Detection of Mutation-Induced, Quinolone-Resistant *Neisseria Gonorrhoeae* among Iranian Women

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ABSTRACT

Background: *Neisseria gonorrhoeae* infection is a major cause of sexually transmitted disease (STD) and remains a major concern in general and community health. Over the past three decades, there have been reports of *N. gonorrhoeae* strains resistant to penicillins, tetracyclines, and quinolones from different countries. The purpose of this study was to investigate *N. gonorrhoeae* drug resistance in view of the widespread use of quinolone antibiotics to low cost and availability, and to search for mutation-induced resistance in order to avoid inappropriate drug use and relapse of infection. Method: This study included 300 women who were referred to obstetrics and gynecology clinics for abnormal vaginal discharge between October 2012 to December 2014 at educational hospitals of Shahid Beheshti University of Medical Sciences, Tehran, Iran. Cultures were prepared for *N. gonorrhoeae* and positive samples were used to develop an antibiogram. Polymerase chain reaction (PCR) and DNA sequencing were used to study genetic resistance to quinolones. Results: Of 300 specimens included in the study, 7 (2.3 %) were positive for *N. gonorrhoeae*. Resistance was determined using a disc diffusion method, with ciprofloxacin resistance (57.1%). A minimum inhibitory concentration (MIC) of ≥1 µg/ml showed moderate resistance compared to ciprofloxacin, and an MIC ≥0.25 ± 0.125 µg/ml indicated intermediate resistance. In this study MIC of one sample was 1 µg/ml, 5 samples 0.25 µg/ml, and 1 sample 0.125 µg/ ml. Genotyping of *gyrA* and *parC* genes identified a point mutation that induced resistance at an MIC ≥1 µg/ml in the amino acid 86 of *gyrA* gene and serine to leucin. Conclusion: In the present study, we found that *N. gonorrhoeae* resistance to quinolones is due to mutation in *gyrA* gene, as reported by other studies worldwide, as well as in Iran.

Key Words: *N. gonorrhoeae*, *quinolone resistance*, *gyr*A gene, *par*C gene.

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INTRODUCTION

Neisseria gonorrhoeae is a gram-negative, non-motile, and aerobic bacterium. The initial site of infection is in the cubic and columnar epithelial cells of the genital tract. In women, the first site of infection is in the endocervical canal, and can lead to bleeding and pelvic pain. Purulent discharge may also involve the Bartholin glands. If untreated, ascending infection can involve the oviducts, leading to salpingitis, ovarian abscesses, and pelvic inflammatory disease [1]. Penicillin, tetracycline, and quinolones have been used for treatment but resistant strains led to the use of third generation cephalosporin [2, 3]. Since the 1990s, several fluoroquinolones with strong activity against gram-negative and gram-positive bacteria have been introduced and used in the treatment of genital and urinary tract infections [4, 5]. These drugs act by inhibiting DNA topoisomerase II (gyrase) and topoisomerase IV in bacteria. Studies have shown that bacteria develop resistance to the effects of quinolone drugs through point mutations in the genes producing DNA gyrase (*gyrA*, *gyrB*) and topoisomerase IV (*parC* ,*parE*) genes [4-6] .Studies on *Escherichia coli* have indicated that mutations in codons 67, 82, 83, 84, and 106 in *gyrA* are responsible for resistance to quinolones [7, 8]. Mutations in codons 80 and 84 in the *parC* gene are

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responsible for resistance [9-11]. The aim of this study was to investigate *N. gonorrhoeae* drug resistance in Iranian women, in view of the widespread use of quinolone antibiotics to low cost and availability, and to search for mutation-induced resistance in order to avoid inappropriate drug use and relapse of infection.

MATERIALS AND METHODS

Ethics statement:

This study was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences.SBMU.REC.1392.416. financially supported by the Infectious Diseases and Tropical Medicine Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran (grant No: 416).

Sampling

Vaginal discharge sampling was performed according to World Health Organization guidelines (using a speculum and dacron swabs), Suspicious colonies were tested for fermentation of glucose, lactose, maltose, and sucrose [12]. Antibiotic susceptibility testing was performed using the Clinical and Laboratory Standards Institute (CLSI) guidelines [13], with disc diffusion and MIC methods applied to positive samples, Reference strain of *N. gonorrhoeae* (ATTC 49226) was prepared, cultured on BHI medium, and used as positive control for culture based and molecular tests. Penicillin, tetracycline, ciprofloxacin, and ceftriaxone discs (Mast, England) were used for susceptibility testing. The MIC for ciprofloxacin (Sigma, Germany) was used for positive samples.

MIC - Agar Dilution: For the MIC, serial antibiotic dilutions consist of 13 plates with dilutions of 512, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, and 0.125 were prepared from antibiotics for each positive sample of *N. gonorrhoeae*.

Polymerase chain reaction (PCR) testing for *N. gonorrhoeae*:

Plasmid DNA was extracted from culture specimens positive for *N. gonorrhoeae* by using Plasmid extraction (Bioneer kit, Korea), and PCR was performed using a primer pair design based on CPPB plasmid genes [14].

In the next step, primers were used to amplify the *gyrA* and *parC* genes, with the specimens determining the sequence.

The following primers were used for PCR sequencing of *N. gonorrhoeae*: *gyrA* and *parC* genes.**0-S23**

primer	Sequence	Expected Amplicon size(bp)
CPPB	Forward: 5' GCT ACG CAT ACCCGC GTT GC3' Reverse: 5' CGA AGA CCT TCG AGC AGA CA3'	**390**
gyrA	Forward: 5' GTT TCA GAC GCC CAA AAG CCC3' Reverse: GGACAACAG CCA TTC CGCAAT3'	**225**
parC	Forward: 5' GTT TCA GAC GCC CAA AAG CCC3' Reverse: 5' GGACAACAG CCA TTC CGCAAT3'	**300**

Performance Standards for

PCR conditions for amplification of the CPPB were as follows: initial denaturation for 5 min at 95 ºC, followed by 35 cycles of 30 s at 94ºC, 1 min at 55 ºC, and 30 s at 74 ºC; the amplification process terminated with 5 min at 94 ºC. Primers of *gyrA* and *parC* genes were used to evaluate resistance in *N. gonorrhoeae*.

PCR conditions for amplification of the 225 bp fragment of the *gyrA* and 303bp fragment of the *parC* gene were as follows: initial denaturation for 5 min at 94ºC, followed by 36 cycles of 45 s at 94 ºC, 45 s at 52 ºC, and 45 s at 72 °C; the amplification process terminated with 10 min at 72 °C.

RESULTS

Among 300 samples, 29 women were with middle age, having abnormal vaginal discharge and a history of recurrent infection with using different classes of antibiotics such as Penicillin, Cephalosporin, Quinolones. 7 cases were positive for ***N.*** *gonorrhoeae* by phenotyping method.

Antibiogram results:

For the 7 positive samples, the results of the antibiogram using disc diffusion were as follows: resistance to penicillin, 71.42%; to tetracycline, 100%; to ciprofloxacin, 57.1%; and to ceftriaxone, 42.85% (Table1).

MIC method results: 1 sample: MIC = 1 µg/ml**,** 5 samples MIC = 0.25 µg/ml**,** 1 sample MIC = 0.125 µg/ml.

Molecular section results: After plasmid extraction, the 7 positive samples were detected by culture underwent PCR analysis, and the 390 bp fragments detected in these 7 samples confirmed the presence of *N. gonorrhoeae* (Figure 1).

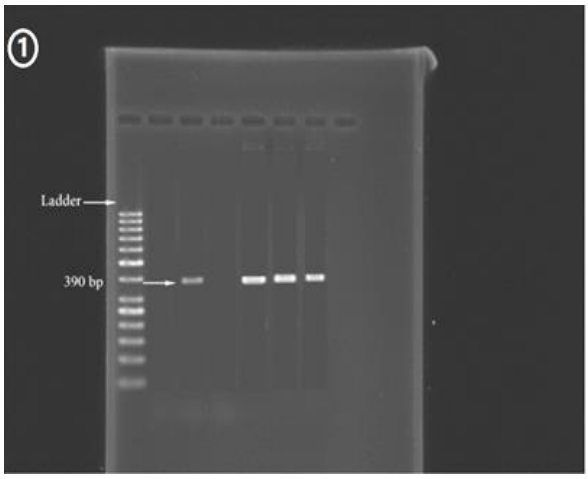

Fig. 1. PCR amplification of CPPB gene from clinical sample; lane 1: DNA ladder 1000bp; lane 3: Positive control; lane 5-7: positive sample.

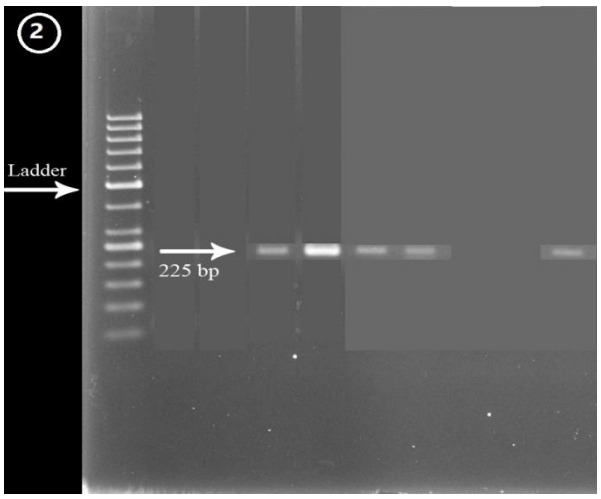

Fig. 2. PCR amplification of gyrA gene; lane 1: DNA ladder 1000bp; lane 10, positive control; lane 4-7 positive sample.

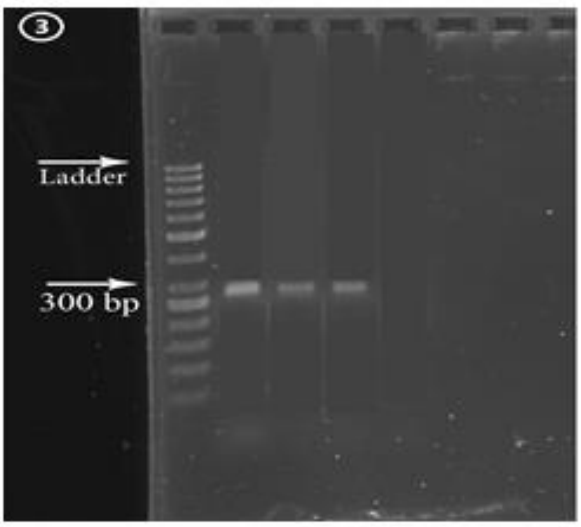

Fig.3. PCR amplification of parc gene; lane 1: ladder 1000bp, lane 4; Positive controle; lane 2, 3 positive sample.

The results of protein BLAST sequencing determined the presence of a single mutation in aa86 of *gyr A* gene, serine changes to leucin.

DISCUSSION

N. gonorrhoeae causes genitourinary tract infections and gonorrhea in humans. Resistant strains of *N. gonorrhoeae* have been reported worldwide for Penicillins, Tetracyclines, and Quinolones. This study focused on resistance to Quinolones, which are widely used for treatment of genitourinary tract infections in Iranian women, as the price is low and the drugs are readily available in all pharmacies. *N. gonorrhoeae* drug resistance has been reported for the past three decades [15], with the first report of resistance to quinolones in the mid-1980s by Tanaka in Japan. Tanaka showed that isolates of *N. gonorrhoeae* in 1993-1994 had MICs ≥1 µg/ml, with 24.4% resistance in 1997-1998. Molecular methods revealed 4-5 point mutations in *gyrA* and *parC* genes [16, 17].

In a study by Yang et al. in Shanghai, 159 *N. gonorrhoeae* isolates from males were sensitive to penicillin, tetracycline, ciprofloxacin, and spectinomycin, and ceftriaxone-phenotypic resistance to ciprofloxacin was found to be present in 98.7% of isolates [18].

In a joint study conducted in India, Pakistan, and Bhutan from 2007-2011, 65 *N. gonorrhoeae* isolates were assessed using the E-test with 8 antibacterial agents, and showed the highest resistance rate for ciprofloxacin at 96%, followed by penicillin G at 68%, erythromycin at 62%, tetracycline at 55%, and azithromycin at 7.7%; all strains were susceptible to ceftriaxone and cefixime [19].

In a large-scale study in 17 European countries from 2006-2008, 3,532 *N. gonorrhoeae* isolates were tested by agar dilution or E-test methods to determine resistance to ciprofloxacin, penicillin, tetracycline, azithromycin, spectinomycin and ceftriaxone. The range of resistance to ciprofloxacin in these 17 countries was 42-52% [20]. Our study showed resistance rates with disc diffusion as follows: penicillin 71.42%; tetracycline 100%; ciprofloxacin 57.1%; and ceftriaxone 42.8%. MIC ≥1 µg/ml was determined in 1 sample, ≥0.25 µg/ml in 5 samples, and ≥0.125 µg/ml in 1 sample. According to the CLSI guidelines, isolates with an MIC ≥1 µg/ml are resistant, those with MIC ≥0.25-0.125 µg/ml show intermediate resistance, and those with MIC ≤0.06 µg/ml are susceptible to ciprofloxacin. MIC testing identified 1 ciprofloxacin-resistant specimen and 6 with intermediate or indeterminate resistance, which could represent low-level resistance, multiple studies have been performed using molecular techniques. A study in Sweden in 2002-2003 used molecular methodology to identify genetic resistance to quinolones, and found that most mutations

occurred between amino acids 90-96, at *Asp95-91ser*, with corresponding MIC >4 mg/l MIC for ciprofloxacin; resistant strains had 3-4 mutations [21]. A study in the USA in 2003, on strains of *N. gonorrhoeae* resistant to quinolones by using real-time and sequencing analysis showed that MIC ≥1 µg/ml may indicate resistance to quinolones due to mutations in *gyrA* and *parC* (100-20). A study in the Far East on 234 *Neisseria* isolates showed that MIC ≥1 µg/ml indicated moderate resistance and MIC ≥4 µg/ml indicated high resistance to ciprofloxacin using induced phenotypic methods. Moreover, study of induced resistance showed that changes in amino acids 91-95 in *gyrA* gene and in amino acid 86 in *parC* resulted in higher resistance [22]. In a study conducted in Taiwan using phenotypic and molecular methods, mutations in the QRDR region averaged 82.2%, indicating that in *gyrA*, amino acid 91 was relocated and that amino acid 95 in *parC* was mutated (95-23) [23]. In a study in 10 hospitals in London using phenotypic and molecular methods, the susceptibility of isolates was measured against penicillin, tetracycline, ciprofloxacin, and spectinomycin, followed by molecular sequencing of the genes. With respect to ciprofloxacin, 4 isolates had a MIC of 16 µg/ml and 2 or more mutations in *gyrA*, while a MIC of ≥1 µg/ml and only 1 mutation was found in the *gyrA* gene [24]. Recent studies in the USA, Germany, and Australia have demonstrated an increase in resistance to ciprofloxacin, cephalosporins, and other classes of antibiotics [23, 24]. The results of the present study show that in 6 samples, mutations responsible for MIC ≥0.25-0.125 µg/ml were not found in *gyrA* or *parC* genes; a sample with MIC ≥1 µg/ml showed mutation in the *gyrA* gene and in amino acid 86, i.e., serine to leucine. An association was found between *gyrA* and MIC ≥1 µg/ml, as shown in research by David et al. in Taiwan; Ison and Martin in London showed a significant association between the MIC value and mutation in *gyrA* and *parC*. With a MIC ≥1 µg/ml, a point mutation occurred in the *gyrA* gene, but with an increase in MIC to 4 µg/ml, the number of mutations in the genes increased to 3-4[24].

CONCLUSION

Inappropriate antibiotic prescribing without cultures, antibiograms, or molecular methods such as PCR, but only on the basis of clinical symptoms is common. Therefore, in order to prevent the spread of these diseases, antibiotic use should be based on proper diagnostic methods, with cultures and antibiograms.

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Table 1: Antibiograme result of patients

Patient number	Penicillin	Tetracycline	Ceftriaxon	Ciprofloxacine	MIC of Ciprofloxacine	Mutation in *gyr A* and *parC* genes
Patient 1	Resistance	Resistance	Resistance	Resistance	0.25 µg/m	Not found
Patient 2	Sensitive	Resistance	Resistance	Resistance	0.25 µg/ml	Not found
Patient 3	Resistance	Resistance	Resistance	Resistance	0.25 µg/ml	Not found
Patient 4	Resistance	Resistance	Resistance	Resistance	1 µg/ml	Aa 86 serine changes to leucin
Patient 5	Resistance	Resistance	Sensitive	Intermediate	0.25 µg/ml	Not found
Patient 6	Intermediate	resistance	Resistance	Intermediate	0.125 µg / ml.	Not found
Patient 7	Resistance	Resistance	Resistance	Resistance	0.25 µg/ml	Not found

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