *N. gonorrhoeae* isolates collected from within and outside Delhi. Further, it was used to determine the correlation between mutations and the level of resistance. All statistical analyses were done using STATA 12.1 (StataCorp LLC, USA) and results were considered significant at $P < 0.05$.

Results

A total of 204 *N. gonorrhoeae* clinical isolates were obtained during the study period. Amongst these, three (1.47%) were susceptible, eight (3.9%) were LS, 73 (35.8%) were resistant and 120 (58.8%) were HLR. Overall, MIC of ≥ 1 µg/ml was seen in 96.4 per cent of isolates. Molecular studies were performed on all less susceptible and susceptible isolates whereas 32 resistant and 70 HLR were included for the same. Therefore, detailed results of 113 isolates are described herein. Table I shows centre-wise distribution of MICs of 113 isolates for ciprofloxacin that were investigated in detail for mutation studies at *gyrA* and *parC* genes. Amongst 113 isolates, three were susceptible, eight were less susceptible and 32 were resistant while HLR was exhibited by 70.

Out of 52 isolates at AIIMS, New Delhi, only three (5.7%) were susceptible to ciprofloxacin whereas three (5.7%) were less susceptible, 13 (25.0%) resistant and 33 (63.5%) demonstrated HLR. Out of 39 isolates from SJH, New Delhi, none were susceptible whereas 4 (12.2%) were less susceptible. Resistance to ciprofloxacin was seen in 14 (35.8%) and HLR in 21 (53.8%) isolates. The other four centres too did not witness any ciprofloxacin susceptible isolates. Amongst 22 isolates, one (4.5%) was less susceptible whereas five (22.7%) were resistant and 16 (72.7%) exhibited HLR.

The results of DNA sequence analysis of *gyrA* and *parC* genes of the WHO reference strains of

Table I. Minimum inhibitory concentration (MIC) values of *N. gonorrhoeae* isolates (n=113) for ciprofloxacin

MIC (µg/ml)	Interpretation category	AIIMS/RML/LBS [†] (n=52)	SJH [#] (n=39)	Others [§] (n=22)	Total
≤0.03	Susceptible	3	0	0	3
0.06-0.5	Less susceptible	3	4	1	8
1-3	Resistant	13	14	5	32
≥4	HLR	33	21	16	70

[†]AIIMS: All India Institute of Medical Sciences, New Delhi; RML: Dr. Ram Manohar Lohia Hospital & PGIMER, New Delhi; LBS: Lal Bahadur Shastri Hospital, Delhi. [#]Safdarjung Hospital, New Delhi. [§]Government Medical College, Srinagar; Institute of Microbiology, Madurai Medical College, Madurai; Grant Medical College & Sir JJ Group of Hospitals, Mumbai; Regional Institute of Medical Sciences (RIMS), Imphal. [¶]HLR, High-level resistance

N. gonorrhoeae (WHO F, M & K) were concordant to the published conclusion. The three ciprofloxacin susceptible isolates encountered in our study did not reveal any alteration in either of the genes. A S91F substitution in *gyrA* gene was demonstrated in all ciprofloxacin non-susceptible isolates. Resistant isolates at all centres (n=32), in addition demonstrated a mutation at codon 95 of *gyrA* gene. Amongst these, D95N was seen in 18/32 (56.2%) and D95G in 14/32 (43.7%) isolates (Table II). Thus, all resistant isolates had a double mutation in *gyrA* gene. Sequence analysis of *parC* gene demonstrated mutations primarily at codons 87, 91 and occasionally at codon 86. Double mutation in *parC* gene was seen in 2/32 (6.2%) resistant isolates, one from AIIMS and the other from SJH, New Delhi. Both had S87N and E91G substitution. Single mutation was seen in 23 (71.8%) resistant isolates, the most common being E91G followed by E91K. Seven isolates did not show any mutation in *parC* gene (Table II).

HLR isolate (n=70), in addition to S91F substitution in *gyrA* gene, also demonstrated a mutation at codon 95. D95N substitution was seen in 49/70 (70%) and D95G

in 21/70 (30%) isolates. In addition to double mutations in *gyrA* gene, all HLR strains except one revealed mutations in *parC* gene. Single mutations were seen in 65 (92.8%) isolates while double mutations were seen in 4 (5.7%) isolates. The most common mutation from isolates from across the country was E91G seen in 30 (42.8%) which was also seen in combination with S87N in two isolates from SJH. Next, in line was E91K, seen in 28 (40%) isolates and also seen in combination with S87N in one isolate from AIIMS. S87N was also seen coupled with E91Q in one isolate from other centres. One isolate from AIIMS had a previously undescribed G85D substitution in the *parC* gene (Table II).

There were no significant variations in mutation pattern in *gyrA* (S91F, $P=1$; D95G, $P=0.8$ and D95N, $P=0.47$) and *parC* genes (S87R, $P=1$; S87N, $P=1$; S87I, $P=0.58$; E91K, $P=0.44$; E91G, $P=0.09$; D86N, $P=1$; E91Q, $P=0.19$ and G85D, $P=1$) in *N. gonorrhoeae* isolates collected from within and outside Delhi.

Discussion

The public health burden of *N. gonorrhoeae* infections is heightened due to continued high rates of

Table II. Centre-wise results of DNA sequence analysis of *gyrA* and *parC* genes

Total no. of isolates	Category ^δ (n)	<i>gyrA</i>				<i>parC</i>								
		S91F (n)	D95G (n)	D95N (n)	No mutation (n)	S87R (n)	S87N (n)	S87I (n)	E91K (n)	E91G (n)	D86N (n)	E91Q (n)	New mutation (n)	No mutation (n)
AIIMS/RML/LBSH^γ														
52	S (3)	0	0	0	3	0	0	0	0	0	0	0	0	3
	LS (3)	3	1	0	0	0	0	0	0	0	0	0	0	3
	R (13)	13	5	8	0	0	1	0	2	6	0	0	0	5
	HLR (33)	33	12	21	0	3	1	2	16	11	0	0	1 (G85D)	0
SJH[#]														
39	S (0)	0	0	0	0	0	0	0	0	0	0	0	0	0
	LS (4)	4	3	1	0	0	0	0	0	0	0	0	0	4
	R (14)	14	7	7	0	0	1	1	2	7	2	0	0	2
	HLR (21)	21	5	16	0	2	2	1	9	9	0	0	0	0
Other centers^{##}														
22	S (0)	0	0	0	0	0	0	0	0	0	0	0	0	0
	LS (1)	1	1	0	0	0	0	0	0	0	0	0	0	1
	R (5)	5	2	3	0	0	0	0	2	3	0	0	0	0
	HLR (16)	16	4	12	0	1	1	0	3	10	0	1	0	1
Total=113	113	110	40	68	3	6	6	4	34	46	2	1	1	19

^δBased on MICs, isolates were categorized as Susceptible (S)= ≤0.03 µg/ml, Less susceptible (LS)=0.06-0.5 µg/ml, Resistant (R)= ≥1 µg/ml and High-level resistance (HLR)= ≥ 4 µg/ml. ^γAIIMS: All India Institute of Medical Sciences, New Delhi; RML: Dr. Ram Manohar Lohia Hospital & PGIMER, New Delhi; LBS: Lal Bahadur Shastri Hospital, Delhi. [#]Safdarjung Hospital, New Delhi. ^{##}Government Medical College, Srinagar; Institute of Microbiology, Madurai Medical College, Madurai; Grant Medical College & Sir JJ Group of Hospitals, Mumbai; Regional Institute of Medical Sciences (RIMS), Imphal

resistance to previously used antimicrobials including fluoroquinolones. High rates of ciprofloxacin resistance have been reported from different parts of the world and more recently from Shanghai¹⁶. Our region itself has reported high rates (83.3%) in 2008¹⁷ and 94 per cent thereafter¹⁸. Therefore, it is not surprising to find 94.6 per cent of our isolates from different parts of India demonstrating resistance to this antibiotic. What is adding to our distress is the high percentage of isolates (58.8%) demonstrating HLR. Perhaps the extensive use of this class of antimicrobials in other infections including enteric fever and urinary tract infection coupled with misuse sees gonococci and other bacteria in an 'antibiotic soup' which accounts for the present scenario¹⁹. A testament to the same is the status report of the Global Antibiotic Resistance Partnership which documents that the units of antibiotic sold annually are highest for quinolone antimicrobials in India²⁰.

We have also investigated the pattern of mutations in QRDR of *gyrA* and *parC* genes in ciprofloxacin-resistant isolates and found that there was no significant variation in the mutation pattern in different parts of our country. A S91F substitution in *gyrA* gene was demonstrated in all ciprofloxacin non-susceptible isolates from all over the country. This is in line with the results obtained in New Delhi alone¹⁰ and also in other parts of the world^{21,22}. It has, therefore, been suggested that this is used as a marker for quinolone resistance for molecular surveillance approaches to complement the existing culture-based methods for AMR surveillance of gonococci. The suitability of the same has been documented²³ which will allow for real-time antimicrobial susceptibility testing. Double mutations in *gyrA* gene were seen in all our resistant isolates, the second being at codon 95. It was either a D95N substitution or D95G but D95N was more frequently encountered all over India. Similar observations have been made by other workers^{18,24,25}.

Sequence analysis of *parC* gene demonstrated mutations at codons 91, 87 and less frequently at 86 and 85. The most common *parC* mutation was E91G which was also seen in combination with other mutations in *parC* gene. However, the percentage of double mutations in *parC* gene in resistant (6.2%) and HLR isolates (5.7%) did not vary significantly ($P=1$). There was no significant association between specific genetic event in *parC* gene and the level of resistance except for E91K, $P=0.04$ (S87R, $P=0.17$; S87N, $P=1$; S87I, $P=1$; E91G, $P=0.51$; D86N, $P=0.09$, E91G, $P=1$; and G85D, $P=1$). Furthermore, in our study, the number of mutations in *parC* gene did not correlate

with the level of ciprofloxacin resistance. This is in contrast to some previous observations which state that *parC* gene has a complementary role in decreasing susceptibility to ciprofloxacin^{26,27}. Our observations match those reported from Brazil²¹ and Russia²². While the former investigated the *gyrA* and *parC* genes only, the latter went on to additionally explore other genes, for example, *por* and *mtrR*. Further, in our study, there was no resistant isolate that demonstrated a mutation in *parC* without a simultaneous mutation in *gyrA*. DNA gyrase has two subunits, GyrA and GyrB, which are encoded by *gyrA* and *gyrB*, whereas topoisomerase IV is encoded by *parC* and *parE* genes. No *parE* analogue has been detected in *N. gonorrhoeae*⁹. Compared to *gyrA* gene, mutations in *parC* were more variable all over the country. We also observed a previously undescribed G85D substitution in *parC* gene, but none can be used as a marker for HLR in our setting. Therefore, we are unable to explain the difference in susceptibility to ciprofloxacin based on sequencing of QRDR of *gyrA* and *parC* genes alone. We, however, cannot rule out the presence of mutations outside the sequenced region, but our data suggest that other mechanisms may have a role to play in HLR to quinolones in *N. gonorrhoeae*.

Considering the seriousness of the situation with regard to treatment of gonorrhoea, there is not only a need to search for new molecules, retool the existing armamentarium but also look into the possibility of individualized treatment taking advantage of molecular diagnostics. They all may be approached in parallel to ensure success in gonorrhoea control.

Our study had some limitations. Firstly, a few of the isolates were taken from centres outside Delhi. Therefore, one needs to be cautious about interpreting these data. Secondly, we did not investigate the possible role of reduced cell permeability and efflux in *N. gonorrhoeae* resistance to fluoroquinolones.

A S91F substitution in *gyrA* gene may be used as a marker for quinolone resistance in *N. gonorrhoeae* in India. Our study also suggests that mutations within the *gyrA* gene are more stable and perhaps more important than those within *parC* gene. Furthermore, other mechanisms may have a role to play in HLR to quinolones in *N. gonorrhoeae*.

Acknowledgment

This work was supported by grant no. IRIS-ID No. 2006-0685E, Indian Council of Medical Research, Department of Health Research, Ministry of Health & Family Welfare, Government of India.

Conflicts of Interest: None.

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