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Original Article

Exploring the Prevalence of *Neisseria gonorrhoeae* in Women with Genitourinary Symptoms in Tehran, Iran

Narjes Noori Goodarzi [1], ***Shaghayegh Zafar*** [1], ***Naghmeh Pourmand*** [2], ***Soheila Ajdary*** [3], ***Mir Saeed Yekaninejad*** [4], ***Mohammad Reza Pourmand*** [1], ***Farzad Badmasti*** [5]

1. *Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran*
2. *Department of Infertility, Yas Hospital Complex, Tehran University of Medical Sciences, Tehran, Iran*
3. *Department of Immunology, Pasteur Institute of Iran, Tehran, Iran*
4. *Department of Epidemiology and Biostatistics, School of Public Health, Tehran University of Medical Sciences,Tehran, Iran*
5. *Department of Bacteriology, Pasteur Institute of Iran, Tehran, Iran*

***Corresponding Author:** Email: mpourmand@tums.ac.ir, fbadmasti2008@gmail.com

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Abstract

Background: *Neisseria gonorrhoeae*, the second most common sexually transmitted infection (STI) worldwide, affects one million people daily. We aimed to investigate the prevalence of gonorrhea in females with genital infections in Tehran, Iran.

Methods: First, a bioinformatic study was conducted to identify a conserved and high-prevalent gene marker for detection of *N. gonorrhoeae*. One desirable marker was selected and a pair of specific primers was designed to amplify it. The reliability of the primer pair was evaluated *in silico* and *in vitro*. Subsequently, 172 patients with genitourinary symptoms were enrolled and an endocervical swab specimen was obtained from each patient to evaluate the presence of *N. gonorrhoeae* in clinical specimens using the specific primers.

Results: Restriction endonuclease subunit S (*resS*, WP_003687768.1) was selected as a specific detection marker. The designed primer pair targeting *resS* showed specific and reliable detection of *N. gonorrhoeae in silico* and *in vitro*. Out of 172 clinical samples, seven (4.06%) cases were infected by *N. gonorrhoeae*. Statistical analysis of clinical manifestations showed that there was a significant association between the occurrence of *N. gonorrhoeae* and dysuria (P= 0.043), pelvic pain (P= 0.017), and fever (P = 0.045).

Conclusion: Three promising markers were introduced for development of point-of-care testing approaches. Moreover, this study highlights a 4% prevalence of gonorrhea among women with genitourinary symptoms in Iran, which reminds the urgent need for routine surveillance and new policies in management of STIs, particularly gonorrhea.

Keywords: *Neisseria gonorrhoeae*; prevalence; Gonorrhea

Introduction

Neisseria gonorrhoeae is the second most common sexually transmitted infection (STI) with considerable economic burden (1). According to the WHO, the incidence of *N. gonorrhoeae* was approximately 82.4 million in 2020 (2).

This organism invades the mucous membranes of the reproductive tract in both males and females (3). While a significant number of infected men exhibit noticeable symptoms, the majority of women remain asymptomatic (4). If not properly treated, *N. gonorrhoeae* can lead to significant complications, particularly in women, fetus and newborns (5). Pelvic inflammatory disease (PID), infertility, and increased risk of ectopic pregnancy are only a number of other subsequent complications of gonorrhea in women (5, 6).

Almost all symptoms of gonococcal infected individuals are predominantly non-specific (7). However, in resource-limited settings, the identification of gonorrhea typically relies on a combination of clinical manifestations and medical history due to lack of proper laboratory diagnostic methods (8). In addition, the overwhelming majority of gonococcal infections are found in low- and middle-income countries (9, 10). Therefore, it is imperative to develop accessible and user-friendly diagnostic methods for the rapid identification of *N. gonorrhoeae* in such settings.

The frequency of *N. gonorrhoeae* has garnered considerable attention in recent years. Despite limited data availability, studies indicated moderate to high rates of gonorrhea in various regions of Iran, ranging from 2 to 8% (11-14). This elevated prevalence underscores the urgency of addressing gonorrhea as a public health concern. Thus, in the current study we aimed to assess the prevalence of gonorrhea among women with STIs attending to Tehran university hospitals. For this purpose, a specific and conserved gene marker was identified through bioinformatic analysis. Then, a pair of specific primers was designed to amplify the targeted marker. Ultimately, after confirming their effectiveness in accurate diagnosis of gonorrhea, they were used to assess the frequency of *N. gonorrhoeae* in clinical samples.

Material and methods

Pan-genome analysis of Neisseria species

On the first step, to identify the homologous coding genes of *N. gonorrhoeae* and other *Neisseria* species, pan-genome analysis was performed using the BPGA (Bacterial Pan-genome Analysis) software (15). To fulfill this purpose, a dataset of pathogenic and normal flora *Neisseria* species was defined compromised of *N. gonorrhoeae* FA 1090, *N. meningitidis* NCTC10025, *N. baciliformis* DSM 23338, *N. cinerea* NCTC10294, *N. elongata* M15910, *N. flavescens* ATCC 13120, *N. lactamica* NCTC10617, *N. mucosa* ATCC 19696, *N. perflava* LPB0400, *N. polysaccharea* M18661, *N. sicca* ATCC 29256, *N. subflava* ATCC 49275, and *N. weaveri* NCTC12742. These 13 species are the most common pathogen or normal flora of throat, genital tract and rectum of human (16). Core-proteins were identified with a cut-off = 0.1 and discarded from the study. The unique proteins of *N. gonorrhoeae* were selected for further studies.

Next, to further confirm the unique genes of *N. gonorrhoeae*, protein sequences were compared to the proteins of *Neisseria* species using the BLASTp tool at the NCBI (https://blast.ncbi.nlm.nih.gov). Proteins showing any resemblance to normal flora *Neisseria* spp. were excluded. The unique proteins were collected for further analysis.

Evaluating the prevalence and conservancy of unique proteins

Then the prevalence of the remaining proteins among 155 *N. gonorrhoeae* strains was assessed using BLASTp at NCBI. The sequence conservancy of each protein was evaluated through BLAST against *N. gonorrhoeae* strains, and the multiple sequence alignment (MSA) of proteins using WebLogo online server (https://weblogo.berkeley.edu/).

Designing specific primers for identification of N. gonorrhoeae

To design appropriate primers to amplify the targeted gene, a conserved specific gene was selected. A set of primers were designed using Primer Premier 6.25 software (PRIMER Biosoft). Thermodynamic properties and secondary structures of the primer pair were confirmed.

In silico and *in vitro* evaluation of primers

To evaluate the specificity of the designed primer pair, *in silico* PCR was conducted against 493 *Neisseria* strains with complete genomes including *N. gonorrhoeae* (n=134), *N. lactamica* (n=44), *N. meningitidis* (n=136), *N. elongata* (n=39), *N. flavescens* (n=26), *N. mucosa* (n=22), *N. subflava* (n=9), *N. polysaccharea* (n=36), *N. perflava* (n=8), *N. baciliformis* (n=8), *N. weaveri* (n=9), and *N. baciliformis* (n=22), using *in silico* PCR UniPro UGENE software. The whole genome sequences were retrieved from NCBI database.

To confirm the efficiency and specificity of the designed primers, a set of non-gonorrhoeae *Neisseria* species were provided from Iranian Biological Research Center and Persian Type Culture Collection, including *N. sicca* ATCC 9913, *N. subflava* CCM 3482, and *N. meningitidis* ATCC 13090, and the presence/absence of the targeted gene marker was evaluated. *N. gonorrhoeae* ATCC 19424 was used as positive control.

Evaluating the prevalence of gonorrhea

Specimen collection

To evaluate the prevalence of gonorrhea, a total of 172 women with clinical manifestations of STIs were enrolled. The study was conducted from October 2023 to June 2024. This study received ethical approval from the Ethical Committee of Tehran University of Medical Sciences (approval code: IR.TUMS.SPH.REC.1403.073).

The endocervical secretions were collected using a sterile speculum and a Dacron swab (Deltalab, Spain), and the swab was placed in 2 ml of PBS. The swab samples were promptly transported to the laboratory within 1 hour. Written consent was obtained from all participants for their involvement in the study. Additionally, a questionnaire form was utilized to document demographic information and clinical manifestations, and risk behaviors of the patients.

Molecular detection of N. gonorrhoeae

For molecular detection of *N. gonorrhoeae*, the genomic DNA of endocervical specimens were extracted using Favorprep™ tissue genomic DNA extraction mini kit (Favorgen Biotech Corporation, Pingtung, Taiwan) according to the manufacturer's protocol. Then, a 255 bp fragment of *resS* gene was amplified using the designed primers. PCR was performed using a 2X Red Mastermix (Ampliqon) in a Sigma thermocycler. The reaction mixture for a total volume of 25 µl consisted of 12.5 µl Mastermix, 10.5 µl double-distilled water, 0.5 µl of each primer (10 pmol), and 1 µl DNA. The thermal cycling conditions were as follows: initial denaturation at 95 ˚C for 7 min, followed by 30 cycles of denaturation at 94˚C for 30 sec, annealing at 55˚C for 60 sec, and extension at 72 ˚C for 45 sec. A final extension step was carried out at 72 ˚C for 7 min. The resulting PCR products were analyzed by gel electrophoresis using a 1% agarose gel stained with FluoroVue™ Nucleic Acid Gel Stain and the bands were visualized using UVITEC Cambridge gel documentation system.

Statistical analyses

Statistical analyses were performed using SPSS version 27.0 (IBM Corp., Armonk, NY, USA). To assess the associations between the occurrence of *N. gonorrhoeae* and demographic data, clinical signs, and symptoms, Fisher's exact test was used. Odds ratios (OR) and their corresponding 95% confidence intervals (CIs) were also calculated. Results with a p-value < 0.05 were considered statistically significant. To address zero cell counts, the Agresti correction was applied to adjust the odds ratios and the corresponding 95% confidence intervals (17). The p-values were adjusted for multiple comparisons using the Bonferroni method (18).

Results

Bioinformatic results

Pan-genome analysis of 13 different *Neisseria* ssp. resulted in identification of 84 unique proteins in *N. gonorrhoeae*. Seventy-two proteins showed partial homology to *Neisseria* spp. other than *N. gonorrhoeae* and excluded from the study. The genomic sequences of remaining proteins were compared to the whole genome of 155 circulating *N. gonorrhoeae* strains. Only three proteins were present in

all strains with an identity > 99%, including resS (WP_003687768.1), DnaB-like helicase C-terminal domain-containing protein (WP_010951190.1), and HNH endonuclease (WP_003706646.1).

Out of three unique eligible proteins, resS (WP_003687768.1) was selected to design a set of primers. MSA of *resS* gene among different *N. gonorrhoeae* strains is represented in Fig. 1. The designed primer pair targeting *resS* with favorable thermodynamic properties is represented in Table 1. *resS* gene (NGO0357) was specifically located on *N. gonorrhoeae* genome and absent in non-gonorrhoeae *Neisseria* species (Fig. 2).

Table 1: The sequence of primers targeting *resS* of *N. gonorrhoeae*

Primer name	Primer sequence	GC%	Melting temperature (Tm)	Annealing temperature (Ta)
ResS-F	5'-GAAGCTAACCGCACGTTACGACAA-3'	50%	57˚C	55 ˚C
ResS-R	5'-ACTGGCAGGACGGATATAGACAACC-3'	52%	59 ˚C	

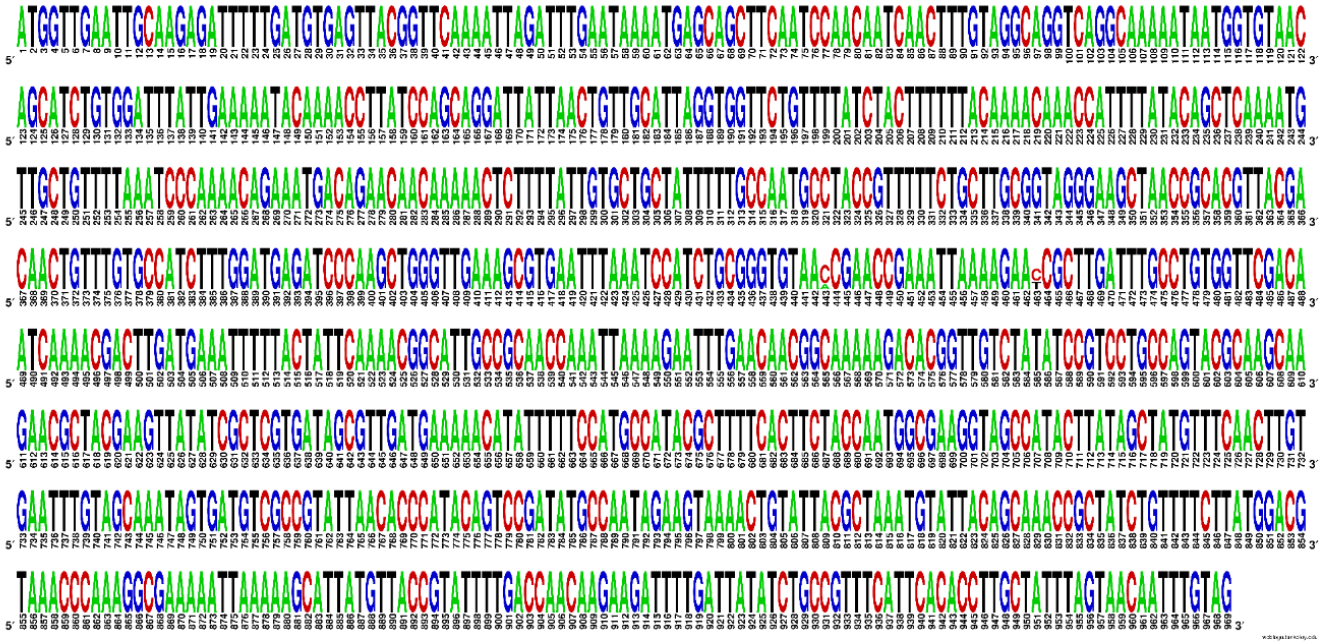

Fig. 1: Sequence conservation of *resS* among 155 *N. gonorrhoeae* strains using WebLogo online server

Evaluating the specificity of designed primers

In silico PCR analysis showed that the 255 bp fragment of *resS* was present in all *N. gonorrhoeae* strains (n=134, 100%), while it was not observed in any non-gonorrhoeae *Neisseria* strains. The desired PCR product was successfully amplified in *N. gonorrhoeae* ATCC 19424, while this fragment was not observed in *N. sicca*, *N. subflava*, and *N. meningitidis* (Fig. 3).

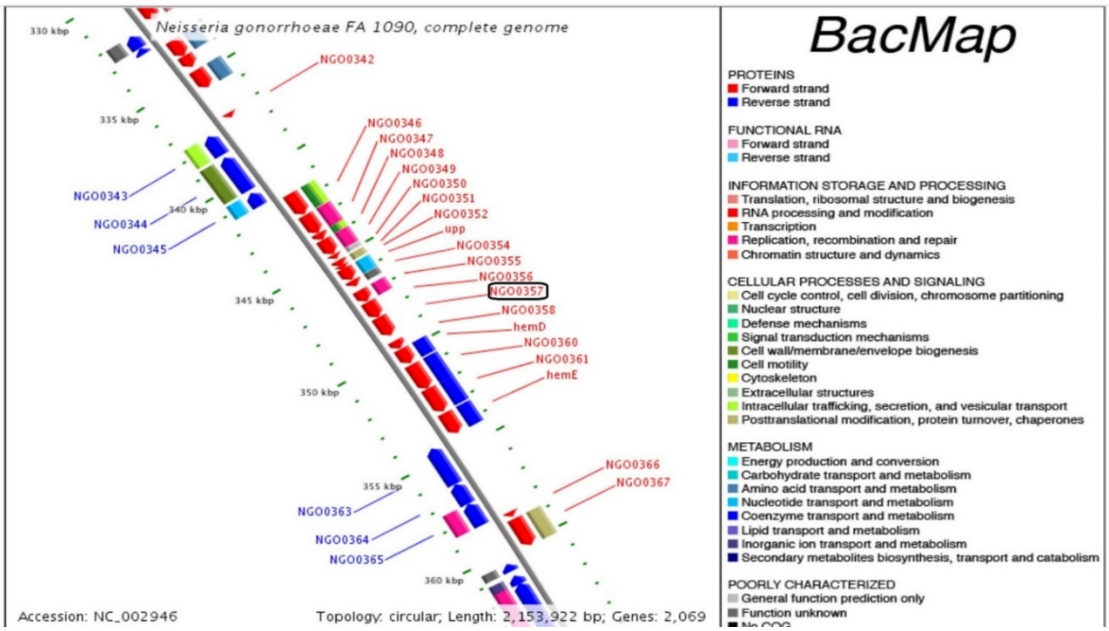

Fig. 2: The *resS* (NGO0357) is a unique gene in *N. gonorrhoeae genomes and not present in non-gonorrhoeae Neisseria species* (http://wishart.biology.ualberta.ca/BacMap/). This gene is involved in Restriction-modification (R-M) system

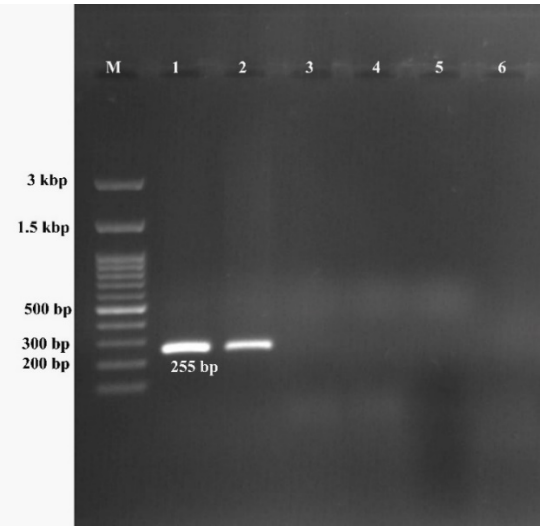

Fig. 3: Amplification of a 255 bp fragment of *resS* gene of *N. gonorrhoeae* using PCR. M: Marker; 1: *N. gonorrhoeae* ATCC 19424; 2: Positive clinical sample; 3: *N. sicca* ATCC 9913; 4: *N. subflava* CCM 3482; 5: *N. meningitidis* ATCC 13090; 6: Negative control

Identification of N. gonorrhoeae in clinical samples

The patients' age ranged from 18-73 years old, and the majority of patients (66.7%) were middle-aged adults, between 32-50 years old. Out of 172 patients, 133 (77.32%) had education levels of high school diploma or lower, 32 (18.6%) held a bachelor's degree, and only seven (4.06%) had a mas-

ter's degree or higher. Only 19 (11.04%) participants used condoms. The most common clinical manifestation that women with STIs suffered from were dysuria (42.44%), discharge (42.44%), vulvar itching (38.37%), pelvic pain (27.32%), and dyspareunia (26.74%). Abortion (30.23%), infertility (10.46%), and ectopic pregnancy (4.65%) were more common fertility complications, respectively.

Among 172 clinical samples, seven positive cases (4.06%) were identified using PCR method. Statistical analysis demonstrated that dysuria, pelvic pain and fever were significantly associated with the incidence of gonorrhea. There was no statistically significant association between *gonorrhea* and other indicators (Table 2).

Table 2: Sociodemographic and clinical manifestations of patients with genitourinary symptoms

Characteristics		Gonorrhea + (n = 7)	Gonorrhea – (n = 165)	*P*-value	Odds ratio	95% CI
Sectors	Gynecology	4 (57.1%)	111 (67.3%)	>0.90	0.64	0.14 to 3.00
	IVF	1 (14.3%)	20 (12.1%)	>0.90	1.20	0.13 to 10.56
	Oncology	2 (28.6%)	34 (20.6%)	>0.90	1.54	0.28 to 8.29
Age groups	18-32	1 (14.3%)	33 (20.0%)	>0.90	0.66	0.07 to 5.73
	33-50	4 (57.1%)	110 (66.7%)	>0.90	0.66	0.14 to 3.08
	Above 51	2 (28.6%)	22 (13.3%)	0.759	2.60	0.47 to 14.23
Educational state	≤ High school diploma	4 (57.1%)	129 (78.2%)	0.579	0.37	0.08 to 1.73
	Bachelor's	3 (42.9%)	29 (17.6%)	0.363	3.51	0.74 to 16.56
	Master's ≥	0 (0.0%)	7 (4.2%)	>0.90	2.48	0.27 to 22.35
Marriage state	Married vs. Divorced	7 (100%)	156 (94.5%)	>0.90	0.51	0.05 to 4.48
Dysuria		6 (85.7%)	67 (40.6%)	0.043	8.77	1.03 to 74.56
Itching		5 (71.4%)	61 (37%)	0.108	4.26	0.80 to 22.64
Discharge		6 (85.7%)	104 (63.0%)	0.424	3.51	0.41 to 29.92
Dyspareunia		2 (28.6%)	42 (25.5%)	>0.90	1.17	0.21 to 6.26
Pelvic pain		5 (71.4%)	42 (25.5%)	0.017	7.32	1.36 to 39.15
Fever		2 (28.6%)	7 (4.2%)	0.045	9.02	1.48 to 54.95
Urinary frequency		2 (28.6%)	29 (17.6%)	0.611	1.87	0.34 to 10.14
Vomit		1 (14.3%)	8 (4.8%)	0.318	3.27	0.35 to 30.51
Barriers		1 (14.3%)	18 (10.9%)	0.566	1.36	0.15 to 11.95
Bleeding during sampling		0 (0.0%)	13 (7.9%)	0.560	1.36	0.15 to 11.72
Infertility		0 (0.0%)	18 (10.9%)	>0.90	0.97	0.11 to 8.21
History of abortion		2 (28.6%)	49 (29.7%)	>0.90	1.17	0.28 to 4.86
Ectopic pregnancy		1 (14.3)	6 (3.6%)	0.256	4.41	0.45 to 42.67
Smoking		0 (0.0%)	11 (6.7%)	0.507	1.61	0.18 to 14.00
Alcoholism		0 (0.0%)	1 (0.6 %)	>0.90	10.31	0.84 to 126.02

The bold values are the indicators that showed a p-value < 0.05

Discussion

Gonorrhea management in resource-limited countries largely relies on syndromic approach (19), which results in overtreatment, missing cases, and contributes to antimicrobial resistance (20). This highlights the need for rapid, accurate, and cost-effective diagnostic methods.

Considering the shortcomings of the common detection methods of *N. gonorrhoeae*, such as microscopy(21), culture (2), DNA probe assays, antigen detection tests, and serology methods (22), in low-

income settings, the PCR assay is the most commonly used and accessible Nucleic Acid Amplification Test (NAAT).

Several NAAT assays have been developed for identification of *N. gonorrhoeae*, so far. 16S rRNA (23), *orf1* (24), 23S rRNA (25), *opa* (26) have been targeted in such assays previously. However, some of these genes are not completely specific to *N. gonorrhoeae*. Conversely, the presence of normal flora *Neisseria* species in genital, rectal, and oral regions can lead to misdiagnosis, resulting in unnecessary antibiotic prescriptions.

Given the complexities associated with the misidentification of gonorrhea and the potential consequences of empirical therapy (27), there is an urgent necessity to develop a new detection method. Moreover, due to the limited number of commercially available point-of-care test with proper sensitivity (28-31), this study aimed to explore a promising approach to fulfill this need.

In our study, to select a specific gene marker, several criteria have been taken into consideration, such as high prevalence and sequence conservancy among circulating *N. gonorrhoeae* strains. We could successfully find three gene markers that were exclusive to *N. gonorrhoeae*, and targeted *resS* to design a specific PCR method. This protein has a type I restriction modification DNA specificity conserved domain. It is associated with Restriction-modification (R-M) systems that protects the bacterial cell against invasion of foreign DNA by endonucleolytic cleavage of DNA that lacks a site-specific modification. The host genome is protected from cleavage by methylation of specific nucleotides in the target sites (32, 33).

The designed assay showed high specificity in detecting *N. gonorrhoeae* in both *in silico* and *in vitro* analyses. In addition, since this approach was able to detect *N. gonorrhoeae* in direct clinical samples, it can be used as a promising method for rapid, easy to use, and cost-effective detection and management of gonorrhea, especially in countries with limited resources. Besides, DnaB-like helicase C-terminal domain-containing protein, and HNH endonuclease were two other potential gene markers for specific detection of *N. gonorrhoeae* which their efficiency needs to be confirmed *in vitro*.

Our study showed approximately 4% of women with genitourinary manifestations suffered from gonorrhea. While specific prevalence rates for Iran may vary across studies, they generally fall within the range reported worldwide (11-13). The prevalence of gonorrhea in other countries varied depending on several factors including risk behaviors, geographical region, age groups, and infection site. The prevalence of *N. gonorrhoeae* among women has been reported to be approximately 0.9 %, 0.3%, 0.9%, and 1.9% in American, European, Western Pacific, and African regions, respectively (34).

Another recent meta-analysis reported the frequency of *N. gonorrhea* in the Middle East and North Africa to be around 1.5 %. However, this rate is higher among women attending infertility clinics (6%) or experiencing miscarriage or ectopic pregnancy (2.8%) (35). Similarly, our previous study showed higher rates of infertility, pregnancy-related complications in women infected with *N. gonorrhoeae* (13). However, no such association was found in this study. This condition might be due to the low number of patients with these complications in the current study.

The asymptomatic nature of gonorrhea in many cases further complicates detection and treatment efforts. Vaginal discharge, dysuria, dyspareunia, lower abdominal pain and pelvic pain are among the most frequently reported symptoms in females infected by gonorrhea (36-38). In our study, dysuria, and pelvic pain were significantly higher in gonorrhea patients. Moreover, a considerable association was observed between fever and gonorrhea. Fever is a major symptom of disseminated gonococcal infections (39-41).

Several factors contribute to high burden of this infection, including inadequate sexual health education, insufficient access to healthcare services, social stigma, and inconsistent condom use (42). Although, no statistically significant relation was observed between educational state or using barriers, with the incidence of gonorrhea in the current study, several studies have reported low education level and condomless sexual practice has been reported as a significant risk factor of gonorrhea (43, 44).

Alcohol consumption and smoking are major risk behaviors of STIs (43, 45). However, the statistical analysis of our data showed no difference in *N. gonorrhoeae* infected and non-infected patients. This condition probably arose from the low proportion of such participants in our study.

Conclusion

Four percent of women with genitourinary symptoms were infected by *N. gonorrhoeae*. The high prevalence of gonorrhea in Iran is often overlooked due to the lack of routine surveillance systems and laboratory diagnostics in healthcare centers. To overcome the limitations of the syndromic approach, this study proposed a promising NAAT targeting *resS* gene. This method offers advantages such as ease of use, cost-effectiveness, and the ability to directly identify pathogens in clinical samples, making it applicable in resource-limited settings. However, in our country new policies are urgently needed for the management of gonorrhea.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

Availability of data and materials

The complete genome sequences of *Neisseria* spp. strains are deposited in the genome database at the NCBI (https://www.ncbi.nlm.nih.gov/datasets/genome/?taxon=482).

Acknowledgements

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Conflict of interest

The authors declare that there are no conflicts of interest.

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