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Study the Incidence of *Herpes Simplex Virus 2* and certain of their Immunological Aspects in Genitourinary Tract Infection among Men in Al-Najaf City /Iraq

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ABSTRACT

This study was planned to evaluate the prevalence and immunological aspects of Herpse simplex virus2 (HSV2) in the semen of men with genitourinary tract infection compared with control group in Al-Najaf City /Iraq. The attempts to investigate the sexually transmitted diseases (STDs) among individuals inhabit in highly restricted religions society could consider a difficult mission, therefore, this paper could provided a novel information about the status of that subject. Primarily, 115 semen samples were collected from cases of genitourinary tract infections, their ages ranged from 20-65 years old during the period from May to October 2015.Fifty samples were also obtained from a comparable 50 healthy persons, all investigated by quantitative real-time polymerase chain reaction (qPCR). RT-PCR directed to certify HSV2 infections. Age groups it was comprised 20:40(50%) with significant statistical differences ($p<0.05$). The investigation of certain immunological parameters as Interleukine-2 (IL-2), Tumour necrosis factor (TNF-α) and secretory Immunoglobulin (sIgA) revealed elevation during the HSV2 infection but without significant statistical differences ($p> 0.05$). We concluded that HSV2 is positively correlated and the patient age may play an important role in increasing the frequency of genitourinary tract infection. RT(PCR)is complementary to cell culture technique in diagnosis of HSV, and it`s preferred to use specific primers of viral virulence factors and different cell lines for monitoring viral pathogenicity and therapeutic approaches is recommend to study .

Keywords: Herpes simlexvirus2, immunological parameters, real time PCR, Semen.

INTRODUCTION

Human herpes viruses cause different infectious diseases, resulting in world-wide health problems. Sexual transmission is a major route for the spread of both herpes simplex virus-1 (HSV-1 and 2). Semen plays an important role in carrying the viral particles that invade the vaginal or rectal mucosa and, thereby, initiate viral replication and increased by about 50- to 100-fold [1]. Human pathogens that can cause infertility may also affect sperm count and quality. Viral infections can be considered as direct and/or indirect cause of male factor infertility[2]. The findings of Monavari *et al*., (2013) suggest that asymptomatic seminal infection of HSV plays an important role in male infertility by adversely affecting sperm count [3]. HSV infects a large population (over 500 million worldwide) and is associated with a variety of diseases, including genital herpes, most of which cases are caused mostly by HSV-2 and partly by HSV-1 [4]. Although the clinical symptoms of HSV-caused diseases can be controlled with antiviral drugs (acyclovir and valacyclovir), these drugs are not strong enough to stop subclinical transmission [5,6]. No prophylactic or therapeutic vaccine against HSV is available. Topical microbicides that

prevent the sexual transmission of viruses could significantly reduce sexually-transmitted diseases. By better understanding the mechanisms employed by HSV in sexual transmission, researchers will be able to design more effective preventive and/or therapeutic drugs against the infection [1]. Our study was planned for the detection of HSV-2 prevalence using real-time PCR and for assessment of immunological parameters in semen specimens.

MATERIALS AND METHODS

2.1 Collection and processing of semen Specimens

The semen samples were collected under consultation of urologist from men who were attended the Al-Sader Medical City/Fertility Centre Laboratory/Al Najaf city for semen analysis either to check their semen abnormality or to check up why they do not have baby for long period of time (> 2 year). Semen analysis was performed according to the WHO,(1999), each semen sample was centrifuged at 2500rpm for 10 minutes. The supernatant was then separated into two sterilized test tube applied to antibiotic pretreatment and divided on two aliquots, One of them is transported to be frozen at -70°C to be used subsequently in virus isolation[7]. Nucleic acid was extracted from the second vial, the residual amount of second vial and the extracted material has been stored at -70 to be ready for amplification of HSV-2 DNA by PCR.

2.2 Viral DNA extraction

As mentioned by Al-Kelaby, 2014, DNA extraction from semen specimens was done by the using of Bosphore® viral DNA extraction spin kit (Bosphore®, Anatolia geneworks, Turkey). This kit is highly compatible with the kit of Bosphore® HSV 1-2 Genotyping Kit v1(Anatolia geneworks, Turkey), which is based on the Real-Time PCR method [8].

The DNA extraction is based on the silica membrane column separation method, involve 4 main steps; lysis, binding, wash and elution, starting with a sample volume of 200µl and DNA recovery (elution) volume was 60 µl.

2.3 DNA purity assessment

The purity of the extracted total DNA were determined by measuring the absorbance ratio at wavelength 260 nm over 280 nm using scandrop spectrophotometer(Analyticajena-Germany). The estimation of DNA purity was done [9]. DNA sample was diluted with TE buffer to 1:10 and the optical density was read with spectrophotometer at wavelength 260nm. For measuring the purity of DNA, reading was taken at wavelength 280 nm . The purity of DNA would be: Pure DNA: A260/A280= 1.8-2.0

2.4 Polymerase chain reaction test (Real-Time PCR method)

This test was achieved by the using of Bosphore® HSV 1-2 Genotyping Kit v1(Anatolia geneworks,Turkey). The genotyping Kit PCR reagents were be stored at -20°C. Repeated thawing and freezing (>3x) had been avoided since it may reduce sensitivity as referred in instructions leaflet of kit. The components were frozen in aliquots. While preparing the PCR; the components had been avoided to exposure for room temperature for more than 10 min. and the detection mix components hadn`t be exposed to light.

Bosphore® HSV 1-2 Genotyping Kit v1 is based on the Real-Time PCR method. Polymerase chain reaction is a technique that is used for amplification of a DNA region. The reaction occurs by the repeating cycles of heating and cooling. The main components of PCR are primers, dNTPs, Taq polymerase enzyme, buffer solution and template.

2.5 Preparing the PCR

All positive controls were added into the PCR reaction together with the samples and the negative control (PCR-grade water). the component below was used for preparing the PCR. It is for only one reaction, these reagent values were multiplied with the sample number to find the values required for the master mix. About 15 µl were Pipetted from the master mix into the PCR tubes, and 10µl of DNA (sample/positive or negative control) were added. the tube then Close capped. finally, to make sure that the solution in each tube was at the bottom of the tube. Centrifugation was done at 8000rpm for about 30 sec. PCR mixture (The total volume 25 µl)was include 12.5 µl PCR Mix, 1.64 µl Detection Mix,10.86 µl dH2O and 10 µl from Sample DNA of negative and positive control.

2.6 Programming the Smart cycler Real-Time PCR Instrument

The thermal protocol for Bosphore® HSV 2 Genotyping Kit v1 is composed of an initial denaturation for activation the Taq DNA Polymerase, a two-step amplification cycle and a terminal hold. The real-time data was collected at the second step of the amplification cycle.

Initial denaturation 95°C 14:30 min. Denaturation 97°C 00:30 min. Annealing and Synthesis 60°C 01:30 min. Hold 22°C 05:00 min. Smart cycler Real-Time PCR Instrument was installed. In order to establish an appropriate link

between the system components, first the thermal cycler and the optical module, and then the PC and the software were be started subsequently. The analytic sensitivity is 1250 copies/ml for HSV2. A region within the Glycoprotein D gene within the HSV2 genome is amplified and fluorescence detection is accomplished using the Cy5 filter.

Before starting a Real-Time PCR reaction using the Bosphore® Kits, the following steps were completed: **a-** Choosing the filter pairs to be used (CY5), **b-** Identification unknown samples, positive and negative controls, **c-** the correct thermal protocol was selected. **d-** The experiment starts by clicking the "Start". By the end of the thermal protocol, the Smart cycler Real-Time PCR Instrument software automatically calculates the baseline cycles and the threshold. CT curve was plotted, with the axes Ct Threshold Cycle and qualitative result report.

2.7 Detection of certain immunological parameters

There are different immunological parameters including IL-2, TNF-α Boster (USA) and sIgA Demeditec(Germany) were enrolled in current study, the principles of assays and their procedures were done according to manual protocol of manufacturer companies (Bostor and Demeditec). Calculation and their interpretations were based on specific standard curve for each parameter.

2.8 Statistical Analysis

Statistical analysis using SPSS 18.0 for Windows was conducted. Inc. Data expressed as mean ± SD, chi-square statistic test. Comparison between virally infected and control samples were performed with independent *t*-test and Mann-Whitney test.In all tests, P <0.05 was considered statistically significant according to the Daniel,1999[10].

RESULTS

3.1 Genitourinary and HSV2 positivity

This study showed that from the total of Genitourinary tract infection patients investigated, HSV2 positive cases was correlated with 40 cases (34.78%), and 10 cases (20%) from the total control group, The *p*-value is .057583. This result is *not* significant at $p < .05$ (Table 1).

Real-time PCR was done and nucleotides segment of the gD region was amplified by the use of thermal protocol for Bosphore® HSV 2 Genotyping Kit v1 as shown in figure 1. Florescence data (CY5 filter) collection during 60^{o}C extension for HSV2 virus, their curves higher than threshold line were positive results and the negative result the curves under than threshold line in quantitative PCR (qPCR) for HSV2 detection.

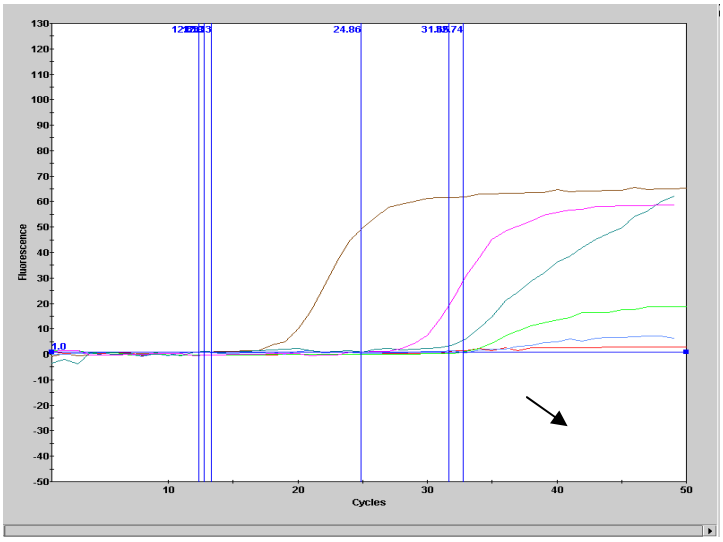

Figure- 1:Polymerase chain reaction for HSV-2 gdD replication: flurescence data (CY5) collection during 60C extention for HSV1 virus, their curves higher than Threshold line were positive results and the negative result the curves under than threshold line in RT- PCR for HSV2 detection

The results revealed that HSV2 infection was more dominant in 31-40 years age group, as it was comprised 20:40(50%) of the total positive cases, followed by the age group of 20-30 years, with incidence of 12:40(30%). Lower incidence was seen among patients of 41-50 years, since they comprise (15%), while only 2 cases (5%) were

recorded among patients of 51-65years of age, with significant statistically differences at p<0.05 as compared with control group (P<0.028004) (Figure 2).

Table 1: Herpse simplex virus2 (HSV2) PCR positivity in Genitourinary cases as compared with control

Cases	Total cases	HSV2 positivity	HSV2 negative cases
Genitourinary patients	115	40 (34.78%)	75(65.22%)
Control group	50	10 (20%)	40 (80%)
Total cases	165	50 (30.3%)	115 (69.7%)

HSV: Herpes simplex virus

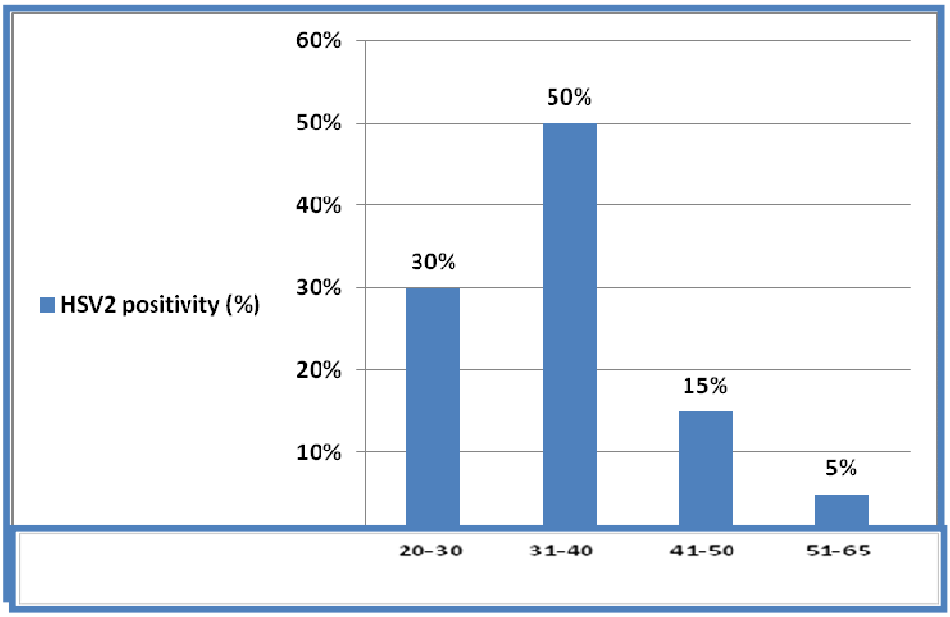

Figure -2: HSV2 positivity according to age groups as detected by real time PCR

Table 2: Semen immunological parameters in Herpse simplex virus2 (HSV-2) DNA positive and negative groups represented by Mean± SD of O.D at 450nm

Source of semen samples	sIgA	IL2	TNF-α	Total
	Mean± SD	Mean ± SD	Mean ± SD	
HSV2 positive Genitourinary cases	219.46±218.66	162.13±83.30	406.86±596.49	19
Control group	128.74±31.54	155.2±139.06	154±23.02	19

HSV2:herpes simplex virus2 IL-2:Interleukine-2, O.D:optical density, sIgA: secretory ImmunoglobulinA, SD:Standard deviation, TNF'alpha:tumour necrosis factor-alpha

Nineteen cases were selected from Genitourinary patients for the evaluation of the immunological aspects correlated with HSV2 infection as compared with control group. The results were shown in table 2. The application of different immunological parameters including IL-2, TNF-α Boster (USA)and sIgA Demeditec(Germany) that included in this study revealed elevation of immune factors during the HSV2 infection period as compared with control group but without significant statistical differences (p>0.05), since Sig. (2-tailed) t - test equal 0.094, 0.955 and 0.083 for sIgA, IL-2 and TNF-α respectively.

DISCUSSION

All of the currently defined viral STDs pathogens have been detected in semen[]11,12. Therefore, semen may be an important carrier in terms of viral sexual transmission. Of the eight human herpesviruses, four have been defined as STD pathogens, and they are EBV, CMV, HSV-1, and HSV-2 [13]. HSV is one of the most common viruses, occurring in all human populations [11]. Results in our study showed that HSV2 positive cases was correlated with 40 cases (34.78%). This result came in agreement with the fact that people who are infected with HSV can shed the virus in their body fluids; for men, those fluids include semen [5]. Semen plays an important role in carrying the viral particle that invades the vaginal or rectal mucosa and, thereby, initiates viral replication [1]. In another study by Kapranos *et al*.,(2003), HSV DNA was detected in 49.5% of semen samples[13]. Other author have also mentioned that HSV-2 was more likely to be the cause of recurrent and atypical genital manifestations (73%) and Real-time

PCR is a sensitive method for diagnosing genital herpes[14]. Most cases of sexually transmitted herpes are caused by HSV-2, but in recent years, the number of cases caused by HSV-1 has risen [15].

Ten cases (20%) from the total control group were also harboring the virus in their semen specimens. This result was also came in agreement with the fact that HSV-2 is the most common cause of genital herpes, which can be asymptomatic at the time of primary, initial, or recurrent infection [16]. Monavari *et al*.,2013 were also detected a considerable prevalence of HSV DNA in semen from asymptomatic infertile males. HSV can be easily transmitted to the partner and cause genital lesions in mothers as well as severe problems such as encephalitis in newborns.[3] Thus, early diagnosis and appropriate anti-viral therapy of asymptomatic genital HSV infection should be purposed. Importantly, HSV may cause asymptomatic persistent infection in the semen with a very low copy number yielding negative cultures[17,18,19]. Therefore, use of molecular techniques such as PCR, increases the possibility of detection of HSV in such cases. The using of sensitive and specific methods like PCR enables us to reduce the abnormal semen parameters and the possibility of infertility as well as to control the transmission HSV infection [3]. Klimova *et al*.,(2010) were observed that seminal HSV infection was more frequently present in male infertile patients than controls, and they revealed that HSV infection was directly correlated with the reduced amount of actively motile sperm.[20] In addition, Abdulmedzhidova *et al*.,(2007) were reported that HSV was detected in 25% of males with infertility and HSV infection was associated with oligospermia and sperm structural abnormality[21]. Kotronias and Kapranos, (1998) were detected HSV-1 and HSV-2 infections in the semen of 21% and 20% of infertile men, respectivelyp22[. As stated above, most cases of sexually transmitted herpes are caused by HSV-2, but in recent years, the number of cases caused by HSV-1 has risen [15]. Expression of HSV thymidine kinase (HSV-tk) in transgenic mouse testis is correlated with sperm structural abnormalities, defects in spermatogenesis and increase in number of apoptotic germ cells[23,24] Moreover, the decrease of HSV-tk levels leads to significant reduction of sperm abnormalities and fertility rehabilitation in mice[23,25]. Interestingly, treatment of HSV positive male infertile patients with anti-viral drugs leads to several healthy pregnancies ([26,22]. Significant importance of HSV infections is observed in *in-vitro* fertilization techniques, and the infections are responsible for the high rate of failed fertilization[27,28].

Additionally, the present results are disagree with Leutscher *et al*., (2005) who stated normally, the level of IL-2 detected at low concentration in seminal fluid, this may lead to suggest that cellular immunity activity is low in the male reproductive tract[29]. But this factor has been rising when semen exposure to infection, otherwise to derive expression of IL-2, a coordination activation of NFAT together with AP-1 transcription factor is necessary, in which , this coordination cannot happen, could be because mounting of intracellular Ca^{2+} level in TM were found only after infection with UPEC[30].

Besides, other reason might be interpreted this circumstance, of noted, TGF-α, is one of immune components which existed in semen, it may be converted to its active form via influence of acidic medium [31], and due presence of infection, the seminal pH were affected and tend to reducing (toward acidity) as appeared in the present study, hence, due to the TGF-α is a key regulator of several aspects of immune responses[32], and it has been identified as a major immune suppressive agent for in human seminal fluid[33]. However, this immune suppressive effects probably inhibits other defense function.

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