RESEARCH ARTICLE

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Expression of Opa, Tfpc, Pora Genes of *Neisseria Gonorrhoeae* Associated With Interleukins 6,8,10 in Some Iraqi Women

Raghad Abdul Wahid Qubian,
Prof Dr Zahrah Adnan Alshammarri*

ABSTRACT

Background: Gonorrhea is a Sexually Transmitted Disease (STD) caused by *Neisseria gonorrhoeae* (*N. gonorrhoeae*) infection, especially in young women. Lactobacillus acidophilus is one of the important bacterial species that prefers an acidic environment, where it ferments sugars and converts them into lactic acid and hydrogen peroxide. It works to inhibit or kill harmful microorganisms in humans, and it opportunistic, causing diseases in humans. The aim of study is diagnosing of Neisseria can be confirmed by molecular detection of the diagnostic and virulence gene by using RT-PCR technology. Measurement the interleukins level 6,8,10 which associated with *Neisseria Gonorrhoeae* infection and refer to degree of infection.

Methods: a total of 200 samples including (170 vaginal swabs from patients and 30 healthy swabs samples as a control sample) were collected from women who visited Al-Ammarah General Hospital in Maysan Governorate, ranging in age from 20 to 42, between October 28, 2022, and May 13, 2023. For the purpose of diagnosing and isolating L. acidophilus and *N. gonorrhoeae*; Visual, microscopic, biochemical testes were used. Also, the levels of interleukin (IL-6, IL-8, IL-10) in serum women with *Neisseria gonorrhoeae* and the expression of Opa, TfpC, and Pora genes were investigated as virulence factors.

Results: The clinical specimens showed that 30 (17.64%) of the total isolates were identified as *N. gonorrhoeae*. The remaining 140 samples (82%) showed negative results in laboratory culture. The levels of interleukins IL-6, IL-8, and IL-10 in the serum of women infected with *N. gonorrhoeae* bacteria appeared very high compared to the control group. The results were analyzed using a quantitative RT-PCR device for expression of the opa, TfpC, and porA genes, and sRNA16 was used as a constant expression gene for comparison (Housekeeping gene), as the results indicated an increase in gene expression in the three genes compared to the control sample. While differences were observed between genes, the TFpC gene gave the highest gene expression, 441.47, compared to the opa and porA genes, which gave 100.723 and 71.02, respectively.
Conclusion: *N. gonorrhoeae* was responding to sexually transmitted diseases and Opa, TfpC, and Pora genes have a role in the virulence of this bacteria.

Keywords: Sexually Transmitted Disease, *Neisseria Gonorrhoea, Lactobacillus Acidophilus*, Tfpc Gene, Por A Gene.

Department of Biology, College of Science, University of Misan, Maysan, Iraq

***Send correspondence to**
Prof Dr Zahrah Adnan Alshammarri
Department of Biology, College of Science, University of Misan, Maysan, Iraq, E-mail: Maysaniraq144@uomisan.edu.iq

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INTRODUCTION

Neisseria gonorrhoeae is a *Gram-negative diplococcus* (*N. gonorrhoeae*) is the pathogen that causes gonorrhea in humans and is an obligatory infection. According to estimates from the World Health Organization[1], there are between (78 - 87) million new cases of gonorrhea each year[2]. The World Health Organization advises doing routine evaluations of the prevalence of gonorrhea in the general public, pregnant women, women visiting family planning clinics, prospective military personnel, and populations at higher risk[3]. According to[4], the primary bacterial species found in the vagina of healthy women is called Lactic Acid Bacteria (LAB), and it is crucial for preserving the ecological balance of the female reproductive system. Because lactobacillus protects the vaginal microenvironment, a reduction in its population causes dysbiosis, which is characterized by a disruption in microbial species and a decline in microbial variety in the vaginal region[5]. Real time PCR is a method used to amplify short stretches of DNA or RNA millions of times outside the body[6].There is related between Neisseria and miscarriage. Miscarriage is a complicated pregnancy and known as a spontaneous ending of pregnancy itself before the baby has attained a level of viability. Miscarriages may be sporadic or recurrent[7].

MATERIALS AND METHODS

• **Collection of specimens:** One hundred and seventy female patients with vaginal discharge (watery, green or yellow in color), pain or burning when urinating, and those with lower stomach pain and uterine hemorrhage during menstruation were included in the sample pool. The samples were taken from women, aged between (20-50), who came to the Al-Amara General Hospital in the province of Maysan between October 2022 and March 2023. In order to identify and isolate *N. gonorrhoeae*, all swabs were put in a transport medium and sent straight to the laboratory in an iced bag.

• **Bacterial isolates**

1. Morphological Examination: In order to investigate the phenotypic patterns (such as shape, color, size, and odor of colonies) as well as their capacity to break down blood, if present, for L. acidophilus and *N. gonorrhoeae*, selective and rich culture media (blood agar, MacConkey agar, chocolate agar, and Thayer-Martin medium) were utilized[8,9].
2. Wet amount technique according to[10].
3. Gram stain: was used Gram stain to detect the bacteria L. acidophilus and *N. gonorrhoeae*, which were isolated on culture media[11].
4. Biochemical Tests: conducted the following biochemical tests to diagnose isolates of *N. gonorrhoeae* bacteria; Catalase Test[12], Oxidase Test, and Motility Test[13]; which Confirmation of diagnosis using the diagnostic system by VITEK 2 system.
5. Determination on Interleukins all interleukins 6,8,10 were diagnostic tool provided by Human Interleukin 6 ELISA KIT, Bioassay Technology Laboratory, Shanghai, China.
6. Genetic Study of Neisseria Isolates

• **RNA extraction:** RNA was extracted using FavorPrep Total RNA Mini Kit and according to the instructions of the supplier company FAVORGEN/Korea.

• **Quantitation of RNA concentration and purity:** RNA concentration and purity were selectively measured for only five Neisseria isolates to the Quantus™ Fluorometer calibration. The standard sample was prepared in the final standard calibration (ng/tube) shown below. Blank samples and standards were prepared as follows: Tris-EDTA buffer (1X TE buffer) was prepared by diluting 20X TE buffer (pH 7.5) to 1X with nuclease-free water. 200 microliters of 20X TE Buffer was taken and the volume was completed to 4 ml with nuclease-free water to prepare 4 ml of 1X TE Buffer. 10 μL of QuantiFluor® Dye solution was mixed with 3,990 mL of 1X TE buffer. The nucleic acid standard was prepared by adding 5 microliters of the standard solution and the sample to 200 microliters of working reagents. A blank tube was prepared by adding 200 μL of working reagent only. The mixture was mixed three times with a pipette slowly, then the tube components were mixed with a vortex at high speed for 10 seconds and incubated at room temperature away from light. RNA purity was measured and the concentration was recorded in nanograms per microliter.

• **Conversion of RNA to cDNA:** RNA serves as a template for the synthesis of complementary DNA (cDNA). The LunaScript Reverse Transcriptase/Biolabs/England RT reagent Diagnostic Kit is designed to perform reverse transcription for real-time RT-PCR.

• Quantitative Detection *Neisseria gonorrhoeae* by Real-Time PCR

• **Master Mix Preparation:** All reaction components were thoroughly dissolved and mixed. The Master Mix was prepared according to the volume allocated for all reaction components common to all reactions or a subset of the reactions to be performed, by following the required volume for each component based on the following table (Table 1).

• **RT-PCR Cycling Program:** Samples were transferred to a thermocycler instrument and programmed according to the annealing step temperature for each primer as listed in (Table 2).

• **Statistical analyses:** Proportions and frequencies were used in descriptive statistics for this study according to[14]. The Chi-square test was conducted in order to study the relationship between the data sets, and the value ($p = 0.05$) was adopted in this study[15].

RESULTS AND DISCUSSION

Distribution Age Groupings Patients and Some Factors: According to the current study as in (Table 3),the

Table 1: Master Mix Preparation.

Components	20µL (Final volume)	Final concentration
Luna Universal qPCR Master Mix(2x)	10µL	2x
Forward primer	0.5µL	0.2µM
Reverse primer	0.5µL	0.2µM
Nuclease-free water	Up to 20µL	
Template cDNA Sample Volume	5	1pg-100ng

Table 2: Time, temperature and number of cycles of the program.

Step	Temp. (°C)	Time	Cycle
Initial Denaturation	95	60 sec.	1
Denaturation	95	15 sec.	40 – 45
Extension	60	30 sec.	
Melt curve	60 - 95	02:00 min	1

Table 3: Prevalence of *N. gonorrhoeae* by age groups and some factors.

Parameters	Categories	No.	%
Age	30-20	18	60
	40-31	9	30
	50-41	3	10
Miscarriage times	0	7	23.3
	1	15	50
	2	5	16.7
	4	3	10
Number of Children	0	2	6.67
	1	1	3.33
	2	6	20
	3	8	26.7
	4	8	26.7
	5	3	10
	6	2	6.66
Chronic diseases	None	26	86.7
	Diabetes	2	6.66
	Diabetes and blood pressure	2	6.67
Address	Rural area	6	20
	City	24	80

high percentage in age group range between(20-30) was 18(60)% and lower rate in age group range between (41-50) was 3(10)% . The miscarriage high rate was between 10 and 50%, and the total time of miscarriages was between one and four. These women did not, however, experience infertility because the percentage of their total number of children (3.33-26.67%) ranged from 1 to 6. That the infection rate with these bacteria was likewise 6.67% in two patients with chronic disorders including diabetes and hypertension, and 86.67% in 26 patients without any chronic illness.finally address was recorded a high percentage in city region 24(80)%, and the lower rate in rural area 6(20)%[16].

It was discovered that the infection rate among women in the Baghdad province between the ages of 20 and 30 and between 30 and 40 was 3.13% after research on the presence of *Neisseria gonorrhoeae* in cervical secretions[17],these study agree with accurent. On the other hand, according to a study conducted in 2022 by researchers Ali and Ghaima, women between the ages of 36 and 45 had the greatest infection rate (4%), with *N. gonorrhoeae*. The findings of the current investigation do not support this. The findings indicate that many women have lower abdomen and pelvic pain, vaginal discharges, and inflammatory eyes in their newborns. It is quite likely that these women have gonorrhoea, but improper treatment and inadequate screening during pregnancy could also contribute to the spread of the infection and the birth of contaminated offspring. The kids have the infection as well[18].

The cultural features of *Neisseria Gonorrhoeae*

- Following 24 and 48 hours of incubation at 37 C°, *N. gonorrhoeae* colonies developed on blood agar. These colonies are smooth, moist, and typically have a grey-white hue numerous researchers have confirmed this, and it might be because Brown Neisseria

is the most sensitive type, needing complex growth media to be supplemented with growth-stimulating substances because it is highly susceptible to toxic substances like fatty acids and cannot grow in normal blood vessels[19].

- After being incubated for 24 hours at 37 °C and with 5–10% CO2, *N. gonorrhoeae* colonies developed on the chocolate agar. They were smooth, mucosal, and seemed to be small, greyish-white, with defined margins and non-zigzag edges. Since gonorrhoea bacteria are extremely sensitive to dehydration and require specific growth boosters, the results of the current study were in line with those of previous investigations, which established that chocolate agar is a rich culture medium used to stimulate the growth of these bacteria in general, these bacteria require X-factor (Hemin) and V-factor (represented by NAD) for their growth[20].
- On the Modified Thayer-Martin agar, *N. gonorrhoeae* colonies have shown up as small, white-grey colonies that tend to be colorless, mucous, and have uneven borders. Their diameters range from 0.5 to 1 mm. According to[21], the findings of this study are consistent with numerous other studies conducted worldwide. The Thayer-Martin agar is the central component of the selective culture media that is crucial for isolating the bacteria responsible for sexual gonorrhoea because it contains four different types of antibiotics (polymyxin, vancomycin, trimethoprim, and nystatin) that help inhibit the growth of both Gram-positive and Gram-negative bacteria—aside from Neisseria—as well as fungi[22].

Average of the Varying Interleukins Among the Groups Under Study: Cytokines made by lymphocytes, phagocytes, and epithelial cells play a role in the regulation of host defenses against *N. gonorrhoeae* According to the study's findings, women infected with *N. gonorrhoeae* had higher serum levels of IL-6, IL-8, and IL-10 than the Control group (Figure 1): 47.11±1.69 vs7.09±1.12; p = 0.0001 for interleukin 6 (IL-6), 1615±197.16 vs 311.47±14.63; p = 0.0001 for interleukin 8 (IL-8), and 164.50±17.24 vs 41.71±3.03; p = 0.0001 for interleukin 10 (IL-10).

When *N. gonorrhoeae* is prevalent in mucosal infections, inflammatory cytokines such as IL-6, IL-8, and IL-10 are generated in local secretions and serum. This was confirmed by the results of the current investigation, which showed elevated levels of IL-6, IL-8, and IL-10; as a result, the findings of the present study are entirely similar to those of previous international studies[23]. During the acute stages of the illness, epithelial cells are the primary producers of IL-6 and IL-8, which are by products of the immunological response to *N. gonorrhoeae* and cause vasodilation and prostaglandin synthesis[24].

• **Gene Expression of Opa, Tfpc and Pora Genes:** In this study, the results were analyzed using a quantitative RT-PCR device for expression of the Opa, TfpC, and Pora genes[25]. sRNA16 was used as a stable expression gene for comparison (Housekeeping gene), as the results indicated an increase in gene expression in the three genes compared to the control sample[26]. While differences were observed between genes, the TfpC gene gave the highest gene expression of 441.47 compared to the opa and porA genes, which gave 100.723 and 71.02, respectively as shown in the following tables (Tables 4, 5, and 6)[27].

RESULT

In reference to the results of the current tables and previous studies, a decrease in the value of the Cycle Threshold (CT) was observed, as the value of CT indicates the first cycle in which the gene begins to be expressed. According to the previous tables, the value of CT was low, indicating high gene expression, through which Bacteria were detected after several cycles, with a large amount of bacteria present compared to Control. The CT value serves as a marker to determine the presence of bacteria and the extent of infection in most cases. The CT values depend on factors such as the method of collecting samples, the source, and the time gap in infection. RT-qPCR is distinguished from other methods of gene expression for its accuracy, sensitivity, and rapid results. High CT values give low gene expression and a low CT value indicates high gene expression. The standard sample gene used in molecular studies has constant expression in cells or tissues under different conditions[25].

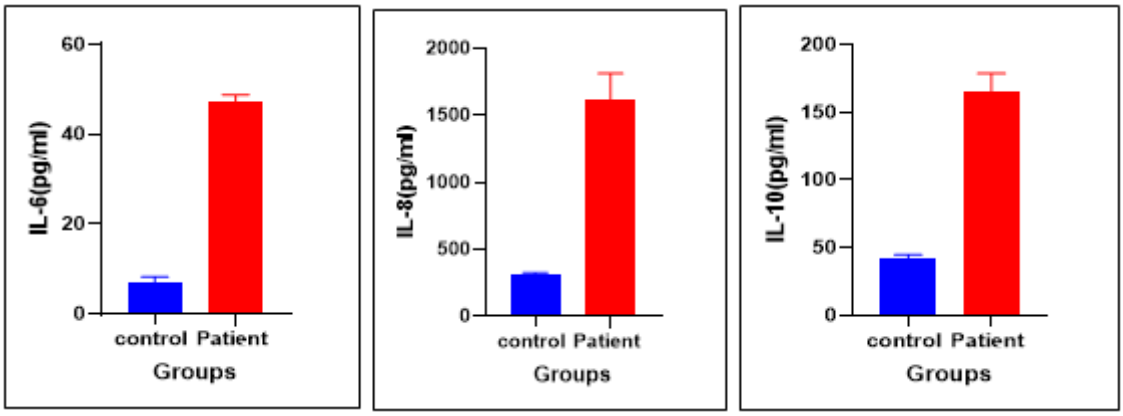

Figure 1: Levels of IL-6, IL-8, and IL-10 in women with *N. gonorrhoeae* infection compared to the control group.

Table 4: Mean of cycle threshold (Ct, ΔCt, ΔΔCT) of Opa gene.

Groups	ΔCT (Mean±SE)	ΔΔCT (Mean±SE)	Fold Change ($2^{-\Delta\Delta CT}$) (Mean±SE)	FP/FC
Control (*Neisseria weaveri*)	12.73±2.61	0.822±0.634	4.32±2.75	1
Patient (*N. gonorrhoeae*)	8.48±2.23	-4.14±1.32	618.687±17.83	100.72
P value	0.0546	0.01	0	0

Table 5: Mean of cycle threshold (Ct, ΔCt, ΔΔCT) of Pora gene.

Groups	ΔCT (Mean±SE)	ΔΔCT (Mean±SE)	Fold Change ($2^{-\Delta\Delta CT}$) (Mean±SE)	FP/FC
Control (*Neisseria weaveri*)	9.83±2.78	0.872±0.43	2.38±1.22	1
Patient (*N. gonorrhoeae*)	4.85±1.88	-4.23±0.788	236.34±19.33	71.02
P value	0.063	0.0223	0	0

Table 6: Mean of cycle threshold (Ct, ΔCt, ΔΔCT) of TfpC gene.

Groups	ΔCT (Mean±SE)	ΔΔCT (Mean±SE)	Fold Change ($2^{-\Delta\Delta CT}$) (Mean±SE)	FP/FC
Control (*Neisseria weaveri*)	13.12±3.63	0.001±0.00	5.65±0.14	1
Patient (*Neisseria gonorrhoeae*)	6.48±2.23	-6.81±1.74	2782±34.73**	441.47
P value	0.0433	0.01	0	0

The results of the current study were consistent with some international studies, as[26] that due to the weak positive predictive value of the Nucleic Acid Amplification Tests (NAATS) for sexual gonorrhea when applied in a low-prevalence environment, PCR technology is used to detect a bacterial gene as a rapid detection in clinical samples. Investigating *N. gonorrhoeae* using selective and enriching culture media is a matter that consumes a lot of time and effort, so many researchers and specialists in most developed countries of the world resort to detecting this bacterium using RT-PCR[27]. Conducted a study at King Edward VIII Hospital in the United Kingdom, which included examining 307 women before giving birth and taking samples from them (cervical swabs). One of the swabs was cultured and the other was used to investigate the opa gene. 16s rRNA showed the highest sensitivity at 62%, followed by opa at 38%, while the positive culture gave an isolation rate of 7.8%[27]. In recent advanced studies, companies have begun producing diagnostic kits to diagnose 16s rRNA and the porA gene together. This is called dual target detection due to its importance in detecting *N. gonorrhoeae* in various samples, even if they are in very small numbers in clinical models such as urine, vaginal and cervical swabs[24]. Therefore, this bacterium can be diagnosed if there is a mixture of other pathogenic bacteria in the sample, such as Lactobacillus and Chlamydia trachomatis[9]. The researchers' results showed that the bacteria isolated from patients in America are phase variable due to the bacteria's expression of the OPa protein, which consists of adhesins and invasins, which have a major role in destroying neutrophil cells. This trait has been utilized in the production[8].

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