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RESEARCH ARTICLE

MOLECULAR *VERSUS*CONVENTIONAL 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 were 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.

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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 (Newmon, 2007; WHO, 2010), 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 case per year (Kenneth, 2008). 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 (Hassan *et al.,* 2011). 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 (Osegua *et al*., 2012). 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 (Al-Janabi *et al*., 2014). 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. (Cook *et al.,* 2005; Ison, 2006). 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.

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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. This study include two types of samples taken from patients suffering from signs and symptoms of gonorrheae.

Urethral swab

1. Microscope examination direct examination and gram stain diagnosis.
2. Cultured on non-selective media (chocolate agar) incubated at 35 to 37C° in a moist atmosphere enriched with CO_2 (3% to 7%) for 24 h.
3. 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 CO_2 (3% to 7%) for 24 h. (Janda and Knapp, 2003; Washington *et al.*, 2006).
4. Biochemical test and characterization was carried out according to Washington *et al.* (2006).

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), (Table1).

Table 1. The sequence and concentration of forward and reverse primers for Detection of *Orf1* gene

Primers	Sequence	Concentration (Pico mole)	Product size(bp)
Forward	CAACTATTCCCGATTG CG	91826	260
Reverse	GTTATACAGCTTCGCC TGAA	87879	

Table 2. The mixture of conventional PCR working solution for detection of *Orf1* gene

Components	Reaction volume (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 Shahcheraghi *et al.* (2010). 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	Temper ature	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 (Mahanta *et al*., 2011). 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 Mahanta *et al.* (2011). 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

Primer	Sequence	Concentration (picomole)	Product size (bp)
SY-254 Forward	GGGTGTTACCAGAAGGCA AA	14880	85
SY-254 Reverse	GAACCGTATCTACCAAAGCAGC	51552	
BPY-2 Forward	ATGATGACGCTTGTCCCCAGAGCC	94399	90
BPY-2 Reverse	CTTCTGTGATCTGGGCTTCGACAC	85126	

Table 5. The mixture of conventional PCR working solution for detection of microdeletions in AZF locus

Components	Reaction volume (μl)
Master mix	12.5
10X primer forwa	1
10X primer revers	1
Template DNA	5
RNase –free wate	5.5
Total reaction volu	25

Table 6. PCR Program for amplification fragment (90bp and 85bp)

No.	Steps	Temperature	Time	No. of cycles
1	Denaturation 1	94C°	30sec	1 cycle
2	Denaturation 2	94C°	5min	35 cycles
3	Annealing	53C°	45sec	
4	Extension 1	72C°	1min	
5	Extension 2	72C°	7min	1 cycle

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 (Ng and Martin 2005).

Culturing

Depending on morphology, round, gray, raised and shiny like dew drop colonies on modified Thayer Martine media (Jawitis 2001; Al-saedi 2013). 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.

Figure 1. The shape of *N.ghonorea* colonies isolated from urethral swab on modified Thayer Martine media

Biochemical tests

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 (Farraj *et al*., 2010; Shahcheraghi *et al*., 2010).

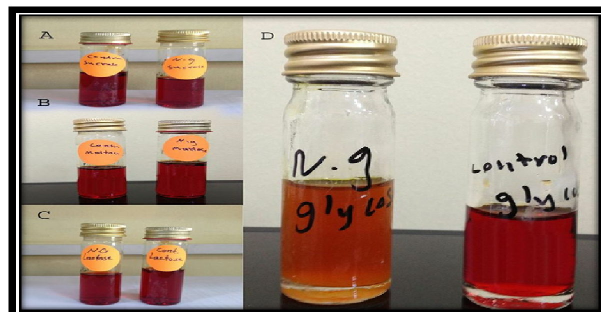

Figure 2. Results of biochemical fermentation sugars for diagnosis of *N. gonorrhea* from urethral swab

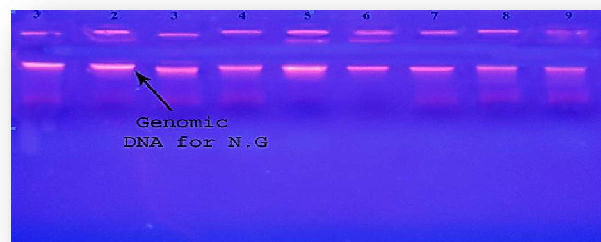

Figure 3. Chromosomal DNA extracted from *N. gonorrhea* isolates

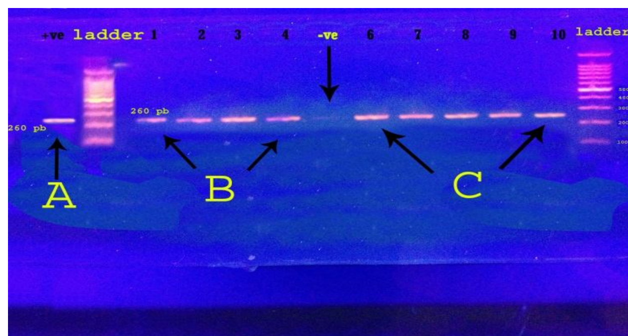

Figure 4. Detection of *N. gonorrhea* gene (260bp).The amplified fragments were separated by electrophoresis on a 1.75% agarose gel, stained with ethidium bromide at 70 volts/cm for 1 hour. photographed under UV light LaneC:+control, Lane DNA ladder, Lanes:1,2,3,4,positive for *Orf1* (260pb), lane :5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

Clear correlation was appeared between PCR technique 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 Alam *et al*. (2002) 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) and Figure (7), respectively.

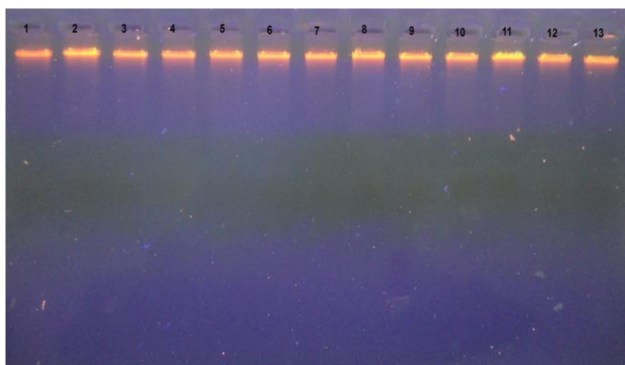

Figure 5. DNA Bands on 0.7% agarosegel at 100 Volts for 20 min. Lane 1-13 Genomic DNA isolated from *N. gonorrhea* patients

Figure 6. 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

These results indicate that the infection with *N. gonorrhea* did not induce microdeletions of *SY 254* and *BPY-2* gene, instead, it may affect the semen quality characteristics which not studied herein. Interestingly, in the present study, no correlation was found between the microdilation Y chromosome in male and *N. gonorrhea* infection. When return back to results of PCR, the members of *N. gonorrhea*, it is worthy to mentioned that all isolates included in this study were taken from male having children. In hypothesis came from finding a correlation between the infection with *N.*

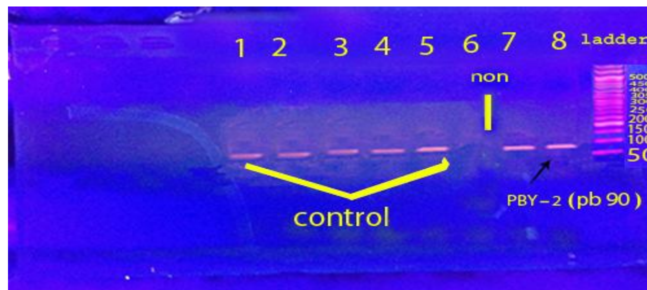

Figure 7. Detection of *BPY-2*(90pb).The amplified fragments were separated by electrophoresis on a 1.75% agarose gel, stained with ethidium bromide at 70 volts/cm for 1 hour. photographed under UV light Lane C:+control, Lane DNA ladder,Lanes:1,2,4,5,6,7,8 positive

gonorrhea and male infertility. From foregoing information we can say that molecular methods can characterized the principle genetic elements in diagnosis of bacteria and fined this correlation since most cases of infertility depend on quality of seminal fluid.

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