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Detection of sexually transmitted disease STDs by using multiplex real-time PCR of SNRPN gene promoter with abnormal semen

Noor H M AL-Hamid, Mays Hussain Albaaj
Assistant Lecturer, Department of Biotechnology, College of Science, University of Baghdad, Iraq

Abstract
Seminal fluid was collected from all males attending a clinical laboratory for routine semen analysis, seminal fluid analysis was performed according to World Health Organization guidelines, all of the 63 infertile males were suffering from reduced sperm concentration (9.42 ± 8.70 million/ml), reduced progressive motility (2.89 ± 5.45%) and abnormal sperm morphology (27.06 ± 16.50%), while control group has higher values thus, there was a highly significant difference in semen parameters between the two groups.
Whole genomic DNA was extracted from all semen samples, DNA concentration and purity were assessed using gel electrophoresis and Nanodrop techniques, DNA integrity was checked using the β-globin gene as an internal control, the targeted region was amplified by polymerase chain reaction (PCR) technique followed by the detection of PCR product (158bp). A successful amplification been has approved when the molecular size of bands linked with the targeted regions.
The number of patients involved in the detection of STDs was (63), STDs detection using a specific kit by multiplex real-time PCR was included the detection of (*Chlamydiatrachomatis/ Neisseriagonorrhoeae/ Mycoplasmagenitalium and Trichomonasvaginalis*), and the number of patients positive for one or two bacteria was 21 of 63 (33.3%). The percentage of *C.trachomatis* infection was (17.4%), *N.gonorrhoeae* was (9.5%), *M.genitalium* was (9.5%) and *T.vaginalis* was (4.7%). There were (5) patients who showed mixed infections (7.9%). The Control group included (13) patients; their results showed only one infection with *C.trachomatis*. The results revealed an association between DNA methylation at *SNRPN* gene promoter, the presence of STDs infections, and reduced semen parameters that could lead to male infertility.

Keywords: sexually transmitted infections, SNRPN genes, Multiplex PCR, abnormal semen's

Introduction

Male Infertility is defined as the inability of a male to achieve pregnancy in a fertile female after 12 months of unprotected intercourse; it is considered a highly prevalent condition around the globe Approximately 15% of couples were suffering from this problem, male factor infertility is responsible for 50% of cases [2-3] Infertility is considered as a multifactorial condition, due to the widespread male infertility or subfertility which is known as the reduced fertility, and in major cases the main causes remain idiopathic, it is crucial to investigate factors affecting male fertility other than sexual disorders, endocrinopathies, obesity, drugs and ejaculatory dysfunction [11]. The analysis of semen reveals a decreased number of spermatozoa (oligozoospermia), decreased motility (Asthenozoospermia) and many abnormal forms on morphological examination (teratozoospermia). Usually, these abnormalities come together and are described asthe oligo-astheno-teratozoospermia syndrome (OAT) [6].
Male infertility is a complex, multi-factorial disorder and the underlying causes often remain unknown, therefore, it is crucial to investigate the probable causes and molecular mechanisms that lead to male idiopathic infertility such as gene mutations, proteome studies of spermatozoa from idiopathic infertile men, the role of epigenetics, post-translational modifications and sperm DNA fragmentation in infertile men [5-13]
The small nuclear ribonucleoprotein polypeptide N (*SNRPN*) gene is an imprinted gene, which is normally methylated at the maternal allele and expressed in a monoallelic way through the paternal allele, abnormal imprinting of this gene due to the methylation may lead to abnormal function or silencing of the gene, hypermethylation of that gene at the promoter region have been observed to be linked with specific sperm abnormalities [4-7].
On the other hand infections of the male reproductive tract have been documented for a long time as a vitaltal factor that interferes with sperm's normal function, transpo,rt and productivity [1]. Different types of microorganisms have been associated with male infertility and the degree of association depends on the type of the infection caused.
A specific mechanism used by each microorganism to affect infertility, either directly by reducing sperms motility, or indirectly by causing an obstruction in the seminal tract *Chlamydia trachomatis,Neisseria gonorrhoeae, Mycoplasma genitalium and Trichomonas vaginalis*, have been detected in the semen of asymptomatic infertile males, these pathogens cause acute and chronic infections, the resultant inflammations in

the male reproductive system, cause a damage in the function of spermatozoa that lead to a reduced sperm quality, decreased sperm count and motility [10]

Materials and Methods

The samples required in this study were seminal fluid from males suffering low fertility and difficulty to conceive, their age ranged from (20-52) years, attending clinical laboratories for routine seminal fluid analysis over a period of two months, November 2017 to January 2018.All the work was done in Baghdad university/Biotechnology department and Genome clinical laboratory (Baghdad/Al-kindi Street) for a period of 4 months.

The patient group consisting of males have no children and a problem in conceiving with a poor seminal fluid quality, the value of sperm count, motility and morphology were below the lower reference limits determined by WHO standard manual of 2010, their age ranged from (20-52) years old. By contrast, males having one child at least and a normal seminal fluid analysis were considered as a control group and their age ranged from (21-45) years old.

2.1 Human Beta-globin gene detection by PCR

Human beta-globin gene detection used in this study as positive internal control to assessand assure the presence and quality of human DNA. Primers for beta-globin gene were designed by (9) using in silico PCR amplification and were downloaded from the website, the primers provided in a lyophilized state by Alpha-DNA Company (Canada). The sequence of forward and reverse primers are F: 5`.. AGTCAGGGCAGA GCCATCTA..3`, R: 5`..CCTCACCACCAACTTCATCC..3` .The amplification reaction were PCR mix 10 µl , primers forward and reverse 0.5µl , template DNA 4µl and nuclease free water 10µl to have total reaction volume 25µl. The PCR amplification were as Initial denaturation at 95 c° for 15 min , Denaturation at 95 c° for 15 sec for 40 cycle , annealing at 58c° for 25 sec , extension at 72 c° for 20 sec and final extension at 72 c° for 5 min [12]

Analysis of PCR product

Agarose gel electrophoresis was used to analyze the product of PCR, agarose gel electrophoresis preparation procedure was followed as mentioned previously. Agarose gel concentration was 2% and DNA ladder of (100bp) used as molecular marker, an amount of 10µl of PCR product and 5µl the ladder were loaded carefully in to the gel wells and run at 90 volt for 1.5 hour followed by staining with EtBr stain for 20 minutes and visualized under UV light by gel documentation system.

Multiplex Real Time PCR for qualitative Detection of Sexually Transmitted Diseases (STDs)

Four types of microorganisms are selected in this study to be detected in semen samples using specific PCR diagnostic kits for sexually transmitted diseases that allow reliable detection of DNA infects from mucosal, epithelial swabs, secretions, urine, semen and synovial fluid.

The AmpliSens® *Neisseria gonorrhoeae/ Chlamydia trachomatis/ Mycoplasma genitalium/ Trichomonas vaginalis* MULTIPRIME-FRT PCR kit contains independent Internal control for checking the efficiency of DNA extraction as well as amplification process. Multiplex STD PCR kits significantly reduce the cost per one analysis and provide a comprehensive picture of possible casual agents. Multiplex STD kits use up to 5 standard dyes (FAM/Green, JOE/Yellow/HEX, ROX/Orange, Cy5/Red and Cy5.5/Crimson/Quasar704) depending on the number of the detected pathogens. So, various Real-Time PCR cyclers can be used. The presence of each bacteria is indicated by sending a signal to different channels that detect a fluorescent dyes specific for each bacteria as shown in Table [1].

Table 1: STDs detection dyes.

Fluorophore	Targets
FAM	Neisseria gonorrhoeae
JOE	Chlamydia trachomatis
ROX	Mycoplasma genitalium
Cy5	IC*
Cy5.5	Trichomonas vaginalis

*Internal control

The results analysis was performed by the software of real time PCR instrument which measures the fluorescent signals accumulated in the channels. It revealed that there were five Quantitation data for cycling channels as showed in Figures (3-5, 3-6, 3-7, 3-8, 3-9), the amplification of each bacterial DNA was recorded as a Ct value.

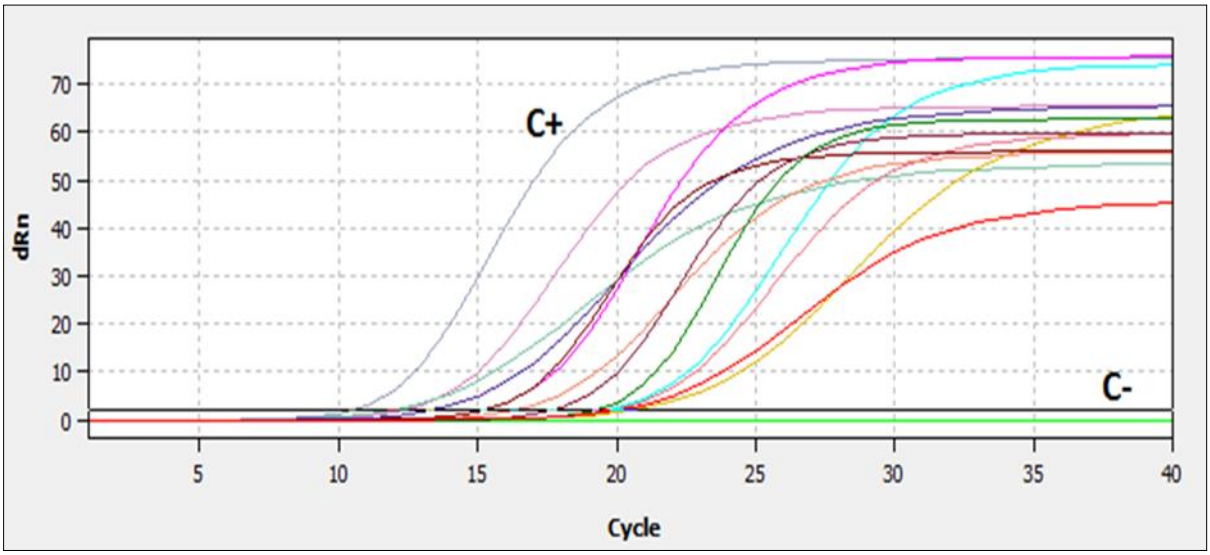

Fig 1: Amplification curves in linear view obtained from target DNA. The channel for Cy5 fluorophore (*IC*). C+ curve: Positive control/C-: Negative control

Result and Discussion

The results of this study indicated that the number of positive and negative results were (21/63) and (42/63) respectively. In general the percentage of *C. trachomatis* infection was (17.4%), *N. gonorrhoeae* was (9.5%), *M. genitalium* was (9.5%) and *T. vaginalis* was 4.7%.

Real-time PCR is a highly sensitive target amplification technique available for STDs detection. Real-time PCR combines fluorescent probes with PCR primers, allowing for accurate quantification of microorganism present in a sample. A commercially available several kits based on real-time PCR that used in STDs detections [29].

The evidence confirms a link between sexually transmitted diseases (STDs) and the burgeoning infertility problem. STDs can adversely affect fertility by three primary mechanisms: [1] pregnancy wastage, [2] neonatal deaths, and (3) obstruction of either male or female reproductive ducts. STDs control programs have been effective in preventing sequelae of disease, including infertility. The first step in developing such a program is to identify the magnitude and distribution of the problem, and to identify the specific agents most commonly involved. Subsequently, a STDs control program emphasizing the prevention of infertility, can be incorporated into the existing health care system whenever STDs are important [21].

There is an argument about the effect of STDs on male infertility, due to the limited information's about it, and the diagnostic tests such as seminal fluid analysis and culture techniques are variable. There must been many steps to delineate the role of STDs in male infertility, include the prospective investigations of infertile couples by using the proper control groups, advanced semen collection and analysis protocols, and precise microbiologic techniques. It is mandatory for health care professionals to spread the awareness of the dangerous effects of STDs and the importance of using contraceptive methods to prevent STDs spread [25].

***Chlamydia trachomatis* and male with abnormal semen**

Chlamydia trachomatis was the most prevalence bacteria among the study groups. (11) Of (63) 17.4% of the infertile males was infected while only (1) of (13) fertile control group was infected. In general the values of seminal fluid parameters (sperm count, PR motility and morphology) of the infected males was reduced. There was no significant difference between infected and un-infected infertile males but the difference was significant between infected infertile and healthy fertile males [8].

A study by [18], suggested an association between *C. trachomatis* infection in men and idiopathic male infertility and revealed that infection was a symptomatic. However, according to the results of this study chlamydial infections appeared to influence semen quality negatively and this statement could be approved by comparing semen parameters of infected samples and control samples. There was a significant difference in sperm count, progressive motility and morphology. The results of the current study came in agreement with a study performed on infertile men in Kuwait [9]. This study indicated the negative effect of chlamydial infection on semen quality. The study also approved that *C. trachomatis* is less frequent among infertile and fertile males in Kuwait, while [28] conclude that *C. trachomatis* was the most widespread sexually transmitted pathogen among males in Tunisia. That difference in the results may be due to the use of different detection techniques of the bacteria, and many studies suggested that chlamydial infection was not associated with reduced sperm parameters (14& 26). Other studies, however, revealed that chlamydial infection correlates with altered and reduced semen parameters such as sperm motility, morphology, Vitality (20 &16). Besides, a study by [23] found that infection by *C. trachomatis* have an impact on sperm concentration, percentage of sperm motility and morphology in patients with prostatitis.

The C. trachomatis was found to be an important factor in sperm pathology, and these results could help to clarify the role of Chlamydia trachomatis in male infertility. There was no significant difference between fertile

and infertile couples in terms of the prevalence of *C. trachomatis* in many other studies. This may be due to the prevalence of other bacteria like *Ureaplama* species in the studied populations [19].

***Neisseria gonorrhoeae* and males with abnormal semen**

The results of this study revealed that the number of positive and negative patients for *N. gonorrhoeae* was 6/63 and 57/63 respectively, while control group showed no infection (0/13). The study showed variation in the results of PCR (depending on *N. gonorrhoeae* detection). There were significant differences for each sperm count, motility, PR motility and morphology. [11]. A study suggested that *N. gonorrhoeae* and *C. trachomatis* are the most widespread sexually transmitted bacteria in the world, Knapp *et al*., (1994) defined *N. gonorrhoeae* as the bacteria that is transmitted sexually and is characterized by asymptomatic or symptomatic infections. In a Jordanian study by (3) involved 93 infertile male and 70 fertile control, DNA of *N. gonorrhoeae* was detected in the semen samples of (6.5%) of the infertile males and none of the controls samples was infected. An association can be noticed between STDs and idiopathic male infertility due to different mechanisms for different pathogens, and some STDs may affect male fertility negatively [15].

***Mycoplasma genitalium* and male with abnormal semen**

The results of this study indicated that the number of positive and negative results were (6/63) and (0/13) respectively. The study showed variation in the results of PCR (depending on *M. genitalium* detection. There were significant differences for each sperm count, motility, PR motility and morphology. There was no significant differences for age. According to (7), the frequency of *M. genitalium* was significantly higher in the infertile men compared with the fertile ones (9.7% vs. 1.2%; $p = 0.001$). Mean cycle threshold (Ct) value was lower in infected infertile than infected fertile men ($p < 0.001$). All semen parameters, except volume, pH, and viscosity, were improved ($p < 0.05$), most of which reached their normal range; leukocytes in seminal fluid decreased ($p = 0.02$), Wives of seven infected infertile men (43.8%) became pregnant 4 months after the treatment completion.

Infections with genital mycoplasmas (*M. hominis* and *M. genitalium*) have been recognized for about a decade as a common sexually transmitted disease (STD) in developed countries (31). Genital mycoplasmas are natural inhabitants of the male urethra, contaminating the semen during ejaculation. However, these microorganisms are potential pathogenic species that play etiologic roles in both genital infections and male infertility (12, 30). *M. genitalium* has seldom been investigated in the semen of infertile men. A study carried out by [17] demonstrated a negative correlation between sperm concentration and the detection of *M. genitalium* in semen samples of infertile men. Limited studies have actually looked at the prevalence of *Chlamydia* and *Mycoplasma*, and examined their role in semen quality in infertile men compared with fertile men [9].

***Trichomonas vaginalis* and male with abnormal semen**

The results of this study indicated that the number of positive and negative results were (3/63) and (60/63) respectively, and for control group (0/13). The study showed variation in the results of PCR depending on *T. vaginalis* detection, there were significant differences for each sperm count, motility, PR motility and morphology. There were no significant differences for age. *Trichomonas vaginalis* infection is a sexually transmitted disease that affects human fertility. In men, trichomoniasis has been related to infertility by deficit of sperm cell quality and function due to physical damage [22]. In other study, *T. vaginalis* was diagnosed in 28.8% of males with urethral discharge and in 8.2% suffering from impotence and infertility. Diagnosis was based on the examination of urethral discharge, urine, semen and prostatic fluid by wet mount, stained films and culture inoculation [1]. Male genital tract infections has been the main controversy in the aspect of male infertility since about 15% of it is due to genital tract infection. *C. trachomatis, N. gonorrhoeae, M. genitalium and T. vaginalis* are the most common genital tract pathogens and are widely studied recently. These infections are hard to be identified because of their infection which are asymptomatic sometimes, and because of culture difficulty and the possible contamination with other pathogens [27].

Conclusion

The relationship between *SNRPN* gene promoter methylation and STDs infections Real time polymerase chain reaction was used in the current study to detect the presence of some STDs in semen samples, also to detect the methylation pattern of *SNRPN* gene promoter. There was a noticeable relationship between STDs infection and *SNRPN* gene methylation in semen samples of the infertile males .The number of methylated samples was 19 sample, 13/19 was infected with one or two of the detected bacteria while only 6/19 was uninfected [24].

Source of funding

Nil

Conflict of interest

Nil

Ethical Clearance

Nil

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