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Research Article

Molecular Versusconventional Diagnosis Of Neisseria Gonorrhoeae Infection And Study Its Role In Azf Locus Microdeletions

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ABSTRACT

Eighty two clinical samples were collected from suspected gonorrhoeae-infected patients (urethra swab and blood sample were taken from each patient). During a period from the beginning of December 2012 to the end of April 2013. In Al- Yarmouk teaching hospital (Baghdad, Iraq) and private laboratories, all of the patients were married and have children compared with 20 samples been taken from apparently healthy control. All samples were bacteriologically examined by traditional methods for detection of N. gonorrhea, 82 isolates were identify by microscopic examination,76 isolates on Modified Thayer martin media and 61 isolates by PCR technique targeting Orf1 gene. The results revealed that all samples which give positive results with Orf1 gene yielded negative results for both SY 254 (85pb), BPY-2(90pb) genes. In the present study show no correlation between Y chromosome (AZF locus) microdeletions and N. gonorrhea infection.

INTRODUCTION

According to world health organization (WHO) estimates, gonorrhoeae remain a significant public health problem challenge and remains a major Sexual transmitted diseases (STI) worldwide. In some countries it is as prevalent as Chlamydia trachomatis [1], the global incidence of gonorrhoeae is approximately 60 million case per year, according to CDC estimate. The incidence of gonorrhoeae in the USA about 700,000 cases per year [2]. It is gained tremendous importance in the last few decades because of its role as a cofactor in increasing HIV infections. Microbial infection has been associated with male infertility for many years. Urogenital infection in male is one of the most important causes of male infertility and accounted for about 40 – 41.4% of male infertility cases worldwide. Acute and chronic infections and consequent inflammation in the male reproductive system may compromise the sperm cell function and the whole spermatogenetic process, causing qualitative and quantitative sperm alterations. In respect to male urogenital tract infection, it was found that asymptomatic bacteriospermia had an important role in male infertility through affecting different sites of male reproductive tract, such as the testis, the epididymis and male accessory gland [3]. Male infertility has been associated with a number of non-genetic and genetic factors. The non- genetic factors include hypogonadotrophic hypogonadism, previous inguinal and scrotal surgery, and environmental factors such as genital infections. Role of bacterial infections on male infertility has always been in the area of controversy due to lack of decisive analysis tools to examine seminal fluid specimens as a result of which these infectious processes leads to deterioration of spermatogenesis, impairment of sperm function and/or obstruction of the seminal tract [4]. Until the late 1980s, laboratory diagnosis of gonorrheae was limited to gram stain and bacterial isolation, which reflected gold standard for definitive diagnosis and confirmatory identification. While, in the early 1990s, nucleic acid tests first became available for routine use. They are more sensitive than culture, offer testing on a wider range of specimen types and are less demanding in specimen quality, transportation and storage [5]. The purpose of this study was to determine the best test (molecular versus conventional methods) for diagnosing Neisseriagonorrheae in suspected gonorrheae- infected patients and study the effect of Neisseriagonorrheae on AZF locus microdeletions.

MATERIALS AND METHODS

Patients

Eighty two patients were involved and special form of information was adopted for each patient including name, age, sex, job, marital state, sexual activity. All patients were suffering from urethral discharge with dysuria and 20 sample were taken from healthy control, who seeking in Al-Yarmouk teaching hospital in Baghdad city during a period from the November 2012 to June 2013. These studies include two types of samples taken from patients suffering

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from signs and symptoms of gonorrhe

Urethral swab

- Microscope examination direct examination and gram stain diagnosis.
- Cultured on non-selective media (chocolate agar) incubated at 35 to 37C° in a moist atmosphere enriched with CO2 (3% to 7%) for 24 h.
- Suspected colonies re-cultured on selective media modified Thayer-Martin, containing antimicrobial agents that inhibit the growth of commensally bacteria and fungi. Incubated at 35 to 37C° in a moist atmosphere enriched with CO2 (3% to 7%) for 24 h. [6] [7].
- Biochemical test and characterization was carried out according to [7].

Molecular study

Isolation of genomic DNA from bacterial culture

Genomic DNA was extracted from bacteria culture using Promega Wizard® Genomic DNA purification Kit (USA), according to the manufacturer's instructions. Subsequently the quality of DNA was assessed by agrose gel electrophoresis using 1%agarose gel stained with ethidium bromide. The purity and concentration of DNA was estimated using Nanodrop at 260 and 280nm.The DNA samples showing the OD 260:280 nm value of 1.6 to 1.9, The purity within this range considered as good quality. Primers were used for detection of Orf1 gene for conformation the identification of the Neisseria gonorrhoea, according to Shahcheraghi et al. (2010) [8].

Table 1: The sequence and concentration of forward and reverse primers for detection of Orf1 gen

Primers Type	Primers Sequence	Concentratiopic omoles in	Product size
Orf1 Forward	CAACTATTCCCGATTGCG	91826	260bp
Orf1 Reverse	GTTATACAGCTTCGCCTGAA	87879	260bp

Table 2: Reaction setup for conventional PCR

Component	Quantity (µl)
Master mix	12.5
10X primer forward	3
10X primer reverse	3
Template DNA	5
RNase –free water	1.5
Total reaction volume	25

PCR reaction was conducted in 25µl of reaction mixture containing 12.5 µl of green master mix, 3µl of each primers, 5µl of DNA template and 1.5 µl of RNase –free water (Table 2). PCR program was adopted from [8]. Amplification was conducted using a master cycler eppendorf programmed with 40 cycler for initial denaturation 94°C for 5 min., Denaturation 94°C for 30 sec, Annealing 52°C for 30 sec, Extention72°C for 1min, final Extension 72°C for 10 min as shown in Table (3).

Table 3: PCR program for Orf1 gene amplification by conventional PCR

No.	Steps	Temperature	Time	No. Of cycles
1	Denaturation 1	94°C	5min	1 cycle
2	Denaturation 2	94°C	30sec	40 cycles
3	Annealing	52°C	30sec	
4	Extension 1	72°C	1min	
5	Extension 2	72°C	10min	1 cycle

AZF Microdeletions

PCR was used for detection of AZ Fcmicrodeletions targeting BPY-2 and SY-254 genes (90 and 85 bp, respectively). As shown below, sequence tagged site (STS) primers were used [9].

Table (4). PCR reaction (25µl) contain 12.5 µl of master mix, 1µl of each 10 × primer (forward and reverse), 5µl of DNA template and 5.5 µl of RNase-free water as shown in Table (5). PCR program for BPY-2 and SY-254 genes fragment amplification (90 and 85 bp, respectively) was adopted from [9]. The program include ; denaturation 1, 94°C for 30 sec. (1 cycl), denaturation 2, 94°C for 5 min, annealing 53°C for 45 sec . and extension 1, 72 °C for 1 min (35 cycle) and extension 2, 72°C for 7 min (1 cycle) as shown in Table (6).

Table 4: Primers used for PCR amplification targeting AZFc region

Primers Type	Primers Sequence	Concentrati on in picomoles	Product size
SY 254 Forward AZFc region	GGG TGT TAC CAG AAG GCA AA	148804	85bp
SY 254 Reverse AZFc region	GAA CCG TAT CTA CCAAAG CAGC	51552	85bp

1-SY-254 primers

2-BPY-2 primers

Table 5: Reaction setup for conventional PCR

Primers Type	Primers Sequence	Concentrati n in picomoles	Produ size
BPY-2 Forwa of AZFc region	ATGATGACGCTTGTCCCCAGAGCC	94399	90bp
BPY-2 Rever 0f AZFc region	CTTCTGTGATCTGGGCTTCGACAC	85126	90bp

Table 6: PCR program

Primers Type	Primers Sequence	Concentrati n in picomoles	Produ size
BPY-2 Forwa of AZFc region	ATGATGACGCTTGTCCCCAGAGCC	94399	90bp
BPY-2 Rever 0f AZFc region	CTTCTGTGATCTGGGCTTCGACAC	85126	90bp

Gel Electrophoresis

PCR products and the ladder marker were resolved by electrophoresis on 2% w/v agarose gels. DNA samples were loaded in the tray of gels and 100 bp marker was included in every gel and run in TBE(1X) buffer, gels were stained with ethidium bromide (0.5 -1µg/ml) and analyzed using UV eliminator The molecular weight identification of resolved band was based on their correspondence to the ladder bands.

RESULTS AND DISCUSSION

Traditional Methods

Microscopic Examination (Gram Stain)

All samples from symptomatic males were examined for detection of N. gonorrhea by gram stain, which appear as diplococcic, kidney-shaped in polymorph nuclear leukocytes, the presence of which is required for the presumptive diagnosis of gonorrhea by culture or nucleic acid test [10].

Culturing

Culturing Depending on morphology, round, gray, raised and shiny like dew drop colonies on modified Thayer Martine media [11] [12]. The results indicate 76 positive samples from 82 samples (Figure 1). It is a method of choice in the identification of N. gonorrhoeae, particularly in a developing country. In addition, isolation of gonococci by culture method is very important in testing the susceptibility pattern that is another key factor in the successful treatment and control of the disease. Other researchers revealed that culturing was not reliable as a sole diagnostic test for N. gonorrhoeae, because of a significant number of false negative results.

Biochemical Tests

Figure 1: The shape of N,ghonorea colonies isolated from urethral swab on modified

Thayer Martine media

The outcome of biochemical tests clarified that seventy six isolates of Neisseria spp. fermented glucose not lactose, maltose and sucrose appeared as yellow surface and bottom Figure (2). DNA was extracted from all strains of N. gonorrhea and genomic DNA was subjected to electrophoresis, no plasmid was seen (Figure 3). Seventy six positive samples which diagnosed by traditional method were analyzed for Conventional PCR for detection of Orf1 gene (260bp), 61 samples gave positive results for Orf1 gene with conventional PCR (Figure 4). Orf1 gene primers were used for the detection of N. gonorrhea in many researches; these primers were demonstrated to be highly sensitive and specific biomarker for detection of N. gonorrhea. It is one of the specific targets to confirm infection, and positive amplification of N. gonorrhea specific DNA may be considered as a direct evidence of the presence of the pathogen (13) (8).

Figure 2: Results of biochemical from urethral swab.

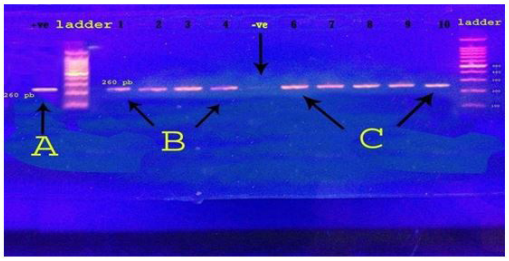

Figure 3: Detection of N. gonorrhea gene (260bp).The amplified fragments were separated by electrophoresis on a 1.75% agarose gel, stained with ethidium bromide at 70 volts/cm for 1 hour, photographed under UV light LaneC:+control, Lane DNA ladder, Lanes:1,2,3,4,positive for Orf1 (260pb), lane :5 negative, lane:7,8, 9,10 positive for Orf1gene (260 pb)

Clear correlation was appeared between PCR techniques and culturing method and biochemical tests, when positive samples with it give appositive amplification results with conventional PCR. In comparing the results with other researchers, it can be conclude that there is similarity with the results of [14] who recorded that the PCR method is sensitive more than culture method in diagnosis of N. gonorrhea. This study was done to detect the possible association between the N. gonorrhea infection and the incidence of microdeletions in Y chromosome (AZF locus). Up to our knowledge either there is a rare or no previous studies in Iraq were conducted to detect the relationship between infertility due to chromosome microdeletion and N. gonorrhea infection in men. DNA was extracted from all blood samples by using one simple protocol, a high yield of purified DNA can be isolated. The DNA quality and integrity were estimated through remarking the DNA bands by electrophoresis on agarose 0.7 % for 20 min. The band appear sharp single not diffused and not have any smear which may result from DNA degradation as shown in Figure (5). The results revealed that all samples which give positive results with Orf1 gene (61 out of 82samples) yielded negative results for both SY- 254 (85pb), BPY-2(90pb) genes as shown in (Figure 6).

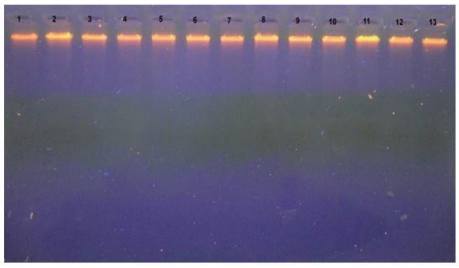

Figure 4: DNA Bands on 0.7% agarosegel at 100 Volts for 20 min. Lane 1-13 Genomic DNA isolated from N. gonorrhea patients

Figure 5: Detection of SY-254 gene(85pb).The amplified fragments were separated by electrophoresis on a 1.75% agarose gel, stained with ethidium bromide at 70 volts/cm for 1 hour. photographed under UV light Lane C:+control, Lane DNA ladder, Lanes: 1,2,3,4,5 positive, lanes:7,8 ,9,10 positive

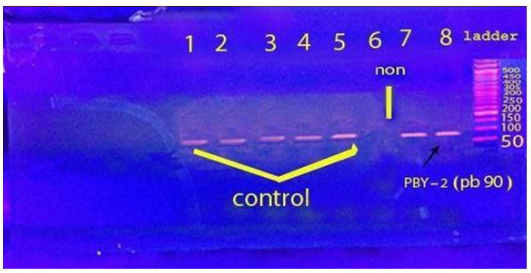

Figure 6: Detection of BPY-2(90pb).The amplified fragments were separated by electrophoresis on a 1.75% agarose gel, stained with ethidium bromide at 70 volts/cm for 1 hour. photographed under UV light Lane C:+control, Lane DNA ladder,Lanes:1,2,4,5,6,7,8 positive.

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