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Immunogenic Study of Human Herpes Virus Type 6 (HHV-6) Infection in Women with Unexplained Recurrent Miscarriage

A Thesis

Submitted to the Council of the College of Science and The Committee of Postgraduate Studies of University of Babylon In Partial Fulfillment of the Requirements for the Degree of Doctorate of Philosophy in Biology / Microbiology

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بِسْمِ ٱللَّهِ ٱلرَّحْمَٰنِ ٱلرَّحِيمِ

﴿ وَقُلِ ٱعْمَلُوا۟ فَسَيَرَى ٱللَّهُ عَمَلَكُمْ وَرَسُولُهُۥ وَٱلْمُؤْمِنُونَ ۖ
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Dedication

To who He taught me a letter in this mortal world

To my father's pure soul May Allah have mercy on him

To My dear mother, may Allah Preserve her as an asset for us

To All respected educational family especially

College of sciences department of biology My respected teachers

To my Dear brothers and sisters

With my love

To my friends

To anyone who help me and see I deserved it

We ask Allah Almighty to make This humble work a beacon for every science student

AWS

2021

Summary

Viral infections during pregnancy have been associated with adverse pregnancy outcomes and birth defects in the offspring .Viruses rarely cross the placental barrier, but when the virus does reach the fetus, it can result in severe birth defects such as microcephaly or even fetal death. Human herpes virus 6 (HHV-6) can integrate into human chromosomes, resulting in genetic transmission from parent to child. Individuals of either sex with inherited chromosomally integrated human herpes virus 6 (iciHHV-6) harbor the virus in every cell.

These experiments were conducted to study the effect of human herpes virus -6 on the pregnant women and abortion cases. This study was done for 100 patients including different ages that range from 17-49 age that sever recurrent miscarriage. Also the study include 50 apparently healthy control **(AHC)**. The specimens were collected during winter from January, 2020 to May , 2021.

Endometrium swab ; Cervical swab ; fetal fluids swab as well as blood specimens were collected and processed to extract viral genome and total DNA gene for screened human herpes virus-6 by using Real Time PCR **(RT-PCR)** and TLR2, IL-10,IL-18 & HLA-G polymorphism by amplification-refractory mutation system **(ARMS).** In addition, estimation serum TLR2, IL-10,IL-18 & HLA-G concentration by enzyme-linked Immunosorbent assay **(ELISA).**

The obtained results of this study are summarized as follows:

There is no significant difference of the mean age between RPL and AHC

A strong positive relationship ($P<0.001$) was found between number of participants; number abortion ; week of abortion and Maternal

age($P < 0.001$). However, there are no significant correlation between number of participants with control maternal age.

Around half patients (55%) have a viral infection with RPL. With no among all the examined apparently healthy infection in control group. There were statistically highly significant differences ($p = 0.01$) between women patients with RPL with viral genome and those without the Viral genome.

The qRT-PCR shows 45.5 % positive while 55.5% negative. Statistically significant differences ($p = 0.04$) among patients group.

In women with RPL, the most commonly affected age with HHV-6 was (30-39 years) which constituted 48% (12 out of 25 cases). Statistical comparison of these HHV-6 in the Patients with Women With RPL according to age stratum revealed significant differences.

The amplified of TLR-2 (rs1898830) result was appeared that the frequency of GG genotypes in women patients with RPL and AHC groups was 17% and 4%, respectively. While ,the frequency of AG genotypes in women patients with RPL and AHC groups reached 38% and 8%, respectively. It was significantly increased in women patients than control. AG genotype increased as rate OR=1.96 compared with GG and AA genotypes among studied groups. both of GG and AG were statistically higher than those of the control group according to the gene expression levels ($P<0.05$), however, AA was statistically lower in patients than control group.

The amplified of IL-10 (rs1518111) result was appeared that the frequency of CC genotypes in women patients with RPL and AHC groups which reached 11% and 0%, respectively it was significantly increased in women patients than control. While ,the frequency of TC genotypes in women patients with RPL and AHC groups which reached 17% and 16%, respectively.TC genotype decreased as rate OR=1.7 compared with CC genotype and equal to TT genotype among studied

groups. According to the results , both of TT and CC were statistically higher than those of the control group according to the gene expression levels (P<0.05).

The amplified of IL-18 (rs187238) result was appeared that the frequency of GG genotypes in women patients with RPL and AHC groups reached 4%,

With no significant differences . While ,the frequency of CG genotypes in women patients with RPL and AHC groups which reached 15% and 6%, respectively. CG genotype increased as rate OR=1.9 compared with GG and CC genotypes among studied groups. According to the results , both of CC and CG were statistically higher than those of the control group according to the gene expression levels (P<0.05) .

The amplified of HLA-G (rs1063320) result was appeared that the frequency of GG genotypes in women patients with RPL and AHC groups which reached 9% and 6% ,respectively .It was significantly increased in women patients than control groups . While ,the frequency of CG genotypes in women patients with RPL and AHC groups which reached 18% and 8%, respectively. It was significantly increased in women patients than control study groups. On the other hand, the frequency of CC genotype in women patients with RPL and AHC groups was 5% and 4%, respectively, that increased in women patients compared with control groups. Finally, was found which CG genotype increased as rate OR=1.8 compared with CC genotype and decreased rate compared with GG genotype among studied groups. According to the results , CC, GG and CG were statistically higher than those of the control group according to the gene expression levels (P<0.05).

The concentration of serum TLR-2, IL10 , IL-18 , and HLA-G were detected by ELISA technique. The mean of serum TLR-2, IL10 , IL-18 , and HLA-G concentration in women patients with RPL and apparently healthy control (AHC) groups were 13.3±1.3; 36.6 ±6.3; 15.8± 2.5 &365.5± 11.3 pg/ml and 12.6± 1.6 ;
32.5± 3.4; 18.9± 3.2 &97.3± 11.3pg./ml, respectively. Significant difference (p=.004) was found by comparing the mean of serum TLR-2, IL10 , IL-18 , and

HLA-G concentration among AHC and women with RPL groups **.**

A strong positive relationship was found between age stratum (17-29 years) and HHV-6 infection in women with RPL. Similarly, there is a positive relationship between number of miscarriage and HHV-6 infection in women with RPL.However, there are no significant correlations among HHV-6 and week of child loss according to the age stratum (17-29 years)**.**

There is a positive relationship (with significant correlation) between number of miscarriage and week of child loss in women with RPL .In addition , significant correlation was found between number of miscarriage and HHV-6 infection in women with RPL . However, there are no significant correlations among HHV-6 and week of child loss according to the age stratum (30-39 years) .

There is a positive relationship (with significant correlation) between age stratum (40-49 years) and week of child loss in women with RPL .In addition , significant correlation was found between number of miscarriage and age stratum (40-49 years) in women with RPL .However, there are no significant correlations between week of child loss and HHV-6 infection in women with RPL . Similarly, there is no relationship (with non-significant correlation) between number of miscarriage and HHV-6 infection in women with RPL.

According to the Spearman correlation , a dendrogram of gene expression and HHV-6 detection was divided significantly ($p<0.01$, $r= 0.7$) into two clusters. The results demonstrated a significant correlation between the gene polymorphism (expression) of IL-18 with viral infection (top cluster). However, the gene expression of both TLR2 and HLAG has less correlation ($p<0.04$, $r=0.4$) with the same infection.

The cluster analyses of the percentage of gene polymorphism in the selected cases samples was performed to study the percentage of similarity between all the IL-10, IL-18, TLR2, HLAG , and HHV-6 and depending on gene expression showed that

IL-18 has a higher similarity (>60%) than the rest variables.

To conclude HHV-6 one of the most recently identified vaginal viruses in Iraqi women patients suffering from recurrent miscarriage. In addition, **TLR2 and HLA-G SNPs** may be associated with the risk of RPL in the Iraqi women. **While , IL-10 and IL-18** SNPs were identified as secondary factors that can also affect the risk of miscarriage.

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List of Abbreviations

Symbol	Description
AHC	Apparently healthy control
CD4	Cluster of differentiation 4
DNA	Deoxy ribose nucleic acid
EIAs	Enzyme immunoassays
ELISA	Enzyme linked immuno sorbent assay
HHV6	Human Herpesvirus 6
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-10	Interleukin 10
IL-18	Interleukin 18
INFs	Interferones
IRF3	Interferon regulatory factor 3
mRNA	Messenger Ribose nucleic acid
NK cell	Natural killer cell
PCR	Polymerase chain reaction
RPL	Recurrent |pregnancy Loss
RT PCR/ qPCR	Real Time Polymerase chain reaction/quantitative
Th2	T-helper 2
TNF-α	Tumor necrosis factor alpha
WHO	World Health Organization

CHAPTER ONE

Introduction

1.1. Introduction

Abortion is the ending of a pregnancy by removal or expulsion of an embryo or fetus. An abortion that occurs without intervention is known as a miscarriage or "spontaneous abortion" and occurs in approximately 30% to 40% of pregnancies (Latt *et al*.,2019).

In addition , recurrent pregnancy loss (RPL), defined as the spontaneous loss of two or more pregnancies or the loss of two or more pregnancies before the 24th week of gestation, presents several still incompletely defined aspects. Among these is the outcome of the successive pregnancy in women with a history of RPL (Carlo *et al*.,2020).

Indeed, there is considerable discrepancy between the reported birth rates and the rates of gestational complications of the successive pregnancy in women with RPL. The likelihood of a live birth in the successive pregnancy in untreated women with RPL has been reported to range 42–86% after three miscarriages and decreases with increasing the number of pregnancy losses, reaching only 23–51% after ≥5 losses (Christiansen *et al*.,2020). This observation suggests that the number of miscarriages—a likely indicator of the gravity of the condition—is a major determinant of the reproductive success of women with RPL; in fact, it has been reported that the live birth rates in the successive pregnancy in women with two consecutive losses is around 75% (Green, and O'Donoghue,2019; Christiansen *et al*.,2020).

In fact, in only around 50% of RPL cases can defined causes/risk factors can be found, including advanced maternal age, genetic abnormalities, selected maternal

autoantibodies, endocrine dysfunctions, and uterine abnormalities .The remaining RPL cases currently are unexplained (uRPL) (Ticconi *et al*.,2019).

Human herpesvirus 6 (HHV-6) was the sixth human herpesvirus discovered. It belongs to the β-Herpesvirinae subfamily. Although horizontal transmission is considered to be the main route of HHV-6 infection, it can be genetically transmitted from parent to child as inherited chromosomally integrated HHV-6 (iciHHV-6) (Miura *et al*.,2018).

The complete HHV-6 genome is integrated into every nucleated cell of an individual with iciHHV-6. Extremely high copy numbers of HHV-6 DNA can be detected in clinical specimens containing nucleated cells, which can lead to a misdiagnosis of active viral infection . Theoretically, a parent with iciHHV-6 has a 50% chance of transmitting the integrated HHV-6 genome to the next generation (Gaccioli *et al*.,2020).

HHV-6 can integrate into human chromosomes, resulting in genetic transmission from parent to child. Individuals of either sex with inherited chromosomally integrated human herpesvirus 6 (iciHHV-6) harbor the virus in every cell. Viral reactivation from the integrated HHV-6 genome can occur in pregnancy (Miura *et al*.,2020).

The exact mechanism by which abnormal immune factors cause recurrent miscarriage is uncertain, but may involve modulation or imbalance between the various cells of the immune systems, especially the T cell subgroups (AlJameil *et al*.,2018).

Toll-like receptors (TLRs) are important receptors involved in innate immunity and play an important role in the regulation of inflammation and immune response.

TLR2 is expressed in placental trophoblast and endometrial glandular epithelial cells as the most representative of the TLRs family of receptors. TLR2 can activate the immune cells at the interface of the mother and child, releasing Th1 type cytokines. Over expression of Th1 may affect the embryo and fetal growth and development, finally leading to abortion (Ying *et al*.,2017).

Interleukins are a group of immunomodulatory proteins leading a variety of immune reactions in human body. In normal pregnancy, a shift to Th2 cytokine production with abundant interleukin-10 (IL-10) is considered important. During pregnancy, IL-10 is produced locally in the feto–placental unit by cytotrophoblasts and decidual T cells, and it up-regulates the human leukocyte antigen (HLA)-G expression of cytotrophoblasts at the feto–maternal barrier (Camil and Viorica ,2014).

Several single nucleotide polymorphisms (SNPs) have been identified in the IL-10 gene promoter region which regulates the expression of IL-10. Among these SNPs located in the promoter region of the IL-10 gene, three single nucleotide polymorphisms (G/A at -1082, C/T at -819, and C/A at -592 positions) in the proximal region, are associated with an increased frequency of spontaneous abortions (Vidyadhari *et al*.,2017).

The IL-18 gene is located on chromosome 11 (11q22.2–22.3), and contains many polymorphisms (SNPs in particular) especially, in the promoter region. SNPs may influence the level of cytokine production and the two polymorphisms in the IL-18 gene promoter seem to affect the transcription and hence the amount of IL-18 (Shadi and Fadel, 2014).

The IL-18 gene is also expressed in the fetal chorion and the maternal decidua's and is thereby present at the materno-fetal interface. The IL-18 level has been shown to increase from the first trimester until the onset of labor. Therefore, a role of IL-18 in pregnancy, labor onset, and pregnancy complications has been suggested (Messaoudi *et al*.,2012).

HLA-G is unique among class I genes because it undergoes alternative splicing to produce four encoding membrane-bound proteins (HLA-G1eG4) and three soluble proteins (HLA-G5eG7). HLA-G gene is located on chromosome 6(6p21.31). It has been reported that HLA-G expression is influenced by 14-bp insertion (ins)/deletion (del) and aþ3142G/C (rs1063320) polymorphisms in the 30untranslated region (30UTR) of HLA-G gene and may have possible implications of clinical significance . The studies focusing on the HLA-G polymorphisms with RSA have been largely inconclusive. Due to genetic risks may vary among diverse populations, repeating previously reports of association of HLA-G variants and RSA in other population is required to find out the genetic risk (Hashemi *et al*.,2017).

1.2. Aim of Study

In view of the a forementioned introduction, the study was designed to determine the percentage of Human Herpes Virus 6(HHV6) as well as TLR2;IL10;IL18 and HLA-G polymorphism in women suffering from recurrent pregnancy loss (RPL) , through achieving the following objectives**:**

1- To determine the percentage of HHV6 in endometrium ; cervical ; fetal fluids swabs as well as blood specimens from individuals who in apparently healthy women to patients with abortion and RPL by RT-PCR.

2- Estimation of the genetic polymorphism for TLR2; IL-10; IL-18 and HLA-G genes in individuals who range from apparently healthy women in patients with abortion and RPL by amplification –refractory mutation system (ARMS).

3- Evaluation the serum concentrations of TLR2; IL-10; IL-18 and HLA-G in apparently healthy women and patients with abortion and RPL by ELISA.

4- Find the association between these TLR2; IL-10; IL-18 and HLA-G genes and HHV6 in women that represent as apparently healthy female peoples as well as patients with RPL .

CHAPTER TWO

Literature of Reviews

2. Literature of Reviews:

2.1 Miscarriage

Miscarriage it is often occurring in the early times of pregnancy (occurs during first or second trimester) were called early miscarriage or may occur at the beginning of the third trimester and called late miscarriage (Pazol *etal*.,2015).Miscarriage can be divided into various types including :

1- Threatened Miscarriage
2- Missed Miscarriage
3- Induced Miscarriage
4- Complete Miscarriage
5- Incomplete Miscarriage
6- Inevitable Miscarriage
7- Spontaneous Miscarriage

2.1.1 Types of Abortion

I. Induced

Most abortions result from unintended pregnancies. A pregnancy can be intentionally aborted in several ways. The manner selected often depends upon the gestational age of the embryo or fetus, which increases in size as the pregnancy progresses. Specific procedures may also be selected due to legality, regional availability, and doctor or a woman's personal preference (Jones *et al*.,2019).

Reasons for procuring induced abortions are typically characterized as either therapeutic or elective. An abortion is medically referred to as a therapeutic abortion when it is performed to save the life of the pregnant woman; to prevent

harm to the woman's physical or mental health; to terminate a pregnancy where indications are that the child will have a significantly increased chance of mortality or morbidity; or to selectively reduce the number of fetuses to lessen health risks associated with multiple pregnancy. An abortion is referred to as an elective or voluntary abortion when it is performed at the request of the woman for non-medical reasons. Confusion sometimes arises over the term "elective" because

"elective surgery" generally refers to all scheduled surgery, whether medically necessary or not(Latt *et al*.,2019).

II. Spontaneous

Miscarriage, also known as spontaneous abortion, is the unintentional expulsion of an embryo or fetus before the 24th week of gestation. A pregnancy that ends before 37 weeks of gestation resulting in a live-born infant is a "premature birth" or a "preterm birth". When a fetus dies in utero after viability, or during delivery, it is usually termed "stillborn". Premature births and stillbirths are generally not considered to be miscarriages, although usage of these terms can sometimes overlap (Stotland,2019).

Only 30% to 50% of conceptions progress past the first trimester. The vast majority of those that do not progress are lost before the woman is aware of the conception, and many pregnancies are lost before medical practitioners can detect an embryo. Between 15% and 30% of known pregnancies end in clinically apparent miscarriage, depending upon the age and health of the pregnant woman. 80% of these spontaneous abortions happen in the first trimester (Cunningham *et al.*,2014).

The most common cause of spontaneous abortion during the first trimester is chromosomal abnormalities of the embryo or fetus, accounting for at least 50% of sampled early pregnancy losses. Other causes include vascular disease (such as lupus), diabetes, other hormonal problems, infection, and abnormalities of the uterus. Advancing maternal age and a woman's history of previous spontaneous abortions are the two leading factors associated with a greater risk of spontaneous abortion. A spontaneous abortion can also be caused by accidental trauma; intentional trauma or stress to cause miscarriage is considered induced abortion or feticide (Horvath, and Schreiber,2017).

2.1.2 Etiology of recurrent miscarriage

1- Endocrine abnormalities
2- Genetic factors
3- Anatomic etiologies
4- Male factors
5- Infectious factors
6- Environmental and Lifestyle factors
7- Inherited acquired thrombophilia
8- Unexplained Etiologies
9- Immunologic etiologies (Besharat *et al*.,2015)

2.2 HUMAN HERPES VIRUS-6 (HHV-6)

2.2.1. Historical Preview:

During 1986, Syed Zaki Salahuddin, Dharam Ablashi, and Robert Gallo cultivated peripheral blood mononuclear cells from patients with AIDS and lymphoproliferative illnesses. Short-lived, large, refractile cells that frequently contained intranuclear and/or intracytoplasmic inclusion bodies were documented. Electron microscopy revealed a novel virus that they named Human B-Lymphotrophic Virus (HBLV) (Kawabata *et al.,2011)*.

Shortly after its discovery, Ablashi *et al.*,(2006) described five cell lines that can be infected by the newly discovered HBLV. They published that HSB-2, a particular T-cell line, is highly susceptible to infection. Ablashi's pioneering research concluded by suggesting that the virus name be changed from HBLV to HHV-6, in accord with the published provisional classification of herpes viruses.

HHV-6 was divided into subtypes. Early research (1992) described two very similar, yet unique variants: HHV-6A and HHV-6B. The distinction was warranted due to unique restriction endonuclease cleavages, monoclonal antibody reactions, and growth patterns (Kawabata *et al.*,2011).

HHV-6A includes several adult-derived strains and its disease spectrum is not well defined, although it is thought by some to be more neurovirulent (Kofman *et al.*,2011). HHV-6B is commonly detected in children with roseola infantum, as it is the etiologic agent for this condition. Within these two viruses is a sequence homology of 95%(Arbuckle *et al.*,2010*;* Harberts *et al.*,2011).In 2012, HHV-6A and HHV-6B were officially recognized as distinct species (Marci *et al.*,2016).

. **2.2.2. Taxonomy and Classification of HHV6**

Herpesvirus was established as a genus in 1971 in the first report of the International Committee on Taxonomy of Viruses (ICTV).The family Herpesviridae was divided into 3 subfamilies (alphaherpesvirinae , betaherpesvirinae and gammaherpesvirinae) and 5 unnamed genera; 21 viruses were recognized as members of the family as the following:

I.α herpesviruses: Herpes simplex virus types 1 and 2, and varicella-zoster virus, which have a short replicative cycle, induce cytopathology in monolayer cell cultures, and have a broad host range.

II.β herpesviruses: Cytomegalovirus, and human herpesviruses 6 and 7, with a long replicative cycle and restricted host range.

III.γ herpesviruses: Epstein-Barr virus and human herpesvirus 8, with a very restricted host range.

Now, there are more than 130 herpesviruses are known, some of them from mammals, birds, fish, reptiles, amphibians, and mollusks. Nine herpesvirus types are known to infect humans (Yaara *et al*.,2020).

HHV-6A, HHV-6B belongs to the β Herpesvirinae subfamily. HHV-6A and HHV-6B were recognized by the ICTV as distinct species in 2012.This genus consisted of 23 viruses among 4 groups (Rizzo *et al*.,2017).

HHV-6A and HHV-6B, which share high genome similarity but differ for some biologic properties, epidemiology, and disease association. Although they are classified as lymphotropic viruses, their in vivo tropism is considerably broader, including T-lymphocytes, macrophages, endothelial cells (ECs), salivary glands,

and brain , thyroid epithelial cells, natural killer (NK) cells , and endometrial cells(Caselli *et al*.,2017).

2.2.3. Morphology and Structure of HHV-6

2.2.3.1 HHV-6 Particles:

The diameter of an HHV-6 virion is about 2000 angstroms. The size of the HHV-6 virion increases from 120 nm to approximately 300 nm after the inclusion of the tegument and envelope (Roizman and Knipe.,2001).

The virion's outer portion consists of a lipid bilayer membrane that contains viral glycoproteins and is derived from that of the host. Below this membrane envelope is a tegument which surrounds an icosahedral capsid, composed of

162 capsomeres. The protective capsid of HHV-6 contains double stranded linear DNA (Kawabata *et al*.,2009).

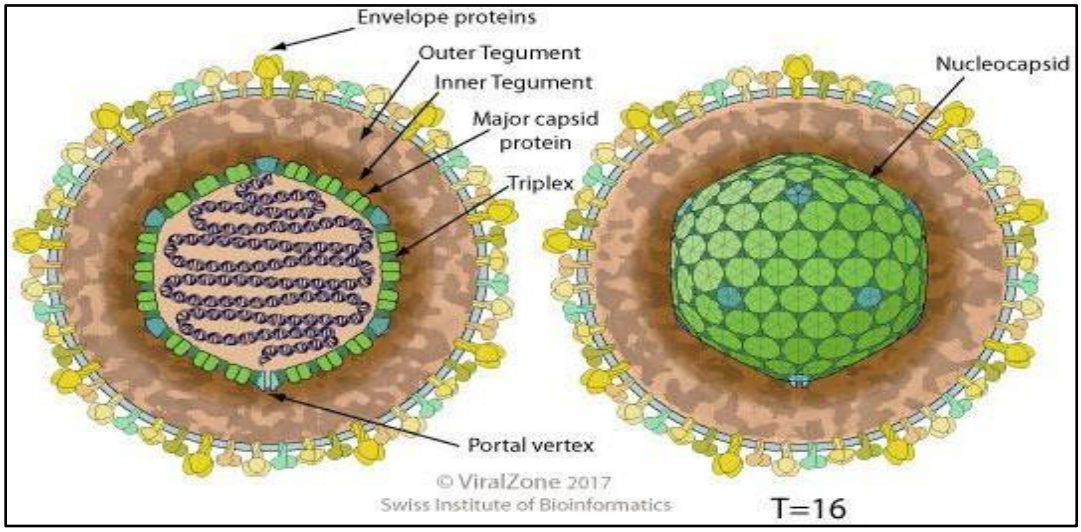


Figure 2.2: HHV-6 structure (Julia *et al*.,2014).

2.2.3.2. HHV-6 Genome Organization

The genomes of HHV-6A and HHV-6B, similar to those of other herpesviruses, consist of large linear double stranded DNA molecules, 160 kb in length, containing a unique segment flanked by direct repeats (Finkel *et al*.,2020).The genetic material of HHV-6 is composed of linear (circular during an active infection), double stranded DNA which contains an origin of replication, two 8–10 kb left and right direct repeat termini, and a unique segment that is 143–145kb (Tang *et al*.,2010).

The direct repeat termini (DR_L and DR_R) possess a repeated TTAGGG sequence, identical to that of human telomeres. Variability in the number of telomeric repeats is observed in the range of 15–180. These termini also contain pac-1 and pac-2 cleavage and packing signals that are conserved among herpesviruses (Arbuckle *et al*.,2011).

The unique segment contains seven major core gene blocks (U27–U37, U38–U40, U41–U46, U48–U53, U56–U57, U66EX2–U77, and U81–U82), which is also characteristic of herpesviruses. These conserved genes code for proteins that are involved in replication, cleavage, and packing of the viral genome into a mature virion. Additionally, they code for a number of immunomodulatory proteins. The unique segment also possesses a block of genes (U2–U19) that are conserved among HHV-6, HHV-7, and Cytomegaloviruses (the betaherpesviruses) (Tang *et al*.,2010; Arbuckle *et al*.,2011).

The annotation of HHV-6 coding capacity has traditionally relied on open reading frame (ORF)-based analyses using canonical translational start and stop sequences and arbitrary size restriction to demarcate putative protein coding genes, resulting in a list of around 100 ORFs for each virus . In recent years, genome

wide-analysis of herpesviruses using short RNA sequencing (RNA-seq) reads, and recently also direct and long-read RNA-seq revealed very complex transcriptomes (Depledge *et al*., 2019; Kara *et al*., 2019; O'Grady *et al*., 2019), and combined with genome-wide mapping of translation, revealed hundreds of new viral ORFs (Bencun *et al*., 2018; Whisnant *et al*., 2019).

Specifically for HHV-6, recent work using proteomics, transcriptomics and comparative genomics on HHV-6B enabled re-annotation of several viral gene products .Taken together, this unforeseen complexity of herpesviruses suggests the current annotations of HHV-6 genomes are likely incomplete (Finkel *et al*.,2020).

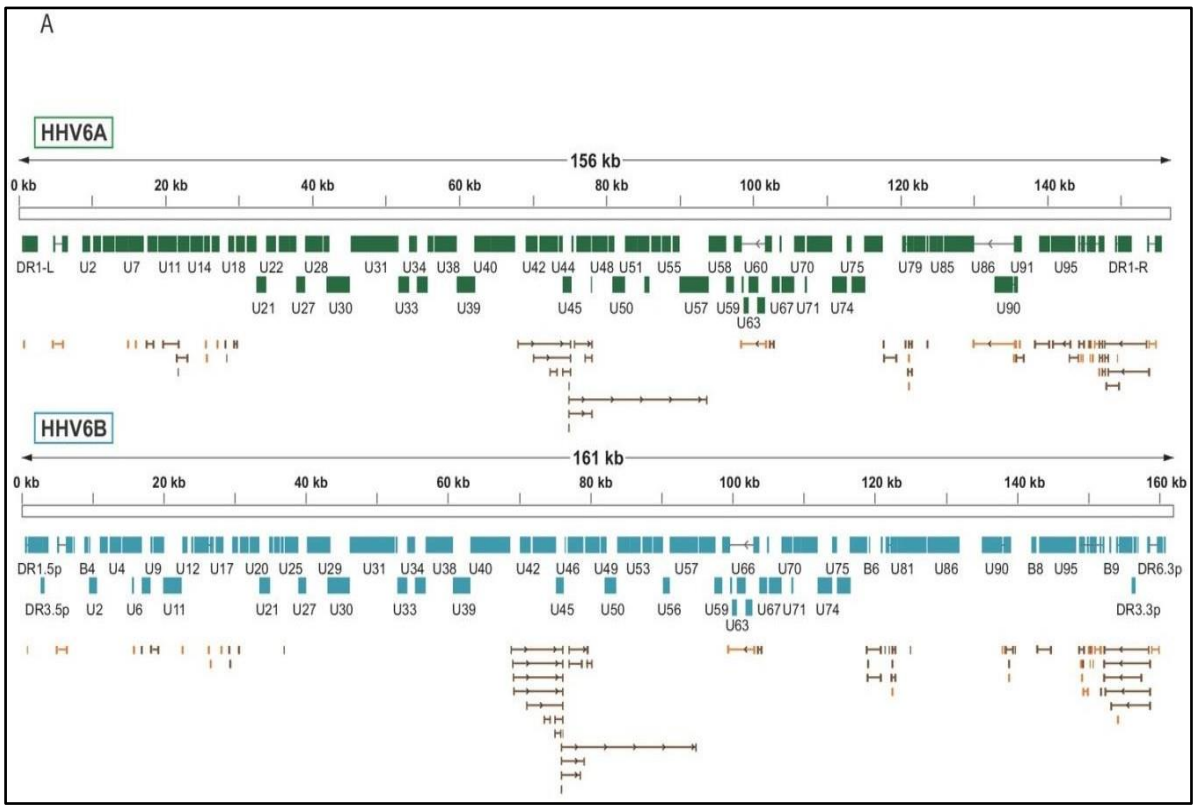


Figure 2.3: Schematic organization of HHV6 A&B genome. Splice junctions mapped using RNA-seq reads are shown throughout the genomes of HHV-6A and HHV-6B. Previously annotated splice junctions are marked in orange and novel splice junctions are marked in brown (Finkel *et al*.,2020).

I.Integrated HHV-6A/B

The extreme end of the viral genome is flanked by two identical directly repeated (DR_L and DR_R) regions of 8–9 kbp. The 5′ end of the DR contains a pac1 sequence (56 bp) while the 3′ end contains pac2 sequence (80 bp), responsible for the cleavage and packaging of the viral genome. Adjacent to pac2 are 15–180 reiterations of TTAGGG telomeric repeats (TMR), identical to human telomeric sequences. Adjacent to pac1 are imperfect TMR (impTMR) Figure (2.4 A) (Collin and Flamand, 2017).

HHV-6A/B integrated genomes remain largely intact with their ORFs conserved. Analysis of cells with ciHHV-6A/B indicates that these viruses are mostly integrated into telomeres with DR_R fused to the chromosome Figure (2.4B) (Tweedy *et al*.,2016).

In fact, HHV-6-A/B DR_R are adjacent to the subtelomeric portion of the human telomere with loss of the pac2 sequence at DR_R and loss of the pac1 sequence at DR_L. Such a structure is compatible with integration occurring by HR events. In addition, at the DR_L end of the integrated genome are impTMR that appear to serve as a template for telomere elongation by the telomerase complex or alternative lengthening mechanisms (Ohye *et al*.,2014). In occasional cases, integrated HHV-6A/B consists of a single DR fused to the chromosome, a structure that is compatible with integration occurring by HR events initiated at DR_L (Figure 2.4 C).

Alternatively, individuals containing multiple contiguous HHV-6A/B copies also exist. Such structure can be explained by integration of a viral concatemer (Figure 2.4 D).By assessing the number of DR present, one can determine the number complete HHV-6A/B's genomes that is/are integrated(Collin and Flamand, 2017).

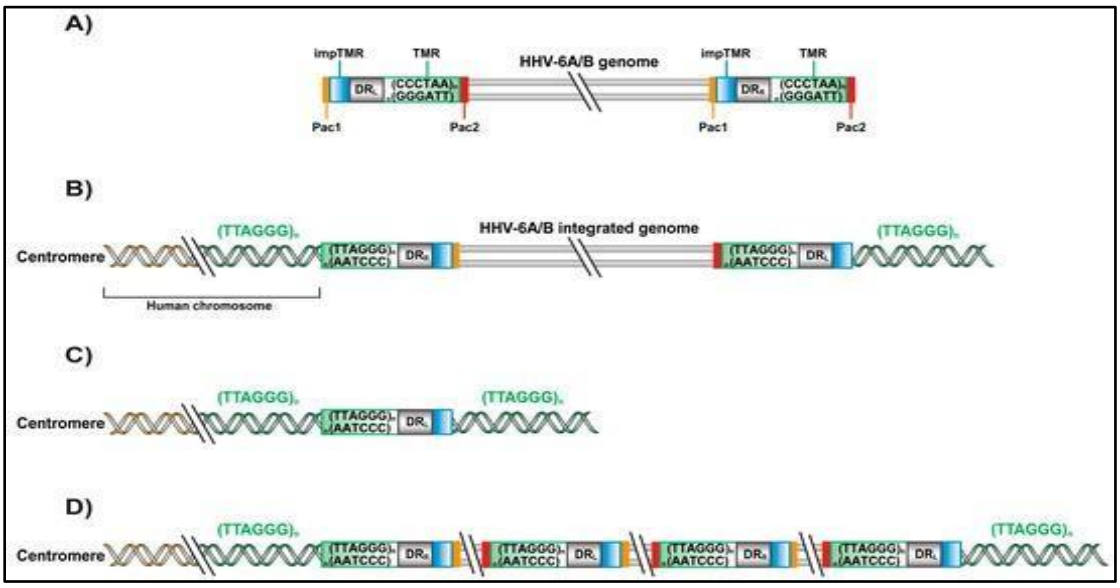


Figure 2.4. HHV-6A/B genomes and their integrated forms. Schematic representation of human herpesvirus 6A and 6B (HHV-6A/B) genomes and the reported integrated forms. (A) The unique region (U) of the 160 kbp HHV-6A/B genomes is flanked by identical direct repeats (DR_L and DR_R) of 8–9 kbp. The DRs possess a pac1 (yellow) and pac2 (red) sequences, adjacent to imperfect telomeric repeats impTMR (blue) and TMR (green) sequences, respectively. The genome is not drawn to scale; (B) Chromosomally integrated HHV-6A/B (ciHHV-6A/B) genome (with loss of pac2 in DR_R and pac1 in DR_L) with elongated telomeres at the DR_L; (C) Single integrated DR_L with elongated telomere; (D) Integrated HHV-6A/B concatemers. Genomes are not drawn to scale (Collin and Flamand, 2017).

II. Excision of Integrated HHV-6

To ensure their long-term maintenance in any given population, integrated viruses must be able to re-initiate a lytic cycle and generate progeny virions. A previous study has reported the presence of an extrachromosomal circular HHV-6 genome with a single DR in cells from an iciHHV-6 subject .This led to the conclusion that the viral genome can be excised from telomeres by one or two t-

loop formation and recombination. To explain the presence of a single DR in the excised viral genome, one hypothesis is that excision occurs by a two-step t-loop formation Figure (2.5A). At each cell cycle, telomeres are shortened and reform a t-loop to protect the chromosomes. First, the telomere sequence at the end of the DR_L could form a t-loop by invasion of the TMR within DR_L itself. This t-loop formation would result in a t-loop excision, forming a telomeric circle with a single DR and an intermediate form of the HHV-6A/B integrated chromosome Figure (2.5B) (Huang *et al*.,2014).

The intermediate form would lack the DR_L but still possess the TMR repeats capable of forming a second t-loop, recombine and excise in the TMR of the DR_R. This first possibility could also explain the presence of a single integrated DR if only one t-loop excision is made. Another possible excision mechanism could be a t-loop formation, recombination and t-loop excision of the DR_L in the DR_R directly. The two possible mechanisms result into a circular viral genome with a single DR that has one pac1 and one pac2 sequence Figure (2.5C). Because ciHHV-6A/B can be excised from chromosomes and form viral episomes, this suggests that integration is possibly a mode of latency for these viruses (Collin and Flamand, 2017).

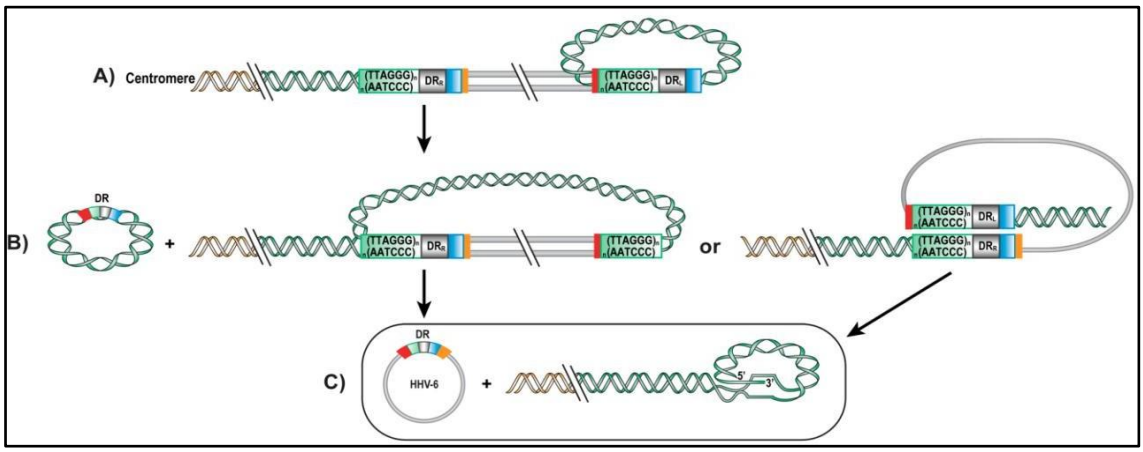


Figure 2.5. Possible mechanisms of HHV-6A/B genome excision from telomeres. Schematic representation of hypothetic processes of HHV-6A/B genome excision from telomeres. (A) Telomeric repeats form a t-loop in the TMR of HHV-6A/B DR_L, followed by recombination and excision, resulting into a first t-loop excision: a telomeric circle and a chromosomally integrated HHV-6A/B lacking a DR but still possessing TMR sequences. (B) A second t- loop formation is made by recombination of the TMR at the end of the genome into HHV-6A/B DR_R, resulting in a fully excised and circular HHV- 6A/B genome containing a single DR with a single pac1, pac2, impTMR and TMR sequence. (C) Invasion of the telomeric repeats into the TMR of the DR_R, resulting into a HHV-6A/B free chromosome and a full viral genome with a complete DR(Huang *et al*.,2014).

2.2.3.3 : HHV6 Proteins

The homotrimeric fusogenic protein gB mediates the viral-host membrane fusion that is required for putting the nucleocapsid into the host cytosol. The gH/gL complex of HHV-6B and HHV-6A are associated with the unique glycoproteins gQ1 and gQ2 encoded in the open reading frame (ORF) U100, resulting in a tetrameric complex gH/gL/gQ1/gQ2, while a trimeric complex gH/gL/gO is also

known including another glycoprotein gO encoded in the ORF U47 (Tang *et al*.,2015; Nishimura *et al*.,2020).

The gH/gL/gQ1/gQ2 complex (hereafter referred to as the 'tetramer') is especially important because gQ1 and the associated gQ2 play critical roles in the interaction with the host receptors (Tang *et al*.,2014 ; Tang and Mori,2015).

U20 is glycoprotein (specific to Roseolovirus) predicted immunoglobulin structure. U20 binds to MHC-1 molecules and prevents antigen presenting cells from presenting HHV-6 peptides — glycoprotein, downregulates HLA I (specific to Roseolovirus).U24 Inhibits proper T cell activation, reducing secretion of cytokines at infection site — phosphorylation target for kinases — glycoprotein M (gM).U94 Involved in transcriptional repression of lytic genes – aids in the specific integration of HHV-6A/HHV-6B into the telomeres — highly expressed during latency (Arbuckle et al.,2011).

2.2.3.4 : Replication Cycle of HHV6

The HHV6 replication cycle takes 72 hours to complete and consists of four general steps as shown in figure (2-6)**:**

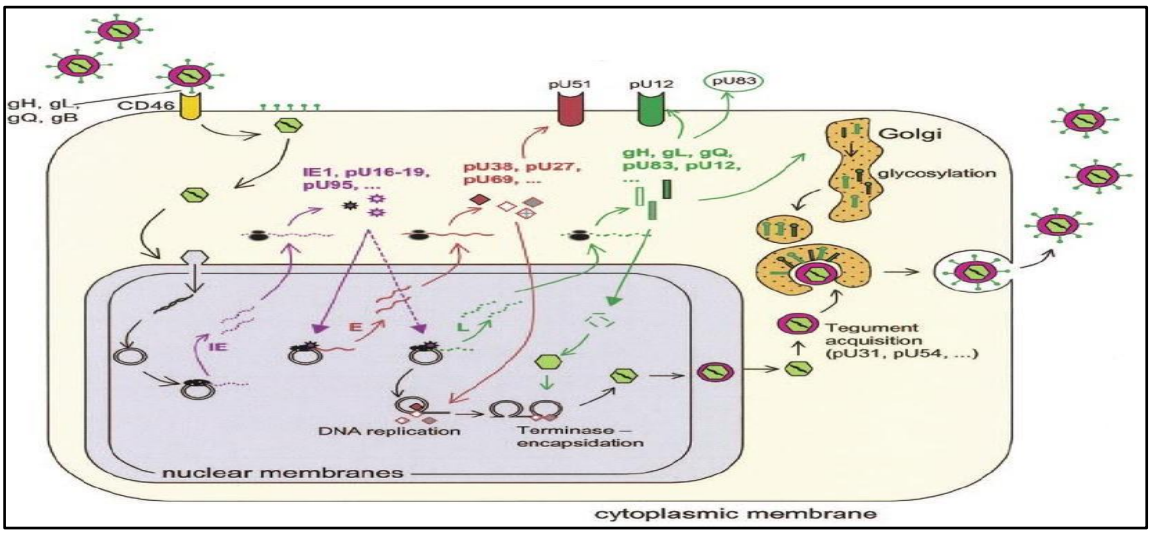


Figure 2.6: Schematic representation of rhinovirus replication cycle (Leen *et al*.,2005).

I.HHV-6 receptor and Host Tropism

HHV-6 infects a wide range of human cells in vitro, but it preferentially replicates in activated $CD4^+$ T lymphocytes .HHV-6A and HHV-6B recognize their specific receptors by differentiating gQ1 and gQ2; the HHV-6A tetramer binds to CD46, which is widely expressed on human cells, whereas the HHV-6B tetramer recognizes human CD134 (hCD134, also known as OX40) which is specifically expressed on activated T cells . A member of the tumor necrosis factor (TNF) receptor superfamily present only on activated T lymphocytes, functions as a specific entry receptor for HHV-6B .The responsible residues in both HHV-6B gQ1 and hCD134 were identified in previous studies, but the interaction is not simple as it depends on the conformation of gQ1 in cooperation with gQ2, and in addition with gH/gL (Tang *et al*.,2014; Nishimura *et al*.,2020).

In addition, to $CD4^+$ T lymphocytes, HHV-6 can infect in vitro $CD8^+$ T lymphocytes (only with HHV-6A), human fibroblasts, natural killer cells, liver cells, epithelial cells, endothelial cells, astrocytes, oligodendrocytes, and microglial cells. However, its capacity to infect continuous T cell lines is limited, and in many cases, it can be obtained only through an adaptation process consisting of serial blind passages of a primary isolate on the target cells. The capability to infect different cell lines is generally higher for HHV-6A than for HHV-6B and appears to be a phenotypic character for discriminating both species. As a whole, no continuous cell line can be recommended for isolation of the virus. The primary isolation of HHV-6 from a human specimen usually requires cocultivation with primary highly susceptible cells consisting of peripheral blood mononuclear cells (PBMCs) or umbilical cord blood lymphocytes (Henri *et al*.,2015).

III. HHV6 Attachment

HHV-6 attaches to its cell receptor by means of a tetrameric viral ligand complex made up of the glycoproteins H (gH), L (gL), Q1 (gQ1), and Q2 (gQ2) (Yamanishi *et al*.,2013).

IV. Entry and Un-coating

Following attachment, HHV-6 entry into cell occurs through a fusion between the viral envelope and the cell membrane by a mechanism which involves gB and gH functions but remains poorly understood. The nucleocapsid is then transported through the cytoplasm to the nucleus, likely using the pathway of the microtubule network. HHV-6 DNA is released into the nucleoplasm (Henri *et al*.,2015).

V. Impacts of HHV6 Gene Expression on Cell Functions

Viral genes are expressed in a temporally ordered manner, starting with immediate early (IE) genes from the IE-A locus, which is constituted of two genetic units, IE1 and IE2 . Those genes are transcribed in the absence of de novo protein synthesis, and this step is followed by the transcription/expression of early (E) and late (L) genes. The replication of the genome occurs after the synthesis of E proteins, which have enzymatic activities dedicated to nucleotide metabolism and DNA synthesis, i.e., phosphotransferase, ribonucleotide reductase, uracil-DNA glycosylase, origin-binding protein, DNA polymerase, polymerase processivity factor, major DNA-binding protein, and helicase-primase complex activities. Viral DNA is assumed to be replicated through a rolling circle process. Progeny DNA is yielded in the form of concatemeric strands, which are cleaved and packaged into capsid precursors thanks to specific cleavage-packaging signals present in the DR_L and DR_R regions (Tsao *et al*.,2009; Yamanishi *et al*.,2013).

In addition, independently of any complete virus-producing process, the expression of certain HHV-6 genes might occur from persisting episomal or ciHHV-6 forms of viral DNA. Many publications have reviewed the formally demonstrated or putative effects of virally encoded gene products on the regulation and modification of cell functions (Flamand *et al*.,2010;Yamanishi *et al*.,2013). As an example, considering the gene products of the IE-A region, IE2 might behave as a general transcriptional activator of many viral and cellular genes, while IE1 interacts with PML bodies.

The products of the DR7 gene appear to demonstrate a cell-transforming activity, presumably through an interaction with p53 . Regarding the U94 gene,

which is analogous to the AAV rep gene, it can bind to the human TATA-binding protein, and its expression in endothelial cells decreases cell migration and angiogenesis . The U95 gene product interacts with the mitochondrial GRIM-19 protein, a component of the oxidative phosphorylation system involved in apoptotic processes . As indicated below, several proteins encoded by the HHV-6 genome have immunomodulatory functions. Taken together, all these features provide molecular bases for understanding the pathological processes associated with acute and chronic HHV-6 infections (Caruso *et al*.,2009).

VI. HHV6 Releasing

The capsids exit the nucleus, acquiring an intermediate envelope by budding through the inner part of the nuclear membrane, are de enveloped by fusion with the external part of this membrane, and appear as tegumentary forms in the cytoplasm. The acquisition of the final envelope carrying viral glycoproteins occurs in the trans-Golgi network, and mature virions are released by exocytosis. The occurrence of a complete replication cycle, which lasts about 3 days, has a major impact on host cell functions and morphology. Infected cells engaged in this virus-producing process ultimately die by apoptosis and/or necrosis(Henri *et al*.,2015; Nishimura *et al*.,2020).

2.2.3.5 : Latency and Reactivation

Like other human herpesviruses, HHV-6 persists indefinitely in its host and is capable of reactivation, meaning the active production of detectable mature virions in some body compartments following a phase of apparently complete clearance. These properties rely on the putative capacity of its genome to be maintained in a nuclear latent form or to drive a low-level productive infection in some cells while

inducing a fully lytic infection in other cells. For other human herpesviruses, such as herpes simplex virus, the latent DNA genome has the form of a covalently closed circular episome associated with cellular nuclear proteins. The existence of such a latent nuclear form has not been demonstrated formally for HHV-6, although an episomal state was shown after experimental infection of cervical carcinoma cell lines.The viral gene U94, which is expressed during latent infection, is assumed to play a major role in the establishment and maintenance of intracellular latency (Tweedy *et al*.,2015).

Other latency-associated transcripts have also been described . Reactivation occurs through the transcription of IE genes in the IE1 and IE2 regions following the likely transactivation effect of cellular and/or viral factors whose nature is still unknown. This reactivation process results in the induction of a replication cycle and the possible appearance of a cytopathic effect (Reynaud *et al*.,2014).

2.2.3.6 : Chromosomal Integration

The integration of the HHV-6 genome into human chromosomes (ciHHV-6) was initially described for transformed cell lines . This phenomenon was further reported to be present in human cells in vivo, including cells which can be transmitted as germinal cells to offspring and hematopoietic stem cells transferred to a transplant recipient . It appears to be a unique feature among human herpesviruses and raises numerous novel questions regarding both pathophysiology and diagnosis (Kaufer and Flamand,2014).

The covalent linkage between viral and cellular DNAs occurs within the subtelomeric region of chromosomes, likely by a mechanism of homologous recombination between telomeric repeat sequences of viral and cellular origins.

The phenomenon has been described for both HHV-6A and HHV-6B and occurs in 0.2 to 1% of the general population in developed countries. It might be generated in the context of de novo infection and is considered by some authors to be the default pathway of HHV-6 latency, including in non-germ line cells and before persistence of viral DNA as an episome (Arbuckle *et al*.,2010).

Although there is no in vivo evidence for that assumption, it must be kept in mind knowing that HHV-6 has the ability to infect sperm cells . Thus, de novo HHV-6 infection of germinal cells might result in individuals harboring the integrated virus in their germ line and transmitting it to their offspring (Godet *et al*.,2014).

Moreover, ciHHV-6 might lead directly to reactivation, as reflected by the production of viral transcripts, proteins, and even transmissible virions. This emphasizes the tight relationship between ciHHV-6, latency, and reactivation. In that context, it is worth recalling the homology between HHV-6 U94 and the human adeno-associated virus (AAV) type 2 rep gene . rep gene products are involved in the site-specific integration of AAV DNA into host cells. Therefore, U94 products might have a pivotal role both in the establishment of latency and in ciHHV-6(Gravel *et al*.,2014).

2.2.4 Transmission of HHV6

Transmission is believed to occur most frequently through the shedding of viral particles into saliva. Both HHV-6B and HHV-7 are found in human saliva, the former being at a lower frequency. Studies report varying rates of prevalence of HHV-6 in saliva (between 3–90%), and have also described the salivary glands as an in vivo reservoir for HHV-6. The virus infects the salivary glands, establishes

latency, and periodically reactivates to spread infection to other hosts (Arbuckle *et al*.,2011; Araujo *et al*.,2011).

Vertical transmission has also been described, and occurs in approximately 1% of births in the United States. This form is easily identifiable as the viral genome is contained within every cell of an infected individual(Araujo *et al.*,2011).

2.2.5 HHV-6 and Disease Association

Several diseases have been associated with HHV-6A/B reactivation in adults, although the causal correlations are still unproven. Most studies on pathogenic association do not specify the HHV-6 virus species, which, however, can be inferred by the reference strains used in the methods(Elisabetta *et al*.,2020).

I. HHV-6A/6B-associated with neurological diseases

In immunocompromised subjects, such as encephalitis, seizures, ataxia, and mild dementia, with a higher prevalence of HHV-6A in cognitive dysfunctions and of HHV-6B in encephalitis and seizures (Pantry *et al*.,2017).

II. HHV-6A/6B correlated to multiple sclerosis

Both HHV-6A and HHV-6B; is predominantly found in peripheral blood mononuclear cells (PBMCs) of patients , systemic sclerosis (HHV-6A) and connective tissue diseases (both HHV-6A/6B) (Caselli *et al*.,2019).

III. HHV-6A correlated with Hashimoto's thyroiditis

HHV-6A was associated with Hashimoto's thyroiditis (Caselli *et al*.,2017).

III. HHV-6A and infertility

HHV-6A was associated with female and male infertility (HHV-6A) (Caselli *et al*.,2017).

IV. HHV-6A and fulminant hepatic failure (HHV-6A/6B) (Caselli *et al*.,2017).

V. HHV-6A and chronic fatigue syndrome (HHV-6A) (Miyagawa *et al*.,2016).

VI. HHV-6A and neoplasia(Miyagawa *et al*.,2016).

VII. HHV-6A and myocarditis, drug reaction with eosinophilia, and systemic symptoms (HHV-6A/6B) (Miyagawa *et al*.,2016).

VIII. HHV-6A and Alzheimer's disease: A possible role of HHV-6A infection has also been suggested in Alzheimer's disease (Rizzo *et al*.,2019).

IX. HHV-6B and COVID-19 patient

Reactivation of HHV-6B has been recently documented in a COVID-19 patient , which may be correlated with the increased expression of the CD134 HHV-6B receptor and the inflammatory cytokine IL-6 . Consistent with this observation, HHV-6-associated diseases such as Pityriasis rosea and Kawasaki's disease increased around ten-fold during the COVID-19 pandemic, compared to previous periods (Elisabetta *et al*.,2020).

2.2.6. HHV6 and Spontaneous Abortion

Miscarriage is the spontaneous loss of pregnancy. There are two types of miscarriage before 12 weeks called early miscarriage and from 12 to 24 weeks called late miscarriage (Giakoumelou *et al*.,2016). HHV-6 has been implicated in

cases of poor pregnancy outcome due to ability of virus to disrupt endometrium epithelial cells function that lead to inhibit the creation of appropriate uterine environment for implantation and fetal growth that lead to infertility and miscarriage (Eliassen *et al* ., 2017).

In another study, the diagnosis of HHV-6A infection in pregnant women, and the detection of HHV-6A DNA in all of fetal tissue, umbilical cord blood samples of healthy newborns, and the placental tissue suggest the possible role of the virus in miscarriage at 24 weeks of gestation (Revest *et al* ., 2011).

In 2016, HHV-6A was found in endometrial biopsies from primary unexplained infertile women, but not in control women. On the contrary, HHV-6B was not found in endometrial biopsies of both groups (Marci *et al* ., 2016).

2.2.7. Laboratory Diagnostic of HHV6

2.2.7.1. Indirect (Serology)

Assays for IgG and IgM detection by immunofluorescence assay (IFA) ; enzyme-linked immunosorbent assay (ELISA) and avidity assays (Ana Lia *et al* ., 2020).

2.2.7.2. Tissue Culture and Cell lines

I. Cell lines and HHV6

The HHV6 detection by culture was performed using different culture systems including JJHan, and HSB-2 cells were cultured in RPMI 1640 supplemented with 10% Nu serum (Corning Cellgro), 10 mM HEPES, and 5 μg/ml plasmocin (Invivogen, San Diego, CA, USA). U2OS and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Corning Cellgro) supplemented

with 10% Nu serum (Corning Cellgro), nonessential amino acids (Corning Cellgro), HEPES . HCT-116, GM847, HEK293T, MCF-7, and NIH 3T3 cells were cultured in the same medium but supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA) instead of Nu serum **(**Gravel *et al* ., 2017).

II.Single-cell cloning integration assay.

Ten thousand cells/well (U2OS, HCT-116, HeLa, GM847, MCF-7, NIH 3T3, and HEK293T) were seeded in 48-well plates **(**Gravel *et al* ., 2017).

2.2.7.3. Antigen detection

Uses conventional equipment, gives evidence of virus gene expression, discrimination between HHV-6A and HHV-6B **(**Agut *et al* ., 2015).

2.2.7.4. Qualitative viral DNA PCR

High sensitivity and specificity, discrimination between HHV-6A and HHV-6B**(**Agut *et al* ., 2015).

2.2.7.5. Quantitative viral DNA real-time PCR

High sensitivity and specificity, discrimination between HHV-6A and HHV-6B, longitudinal follow-up studies, comparison of viral loads in blood versus organs**(**Agut *et al* ., 2015).

2.2.7.6. Detection of viral transcripts by RT-PCR

Distinction between active and latent infections, recognition of active infection in ciHHV-6 subjects **(**Agut *et al* ., 2015).

2.2.7.7. Droplet digital PCR

A Precise method to measure the nucleic acid amounts and identification of ciHHV-6

(Agut *et al* ., 2015).

2.2.8. Immune Response to HHV6

2.2.8.1. Innate Immune Responses To HHV6 Infection

Both HHV-6A and -6B establish a latent infection in the host following resolution of primary infection. Reactivations in the adult have been associated to the development of multiple symptomatic diseases often characterized by immune dysregulation (multiple sclerosis, Sjögren's syndrome, autoimmune thyroiditis, and others). Both viruses are considered lymphotropic, showing an elective tropism for CD4+ T-lymphocytes and being able to infect several different cell types of the immune system, including NK cells (Rizzo *et al*., 2017).

Interestingly, *in vivo* and *in vitro* evidences indicate that HHV-6A/6B interfere with the immune system of the infected host in several ways . They can modulate surface antigens important for T-cell activation, such as human leukocyte antigen (HLA) class I molecule expression in dendritic cells; they also can affect cytokine and chemokine productions, including selective suppression of IL-12, affecting the generation of effective cellular immune responses . Furthermore, we recently observed that HHV-6A infection induces the expression of the tolerogenic non-classical class I HLA-G molecule in primary human mesothelial cells, leading to impairment of NK cell recognition and killing of infected cells . With reference to the NK cell component of the immune response, HHV-6A was reported to establish a productive infection in CD3-negative NK cell clones, leading to the *de novo* expression of CD4 on the NK cell surface , and HHV-6B was recently

shown to induce down-modulation of the activating NKG2D ligand in infected cells (Schmiedel *et al*., 2016).

Notably, it has been recently reported that NK cells may be directly involved in the onset and progression of autoimmune diseases, through their potential autoreactivity or through their interaction with the other immune cells, thus supporting the hypothesis of a correlation between HHV-6A/6B infection, NK cell function and autoimmunity (Rizzo *et al*., 2017).

2.2.8.2. Adaptive Immune Responses To HHV6 Infection

I. Cellular Response to HHV-6

Information on HHV-6-specific T cell responses is still limited, in particular regarding CD8 T cells . It was shown early that healthy virus carriers have CD4 T cells that respond to HHV-6 lysate or infected cells (Becerra *et al*.,2014).

Target antigens and epitopes of the specific CD4 T cell response were identified first in a study on six selected structural proteins , and more recently by a proteomic approach that has identified ten viral antigens targeted by CD4 T cells (Becerra-Artiles *et al*.,2015).

Responses to five HHV-6B proteins have been investigated so far, and a number of epitopes from these proteins that are presented by infected cells were identified Both CD4+ and CD8+ HHV-6 specific T-cells have been isolated from the PBMCs of healthy donors. In the case of both CD4+ and CD8+ the frequency of HHV-6 specific T-cells is low, but the responding population could be expanded in vitro . Expanded CD4+ and CD8+ populations characteristically secrete IFNγ and TNFα,

while also performing cytolytic functions mediated by the secretion of perforin (CD4+) or granzyme B (CD8+) (Martin *et al*.,2018).

Additionally, CD8+ clones were found to also secrete granulocyte macrophage stimulating factor. Studies on T-cell response to HHV6 have identified 8 HLA-DR restricted CD4+epitopes and 5 HLA-A restricted CD8+ epitopes (Becerra-Artiles *et al*.,2015).

II. Antibody Response to HHV-6

During primary infection, anti-IgG and anti-IgM antibodies are produced, with IgM antibodies being the first to be detected . IgG titer begins to increase about one week post infection and peaks a week later. Additionally, there is an increase in IgG avidity over the course of infection (Ward ,2013).

Interestingly, 57% of individuals with CIHHV-6 had antibodies to the IE-A antigen present in the serum, while IE-A antibodies were undetectable in non-CIHHV-6 individuals. On the other hand, 14% of CIHHV-6 versus 60% of the healthy individuals had an antibody response to gB . Glycoprotein B is considered one of the major neutralizing epitopes and variant specific gB antibodies have been detected . The absence of gB antibodies or reduced antigB titers may reduce the ability of CIHHV-6 individuals to mount an immune response to an exogenous HHV6 infection(Agut *et al*.,2015).

2.3. Toll -Like Receptor-2 (TLR2)

2.3.1 Definition

TLR2 is a protein that in humans is encoded by the TLR2 gene.TLR2 has also been designated as CD282 (cluster of differentiation 282). TLR2 is one of the toll

Like receptor and plays a role in the immune system (*Nachtigall et al*.,2014).

2.3.2. Location of TLR 2

TLR2 is expressed on microglia, Schwann cells, monocytes, macrophages, dendritic cells, polymorphonuclear leukocytes (PMNs or PMLs), B cells (B1a, MZ B, B2), and T cells, including Tregs (CD4+CD25+ regulatory T cells) (Cario ,2008).

2.3.3. Structure of TLR 2

The N-terminal ectodomains (ECDs) of TLR 2 (human or mouse) have been reported (table 2-1) (Istvan et al.,2011).

Table 2.1:Main features of the human TLR-2 molecules (Istvan *et al*.,2011)**.**

TLR2	Residues	LRRs[a]	N-linked glycosylation sites[b]	Accession code
2	784	19	3 (4)	O60603

[a]The number of LRRs in the extracellular domain do not include the LRR-NT or LRR-CT motifs.

[b]Number of N-glycosylation sites observed in the crystal structure or predicted by the NetNGlyc server 1.0 in parentheses (http://www.cbs.dtu.dk/services/NetNGlyc/).

All ECDs assume the typical horseshoe-shape, but their structures cannot be superimposed because of variations in curvature. In the known structures, the glycans are distributed throughout the molecule, except for the lateral face formed

by the ascending loops of the LRRs (Jin *et al*., 2007). This glycan-free face is involved in dimerization upon ligand binding in the known TLR/ligand structures (Istvan *et al*.,2011).

2.3.4. Function of TLR 2

TLR2 is a membrane protein, a receptor, which is expressed on the surface of certain cells and recognizes foreign substances and passes on appropriate signals to the cells of the immune system (Nachtigall *et al*.,2014). In the intestine, TLR2 regulates the expression of CYP1A1, which is a key enzyme in detoxication of carcinogenic polycyclic aromatic hydrocarbons such as benzo(a)pyrene (Do KN *et al* .,2012).

TLR2 recognize many bacterial, fungal, viral ,and certain endogenous substances. In general, this results in the uptake (internalization, phagocytosis) of bound molecules by endosomes/phagosomes and in cellular activation; thus such elements of innate immunity as macrophages, PMNs and dendritic cells assume functions of nonspecific immune defense, B1a and MZ B cells form the first antibodies, and specific antibody formation gets started in the process. Cytokines participating in this include tumor necrosis factor-alpha (TNF-α) and various interleukins (IL-1α, IL-1β, IL-6, IL-8, IL-12). Before the TLRs were known, several of the substances mentioned were classified as modulins. Due to the cytokine pattern, which corresponds more closely to T_h1, an immune deviation is seen in this direction in most experimental models, away from T_h2 characteristics. Conjugates are being developed as vaccines or are already being used without a priori knowledge(Nachtigall *et al*.,2014).

2.3.4.Relationship Between TLR 2 Polymorphism And Abortion

Toll-like receptor single-nucleotide polymorphisms induce individual variation in the quality of identification of infectious agents during pregnancy. Mutant TLR SNPs are often associated with imbalance in the system of innate immunity and, as a result, an increase in mother organism sensitivity to the infections and the development of chronic inflammatory processes, which increase the risk of miscarriage (Skevaki *et al*.,2015; Razdaibiedina *et al*.,2018).

TLR2 gene plays an important role in pathogen recognition or other inflammatory stimuli, initiating the signalling cascades and directing the interactions between the immune and reproductive systems (Amjadi *et al*.,2014).

Expression of TLR2 gene is genetically controlled and the patients heterozygous for guanine (G) 2258A had reduced production levels of IFN-γ suggesting that altered TLR2 responsiveness might contribute to the course of infections (Dalgic et al.,2011).and weakened TLR2-IFN-γ signalling have been in differential clinical presentations (Woods *et al*.,2009).

2.4 .Interleukin -10 (IL-10)

2.4.1. Definition

Interleukin 10 (IL-10), also known as human cytokine synthesis inhibitory factor (CSIF), is an anti-inflammatory cytokine. In humans, interleukin 10 is encoded by the IL10 gene. IL-10 signals through a receptor complex consisting of two IL-10 receptor-1 and two IL-10 receptor-2 proteins. Consequently, the functional receptor consists of four IL-10 receptor molecules. IL-10 binding

induces STAT3 signalling via the phosphorylation of the cytoplasmic tails of IL-10 receptor 1 + IL-10 receptor 2 by JAK1 and Tyk2 respectively (Mosser *et al*.,2008).

2.4.2. Gene and protein structure

The IL-10 protein is a homodimer; each of its subunits is 178-amino-acid long. IL-10 is classified as a class-2 cytokine, a set of cytokines including IL-19, IL-20, IL-22, IL-24 (Mda-7), IL-26 and interferons type-I (IFN-alpha, -beta, -epsilon, -kappa, -omega), type-II (IFN-gamma) and type-III (IFN-lambda, also known as IL-28A, IL-28B, and IL-29) (Lazear *et al*.,2015).

2.4.3. Expression and synthesis

In humans, IL-10 is encoded by the IL10 gene, which is located on chromosome 1 and comprises 5 exons, and is primarily produced by monocytes and, to a lesser extent, lymphocytes, namely type-II T helper cells (T_H2), mast cells, $CD4^+CD25^+Foxp3^+$ regulatory T cells, and in a certain subset of activated T cells and B cells. IL-10 can be produced by monocytes upon PD-1 triggering in these cells. IL-10 upregulation is also mediated by GPCRs, such as beta-2 adrenergic and type 2 cannabinoid receptors(*Ağaç et al*.,2018).

The expression of IL-10 is minimal in unstimulated tissues and seems to require triggering by commensal or pathogenic flora. IL-10 expression is tightly regulated at the transcriptional and post-transcriptional level. Extensive IL-10 locus remodeling is observed in monocytes upon stimulation of TLR or Fc receptor pathways. IL-10 induction involves ERK1/2, p38 and NF-κB signalling and transcriptional activation via promoter binding of the transcription factors NF-κB and AP-1(Saroz *et al*.,2019).

IL-10 may autoregulate its expression via a negative feed-back loop involving autocrine stimulation of the IL-10 receptor and inhibition of the p38 signaling pathway. Additionally, IL-10 expression is extensively regulated at the post-transcriptional level, which may involve control of mRNA stability via AU-rich elements and by microRNAs such as let-7 or miR-106(Schulte *et al*.,2011).

2.4.4.Function

IL-10 is a cytokine with multiple, pleiotropic, effects in immunoregulation and inflammation. It downregulates the expression of Th1 cytokines, MHC class II antigens, and co-stimulatory molecules on macrophages. It also enhances B cell survival, proliferation, and antibody production. IL-10 can block NF-κB activity, and is involved in the regulation of the JAK-STAT signaling pathway (Iyer *et al*.,2012).

IL-10 was initially reported to suppress cytokine secretion, antigen presentation and CD4+ T cell activation. Further investigation has shown that IL-10 predominantly inhibits lipopolysaccharide (LPS) and bacterial product mediated induction of the pro-inflammatory cytokines TNFα, IL-1β, IL-12, and IFNγ secretion from Toll-Like Receptor (TLR) triggered myeloid lineage cells(Sabat *et al*.,2010).

2.4.5.Relationship between IL-10 polymorphism and abortion

Numerous studies to date have investigated the association between IL-10 gene polymorphisms and URSA and yielded inconsistent results. Therefore, we performed this meta analysis to further clarify the association between IL-10 polymorphisms (-1082A/G,-819C>T, -592C>A, IL-10 haplotypes) in the promoter region and URSA risk **(**Jung *et al*.,2015; Chenglei *et al*.,2018**).**

Several studies have analyzed the possible correlations between 3 polymorphisms present at the level of the promoter of the codifying gene for IL10 and the abortion disease, namely polymorphisms -592C/A, -819C/T and -1082A/G. Conclusions varied based on the researched population groups (Kamali *et al*.,2005; Bohiltea *et al*.,2014).

The-1082A>G polymorphismis located within a putative Ets transcription factor binding site, whereas the -819C>T lies within a putative positive regulatory region.The-592C>A is located within a putative STAT-3 binding site and negative regulatory region (Chenglei *et al*.,2018).

2.5. Interleukin -18 (IL-18)

2.5.1 Definition

Interleukin-18 (IL18, also known as interferon-gamma inducing factor) is a protein which in humans is encoded by the IL18 gene. The protein encoded by this gene is a proinflammatory cytokine. Many cell types, both hematopoietic cells and non-hematopoietic cells, have the potential to produce IL-18 (Yasuda et al.,2019).

Originally, IL-18 production was recognized in Kupffer cells, liver-resident macrophages. However, IL-18 is constitutively expressed in non-hematopoietic cells, such as intestinal epithelial cells, keratinocytes, and endothelial cells. IL-18 can modulate both innate and adaptive immunity and its dysregulation can cause autoimmune or inflammatory diseases(Baker *et al*.,2019).

2.5. 2.Processing

Cytokines usually contain the signal peptide which is necessary for their extracellular release. In this case, IL18 gene, similar to other IL-1 family members, lacks this signal peptide. Furthermore, similar to IL-1β, IL-18 is produced as a biologically inactive precursor. IL-18 gene encodes for a 193 amino acids precursor, first synthesized as an inactive 24 kDa precursor with no signal peptide, which accumulates in cell cytoplasm. Similarly to IL-1β, the IL-18 precursor is processed intracellularly by caspase 1 in the NLRP3 inflammasome into its mature biologically active molecule of 18 kDa (Kaplanski, 2018).

2.5 3. Receptor and Signaling

IL-18 receptor consists of the inducible component IL-18Rα, which binds the mature IL-18 with low affinity and the constitutively expressed co-receptor IL-18Rβ. IL-18 binds the ligand receptor IL-18Rα, inducing the recruitment of IL-18Rβ to form a high affinity complex, which signals through the toll/interleukin-1 receptor (TIR) domain. This signaling domain recruits MyD88 adaptor protein that activates proinflammatory programs and NF-κB pathway. The activity of IL-18 can be suppressed by extracellular interleukin 18 binding protein (IL-18BP) that binds soluble IL-18 with a higher affinity than IL-18Rα thus prevents IL-18 binding to IL-18 receptor(Jia and Han, 2018).

IL-37 is another endogenous factor that suppresses the action of IL-18. IL-37 has high homology with IL-18 and can bind to IL-18Rα, which then forms a complex with IL-18BP, thereby reduces the activity of IL-18. Moreover, IL-37 binds to single immunoglobulin IL-1 receptor related protein (SIGIRR), also

known as IL-1R8 or TIR8, which forms a complex with IL-18Rα and induces an anti-inflammatory response. The IL-37/IL-18Rα/IL-1R8 complex activates the STAT3 signaling pathway, decreases NF-κB and AP-1 activation and reduces IFNγ production. Thus, IL-37 and IL-18 have opposing roles and IL-37 can modulate pro-inflammatory effects of IL-18 (Kaplanski,2018).

2.5.4. Function

IL-18 belongs to the IL-1 superfamily and is produced mainly by macrophages but also other cell types, stimulates various cell types and has pleiotropic functions. IL-18 is a proinflammatory cytokine that facilitates type 1 responses. IL-18 in combination with IL12 acts on CD4, CD8 T cells and NK cells to induce IFNγ production, type II interferon that plays an important role in activating the macrophages or other cells. The combination of this IL-18 and IL-12 has been shown to inhibit IL-4 dependent IgE and IgG1 production and enhance IgG2a production in B cells (Zouboulis and Altenburg,2021).

Importantly, without IL-12 or IL-15, IL-18 does not induce IFNγ production, but plays an important role in the differentiation of naive T cells into Th2 cells and stimulates mast cells and basophils to produce IL-4, IL-13, and chemical mediators such as histamine (*Yasuda et al.,2019)*.

2.5.5. Relationship Between IL-18 Polymorphism And Abortion

IL-18 plasma concentrations are higher in women with successful pregnancies than in women with RPL. Variants of genes alter the corresponding protein expression levels. Therefore, it was necessary to assess the global frequencies of the variant alleles of interleukins that might cause RPL (Barbaux *et al*.,2007).

Single-nucleotide polymorphisms (SNPs) are variations at a single nucleotide position in DNA sequence among individuals. If more than one percent of the population carries the different nucleotide at a specific position in the DNA sequence, this variation is defined as a SNP. They can occur in non-coding regions, such as the promoters, and coding regions, such as the gene body at a total frequency of approximately every 200–1000 bases. SNPs in promoters are suspected to affect transcription factor binding, which may in turn influence interleukin-18 production and thus be associated with RPL. While SNPs primarily originate as genetic adaptions, genetic recombination and mutations. The biggest difference between SNPs and mutations is that SNPs are inherited (Zhang *et al*.,2017).

2.6. Major histocompatibility complex antigen, class I, G (HLA-G)

2.6.1 Definition

HLA-G also known as human leukocyte antigen G (HLA-G), is a protein that in humans is encoded by the HLA-G gene. HLA-G belongs to the HLA nonclassical class I heavy chain paralogues. This class I molecule is a heterodimer consisting of a heavy chain and a light chain (beta-2 microglobulin). The heavy chain is anchored in the membrane (Darbas *et al*.,2021).

HLA-G is expressed on fetal derived placental cells. The heavy alpha chain is approximately 45 kDa and its gene contains 8 exons. Exon one encodes the leader peptide, exons 2 and 3 encode the alpha1 and alpha2 domain, which both bind the peptide, exon 4 encodes the alpha3 domain, exon 5 encodes the transmembrane region, and exon 6 encodes the cytoplasmic tail. Exon 7 and 8 are not translated due to a stop codon present in exon 6 (Castelli *et al*.,2011).

There are two types of NK cells , peripheral blood NK cells (pb NK cells) ,from 90% in peripheral blood expressing CD56 ,CD16 on their surface and have cytotoxic activity ;and the other type is uterine NK cells (uNK cells) ,represent 90% in uterine , expressing CD56 CD16 on their surface and produce certine cytokines (IFN-Ɣ,TNF-α , IL-10 and 6M-CSF), also express immunoglobulin – Like Transcript Receptor that binds with its ligand HLA-6 express on placental trophoblast cells (Sacks,2014).

2.65.2 Function

HLA-G may play a role in immune tolerance in pregnancy, being expressed in the placenta by extravillous trophoblast cells (EVT), while the classical MHC class I genes (HLA-A and HLA-B) are not. As HLA-G was first identified in placenta samples, many studies have evaluated its role in pregnancy disorders, such as preeclampsia and recurrent pregnancy loss (Michita *et al*.,2016).

Its downregulation is related to HLA-A and -B downregulation results in protection from cytotoxic T cell responses, but would in theory result in a missing self response by natural killer cells. HLA-G is a ligand for NK cell inhibitory receptor KIR2DL4, and therefore expression of this HLA by the trophoblast defends it against NK cell-mediated death (Lash *et al*.,2010).

However, a large family with several members bearing only "null" HLA-G alleles has been found. None of these homozygous subjects have pregnancy or birth difficulties; nor do they present immunodeficiencies, autoimmune diseases, or tumors. It is striking that this "null" allele (HLA-G*01:05N), while it is quite frequent in some populations, like in Iranians, it is almost absent in some Amerindian populations (Arnaiz-Villena *et al*.,2013).

Also, some higher primates do not show all MHC-G isoforms. In addition,

Cercopithecinae middle-sized Old World monkeys do not bear full MHC-G molecules since all of these monkeys present stop codons at MHC-G DNA (Rebmann *et al*.,2010).

2.6.3. Relationship Between HLA-G Polymorphism And Abortion

During pregnancy, maternal and fetal immune cells are in close contact. It has been suggested that the human leukocyte antigen G (HLA-G), contributes to the maintenance of pregnancy by the protecting semi-allogeneic fetus against the cytotoxic activity of maternal natural killer cells .HLA-G proteins contribute towards improvement in the survival of the allogeneic fetus by inhibiting maternal T-cell proliferation, inducing IL-10 production in peripheral blood monocytes and placental trophoblast cells, and severe angiogenesis regulation (Divya *et al*., 2015).

Polymorphisms in the 5′ URR and 3′ UTR regions of the HLA-G gene has been found to be associated with differences in HLA-G expression, which may affect HLA-G function and may play an important role in the regulation of pregnancy (Berger *et al*., 2010). A 14bp insertion–deletion polymorphism (5′-ATTTGTTCA TGCCT-3′) in the 3′ UTR of the HLA-G is located at position 3741 in exon 8, which has been associated with pregnancy outcome (Christiansen *et al*., 2012).

Reports have been conflicting on the association of HLA-G polymorphisms with pregnancy outcomes (idiopathic RSA, sporadic spontaneous abortions and preeclampsia) in different ethnic groups (Yie et al., 2008; Larsen et al., 2010).

In some studies, HLA-G polymorphisms have been shown to be associated with pregnancy outcomes, whereas other studies have found no association .Furthermore, HLA-G polymorphisms in 5′URR have not been fully explored in among Indian populations experiencing idiopathic RSA. This present study has

been undertaken to fill this gap (Larsen *et al*., 2010; Divya *et al*., 2015).

The studies on Americans, German and Indian women support the association of particular HLA-G genotypes with RSA Aldrich *et al*., (2001); Pfeiffer *et al*., (2001) and Abbas *et al*., (2004), respectively.

2.7. Diagnostic of TLR-2; IL-10; IL-18 and HLA-G Polymorphism

1. Single Strand Conformation Polymorphism (SSCP)

SSCP analysis is a simple and sensitive technique for mutation detection and genotyping. The principle of SSCP analysis is based on the fact that single-stranded DNA has a defined conformation. Altered conformation due to a single base change in the sequence can cause single-stranded DNA to migrate differently under no denaturing electrophoresis conditions (Satyanarayan *et al*.,2019).

2. Restriction Fragment Length Polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) is a technique that exploits variations in homologous DNA sequences, known as polymorphisms, in order to distinguish individuals, populations, or species or to pinpoint the locations of genes within a sequence. The term may refer to a polymorphism itself, as detected through the differing locations of restriction enzyme sites, or to a related laboratory technique by which such differences can be illustrated. In RFLP analysis, a DNA sample is digested into fragments by one or more restriction enzymes, and the resulting restriction fragments are then separated by gel electrophoresis according to their size (Reema and Ganesh,2019).

3. Amplification Refractory Mutation System (ARMS)

In ARMS technique, one PCR comprises one allele-specific oligonucleotide primer at 5′-end and a common primer at 3′-end. If the presence of an amplified mutant is detected by agarose gel electrophoresis, it suggests that the target sequence contains the mutant allele. Similarly, if the result displays an absence of the amplified mutant, it indicates the presence of the normal DNA sequence on that specific point.In the same way, a normal primer at 5′-end together with a common primer at 3′-end was used in another PCR. If normal amplified product is present, it reveals the existence of a natural DNA sequence, whereas if normal amplified product is absent, then it reveals the presence of a mutant allele (Lisha *et al*.,2018).

4.Sequencing of PCR Products

The term DNA sequencing refers to methods for determining the order of the nucleotides bases adenine,guanine,cytosine and thymine in a molecule of DNA. The first DNA sequence were obtained by academic researchers,using laboratories methods based on 2- dimensional chromatography in the early 1970s. By the development of dye based sequencing method with automated analysis,DNA sequencing has become easier and faster. The knowledge of DNA sequences of genes and other parts of the genome of organisms has become indispensable for basic research studying biological processes, as well as in applied fields such as diagnostic or forensic research (Hao and Stephen ,2018**)**.

CHAPTER THREE

MATERIALS & METHODS

3. Materials and Methods

3.1. Subjects

3.1.1 Patients population

This case control study was done for a one hundred-fifty specimens collected from female patients subjected to recurrent pregnancy loss *and* apparently healthy persons as control group from general hospitals as well as many private clinical in Diyala province and Middle Euphrates -Iraq. The age range of the study population was 17 years to 49 years. The specimens were collected during period from February 2020 to September 2021.

Endometrium ; Cervical swabs ; fetal fluids swabs as well as Blood from each study group of female patients suffering from recurrent pregnancy loss should be enrolled, that classify into:-.

1. One –hundred endometrium ; cervical swabs ; fluids swabs as well as blood specimens from women suffering from recurrent Miscarriage .

2. Fifty blood and cervical swabs specimens of apparently healthy persons as control group.

All these specimens were submitted for genetic part for screening human Herpes virus-6 (HHV-6) in patients and apparently healthy person control groups by real time polymerase chain reaction (RT-PCR). the second part for detection SNPs of TLR2rs1898830 , IL10 rs4129009 ,IL18, and HLA-G gene polymorphism by amplification –refractory mutation system (ARMS).

3.1.2 Specimens Collection

Endometrium and\or cervical swabs ; fetal fluids swab as well as blood specimens were collected from patients and AHC by using three swabs for each patient:

The first is the flocked swab regular for endometrium ; cervical swabs collection, according to Catalog Number 21031 (Heinz, Herenz; Germany). The second one is the flocked swab for fetal fluids swabs collection, according to Catalog Number 80503CS (Copan, Italy).

The three swabs were taken and mixed together in a 3-ml universal transport medium (UTM) tube which provided with the flocked swab regular Catalog Number 21031 (Heinz, Herenz; Germany).

Each specimens was aliquot into three 1.8 cryotube (Nunc-Kamstrup, Denmark) and stored at (-20°C) at the Virology Research Unit, College of Science, Babylon University . 5ml venous blood were collected aseptically from all patients by using gel tubes and EDTA tubes for gating blood serum and buffy coat ,respectively; then stored at (-20°C).

I.Inclusion Criteria

Women with age ranged from 17 to 49 years old with unexplained miscarriage till 24 weeks of pregnancy were taken as cases, while women with full-term pregnancy during the conduction of cesarean operation have more than one successful pregnancy were taken as controls.

II.Exclusion Criteria

Women with other causes of miscarriage such as endocrine disorder (diabetes mellitus, thyroid disorder), anatomical causes acquired or congenital thrombophilia and other infection causes miscarriage such as toxoplasmosis, cytomegalovirus infection, rubella and herpes simplex-1 and 2 (HSV1 &HSV2).

3.2 Study Design

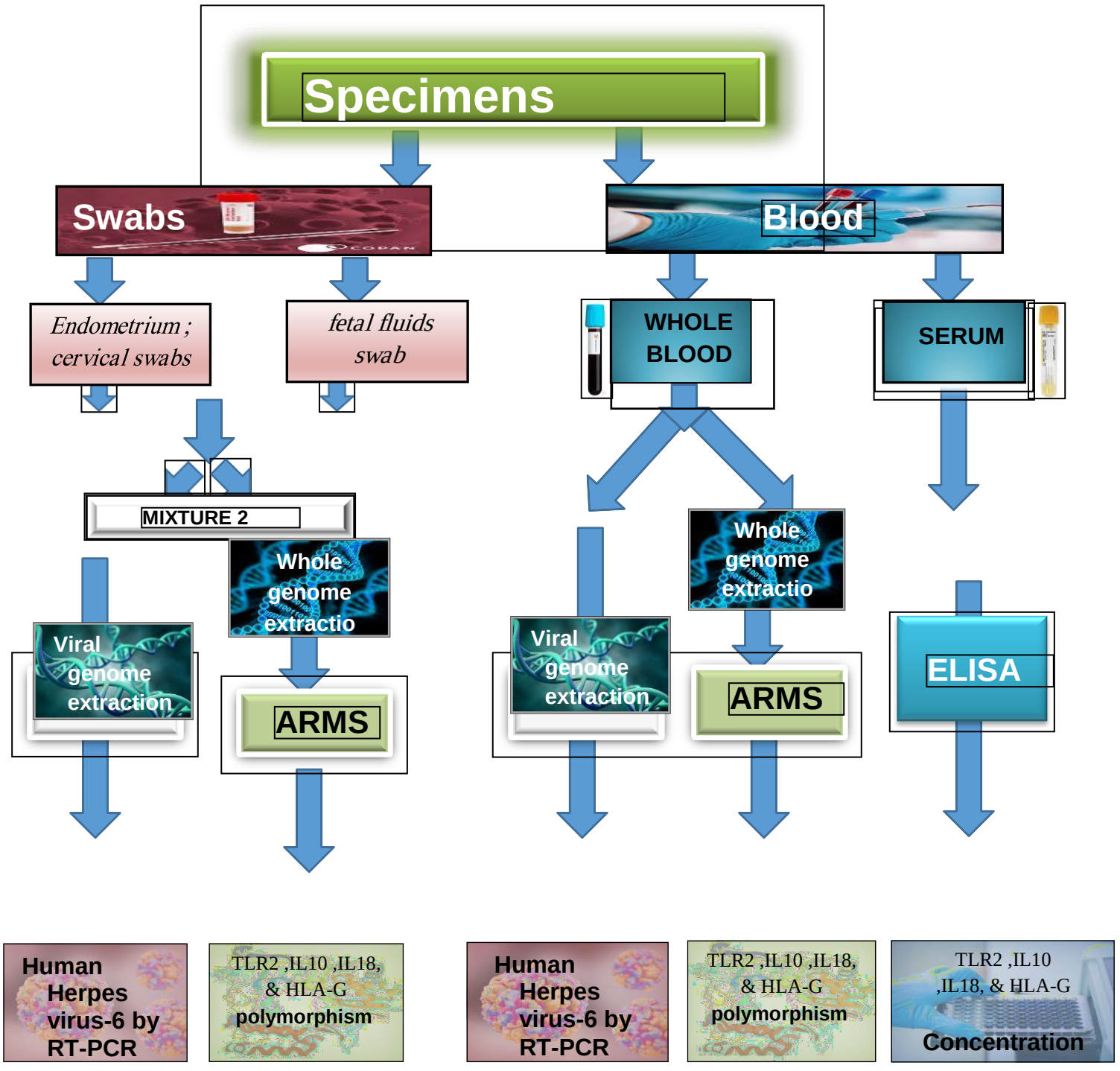


Figure 3.1: Study Design

3.3. Materials

3.3.1. Instruments and Equipment

Instruments and equipment used in this study are listed in table (3-1 A and B).

Table 3.1.A: Instruments used in this study with their Manufacturer Company and origin

Instruments	Manufactured Company (Origin)
Centrifuge	Hettichzentrifuge, Sigma / Germany
Deep freeze (-20 C)	Bosch / Germany
Electrophoresis system	Germany
ELISA reader	Bio Tech (USA)
Fume hood	UK
Hot plate	Memmert / Germany
Incubator (model IB-909)	
Micro centrifuge	Hettichzentrifuge, Sigma / Germany
Multibiodrop	England
Real time Qiagen device Rotor Gene Q MDx	QIAgen / Swaziland
Real time soft wear system	
Refrigerator	Arcelik / Turkey
Sensitive balance	Sartoris / Germany
Timer with alarm	Junghans / Germany
Ultraviolet imaging device	Motic / USA
Water bath (1-A)	Memmert / Germany

Table 3.1.B: Equipment used in this study with their Manufacturer Company and origin.

Equipment	Manufactured Company (Origin)
0.2ml PCR Tubes with Flat Caps	Extragene / Taiwan
1.5ml clear Microtubes	
Disposable plastic container	China
Disposable tips	Extragene/Taiwan
Eppendorf tubes with different size 2ml, 1.5ml, 0.5 ml	
Falcon with different size 15ml , 50 ml	China
Flask with different size 25cm^2 , 75cm^2	
Gel loading tips	Bio Basic – Canada
Gloves	
Micropipettes various sizes(1000,100,10) µl	Extragene/Taiwan
Plastic scraper	China
VIR-Swab	Heinz Herenz / Germany
	Copan / Italy

3.3.2 Reagents and Buffers:

Reagents and buffers used in this study are listed in table (3-2)

Table 3.2: Reagents and buffers used in this study with their Manufacturer Company and origin.

Reagents	Manufactured Company (Origin)
10X TBE DNA Sequencing Grade	Intron /S. Korea
Absolute Ethanol alcohol	Merck – Germany
Agarose E	Conda / Spain
DNA Ladder(100bp)	Intron /S. Korea
Proteinase K	Intron /S. Korea
Rad Safe Nucleic Acid staining solution	Zymo Research / USA
RNase A	

3.3.3 Kits and Marker

Kits and marker used in this study with their Manufacturer Company and origin are listed in table (3-3).

Table 3.3: Kits and markers used in this study with their Manufacturer Company and origin

Kits	Manufacturer Company/ Origin	CAT.NO=
G-Spin Total DNA Extraction Kit	Intron / Korea	14001
Human TLR2	Boster Biological Technology \USA	EK0906
Human IL-10	Boster Biological Technology \USA	EK0416
Human IL-18	Boster Biological Technology \USA	EK0864
Human HLA-G	Boster Biological Technology \USA	M 01235-2
Viral Nucleic Acid Extraction kit	Intron / Korea	17151
Human Herpes Virus Real-Time PCR kit	AmpliSens \Russia	R-V10-T(RG,iQ,Mx)-CE

3.3.4 Primes of TLR-2(rs1898830) , IL-10 rs1518111 , IL-18 rs187238, and HLA-G rs1063320 SNPs.

Primers sets used in this study to detect the SNPs of TLR-2(rs1898830) , IL10 rs1518111 , IL18 rs187238, and HLA-G rs1063320& rs1610696 polymorphism with their product size and source as well as origin are listed in table (3-4).

Table 3.4: Primers sets that used for detection of TLR2 ,IL10 ,IL18, and HLA-G gene polymorphism.

Gene	Sequence (5′-3′)	Product size (bp)	Source/origin
TLR-2(rs1898830) IF	GGAATAGTAAAATAAATCCAGAGAAAGC	**A allele: 162** **G allele: 108** **two outer primers: 270**	IDT / USA
TLR-2(rs1898830) IR	ATTCTTATATTATTATTTCCCCTGTGCC		IDT / USA
TLR-2(rs1898830)OF	ATGAAGAGTGACGAAAAATGAATGA		IDT / USA
TLR-2(rs1898830)OR	GTACCAGCTTTTATTGTCTTGCCA		IDT / USA
IL10 rs1518111 (IF)	GGCGCTGTGTAAGTAGCAGATCAGGTT	**T allele: 183** **C allele: 133** **two outer primers: 316**	IDT / USA
IL10 rs1518111 (IR)	GGGGCAGCTGCAAGGGACAG		IDT / USA
IL10 rs1518111 (OF)	CACGTTTCTATGGTTCCAAATTTCA		IDT / USA
IL10 rs1518111 (OR)	CGCCTGCTCAAAGAGAAATGAGCA		IDT / USA
IL18 rs187238 (IF)	CCCAACTTTTACGGAAGAATAG	**G allele: 178** **C allele: 210** **two outer primers: 388**	
IL18 rs187238 (IR)	ATGTAATATCACTATTTTCATGAATTG		IDT / USA
IL18 rs187238 (OF)	TTGTAAAATAAGAAAAAGTTTTGTGC		IDT / USA
IL18 rs187238 (OR)	GGAGAGAATGAGGAAGAAGGT		IDT / USA
HLA-G rs1063320 (IF)	TTAATACAGAAGTAAGTTATAGCTCAGAGC	**C allele: 245** **G allele: 178** **two outer primers: 423**	IDT / USA
HLA-G rs1063320 (IR)	TCTCTGTCTCAAATTTGTGCTC		IDT / USA
HLA-Grs1063320 (OF)	ACTGTGGAAAGTTCTCATGTCTT		IDT / USA
HLA-Grs1063320 (OR)	ATTTTCTGTAGTGTGAAACAGCTG		IDT / USA

3.4. Methods

3.4.1 Human Herpes virus detection by Real Time –PCR POWER CHEK :

I. Principle of the Method:

Real-time PCR (qPCR) is based on two major processes: **Firstly** ,isolation of viral genome (DNA) from specimens, and **Secondly**, Real Time amplification for each specimens. In real-time PCR (qPCR), the accumulating amplified product can be detected at each cycle with fluorescent dyes. This increasing signal allows to achieve sensitive detection and quantification of pathogens.

3.4.1.1. Extraction of Viral Nucleic Acid from Clinical Specimens :

By using specific viral DNA/RNA extraction kit (Intron/Korea); the viral genomic was extracted ,purifying and migrated using agarose gel from the endometrium; cervical swabs and fetal fluid as a first step to amplify the target Herpes virus DNA.

I. Specimens preparation:

Endometrium and\or cervical swabs ; fetal fluids swab were mixed together in 3 ml liquid viral transport media tube (UTM),each specimen was aliquot into three cryotube containing 1000 µl of the sample which stored at (-20ºc) until genome extraction. After that, required part of specimens were taken and centrifuged at 10000 g/min for 5 minutes, discarded the supernatant except 100 µl of the solution was left to be used in re-suspension of the pellet for RNA/DNA extraction.

II. Assay Procedure:

The procedure was carried out in accordance with the manufacturer's instruction with some modification as following:

1. Three hundred (300) µl from cell-culture supernatant or blood plasma was transferred into 1.5ml micro centrifuge tube.
2. A 600 µl Lysis buffer was added, then the lysate mixed by vortex for (25sec). Mixture was incubated at room temperature for (15 min). DNase (20) µl (modification).
3. Six hundred µl from binding buffer was added, and completely mix well by gently vortex. This step is conducive efficient passage of cell lysates through a column and to increase binding onto column resins and important for effective deproteinization.
4. The lysates of cell was placed in a spin column that provided 2m collection tube.
5. Loaded lysates on the column and centrifuged for 2 min at 13,000 g.
6. Discarded solution in collection tube and place the spin column back in the same (2ml) collection tube .
7. A 500 µl of washing buffer A was added to spin column and centrifuged for 2 min at 13,000 g.
8. The solution was discarded in collection tube and places the spin column back in the same (2ml) collection tube and centrifuged for 2 min at 13,000 g and then Discarded solution .
9. Five hundred µl of washing buffer B was added to the spin column and centrifuged for 1min at 13,000 g.
10. The solution was discarded in collection tube and places the spin column back in the same (2ml) collection tube. Centrifuged for 1min at 13,000g. It is important to dry the membrane since residual ethanol may interfere with downstream reactions.

11. Placed spin column in an RNase-free (1.5ml) microcentrifuge tube; then 50 µl of Elution buffer was added directly onto the membrane and was incubated at RT for 2min, then centrifuged for 2min at 13,000 g.
12. At this stage the supernatant was containing viral genome (RNA).

3.4.1.2. Estimation of the extracted RNA and DNA concentration and purity:

After extraction of viral RNA and DNA from samples ; the concentration of RNA and DNA yield and purity were measured by using Mlite bio drop (England) , by appling 5 µl of the extracted RNA and DNA in the instrument curette. Extracted with purity in between (1.7-1.9)at absorptionwave length 260/280 was included in this study ,otherwise ; RNA/DNA extraction of the sample was repeated.

3.4.1.3. Detection of Human Herpes virus -6 (HHV-6) infection by Real Time –PCR det-qPCR:

Human Herpes virus Detection by Real-time RT-PCR assay is a qualitative Duplex one-step Real-time RT-PCR test, AmpliSens® HHV6-screen-titre-FRT PCR kit is an in vitro nucleic acid amplification test used for qualitative detection of human herpes virus type 6 (HHV6) DNA in the clinical material (whole human blood and swabs) using real-time hybridization-fluorescence detection of amplified products.

1.Provided Materials

Provided materials of AmpliSens® HHV6-screen-titre-FRT PCR kit used for HHV-6 detection are listed in table (3-5).

Table 3.5: Provided materials of AmpliSens® HHV6-screen-titre-FRT RT-PCR

Reagent		*Description*	*Volume, ml*	*Quantity*
PCR-mix-1-FRT *HHV*-6 / Glob		clear liquid from colorless to light lilac colour	0.6	2 tubes
PCR-mix-2-FRT		colorless clear liquid	0.3	2 tubes
Polymerase (TaqF)		colorless clear liquid	0.03	2 tubes
RNA-buffer		colorless clear liquid	0.6	1 tube
DNA calibrators	KSG1	colorless clear liquid	0.2	1 tube
	KSG2	colorless clear liquid	0.2	1 tube
Negative Control (C–)*		colorless clear liquid	1.2	2 tubes
Positive Control DNA *HHV*-6 and human DNA**		colorless clear liquid	0.2	2 tubes

2. Additionally Required Materials, Reagents And Devices

1- Nuclease free water

2- Disposable powder-free gloves and laboratory coat

3- Pipettes (capacity 0.5~10 µl , 2~20 µl , 20~200 µl , 200~1000 µl) with aerosol barriers

4- Sterile pipette filter tips with aerosol barriers

5- DNA Isolation Kit

6- Ice maker

7- Vortex mixer

8- Clean bench or PCR box

9- Desktop centrifuge with rotor for 2 ml reaction tubes

10- Real -time thermocyclers (for example, PowerAmp96TM series, AB7500 StandardTM, AB7500 FastTM, Roche LC480TM, Bio-Rad

CFX96TM, Agilent Mx3005PTM and Qiagen RotorGene QTM etc. with FAM and Cy5 detection channels).

11- Disposable polypropylene microtubes for PCR.

I. Preparing tubes for PCR

The total reaction volume is 25 µl, the volume of DNA sample is 10 µl. The type of tubes depends on the type of PCR real-time instrument. Use disposable filter tips for adding reagents, cDNA and control samples into tubes.

1. Prepared the mixture of PCR-mix-2-FRT and Polymerase (TaqF). For this purpose transferred the content of one tube with Polymerase (TaqF) (30 µl) into the tube with PCR-mix-2-FRT (300 µl). Vortexed carefully avoiding foaming. Marked the tube with the mixture preparation date.

2. The reaction mixture was prepared. Note that, for analyzed of even one DNA sample in the qualitative format, it is necessary to run two controls of amplification:

1. Positive Control of Amplification (KSG2) .

2. Negative Control of Amplification (RNA-buffer).

3. Mixed PCR-mix-1-FRT HHV-6 / Glob and the mixture of PCR-mix-2-FRT and polymerase (TaqF) prepared earlier in an individual tube in the following proportion:

- 10 µl of PCR-mix-1-FRT HHV-6 / Glob.
- 5 µl of PCR-mix-2-FRT and polymerase (TaqF) mixture.

Calculate the required number of reactions including the test and control samples as shown in table (3-6).

Table 3.6: Scheme of reaction mixture preparation.

Total reaction volume is 25 µl including the volume of DNA sample – 10 µl.			
Reagent volume per 1 reaction, µl		10,0	5,0
Number of clinical samples		PCR-mix-1-FRT *HHV-6* / Glob[1]	Mixture of PCR-mix-2-FRT and polymerase (TaqF)[1]
For quantitative analysis	For qualitative analysis		
1	4	70	35
2	5	80	40
3	6	90	45
4	7	100	50
5	8	110	55
6	9	120	60
7	10	130	65
8	11	140	70
9	12	150	75
10	13	160	80
11	14	170	85
12	15	180	90
13	16	190	95
14	17	200	100
15	18	210	105
16	19	220	110
17	20	230	115
18	21	240	120
19	22	250	125
20	23	260	130
21	24	270	135
22	25	280	140
23	26	290	145
24	27	300	150
25	28	310	155
30	33	360	180

4. The control was prepared as the following :

C–: Added 100 µl of Negative Control (C–) to 1.5ml microcentrifuge tube labelled C– (Negative Control of Extraction).

PCE: Added 90 µl of Negative Control (C–) and 10 µl of Positive Control DNA HHV-6 and human DNA to 1.5ml microcentrifuge tube labeled PCE (Positive Control of Extraction).

5. Tacked the required number of tubes for amplification of test and control DNA samples. Transfered 15 µl of the prepared mixture to each tube.

6. Added 10 µl of DNA samples obtained at the DNA extraction stage into each tube with the reaction mixture.

7. Control tubes were used for qualitative analysis:

NCA: Added 10 µl of RNA-buffer to the tube labeled NCA (Negative Control of Amplification).

C+: Added 10 µl of KSG2 to the tube labeled C+ (Positive Control of Amplification).

C–: Added 10 µl of the sample extracted from the Negative Control (C–) reagent to the tube labeled C– (Negative control of Extraction).

PCE: Added 10 µl of the sample extracted from the Positive control DNA HHV-6 and human DNA reagent to the tube labeled PCE (Positive control of Extraction).

II. Amplification

A temperature profile was created on instrument as follows the table (3-7).

Table 3.7: AmpliSens-1 amplification program

Step	*Temperature, °C*	*Time*	Cycles
Hold	**95**	15 min	1
Cycling 1	**95**	5 s	5
	60	20 s	
	72	15 s	
Cycling 2	**95**	5 s	40
	60	20 s *Fluorescence acquiring*	
	72	15 s	

1-Fluorescent signal was detected in the channels for the FAM and JOE fluorophores.

2. Adjusted the fluorescence channel sensitivity according to the Important Product Information Bulletin.

3. Inserted tubes into the reaction module of the device.

4. The amplification program was run with fluorescence detection.

5. Analyzed results after the amplification program was completed.

III. Data Analysis

Analysis of results was performed by the software of the real-time PCR instrument used by measuring fluorescence signal accumulation in two channels:

 The signal of β-globin gene DNA (IC Glob) amplification product was detected in the channel for the FAM fluorophore.

 The signal of the HHV6 DNA (Positive Control DNA HHV-6 and human DNA) amplification product was detected in the channel for the JOE fluorophore.

Results were interpreted by the crossing (or not-crossing) the fluorescence curve with the threshold line set at the specific level that corresponds to the presence (or absence) of a Ct value of the DNA sample in the corresponding column of the results grid.

I. Principle of interpretation is the following:

HHV6 DNA was detected if the Ct value determined in the results grid in the channel for the JOE fluorophore does not exceed the boundary value. Moreover, the fluorescence curve of the sample should cross the threshold line in the area of typical exponential growth of fluorescence.

HHV6 DNA is not detected if the Ct value was not determined (absent) in the results grid in the channel for the JOE fluorophore (the fluorescence curve does not cross the threshold line), whereas for qualitative analysis the Ct value in the channel for the FAM fluorophore does not exceed the boundary Ct value specified in the Important Product Information Bulletin .

The result of analysis was invalid if the Ct value was not determined (absent) in the results grid or if it is greater than the boundary Ct value in the channel for the JOE fluorophore whereas for qualitative analysis the Ct value in the channel for the FAM fluorophore exceeds the Ct value specified in the Important Product Information Bulletin .

The result of analysis was equivocated if the Ct value in the channel for the JOE fluorophore exceeds the boundary Ct value specified in the Important Product Information Bulletin. It was necessary to carry out the additional analysis for such sample with two repeats. If a reproducible positive result was obtained, the result was considered positive. If the obtained Ct values are not reproducible in two repeats, the result was considered equivocal.

For qualitative analysis, the negative result was considered unreliable if the Ct value in the channel for the FAM fluorophore exceeds the boundary Ct value specified in the Important Product Information Bulletin.

2. Qualitative Control Results

The result of the analysis is considered reliable only if the results obtained for controls C–, PCE, NCA, C+, KSG1, and KSG2 are correct . For qualitative analysis results for positive control are shown in table (3-8)

Table 3.8: Qualitative analysis results for positive control.

control	Amplification results in the channel for the fluorophore	
	FAM	**JOE**
C-	Absent	Absent
PCE	*Ct* boundary value	*Ct* boundary value
NCA	Absent	Absent
C+	*Ct* boundary value	*Ct* boundary value

3.4.2. Detection of TLR-2(rs1898830) , IL10 rs1518111 , IL18 rs187238, and HLA-G rs1063320& rs1610696 Gene Polymorphism by ARMS:

Total DNA for SNPs of TLR-2(rs1898830) , IL10 rs1518111 , IL18 rs187238, and HLA-G rs1063320& rs1610696 polymorphism were extracted from peripheral blood and swabs of female patients using ARMS technique.

3.4.2.1. Principles of Assay

There are two major processes to isolate of total DNA from specimens and Polymerase chain reaction (ARMS-PCR) is allows the amplification of a target region from a DNA template by using specific oligonucleotides.

3.4.2.2. Extraction of Total DNA from Clinical Samples:

The G-spin™ Total DNA Extraction Mini Kit is suitable for use with deferent swabs and fresh or frozen whole blood which has been treated with citrate, heparin, or EDTA. Pre-separation of leukocytes is not necessary • Purification does not require phenol/chloroform extraction or EtOH precipitation, and provides the simplest protocols. DNA is eluted in Buffer CE , TE (10:1), 10mM Tris (pH 7.5 ~ 8) or water, is prepared for direct addition to PCR or other enzymatic reactions. Alternatively, it can be safely stored at (–20°C) for later use. The purified DNA is protein-free, nucleases-free and does not include other contaminants or inhibitors. G-

spin™ Total DNA Extraction Mini Kit is optimized for extraction of (20-30kb) DNA fragments and able to extract up to 50 kb fragments.

All reagents required for the total DNA extraction were provided with DNA extraction kit (G-Spin total DNA Extraction kit, Cat .No. 14001 Intron / Korea).

Table 3.9 : List of reagents and buffers of G-Spin total DNA extraction kit used in this study.

Label	**Contents 50 Columns**	**Contents 200 Columns**
Buffer CL	25 ml	90 ml
Buffer BL 1	25 ml	90 ml
Buffer WA1	40 ml	160 ml
Buffer WB 2	14 ml	56 ml
Buffer CE 3	20 ml	40 ml
Spin Column4 / Collection Tube5	50 ea	200 ea
RNase A (Lyophilized powder)6	3 mg x 1 vial	3 mg x 4 vials
Proteinase K (Lyophilized powder)6	22 mg x 1 vial	22 mg x 4 vials

3.4.2.3. Assay Procedure:

The procedure was carried out in accordance with the manufacturer's instruction as following:

I. Extraction of Total DNA From Swabs

1. A 300 µl of swabs was placed into a (1.5 ml) micro-centrifuge tube.

2. Added (400 µl) of CL Buffer, (20 µl) of proteinase K solution and (40 µl) of RNase A into sample tube and mixed by vortexing vigorously. Then incubated the lysate at (56°C) for 30 min.
3. A 1.5 ml tube was centrifuged briefly (to remove drops from the inside of the lid.
4. Added (400 µl) of Buffer BL into the lysate, and mixed well by gently inverting 5 - 6 times. After mixing, was incubated the lysate at (70°C) for 5 min.
5. A 1.5 ml tube was centrifuged briefly to remove drops from the inside of the lid.
6. A 400 µl of absolute ethanol was added into the lysate, and mixed well by gently inverting 5 - 6 times or by pipetting. DO NOT vortex. After mixing, briefly centrifuged the (1.5 ml) tube to remove drops from inside of the lid.
7. A 800 µl of the mixture from step 7 was applied carefully (to the Spin Column (in a 2 ml Collection Tube) without wetting the rim. Closed the cap and centrifuged at 13,000 g for 1 min. Discarded the filtrate and placed the spin column in a 2 ml collection tube (reused).
8. Repeated step 8 by applied up to 600 µl of the remaining mixture from step 7 to the spin column. Discarded the filtrate and placed the spin column in a new (2 ml) collection tube.
9. Carefully applied the mixture from step 7 to the spin column (in a 2 ml collection tube) without wetting the rim, then closed the cap, and centrifuged at 13,000 g for 1 min. Discarded the filtrate and placed the spin column in a new (2 ml) collection tube (additionally supplied).
10. Seven hundred µl of Buffer WA was added to the spin column without wetting the rim, and centrifuged for 1 min at 13,000 g. Discarded the flow-through and reused the collection tube.
11. After then was added (700 µl) of Buffer WB to the spin column without wetting the rim, and centrifuged for 1 min at 13,000 g. discarded the

flow-through and placed the column into a new (2.0 ml) collection tube (additionally supplied), then agained centrifuged for additional (1 min) to dry the membrane. discarded the flow-through and collection tube altogether.

12. Placed the spin column into a new (1.5 ml) tube (not supplied), and added (30 - 100 µl) of buffer CE directly onto the membrane. Then incubated for 1 min at room temperature and then centrifuged for 1 min at 13,000 g to elute.

II .Extraction of total DNA from Blood Samples

1. A 200 µl of whole blood was pipeted into a (1.5 ml) microcentrifuge tube.
2. Added 40 µl proteinase K Solution and 5 µl of RNase A solution into sample tube and gently mixed.
3. A 200 µl of Buffer BL was added into upper sample tube and mixed thoroughly. This step is important in order to ensure effective decomposition, and it is important that the BL sample and buffer blood are thoroughly mixed to give a dissolution solution.
4. Placed the mixture at Room Temperature for (2minutes).
5. The lysate was incubated at 56°C for 10 min. For complete lysis, mixed 3 or 4 times during incubation by inverting tube. If it lysis perfectly, the red color of lysate became the dark green.
6. The 1.5 ml tube was centrifuged briefly to remove drops from the inside of the lid.
7. Added (200 µl) of absolute ethanol into the lysate, and then mixed well by pulse vortex. After mixing, briefly centrifuged the (1.5 ml) tube to remove drops from inside of the lid. This step is an equilibration step for binding genomic DNA to column membrane. Also, this step conduces to pass efficiently cell lysate through a column.

8. The mixture from step 7 was applied carefully to the spin column (in a 2 ml collection tube) without wetting the rim, close the cap, and centrifuged at 13,000 g for 1 min . Discarded the filtrate and placed the spin column in a new (2 ml) collection tube (additionally supplied).
9. Seven hundred µl of Buffer WA was added to the spin column without wetting the rim, and centrifuged for 1 min at 13,000 g. Discarded the flow-through and reused the collection tube.
10. Seven hundred µl of Buffer WB was added to the spin column without wetting the rim, and centrifuged for 1 min at 13,000 g. Discarded the flow-through and placed the column into a new (2.0 ml) collection tube (additionally supplied),.Then was centrifuged again for additional 1 min to dry the membrane. Discarded the flow-through and collection tube altogether.
11. Placed the spin column into a new (1.5 ml) tube (not supplied), and added (50 µl) of Buffer CE directly onto the membrane. Then incubated for (1 min) at room temperature and then centrifuged for (1 min) at (13,000 g) to elute.

3.4.2.4. Measurement of concentration and purity of extracted DNA

The DNA quantity and purity was determined using a spectrophotomete (Nano drop) at the absorbance at 260nm and 280nm respectively. The concentration of DNA was estimated at ng/ml and the purity calculated as 260/280 ratio, when the DNA solution is pure the ratio ranged 1.2-2.

3.5. Polymerase Chain Reaction (PCR)

3.5.1 Primers Pairs Dilution

The primers source were from IDT / USA . Bioneer' primers are commonly transported in a lyophilized state. The units of a lyophilized primer are given as a mass, in Pico moles. To create a stock of primers, one would reconstitute the primer in sterile, nuclease-free H_2O. The company supplies the amount of sterile, nuclease-free H_2O to be added to each primer

to obtain master stock (100mol/ul) that will be used again to obtain working stock. As following: The tube was spin down before opening the cap, then the desired amount of water was added according to the oligos manufacturer to obtain a 100 pmol/µl (Master Stock). Vortex properly for re-suspend the primers evenly. Then 10µl of the master stock was transfered to a 0.2ml Eppendorf tube that contains 90µl of sterile, nuclease-free H_2O (Working Stock). The master stock is stored at -20 C° and the working stock is stored at -20 C°. Finally, the working stock was thawed on ice and vortex before using in PCR and then stored at -20 C°.

3.5.2. PCR Experiments

PCR amplification was done using conventional thermal cycler (Biometra - Germany) as follows: Template DNA (about 2 µl) was added into PCR master mix tubes. Forward and reverse primers were added 1 µl into PCR master mix tubes(for each one). Distilled water was added to PCR Premix tubes to a total volume of 25 µl as shown in the table (3-10).

Table 3.10 : Recommended volumes and concentration for applying PCR into AccuPower® PCR tubes.

No.	Content of PCR Reaction Mixture	Volume/ µl
1	Master mix	10 µl
2	Forward primers (each one of snps)	4 µl
3	Reverse primers (each one of snps)	4 µl
4	Template DNA	2 µl
5	Nuclease free water	5 µl
Total		**25 µl**

3.5.3. Thermal Cycles Condition

Reactions were placed in a thermal cycler (Biometra-Germany) that had been preheated to 94°C and beforehand set up to the desired cyclic conditions. The target regions of SNPs TLR-2(rs1898830) , IL10 rs1518111 , IL18 rs187238, and HLA-G rs1063320& rs1610696 polymorphism were amplified using specific primers according to mention conditions as shown in table (3-11).

Table 3.11: Amplification conditions of TLR2,IL-10,IL-18 and HLA-G gene in patients with recurrent miscarriage .

Gene	Initial denaturation	Denaturation	Annealing	Extension	Final extension	No. of cycles
TLR2(rs1898830)	95C^0 /5 min	95C^0 / 30 sec	58 C^0 /30 sec	72 C^0 /30 sec	72 C^0 /10min	40
IL10(rs1518111)	95C^0 /5 min	95C^0 / 30 sec	63 C^0 /30 sec	72 C^0 /30 sec	72 C^0 /10min	40
IL-18(rs187238)	95C^0 /5 min	95C^0 / 30 sec	55 C^0 /30 sec	72 C0 /30 sec	72 C^0 /10min	40
HLAG(rs1063320)	95C^0/5 min	95C^0 / 30 sec	58 C^0 /30 sec	72 C^0 /30 sec	72 C^0 /10min	40
HLAG(rs1610696)	95C^0 /5 min	95C^0 / 30 sec	51 C^0 /30 sec	72 C^0 /30 sec	72 C^0 /10min	40

PCR products of target regions TLR-2(rs1898830) , IL10 rs1518111 , IL18 rs187238, and HLA-G rs1063320& rs1610696 polymorphism were electrophoresed on 1.5% agarose at 75 V for 1h and visualized by safe stain. Photos were taken using gel documentation system.

3.6. Agarose Gel Electrophoresis Technique

The agarose gel electrophoresis was perfomed according to the method of Robinson and Lafleche (2000). This technique was used to detect viral genomic DNA extracts, and PCR products.

3. 6.1 Preparation of Solutions and Buffers

I . Loading Buffer

The buffer was prepared from 0.25 % Bromophenol blue and 40% sucrose and stored at 4° C (Sambrook and Rushell, 2001).

II . TBE Buffer (1X)

To prepare 500 ml of 1X TBE buffer, 50 ml of TBE (10X) stock solution was mixed with 450 ml of dH_2O. The pH value was adjested to 8 with concentrated HCl or 0.5 M tris base solution. Then the volume was completed to 500ml with dH_2O.

3.6.2 Gel electrophoresis protocol

1. Device setup: The casting gates were sited on the ends of the gel tray and locked in place firmly against casting tray. This was done by engaging the "claws" of the gate in the recess of the side wall of the tray. The comb was sited into the slots of the gel tray, (1.0 mm above the base of gel casting tray) so that the sample wells are near the cathode.

2. Gel dissolving: A1g of agarose was dissolved in 100ml of 0.5 X TBE solution by mealting to 100°C to prepare 1% agarose gel for migrated genomic DNA extracts. Whereas, 1% or/and 2% agarose gel was prepared in 1X TBE buffer for migrated PCR products or digested DNA by restriction enzyme respectively.

3. Gel casting: After agarose gel dissolving completely, it let to cooling to approximately 60°C and 2-3 µl of the safe stain stock solution was added, then slowly pour the agarose into the gel- casting tray, and any air bubbles were removed. The comb was positioned at approximately 1.5 cm from one edge of the gel. The agarose was allowed to solidify at room temperature at least 30 min. After that, the claws were disengaged from the gel tray and the comb was separated gently. Then the gel was placed in the gel tank in such a way that the wells should be on end with the cathode. 1X TBE buffer

(depending the purpose) was added to the buffer tank until it was about 5 mm above the top of the gel.

4. Loading the samples: Each 5µl of the genomic DNA sample was mixed with 3µl loading dye briefly and loaded into the wells. Whereas, the PCR products were loaded without loading dye because of the PCR master mix contained loading dye.

5. Gel electrophoresis conditions: After sample loading the electric field was turned on at 5 V/cm (75V) for 60-120 min until bromophenol blue dye before reached at the end edge of the gel.

6. The gel was photographed using gel documentation system (Cleaver Scientific - UK).

3.7. Evaluation the levels of TLR-2, IL-10, IL-18 and HLA-G Concentration in Blood Serum of Patients and AHC.

The concentration of TLR-2, IL-10, IL-18 and HLA-G in the serum of female patients with recurrent miscarriage according to the Manual Procedure of Boster Biological Technology Company / USA were evaluated by enzyme linked immunosorbent assay (ELISA).

I.Assay Principle

This kit is an Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with Human TLR-2, IL-10, IL-18 and HLA-G TLR-2, IL-10, IL-18 and HLA-G antibody. TLR-2, IL-10, IL-18 and HLA-G present in the sample is added and binds to antibodies coated on the wells. And then biotinylated Human TLR-2, IL-10, IL-18 and HLA-G Antibody is added and binds to TLR-2 or IL-10 or IL-18 or HLA-G in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated TLR-2, IL-10, IL-18 and HLA-G antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of Human TLR-2, IL-10, IL-18 and HLA-G. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm .

I. Reagent Provided

Reagent provided of ELISA kits to evaluate TLR-2, IL-10, IL-18 and HLA-G are listed in table (4-12).

Table 4.12 : Reagent provided of ELISA kits to evaluate TLR2,IL10, IL-18 and HLA-G

Components	Quantity (96T)	Quantity (48T)
Standard Solution (1600pg/ml)	0.5ml x1	0.5ml x1
Pre-coated ELISA Plate	12 * 8 well strips x1	12 * 4 well strips x1
Standard Diluent	3ml x1	3ml x1
Streptavidin-HRP	6ml x1	3ml x1
Stop Solution	6ml x1	3ml x1
Substrate Solution A	6ml x1	3ml x1
Substrate Solution B	6ml x1	3ml x1
Wash Buffer Concentrate (25x)	20ml x1	20ml x1
Biotinylated HumanTLR2; IL-10 ; IL-18;HLA-G Antibody	1ml x1	1ml x1
User Instruction	1	1
Plate Sealer	2 pics	2 pics
Zipper bag	1 pic	1 pic

II. Assay Procedure

1. Prepare all reagents, standard solutions and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature.
2. Determine the number of strips required for the assay. Insert the strips in the frames for use. The unused strips should be stored at 2-8°C.
3. Add 50µl standard to standard well. **Note**: Don't add antibody to standard well because the standard solution contains biotinylated antibody.
4. Add 40µl sample to sample wells and then add 10µl anti-IL-10 antibody

to sample wells, then add 50µl streptavidin-HRP to sample wells and standard wells (Not blank control well). Mix well. Cover the plate with a sealer. Incubate 60 minutes at 37°C.

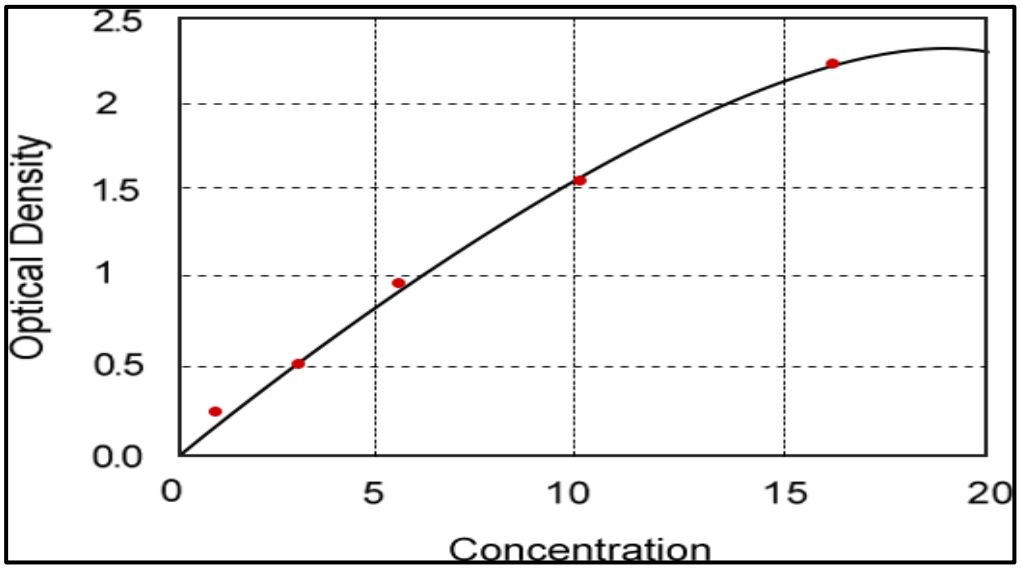
5. Remove the sealer and wash the plate 5 times with wash buffer. Soak wells with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspirate or decant each well and wash 5 times with wash buffer. Blot the plate onto paper towels or other absorbent material.
6. Add 50µl substrate solution A to each well and then add 50µl substrate solution B to each well. Incubate plate covered with a new sealer for 10 minutes at 37°C in the dark.
7. Add 50µl Stop Solution to each well, the blue color will change into yellow immediately.
8. Determine the optical density (OD value) of each well immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

IV .Calculation of Results

Construct a standard curve by plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis and draw a best fit curve through the points on the graph. These calculations can be best performed with computer-based curve-fitting software and the best fit line can be determined by regression analysis.

V.Typical Data

The standard curve is only for demonstration purposes. A standard curve should be generated for used all parameters of the study.



3.8. Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 3.06. Standard deviations are plotted as error bars for the data points on all figures Two way ANOVA, One-way ANOVA, and Chi square (X^2) were done to establish relationships of expression immunological variables levels according to the ELISA test results between women with and without clinical spontaneous abortion.

The correlation matrix between the selected variables and HHV-6 infection in current study was estimated by using Spearman's correlation coefficient analysis. Correlation coefficients were considered significant at *P-values* less than 0.05 by using using GraphPad Prism version 3.06. Asterisk (*****) indicates that the differences was statistically significant when compared with control group with patient groups. Chi square test (X^2)was used to compare the selected groups ** $p<0.01$; *** $p<0.001$. Hierarchical cluster analysis based on similarity coefficient was used in this study to identify the relatively homogenous results using expression immunological variables levels according to the ELISA test results between women with and without clinical spontaneous abortion using PRIMER-E7 software package (http://www.primer-e-.com/)(Clarke and Gorley, 2014).

CHAPTER FOUR

THE RESULTS

4. The Results

4.1 Study population According to Their Age.

Table (4-1) shows the mean age groups of the study population.The mean age of patients with recurrent pregnancy loss **(RPL)** was (30.70±12.4**1** years) was more than the mean age of the apparently healthy control (AHC) (28.67 ± 11.17 years).There are non-significant statistical differences (p=0.47) between **RPL** and AHC grops.

Table 4.1 : Distribution of Patients with RLP and AHC according to Their Age.

Study groups	No.	Mean of age (years)	S. D	S. E	Range(years)	
					Minimum	Maximum
AHC	50	28.67	11.17	2.403	21	44
RPL	100	30.70	12.41	1.979	17	49
Total	150	P-value = 0.3				

4.2 . A Comparison of the Pattern of Miscarriage Between Women With and without Clinical Spontaneous Abortion

A strong positive relationship was found between number of participants; number abortion ; week of abortion and maternal age($P < 0.001$). However, there are no significant correlation between number of participants with control maternal age (and as illustrated in Table (4- 2).

Table 4.2: A comparison of the pattern of miscarriage between women with and without clinical spontaneous abortion.

Variables	Study groups (cases)		
Maternal age (Y*)	**17-29** **No.**(42)	**30-39** **No. (38)**	**40-49** **No.(20)**
Number of participants			
Means (SEM)	**23(±0.7)**	**33(±0.3)**	**44(±0.6)**
Number abortion			
Means (SEM)	**1.6(±0.4)*** **	**2.5(±0.1)** **	**4.5(±0.9)** **
Week of abortion			
Means (SEM)	**12(±0.9)**	**13(±0.6)**	**14(±0.7)**

NF : Not found: * *P* < 0.001 : ** *P* <0.01**

4.3 Detection of Human Herpes Virus -6 (HHV-6) by Real-Time Polymerase Chain Reaction Technique (RT.PCR)

4.3.1 Extraction Nucleic Acid by Specific Viral DNA/RNA Extraction Kit:

Out of 100 Endometrium ; Cervical swabs ; fetal fluids swabs as well as Blood specimens involved in this study 55 (55%) were found to have a viral infection with **RPL** as shown in Table (4-3) and figure (4-1).

While, no viral nucleic acid was detected among all the examined apparently healthy specimens (50) that were used as control group . There were statistically highly significant differences (p = 0.01) between women patients with Recurrent Pregnancy Loss with viral genome and those without the Viral genome (Table 4-3).

Table 4.3 Percentage of Viral Genome Extraction of Women Patients with RPL And AHC Groups.

Viral Genome		Study Groups		Pearson Chi-Square (P-value)
		RPL No. (100)	AHC No. (50)	
Positive	**N**	55	0	***P=0.01*** ***Hig.sign.*** ***(P>0.05)***
	%	55%	0.00%	
Negative	**N**	45	50	
	%	45%	100%	
Total	**N**	100	50	
	%	100%		

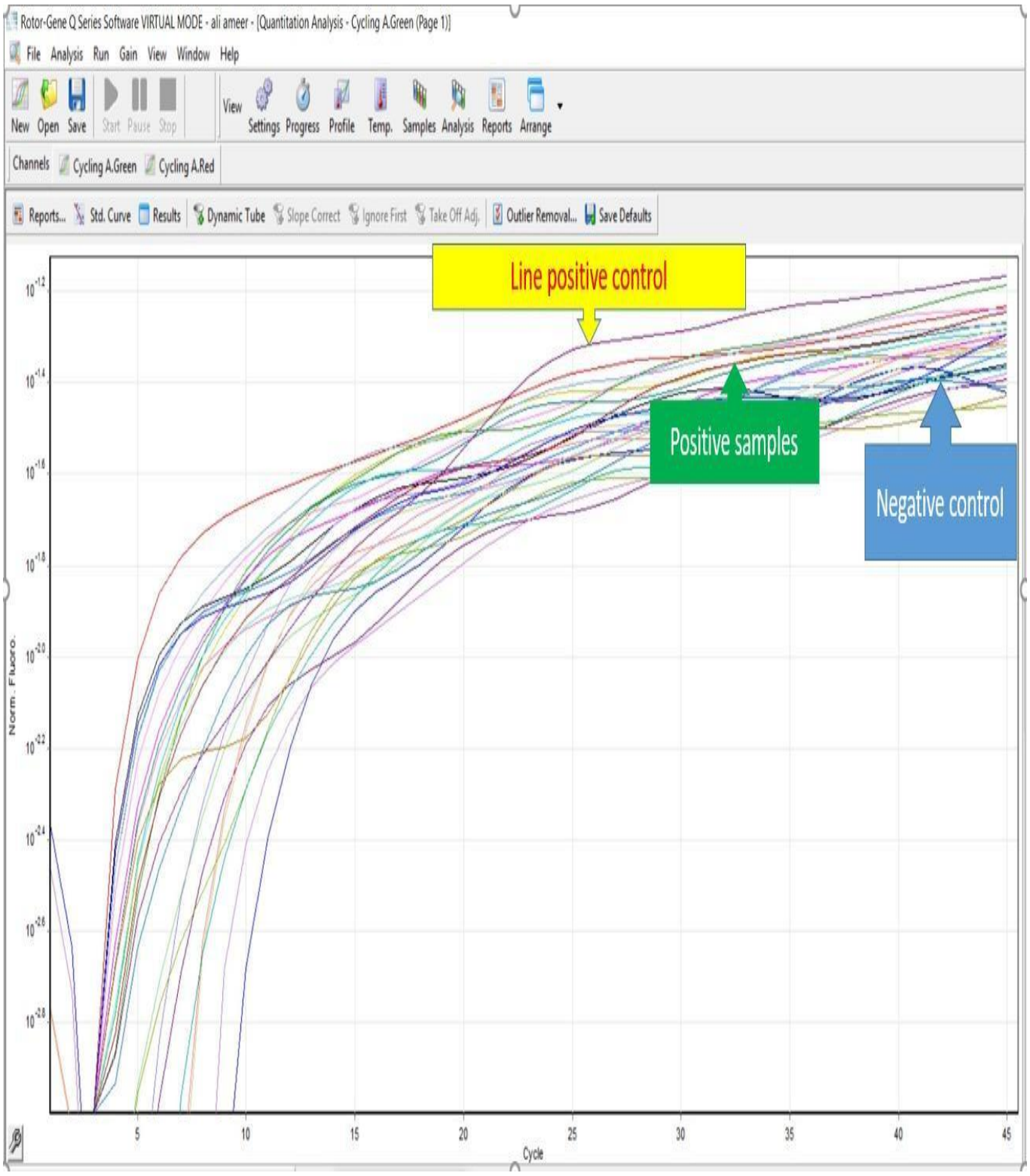
4.3.2. Detection of HHV-6 –DNA By Qualitative qRT.PCR:

The positive result according to qRT-PCR shows 45.5 % (25 out of 55 cases) as positive while 55.5% (30 out of 55 cases) as negative, as shown in Table (4-4) as well as Figures (4-2-A, & B). Statistically significant differences (p = 0.04) among patients group.

Table 4.4 Percentage of HHV-6 Positive Signals in Women Patients with RPL by Using qRT.PCR Technique.

Total Viral genome	No.	%	Chi-Square (P-value)
Positive	**25**	44.5	***P ≤ 0.04***
Negative	30	55.5	
Total	55	100	

according to detection kit (qRT-PCR) of HHV-6 which used in this study the curves that represent positive samples appear between the line positive control and line negative control.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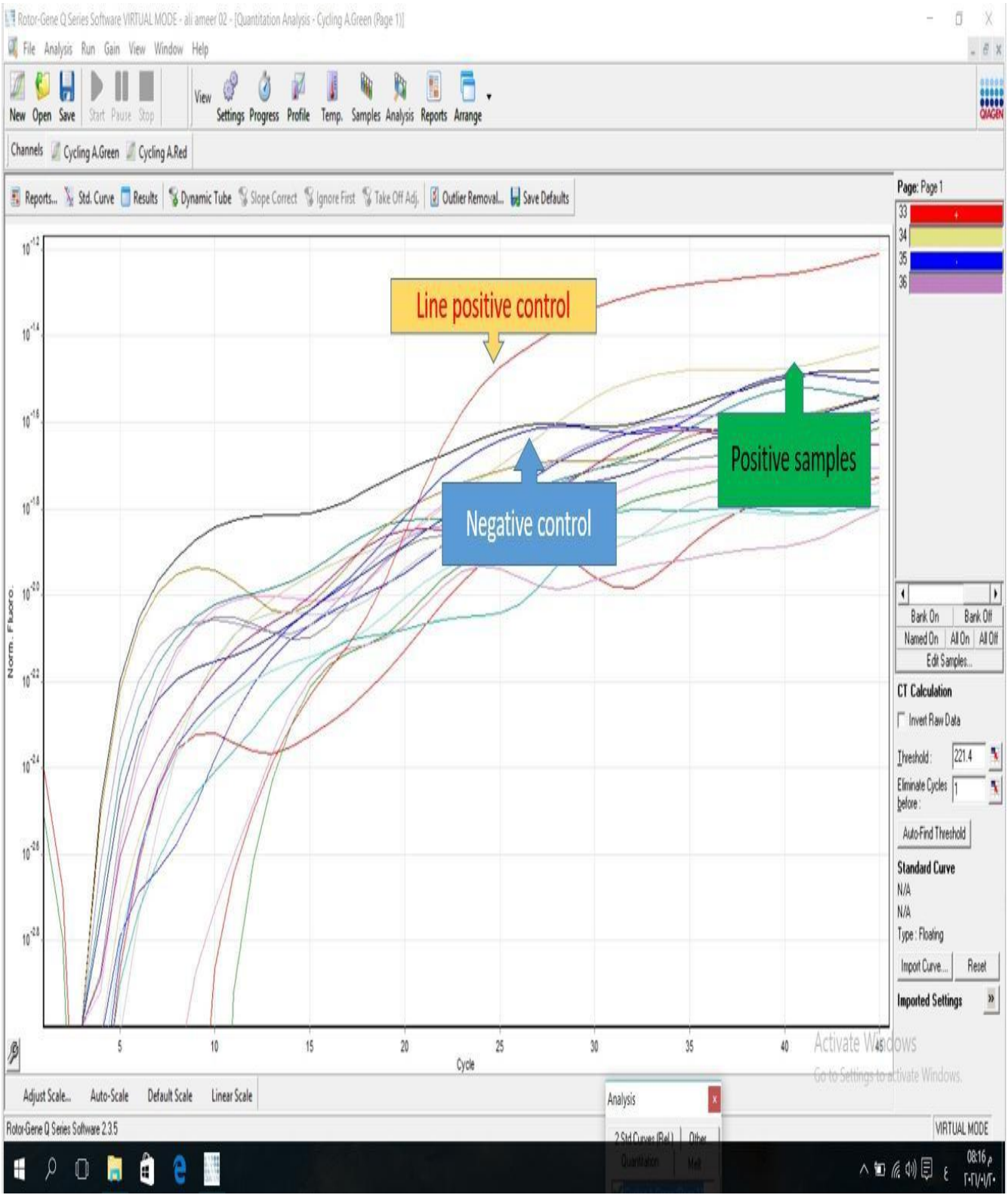
File Analysis Run Gain View Window Help
New Open Save Start Pause Stop
View Settings Progress Profile Temp. Samples Analysis Reports Arrange
Channels Cycling A.Green Cycling A.Red
Reports... Std. Curve Results Dynamic Tube Slope Correct Ignore First Take Off Adj. Outlier Removal... Save Defaults
Line positive control
Positive samples
Negative control
Norm. Fluoro.
Cycle

A



B

Figure 4. 2 A & B: Detection of HHV-6 by qRT-PCR.

4.3.3 HHV-6 in the Patients with RPL According to the Age .

In women with RPL, the most commonly affected age by DNA -HHV-6 was (30-39 years) which constituted 48% (12 out of 25 cases), while the age stratum (17-29 years) was constituted 32% (8 out of 25 cases), followed by 20% (5 out of 25) in age stratum (40 – 49 years) Table (4-5).

Statistical comparison of HHV-6 infection for Women With RPL according to age.

Table 4. 5 Frequency of HHV-6 RT-PCR Signal Among The Patients With Women With RPL According to the Age Stratum

	Years	HHV-6			P value
		No.	Positive	Negative	
Age Stratum	17-29	18 32.7%	8 32%	10 33.3%	
	30-39	25 45.5%	12 48%	13 43.3%	
	40-49	12 21.8%	5 20%	7 23.4%	
Total		55 100%	25 45.5%	30 54.5%	

4.4 Gene Polymorphism of TLR-2(rs1898830) , IL10 (rs1518111), IL-18 (rs187238), and HLA-G(rs1063320) SNPs:

4.4.1. Extraction Total Genome DNA from the Endometrium ; Cervical swabs ; fetal fluids Swabs as well as Blood Specimens:

Genome DNA extraction kit (G-Spin total DNA Extraction kit, Intron / Korea) the whole (total) genomic (DNA) was extracted
,purifying and migrated using agarose gel from the endometrium ; Cervical swabs ; fetal fluids swabs as well as blood specimens of women patients with RPL as well as apparently healthy control groups as a first step to amplify the target the two single nucleotide polymorphisms (SNPs) TLR.2 ; IL-10 ;IL-18 and HLA-G genes polymorphism .

After using the gel electrophoresis technique the result was almost the appearance of the DNA in 100 patients with women with RPL and 50 as AHC samples as shown in (Figure 4-3).

4.4.2 Genotyping of TLR-2(rs1898830) Gene Polymorphisms In RPL and AHC

The amplified of TLR-2 (rs1898830) target sequences of studied groups were detected by ARMS technique are summarized in (table 4-8) and figure (4-4) .The result of amplified appeared the presence of two bands (G Allele= 162 bp and A Allele= 108 bp (due to the presence of the G>A mutation. Whereas the wild type was identified by a single **270** bp fragment.

It can be seen that the frequency of GG genotypes in women patients with RPL and AHC groups (which reached 17% and 4%, respectively) it was significantly increased in women patients than control. While ,the frequency of AG genotypes in women patients with RPL and AHC groups which reached 38% and 8%, respectively. It was significantly increased in women patients than control.

On the other hand, the frequency of AA genotype in patients with RPL and AHC groups was 16% and 7%, respectively, that decreased in women patients compared with control group.

Finally, was found which AG genotype increased as rate OR=1.96 compared with GG and AA genotypes among studied groups.

According to the results , both of GG and AG in patients were statistically higher than those of the control group according to the gene expression levels ($P<0.05$), however, **AA** was statistically lower in patients than control group Table (4-6).

Table 4.6: Comparison between patients with and without clinical spontaneous abortion based on percentages of TLR-2 expressed gene polymorphism.

Genotype	Study groups RPL		*P value*	OR [Control]	95% C.I for OR [Control]		OR [Patients]	95% C.I for OR [Patients]	
TLR2	Control N=50	Patients N=100			Lower	Upper		Lower	Upper
AG	8%	38%	**0.001**	1.69	1.65	2.2	1.96	1.96	1.67
GG	4%	17%	**0.001**	1.9	1.9	2	1.81	1.7	1.89
AA	16%	7%	**0.01**	1.96	1.87	1.99	1.9	1.88	1.91

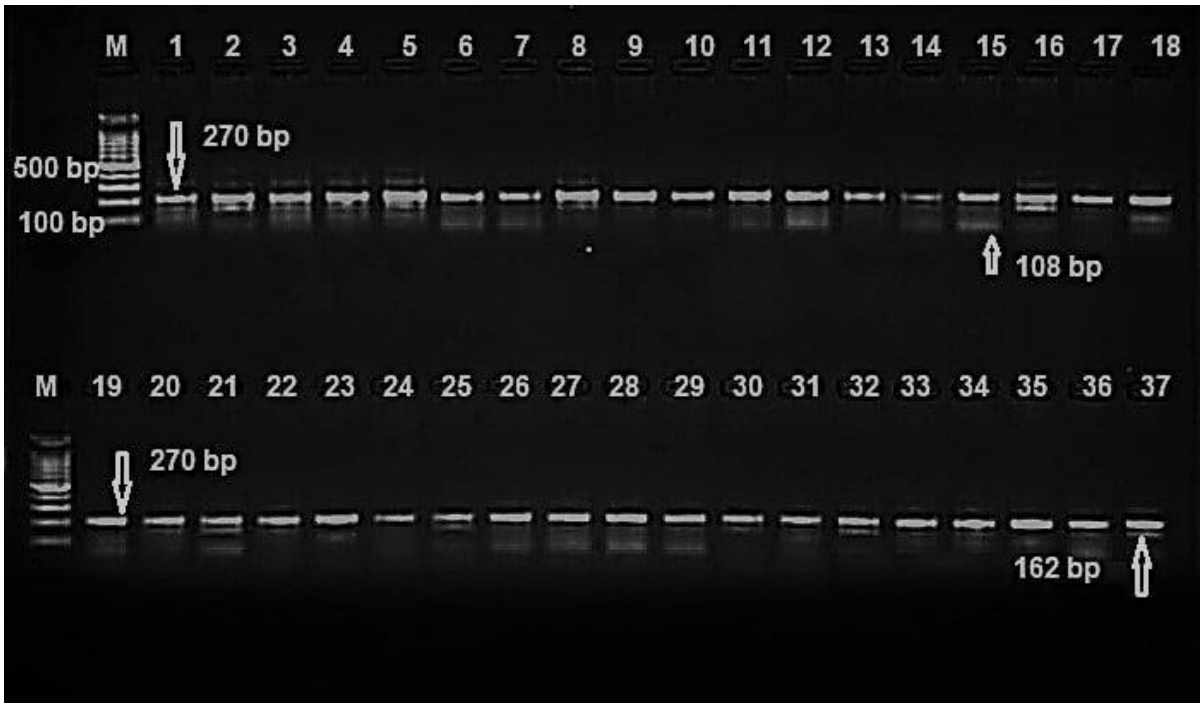


Figure 4.4: Allele typing patterens of TLR.2 rs1898830 gene using PCR-ARMS; Showed a heterozygous allele (AG) had a two band (108 and 162pb) molecular size in women with RPL. While ,homozygous allele had a single band with 270 bp molecular size. M: DNA ladder 100-1100 bp .The amplified products using PCR-ARMS migrated into 3% agarose, 75V, 20 mA for 120 min; 15 µl in each well; stained with ethidium bromide.

4.4.3 Genotyping of IL-10 (rs1518111) Gene Polymorphisms

The amplified of IL-10 (rs1518111) target sequences of studied groups were by ARMS technique are summarized in table (4-7) and figure (4-5) .The result of amplified and appeared the presence of two bands (T Allele= 183 bp and C Allele= 133 bp (due to the presence of the T>C mutation. Whereas the wild type was identified by a single 316 bp fragment.

It can be seen that the frequency of CC genotypes in women patients with RPL and AHC groups which reached 11% and 0%, respectively it was significantly increased in women patients than control.

While ,the frequency of TC genotypes in women patients with RPL and AHC groups which reached 17% and 16%, respectively.

It was non- significantly difference in women patients compared with control group. On the other hand, the frequency of TT genotype in women patients with RPL and AHC groups was 9% and 4%, respectively, that increased in women patients compared with control group (Table 4-7).

Finally,it was found that TC genotype decreased as rate OR=1.7 compared with CC genotype and equal to TT genotype among studied groups. According to the results , both of **TT** and **CC** were statistically higher than those of the control group according to the gene expression levels ($P<0.05$) (Table 4-7).

Table 4.7: Comparison between women with and without clinical spontaneous abortion based on percentages of IL-10 expressed gene polymorphism.

Genotype	Study group		*P value*	OR [Control]	95% C.I for OR [Control]		OR [Patients]	95% C.I for OR [Patients]	
IL-10	Control N=50	Patients N=100			Lower	Upper		Lower	Upper
TT	4%	9%	0.08	1.94	1.9	**2**	**1.7**	**1.8**	**1.9**
CC	0	11%	**0.001**	1.9	1.8	**2.04**	**1.8**	**1.9**	**2**
TC	16%	17%	**0.01**	1.8	1.7	**1.9**	**1.7**	**1.67**	**1.95**

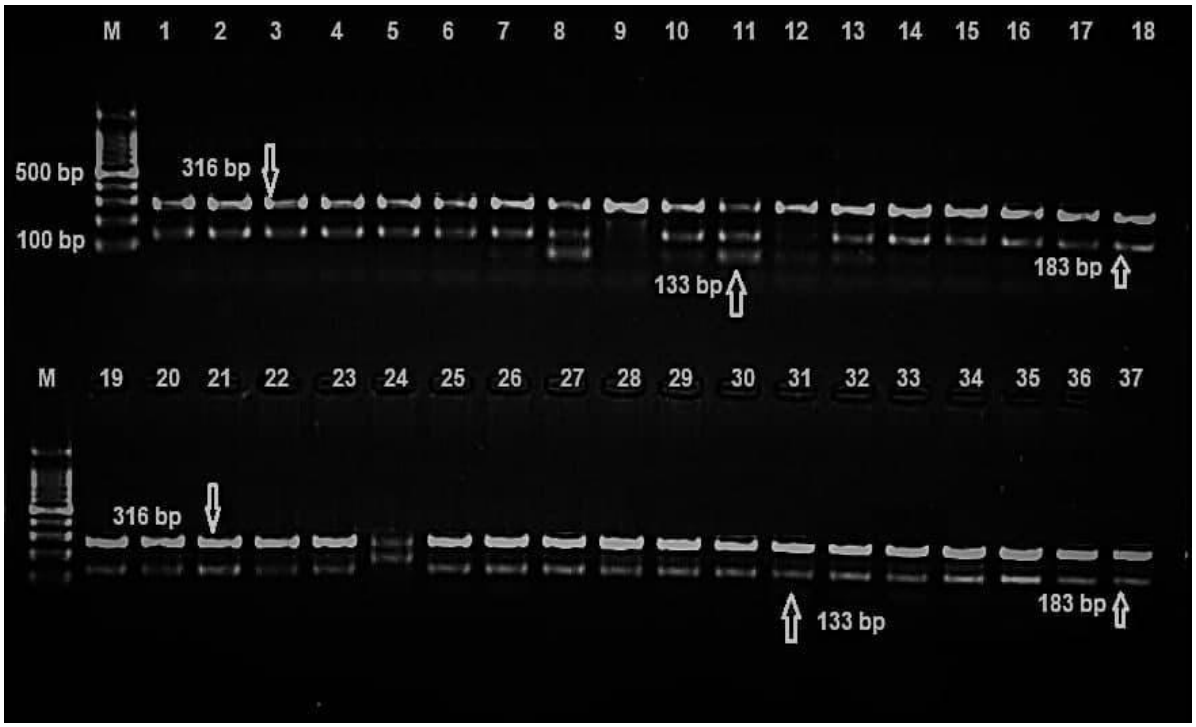


Figure 4.5: Allelo typing patterens of IL-10 gene using PCR-ARMS; Showed a heterozygous allele (TC) had a two band (183 and 133 pb) molecular size in women with RPL. While ,homozygous allele had a single band with 316 bp molecular size. M: DNA ladder 100-1100 bp .The amplified products using PCR-ARMS migrated into 3% agarose, 75V, 20 mA for 120 min; 15 µl in each well; stained with ethidium bromide.

4.4.4 Genotyping of IL-18 (rs187238) Gene Polymorphisms

The amplified of IL-18 (rs187238) target sequences of studied groups were by ARMS technique are summarized in table (4-8) and figure (4-6) .The result of amplified was appeared the presence of two bands (G Allele= 178 bp and A Allele= 210 bp (due to the presence of the G>C mutation. Whereas the wild type was identified by a single 388 bp fragment.

It can be seen that the frequency of GG genotypes in women patients with RPL and AHC groups which reached 4%, in both them .It was non significantly in women patients and control groups .

While ,the frequency of CG genotypes in women patients with RPL and AHC groups which reached 15% and 6%, respectively. It was significantly increased in women patients than control study groups.

On the other hand, the frequency of CC genotype in women patients with RPL and AHC groups was 16% and 2%, respectively, that increased in women patients compared with control groups. Finally, was found which CG genotype increased as rate OR=1.9 compared with GG and CC genotypes among studied groups.

According to the results , both of **CC** and **CG** were statistically higher than those of the control group ($P<0.05$) according to the gene expression levels Table (4-8).

Table 4.8: Comparison between patients with and without clinical spontaneous abortion based on percentages of IL-18 expressed gene polymorphism.

Genotype	Study group		*P value*	OR [Control]	95% C.I for OR [Control]		OR [Patients]	95% C.I for OR [Patients]	
IL-18	Control N=50	Patients N=100			Lower	Upper		Lower	Upper
CG	6%	15%	**0.01**	1.87	1.9	2	1.9	1.68	2
GG	4%	4%	0.09	1.92	1.8	1.9	1.87	1.9	1.9
CC	2%	16%	**0.001**	1.9	1.89	2.4	1.78	1.8	1.8

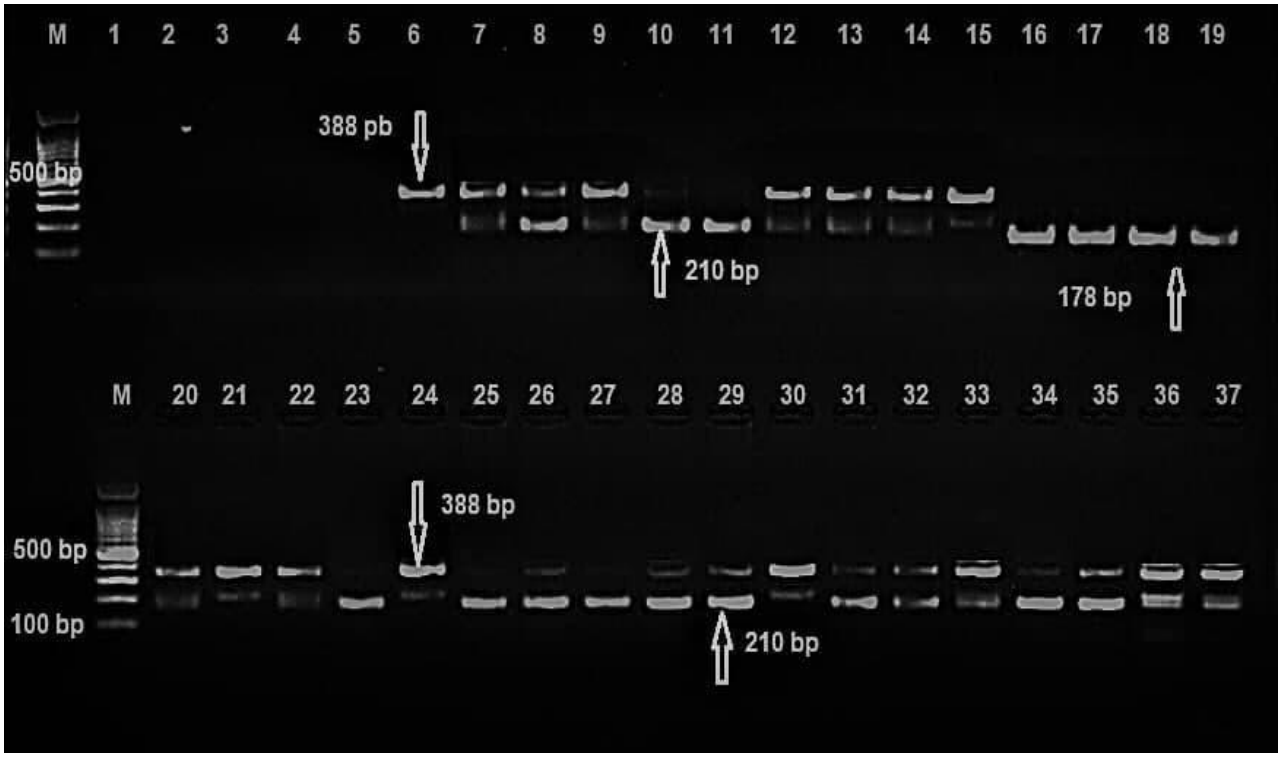


Figure 4.6: Allele typing patterens of IL-18 gene using PCR-ARMS; Showed a heterozygous allele (CG) had a two band (210 and 178 pb) molecular size in women with RPL. While ,homozygous allele had a single band with 388 bp molecular size. M: DNA ladder 100-1100 bp .The amplified products using PCR-ARMS migrated into 3% agarose, 75V, 20 mA for 120 min; 15 µl in each well; stained with ethidium bromide.

4.4.5 Genotyping of HLA-G (rs1063320) Gene Polymorphisms

The amplified of HLA-G (rs1063320) target sequences of studied groups were by ARMS technique are summarized in table (4-9) and figure (4-6) .The result of amplified was appeared the presence of two bands (G Allele= 178 bp and C Allele= 245 bp (due to the presence of the G>C mutation. Whereas the wild type was identified by a single 423 bp fragment.

It can be seen that the frequency of GG genotypes in women patients with RPL and AHC groups which reached 9% and 6% ,respectively .It was significantly increased in women patients than control groups .

While ,the frequency of CG genotypes in women patients with RPL and AHC groups which reached 18% and 8%, respectively. It was significantly increased in women patients than control study groups.

On the other hand, the frequency of CC genotype in women patients with RPL and AHC groups was 5% and 4%, respectively, that increased in women patients compared with control groups.

Finally, was found which CG genotype increased as rate OR=1.8 compared with CC genotype and decreased rate compared with GG genotype among studied groups.

According to the results , CC, GG and CG were statistically higher than those of the control group according to the gene expression levels (P<0.05) (Table 4-9).

Table 4.9: Comparison between patients with and without clinical spontaneous abortion based on percentages of HLA-G expressed gene polymorphism.

Genotype	Study group		*P value*	OR [Control]	95% C.I for OR [Control]		OR [Patients]	95% C.I for OR [Patients]	
HLA-G	Control N=50	Patients N=100			Lower	Upper		Lower	Upper
CG	8%	18%	**0.01**	1.82	1.98	2.02	1.8	1.9	1.9
GG	6%	9%	0.07	1.9	1.8	2	1.9	1.68	1.8
CC	4%	5%	0.1	1.8	1.9	1.9	1.7	1.7	1.78

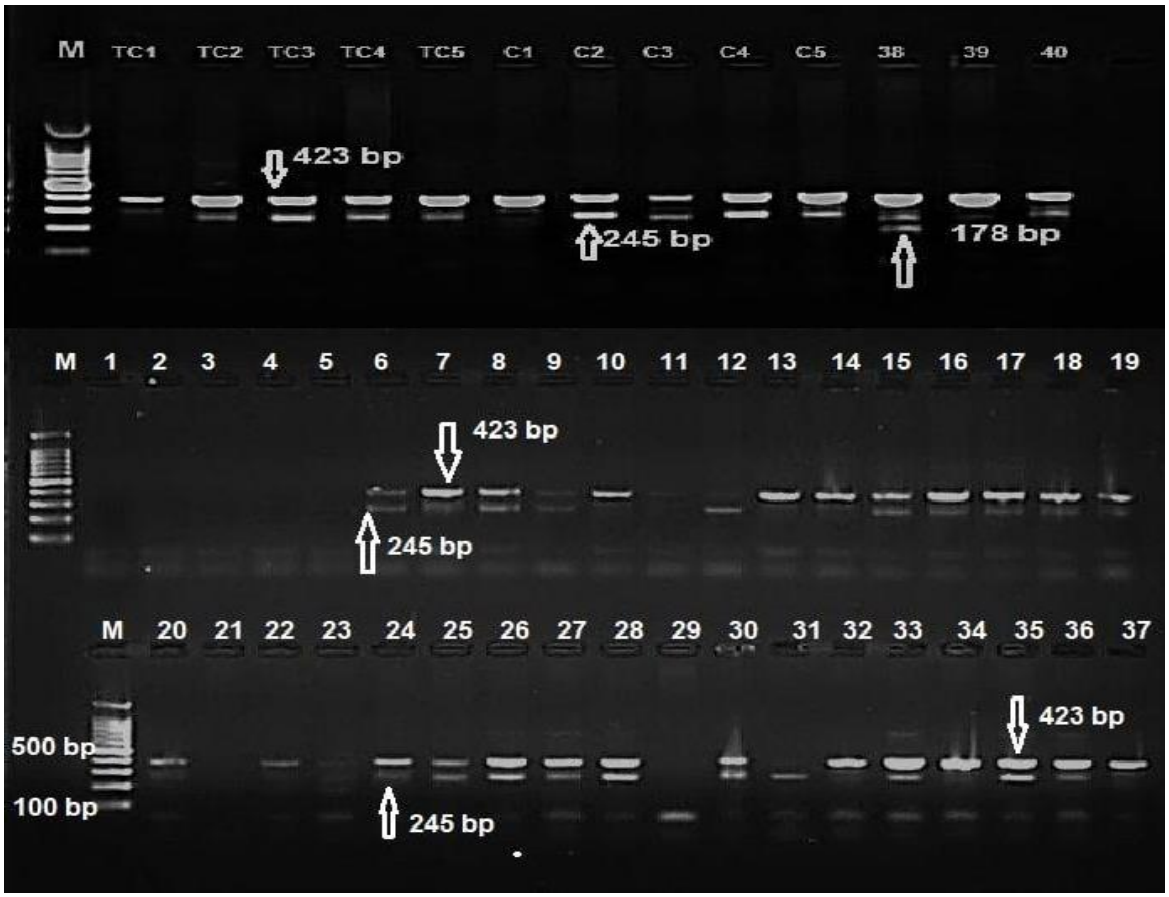


Figure 4.7: Allele typing patterens of HLA-G gene using PCR-ARMS; Showed a heterozygous allele (CG) had a two band (245 and 178 pb) molecular size in women with RPL. While ,homozygous allele had a single band with 423 bp molecular size. M: DNA ladder 100-1100 bp .The amplified products using PCR-ARMS migrated into 3% agarose, 75V, 20 mA for 120 min; 15 µl in each well; stained with ethidium bromide.

4.5. Evaluation of Serum TLR-2, IL10 , IL-18 , and HLA-G concentration By ELISA Among Study Population:

Table (4-10) showed the concentration of serum TLR-2, IL10 , IL-18 , and HLA-G were detected by ELISA technique. The mean of serum TLR-2, IL10 , IL-18 , and HLA-G concentration in women patients with RPL and apparently healthy control (AHC) groups were 13.3±1.3; 36.6 ±6.3; 15.8± 2.5 &365.5± 11.3 pg/ml and 12.6± 1.6 ; 32.5± 3.4; 18.9± 3.2 &97.3± 11.3pg./ml, respectively.

Highly significant difference (p=.004) was found by comparing the mean of serum TLR-2, IL10 , IL-18 , and HLA-G concentration among AHC and women with RPL groups (Table 4-10).

Table 4.10: Results of serum TLR-2, IL10 , IL-18 , and HLA-G concentration by ELISA for AHC and women patients with RPL

Immune Variables	Control (pg/ml)	Patients (pg/ml)
TLR2	12.6± 1.6	13.3±1.3
IL-10	32.5± 3.4	36.6 ±6.3
IL-18	18.9± 3.2	15.8± 2.5
HLAG	97.3± 11.3	365.5± 11.3
P value	***P*<0.01**	

4.6.Correlation between studied markers and HHV6 infection among study population:

4.6.1 The correlation of women with a clinical spontaneous abortion (17 - 29 years) and the pattern of miscarriage

A strong positive relationship (with statistically significant correlation) was found between age stratum (17-29 years) and HHV-6 infection in women with RPL. Similarly, there is a positive relationship (with significant correlation) between number of miscarriage and HHV-6 infection in women with RPL (Table 4-11).

However, there are no significant correlations among HHV-6 and week of child loss according to the age stratum (17-29 years) of study population (Table 4-11) .

Table 4.11 : The correlation of women with a clinical spontaneous abortion (17 -29 years) and the pattern of miscarriage

	17-29 Years	Week of child loss	Number of Miscarriage	HHV-6
17-29 Years	1	0.2	**0.8*****	**0.4****
Week of child loss	0.2		0.2	0.1
Number of Miscarriage		0.2		**0.4****

P-value <0.05 was considered statistically significant (are shown in **bold**): * $P < 0.05$: ** $P < 0.01$: *** $P < 0.001$.

4.6.2 The correlation of women with a clinical spontaneous abortion (30 - 39 years) and the pattern of miscarriage

There is a positive relationship between number of miscarriage and week of child loss in women with RPL .In addition , significant correlation was found between number of miscarriage and HHV-6 infection in women with RPL Table (4-12). However, there are no significant correlations among HHV-6 and week of child loss according to the age stratum (30-39 years) of study population Table (4-12) .

Table 4.12 : The correlation of women with a clinical spontaneous abortion (30 -39 years) and the pattern of miscarriage

	30-39 Years	Week of child loss	Number of Miscarriage	HHV-6
30-39 Years	1.0	-0.2	0.1	0.0
Week of child loss			**0.4****	0.0
Number of Miscarriage		**0.4****		**-0.4****

P-value <0.05 was considered statistically significant (are shown in **bold**): * $P < 0.05$: ** $P < 0.01$.

4.6.3 The correlation of women with a clinical spontaneous abortion (40 - 49 years) and the pattern of miscarriage

There is a positive relationship (with significant correlation) between age group (40-49 years) and week of child loss in women with RPL .In addition , significant correlation was found between number of miscarriage and age stratum (40-49 years) in women with RPL Table (4-13).

However, there are no significant correlations between week of child loss and HHV-6 infection in women with RPL . Similarly, there is no relationship (with non-significant correlation) between number of miscarriage and HHV-6 infection in women with RPL (Table 4-13) .

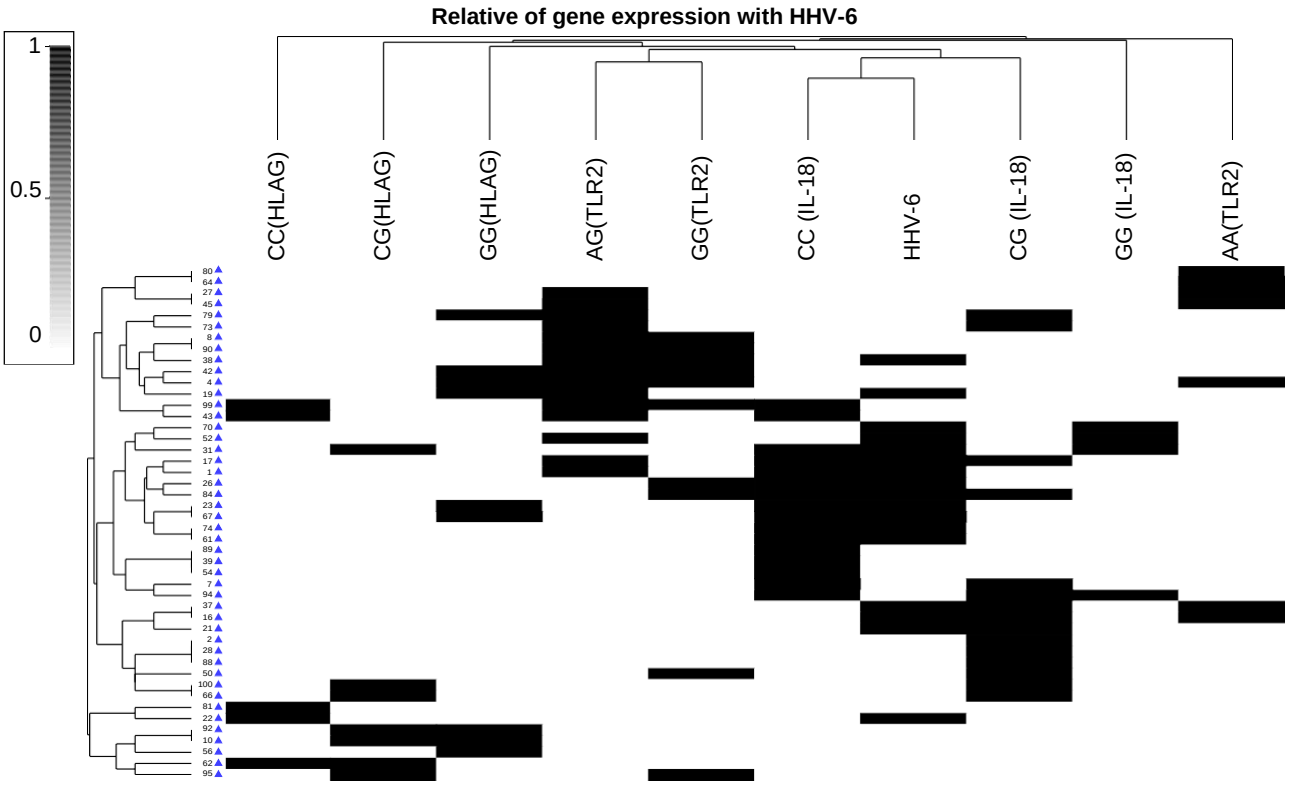
Table 4.13 : The correlation of women with a clinical spontaneous abortion (40 -49 years) and the pattern of miscarriage

	40-49 Years	Week of child loss	Number of Miscarriage	HHV-6
40-49 Years	1.0	**-0.6****	**-0.7****	0.3
Week of child loss			0.3	-0.1
Number of Miscarriage		0.3		-0.4

P-value <0.05 was considered statistically significant (are shown in **bold**): * $P < 0.05$: ** $P < 0.01$: *** $P < 0.001$

4.7 The correlation between molecular markers and HHV-6 infections

According to the Spearman correlation , (Figure 4-8) shows a dendrogram for gene expression and HHV-6 detection was divided significantly ($p<0.01$, $r= 0.7$) into two clusters. The results demonstrated a significant correlation between the gene polymorphism (expression) of IL-18 with viral infection (top cluster). However, the gene expression of both TLR2 and HLAG has less correlation ($p<0.04$, $r=0.4$) with the same infection. Additionally, the patient's samples were clustered into four categories (left cluster) depending on the level of gene expression in each sample.



Figur 4.8 Spearman's rho statistical testing to evaluate studied molecular markers in relation with HHV-6 infections in women with clinical spontaneous abortion.

Furthermore, the cluster analyses of the percentage of gene polymorphism in the selected cases samples divided them into three groups .In the group A , six samples displays 65% of similarity according to the gene polymorphism of the selected variables (IL-10, IL-18, TLR2, HLAG , and HHV-6) . Whereas the most samples in the cluster B and C gave more than 80 % of similarity (with an exception of few samples) .

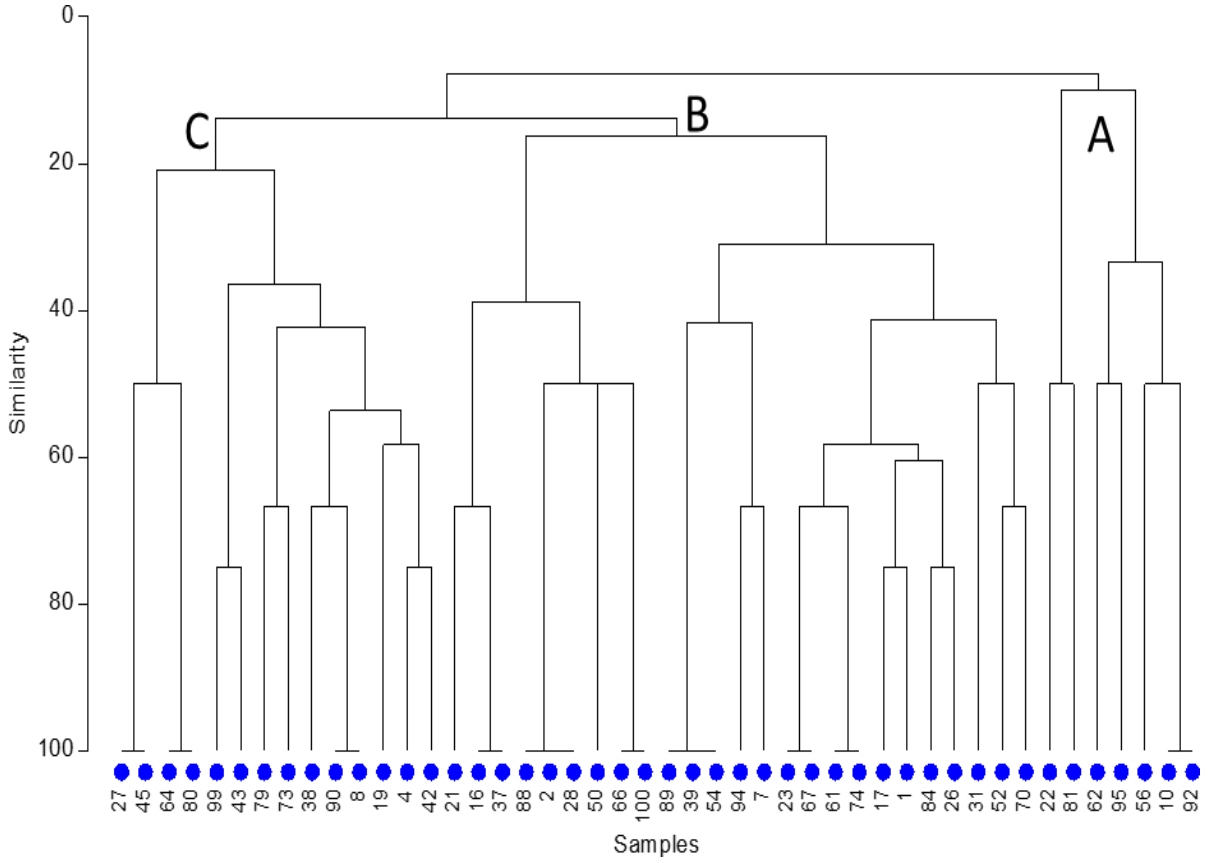


Figure 4.9 Dendrogram showing the similarity (%) between cases based on expression genes of IL-10, IL-18, TLR2, HLAG (gene polymorphism) and HHV-6 infection in women with clinical spontaneous abortion.

Chapter Five
Discussion

5.Discussion:

5.1 Recurrent Pregnancy Loss

Miscarriage is a common outcome of pregnancy, but the rate is challenging to estimate because of inconsistent registration and documentation. The provision of abortion services has changed in the past decade. Medication abortion accounts for at least half of all abortions in the majority of countries. Also in the majority of countries, over 90% of all abortions were completed before 13 weeks, and more than two-thirds of abortions occurred before the first 9weeks of gestation. Over the past 10 years there has been an increase in both the proportion of abortions that were medication abortions, and the proportion that were obtained before 9weeks gestation (Popinchalk and Sedgh ,2019).

In more than half of the cases, the causes of abortion have been genetic disorders and chromosomal abnormalities (Vaiman,2015). Nevertheless, other factors affecting abortion are as follows: uterine abnormalities, infectious diseases and untreated diseases of the mother ,the age of the mother during pregnancy, previous history of abortion, age at the first menstruation , menstrual disorders , use of contraceptive drugs , environmental conditions and mother's lifestyle such as smoking and use of caffeine , being exposed to cigarette smoke , stress , exposure to mobile phone radiation, and low socioeconomic and employment status , which are effective in the occurrence of abortion (Kaur *et al*.,2016; Volgsten *et al*.,2018 ; Poustchi *et al*.,2018).

5.2 A Comparison of The Pattern of Miscarriage Between Women with and Without Clinical Spontaneous Abortion

The most of the spontaneous abortions occur in the early weeks of pregnancy, and therefore, it can be confused with menstrual bleeding. Generally, it is very difficult to determine the rate of spontaneous and unwanted abortions because in countries where legal abortion is prohibited, there is a possibility of false report. Besides, the study of spontaneous abortion in low- and middle-income countries is also very challenging because most abortions have not been reported to and recorded in their official health system (Dellicour *et al*.,2016).

In this study , a strong positive relationship was found between number of participants; number abortion
; week of abortion and Maternal age($P < 0.001$). However, there are no significant correlation between number of participants with control maternal age (and as illustrated in Table 4-2). Although the total burden related to maternal abortion and miscarriage is less than 0.9% in Iraq, the emotional complications of abortion (such as depression) as well as its physical complications may face the families and women with different psychosocial problems.

Accordingly, the risk for abortion in women with the first marriage and pregnancy at age greater than 40 years old more than the other age groups; This finding was also consistent with previous researches by Cohain *et al*.,(2017) and Kebede *et al*.,(2018).

Scientists believe that marriage and pregnancy of a mother at older ages increase the risk of abortion, fetal and chromosomal problems, and pregnancy-related complications (Nabti *et al*., 2017).Therefore, as it has

been recommended, it is necessary to have regular check-ups and tests on the natural development of fetuses in pregnant women of older ages.

Extremes of age increase the risk of pregnancy loss, with age >35 years being the most significant risk factor because of the strong association with fetal chromosomal abnormalities . In a national prospective cohort study of over 421,000 pregnancies, the risk of miscarriage (after excluding induced abortions) was lowest (10 percent) in individuals age 25 to 29 years and rose to a high of 57% for people age ≥45 years (Magnus *et al*.,2019).

Various causes of maternal morbidity, such as endocrinopathies, cardiovascular disease, and metabolic disorders, are associated with pregnancy loss . These may also be considered modifiable risk factors, as well-controlled maternal conditions are far less likely to result in loss. While any medical condition that negatively impacts maternal health can have potential reproductive consequences.

Bacterial, protozoan, and viral infectious agents have been associated with increased risk of miscarriage; the exact mechanisms are not full

known .Untreated syphilis leads to a 21 percent increased risk of fetal loss and stillbirth (Giakoumelou *et al*.,2016). As compared with uninfected pregnant individuals, maternal viral infections have been associated with fetal loss rates nearly 8 %for parvovirus B19, nearly 6 percent for Zika virus, and 2.5% for cytomegalovirus (Xiong *et al*.,2019). However, maternal infection with HIV or toxoplasmosis does not appear to be associated with an increased risk of pregnancy loss (Ghasemi *et al*.,2016).

On the other hand, abortion is closely related to the cultural and religious factors, and Iraq is a multicultural country with different ethnicities. This study seeks to determine the lifetime prevalence of abortion and its risk factors in women 20-49 years old who have participated in the first cohort study among Iraqi female patients.

5.3 Detection of HHV-6 in Women Patients with RPL by RT-PCR

Although causal relationships between abortion and infections are difficult to establish, the detection rate of several viruses such as HHV-6, B19V, CMV, and HSV during pregnancy is an important way to analyze their relationship with first-trimester spontaneous abortion.It is generally thought that the optimal specimen type for detecting HHV-6 is fetal swab ; cervical swab as well as blood specimens, although this may not be true for all HHV-6 and detection techniques. Given the complexity and invasive nature of the procedure used to acquire swabs specimens have emerged as an alternative specimen type .Fetal swab ; cervical swab as well as blood specimens were shown to be superior or equivalent to specimens for RT-PCR in many studies (Francesca *et al*., 2020).

Out of 100 Endometrium ; Cervical swabs ; fetal fluids swabs as well as Blood specimens involved in this study 55 (55%) were found to have a viral infection with **RPL** of them were discovered to have viral infection by using Real Time PCR technique table (4-3). A study done by Sayyadi-Dehno *et al.,* (2019) and Miura *et al*., (2021) and for aborted women, which revealed (38.2%) and (34.7%) positively viral infected women, respectively that support current study in the total of viral infection.

It may be possible that the quantity or the physical status of viral DNA in the gestational tissue of pregnancy loss does not permit its easy detection and requires the use of the more sensitive and specific nested PCR technique. In addition, it remains unclear whether Endometrium ; Cervical swabs ; fetal fluids swabs as well as blood specimens are optimal for detection of most aborted viral by molecular technique such as multiplex RT-PCR or RT-PCR.

5.3.1 Detection of HHV-6 Genome in Aborted Women by Real Time PCR (qRT-PCR)

HHV-6A is an etiological agent or a risk factor in a portion of these cases. A few previous reports have tied HHV-6A infection to spontaneous abortion and neonatal hypotonia (Revest *et al* .,2011; Marci *et al* .,2016; Eliassen *et al* .,2017).

The concept of the relationship between HHV-6 and spontaneous abortion is based on the identification of HHV-6 genome sequences in fetal tissues, umbilical cord blood, and villous tissue and antigen has also been found in villous tissue (Revest *et al* .,2011).

As this is up to what we know the first study in Mid-Euphrates of Iraq for detection of HHV-6 DNA in aborted women, it's reasonable to compare the present result with relatively similar world-wide studies done for similar purposes of detection HHV-6-DNA in the endometrium swab, fetal swab , cervical swab as well as blood specimens by RT-PCR.

In the current study , DNA of HHV-6 was studied and detected in fetal swab ; cervical swab as well as blood specimens in a frequency that has showed significant differences among study groups (Table 4-4). A follow up study revealed similar results for women who developed PR in the first 15 weeks of pregnancy, with 57% of 14 pregnancies ending in miscarriage (Drago *et al* ., 2014).

In addition ,the present result of HHV6 is compatible with Drago *et al*.,2014 and Miura *et al*., (2021) who found HHV6 in 57% and 27.6% in spontaneous aborted women, respectively. Furthermore , a recent British study found that women with iciHHV6+ fetuses have a 2.5 to 3-fold increased risk of preeclampsia .HHV-6 RNA transcripts were found in 6.1% of cases of preeclampsia and only 2.2% of other pregnancies (Gaccioli *et al*., 2020).

Mary *et al*., (2007) who found the pregnant women with HHV-6 DNA present in cervical swabs had a greater odds of having HHV-6 DNA present in the blood than did pregnant women with negative cervical swabs (odds ratio, 12.9; P=.0009).

Italian investigators have also tied HHV-6A infection to unexplained female infertility (Marci *et al*., 2016) and demonstrate that HHV-6A

infection of endometrial endothelial cells result in a disruption of the maternal immune system (Bortolotti *et al*., 2020). Endometrial cells infected with HHV6A are less receptive to trophoblast cells (Bortolotti *et al*., 2020).The authors hope that this finding will help physicians address a subset of recurrent spontaneous abortions which are unexplained in 50% of cases.

Previous reports showed that HHV-6 DNA has been detected in genital tract secretions from pregnant and non-pregnant women ; HHV-6 DNA and antigens have been identified in biopsies of archived cervical samples (Baillargeon *et al.*,2000;Ohashi *et al.*,2002), and these suggest that the female genital tract may be the secondary site for HHV6 persistence.

Human herpesvirus 6 (HHV-6) can integrate into human chromosomes, resulting in genetic transmission from parent to child.Individuals of either sex with inherited chromosomally integrated human herpesvirus 6 (iciHHV-6) harbor the virus in every cell. Viral reactivation from the integrated HHV-6 genome can occur in pregnancy (Miura *et al.*,2021). The authors suggest two possible mechanisms by which reactivated HHV-6 from an integrated viral genome could cause spontaneous abortion or preeclampsia: (1) direct cellular damage or (2) bystander effects (such as upregulation of inflammatory cytokines) induced by the viral reactivation.

The effect of virus on the pregnancy outcome related with other factors such as hormonal effect (Marci *et al.*,2016), immunological statues of individual (Ozkan *et al.*,2014;Seshadri *et al.*,2014) that lead to reactivate of virus, and inhibit the creation of appropriate uterine environment for

implantation and fetal growth that lead to miscarriage (Eliassen *et al.*,2017).

Nigro *et al.*,(2011) who explained the recurrent abortion due to maternal infections transmissible in utero at various stage of gestation can be caused by a wide array of organisms including HHV-6.

Viral DNA has also been detectable in vaginal swabs with an incubation period of about 10 days suggesting that horizontal transmission from mothers to babies is possible (Pass,2004). Maternal-fetal infection with HHV-6 has also been described and may be linked with a higher rate of fetal loss (Gravel *et al.*,2013).

Similarly, a group from the United Kingdom investigated occurrence of viral infection in fetal death and detected viral DNA in 34% of tissue samples including detection of HHV-6 and HHV-7 in 5 samples (Al-Buhtori *et al.*,2011). Another group analyzed over a thousand samples from multiple sites including tissue biopsies for detection of HHV-6 by PCR and identified a case of primary HHV-6A seroconversion occurring in a young pregnant woman with subsequent transmission to the fetus and unfortunately a spontaneous abortion at 24weeks(Revest *et al.*,2011).

The majority of congenital HHV-6 infections are thought to be secondary to chromosomal integration of the virus into different human chromosomes within the whole genome, which is a proven phenomenon (Hall *et al.*,2008). Thus, there are 5 possibilities of congenital HHV-6 infection in an infant like our patient:

1. She had ciHHV-6 but no active infection; however, these patients would be asymptomatic with evidence of HHV-6 DNA but no IgM antibodies.
2. She had HHV-6 with active infection from a HHV-6+ mother who reactivated the virus during pregnancy and subsequently transmitted the active virus, but it is impossible to differentiate active infection in a HHV-6 patient using PCR methods alone.
3. She did not have HHV-6 but had evidence of active infection from a HHV-6 mother who reactivated and transmitted the replicating HHV-6 virus transplacentally.
4. She acquired it transplacentally from a mother re-infected with HHV-6 or whose latent HHV-6 reactivated with no evidence of maternal HHV-6.
5. She acquired it postnatally from another person in the NICU about 2weeks prior to the onset of symptoms.

The opinion of authors of current study , the differences in percentages of HHV-6 detection among the present as well as these studies could be attributed to the site of infection ,genetic as well as environmental factors, sample size, the quality & sensitivity of the techniques used in these studies. However, most of the studies done in this respect have included a small numbers of recurrent miscarriage cases and for better revealing of the importance of HHV6 in spontaneous aborted women ,this may need enrollment of large case-control studies.

5.3.1.1 Correlation between number of abortion; age stratum and HHV6 infection among study population

In the current results, a strong positive relationship (with highly significant correlation) between number of miscarriage ; age stratum in women with RPL according to the HHV-6 infection. These results consistent with Miura *et al*., (2021) who found the spontaneous abortion rate was significantly higher in women with HHV-6 than in women without iciHHV-6 but whose offspring carried paternally inherited iciHHV-6 or women in families without iciHHV-6. Furthermore, 38% of women with iciHHV-6 reported ≥2 spontaneous abortions compared with none of those whose offspring had paternally inherited iciHHV-6 and 6% of couples without iciHHV-6 (*P*=0.002).

In addition , multivariable analyses showed that mothers with iciHHV-6 (odds ratio [OR], 6.41; 95% CI, 1.10-37.4; *P* =.001) and maternal age of 40 years or older at the most recent pregnancy (OR, 3.91; 95% CI 1.30-11.8; *P* =.016) were significantly associated with recurrent pregnancy loss (Miura *et al*., 2021).

On other hand, results of the current study differed from these of Cusini and Ghislanzoni, (2001) ; Mohammad and Salman (2014), and Amar *et al.*,(2015) they fixed most of positive cases at ≥40 years of age. Results also disagreed with that of Hassan *et al.*,(2014) he established that pregnant women at the age between 30-39 years become more susceptible to infection than other periods of age. These results agreed with Salman, (2007) when reported that the maximum rate of viral abortion occurs at the second and third trimester more highly than first

trimester, and differed from results of Kapranos and Kotronias,(2009) and Hasan *et al*.,(2014) who established high rate of viral detection in serum samples aborted women at the first trimester. Baud *et al.*,(2008) established the infection as a causative agent of 15% and 66% of early and late abortion ,respectively.

Maternal age at the most recent pregnancy of 40 years or older and mother with HHV-6 were only risk factors for two or more spontaneous abortion identified by multivariable analyses. Since older maternal age is a reasonable risk factor for spontaneous abortion (Magnus *et al*.,2019)the reliability of this study is considered to be high.Furthermore ,it is well known that older maternal age , genetic abnormalities ,selected maternal antibodies , endocrine dysfunction , and uterine abnormalities are risk factors for spontaneous abortion(El Hachem *et al*.,2017).

There are many reasons that may explain the high incidence of HHV6 in women at age between 20 to 29 years, first: this age period considered the typical reproductive age of the women. Second, women at this age are more susceptible to chronic infection such as Herpes viruses (Srirup *et al.*,2012).Finally, occurrence of primary or recurrent Herpes viruses infection reaches to the peak at this age period (Duran *et al.*,2004).

5.4 Genotyping of TLR-2(rs1898830) Gene Polymorphisms In RPL and AHC

Spontaneous abortion is a complex, multifactorial pathology, where various genetic, neural, endocrine, and immunological factors are involved .Cytokines, Toll-like receptors, and progesterone receptors play critical roles in embryonic implantation and development. A delicate, stage-specific equilibrium of these proteins is required for successful pregnancy outcome. Recurrent pregnancy loss is a serious complication of pregnancy and failure of the innate immune system, one part of which are toll-like receptors (TLRs). May be link between variant of *TLR-2* with recurrent pregnancy loss (Anastasiia *et al*.,2018; Bahia *et al*.,2020).

The current results of TLR-2 (rs1898830) amplified was found the frequency of GG genotypes in women patients with RPL and AHC groups which reached 17% and 4%, respectively. It was significantly increased in women patients than control. While ,the frequency of AG genotypes in women patients with RPL and AHC groups which reached 38% and 8%, respectively. It was significantly increased in women patients than control. These results compatible with study of Bahia *et al*.,2020 who found the minor allele frequency of TLR-2 rs1898830 was significantly more frequent in recurrent pregnancy loss patients than in controls. Significantly higher frequencies of homozygous (2/2) *TLR-2* rs1898830 (14.1% vs. 8.9%) genotype carriers were seen between recurrent pregnancy loss cases and control women. Haploview analysis identified 1-locus TLR-2 haplotype (GC) that was positively associated with recurrent pregnancy loss.

Also current result consistent with Anastasiia *et al*.,(2018) who found the frequency of GG & GA genotypes in women patients with RPL and AHC groups which reached 0.925 ; 0.075 and 0.986 ;0.014, respectively. It was significantly increased in women patients than control. Estimated genotype frequencies as well as corresponding odds ratios and 95% condence interval (signicance level of 0.05) in the group of women with miscarriage and the control group.

In addition, He M *et al*.,(2017) who found higher protein expression levels of TLR-2 in the placentae and deciduas in the abortion-prone group than in the normal pregnant group.

Furthermore, these current result was disagree with result of Nasrin *et al*.,(2019) who showed by the flow cytometry no significant differences in the percentage of TLR2 expression in both the RPL and control groups.

Since pregnancy is delicately balanced between inflammation and intrauterine infections, immune and endocrine factors should always be in stage-specific equilibrium to avoid pregnancy loss (Axton *et al*.,2009).And hence, genetic variation in genes that encode proteins, which are involved in immune, endocrine, and neural mechanisms is an important factor at determining spontaneous abortion risk (Sykes *et al*.,2012 ;Sharma *et al*.,2013).

SNPs in Toll-like receptor gene can have severe impact on the pregnancy outcome (Manuck *et al*.,2010). Mutant SNPs TLR2, gene was found to has statistically significant effect on the risk of miscarriage with help of multiple logistic regression. (e multiple logistic regression analysis has also shown that the following double interactions showed statistically significant association with the risk of miscarriage: IL-10; IL-

8; and HA-G. Moreover, one triple genetic interaction was significantly associated with the risk of miscarriage: IL-10, and IL-18 (Anastasiia *et al*., 2018).

Effects of SNPs in TLR2 was identified as statistically significant by multiple logistic regression. TLR2 SNP is associated with imbalance in the system of innate immunity and, as a result, an increase in mother's organism sensitivity to the infections and miscarriage risk.

5.5 Genotyping of IL-10 (rs1518111) Gene Polymorphisms In RPL and AHC

A successful pregnancy depends on maintaining equilibrium between immunity mediated by Th1 cells and that mediated by Th2 cells. A predominance of Th2 immunity was observed, that means the stimulation of cytokines synthesis, produced by Th2cells, among which IL10 have an extremely important role Also the physiological evolution of pregnancy depends on the reduction of cytokine synthesis produced by Th1 cells aswell .The cytokine production is under genetic control (Costeas *et al*.,2004; Camil *et al*.,2014).

In the current results ,it was found the frequency of CC genotypes in women patients with RPL and AHC groups which reached 11% and 0%, respectively it was significantly increased in women patients than control. While ,the frequency of TC genotypes in women patients with

RPL and AHC groups which reached 17% and 16%, respectively. It was non- significantly in women patients compared with control group. On the other hand, the frequency of TT genotype in women patients with RPL and AHC groups was 9% and 4%, respectively, that increased in women patients compared with control group . Finally, was found which TC genotype decreased as rate OR=1.7 compared with CC genotype and equal to TT genotype among studied groups. According to the results , both of **TT** and **CC** were statistically higher than those of the control group according to the gene expression levels ($P<0.05$)**.**These results consistent with Camil *et al*.,(2014) who demonstrated a role for -819 C/T but not for -592 C/AIL10,-1082 A/G IL10 polymorphisms in idiopathic recurrent spontaneous abortion (RSA) in Romanian population. Frequency of genotype-592 CC/-819 CC was higher in the control group than in experimental group (p=0.005).

In addition ,current results is agreement with Qaddourah *et al*.,(2014) who found A higher minor allele frequency (MAF) of IL-10 rs1518111 (P ¼ 0.03) was in seen RPL cases; but the MAFs of the remaining SNPs were comparable between cases and controls. Setting the homozygous major allele genotype (1/1) as the reference, significantly higher frequencies of heterozygous IL-10 rs1554286 and IL-10 rs1800872, and homozygous rs1800896 genotype carriers, and a reduced frequency of homozygous IL-10 rs1518111 genotype carriers, were seen in RPL cases, while the distribution of the remaining genotypes were comparable between cases and controls.

IL-10 plays a dual immunosuppressive/anti-inflammatory role during pregnancy; it establishes a Th2 cytokine environment while reducing Th1 cytokine expression, which has been highlighted by the findings that

increased maternal IL-10 production is associated with successful pregnancy, whereas low levels are linked with recurrent fetal loss (Brogin *et al*., 2012).

Furthermore , a study of Kamali-Sarvestani *et al*.,(2005) who showed the correlation between polymorphisms IL-10 -592 C/A and -819 C/T and early spontaneous abortions; another study Zammiti *et al*.,(2008) was performed on the population in Iran, has demonstrated the existence of the correlation only between the polymorphism -592 C/A and recurrent abortions. There were also studies that concluded that there are no correlations between any of the 3 described polymorphisms and the etiology of spontaneous miscarriages.

A predominance of Th2 immunity was observed, that means the stimulation of cytokines synthesis, produced by Th2cells, among which IL10 have an extremely important role. Also, the physiological evolution of pregnancy depends on the reduction of cytokine synthesis produced by Th1 cells as well (Camil *et al*.,2014).

Conflicting results have been reported on the association of IL-10 polymorphism with RPL. For IL-10 rs1800896 (–1082A.G), no significant association with RPL was found in this study. This was disagreement with Argentinian (Prigoshin *et al*., 2004), Brazilian (Daher *et al*., 2003), Cypriot (Costeas *et al*., 2004), Tunisian (Zammiti *et al*., 2006) studies, but in apparent agreement with a study on 200 RPL and 300 control North Indian women, which documented strong association of IL-10 rs1800896 (P ¼ 0.0004) with RPL (Parveen *et al*., 2013) and IL-10 (-1082G/A, –819T/C) (P = 0.01, 95% CI 0.80[0.67,0.96]; P<0.01, 95% CI 0.66[0.49,0.89]) (Zhang *et al*.,2017).

Several replicative studies that followed also found no association between the IL-10 polymorphism and risk of recurrent pregnancy loss (Alkhuriji *et al*., 2013 ; Ma *et al*., 2017) these results was incompatible with current results.

In so far as its production varies as per the specific polymorphism, the role of IL-10 in RSA pathogenesis remains controversial . It was suggested that increased IL-10 expression was associated with successful pregnancy, whereas low levels were linked with recurrent fetal loss (Crilly *et al*.,2003). Others suggested theopposite, that enhanced IL-10 production was seen in recurrent pregnancy loss cases compared with fertile women (Prigoshin *et al*.,2004; Camil *et al*.,2014).

IL-10 takes part in the immunosuppressive response in congestion; high maternal IL-10 levels are associated with successful pregnancy and vice versa. The IL-10 polymorphism alters IL-10 secretion(Qaddourah *et al*.,2014).

Therefore, a SNP meta-analysis identifying the potential target out of a large population of SNPs and providing clinical evidence for relative gene promoter regulations is required. It can even provide clues about DNA folding, interactions, secondary structure formation and transcription complex function(Lupianez *et al*.,2015).

A successful pregnancy depends on immune balance, including immunotolerance, the immune response and relative cytokines levels. The corresponding immune cells that reside at the interface between the placenta and the uterus are subject to a superimposed layer of regulation by maternal immune cells. These cells not only foster placental function and development but also reduce the possibility of the placenta attacking the fetus. Abnormal decidual leukocytes lead to RPL, intrauterine growth restriction, preeclampsia, etc. These leukocytes also secrete interleukins that act in placental immunoregulation (Erlebacher ,2013).

All of these pregnancy-assisting functions of interleukins are based on normal interleukin expression and sequence and appropriate regulation, such as with DNA methylation and SNPs. In some situations, SNPs are located in the gene promoter; sometimes, they also appear within gene bodies and other non-coding regions. In these sensitive areas, even one base alteration will change relative protein binding and transcription

levels. These changes perhaps will become lethal to the sensitive immune balance between mother and fetus.

5.6 Genotyping of IL-18 (rs187238) Gene Polymorphisms In RPL and AHC

Production of cytokines and the distribution of the immune cells during pregnancy appeared to be critical in successful pregnancy. As a member of the IL-1 family of cytokines, interleukin-18 (IL-18) is produced by a variety of immune and non-immune cells, including endothelial cells, keratinocytes, intestinal epithelial cells, macrophages and dendritic cells (Yue *al*., 2015). As a pleiotropic proinflammatory cytokine, IL-18 is actively involved in the regulation of immune responses and enhances either Th1 or Th2 differentiation depending on the immunologic milieu. A few studies have shown that polymorphisms of IL-18 gene may be involved in the association of recurrent miscarriage (Galbiati *et al*.,2014; Weidlich *et al*.,2014).

In the current results ,was found the frequency of GG genotypes in women patients with RPL and AHC groups which reached 4%, in both them .It was non significantly in women patients and control groups.While ,the frequency of CG genotypes in women patients with RPL and AHC groups which reached 15% and 6%, respectively. It was significantly increased in women patients than control study groups. On the other hand, the frequency of CC genotype in women patients with RPL and AHC groups was 16% and 2%, respectively, that increased in women patients compared with control groups. Finally, was found which CG genotype increased as rate OR=1.9 compared with GG and CC

genotypes among studied groups. According to the results , both of **CC** and **CG** were statistically higher than those of the control group according to the gene expression levels ($P<0.05$). These results are in agreement with study of Ting *et al*.,(2019) who found the IL-18 heterozygous CG genotype had an OR of 0.6266 (0.4859–0.8081) (P = 0.0003) and an adjusted OR of 0.7342 (0.4412–0.8423) (P = 0.0016), while the homozygous GG genotype had an OR of 0.3878 (0.2112–0.7119) (P = 0.0022) and an adjusted OR of 0.5424 (0.1768– 0.7865) (P = 0.0014).

In addition, Yue *al*.,(2015) who found significant differences in the distribution of IL-18 rs187238 genotype are observed between aborted women patients and controls. IL-18 rs187238 variant exhibits significant association with recurrent miscarriage (RM) in any of three genetic models (additive model $p = 1.05 \times 10^{-4}$, dominant model $p = 0.025$, recessive model $p = 2.43 \times 10^{-5}$).

Specifically, variants in the promoter region of IL-18 gene influence the level of IL-18 protein production . IL-18, originally identified as an IFN-γ-inducing factor, is a pro-inflammatory cytokine with a unique capacity to induce T helper 1 or T helper 2 cells polarization depending on the immunologic context . The high production of IL-18 has been implicated in the pathogenesis of several immune-mediated processes (Messaoudi *et al*.,2012). Successful pregnancy depends on the cytokine environment, which can either be protective or harmful to the conceptus . Levels of IL-18 were significantly lower in women with RM than those without RM .Research also shows that the production of IL-18 is mediated by cleavage of caspase-1. Expression of both caspase 1 and IL-

18 are essential for pregnancy stability in pigs (Chatterjee *et al*.,2014; Yue *al*., 2015).

Some studies have been reported on the association of IL-18 variants with recurrent miscarriage but with inconclusive findings .IL-18 −105G/A (rs360717) and IL-18 −656C/A (rs1946519) IL-18 variants are significantly associated with recurrent miscarriage in 470 Tunisian women comprising 235 RM cases and 235 multi-parous controls. For IL-18−137G/C (rs187238), −119A/C (rs360718)**,** no significant association with RM was found in this study (Messaoudi *et al*.,2012).

This was an agreement with a case-control study in Arabic population from Bahraini . Both rs360717 and rs1946519 IL-18 single-nucleotide polymorphisms, but not rs360718 and rs187238, showed significant association with RM under additive, dominant, and recessive models. Lower serum IL-18 levels were seen between patients and controls and were more pronounced in rs360717 and rs1946519 heterozygous and homozygous genotypes (Al-Khateeb *et al*.,2011).

Meanwhile, in three ethnic populations with RM, including southern Iranian, Arabic population from Bahraini and Slovenian women, IL-18 gene promoter polymorphisms at positions −607 (rs1946518) and −137 (rs187238) did not confer susceptibility to RM (Ostojic *et al*.,2007; Al-Khateeb *et al*.,2011).

As discussed above, the inconsistency in study results may be due to study size or to genetic and/ or geographical features of the population from which the subjects were recruited.

5.7 Genotyping of HLA-G (rs1063320) Gene Polymorphisms In RPL and AHC

HLA-G molecules modulate the immune system by inhibiting the activity of cytotoxic T lymphocytes and NK cells, causing apoptosis of activated CD8+ T and CD8+ NK cells, as well as by inhibiting the proliferation of allogenic CD4+ T cells. HLA-G could also inhibit the transcription processes in the NK cells, thus protecting the extravillous trophoblast. All these functions of HLA-G illustrate its crucial role in the modulation of the maternal immune response, ensuring tolerance towards the semi-allogenic fetus in order to avoid miscarriage(Ferreira *et al*.,2017).

The present results of HLA-G (rs1063320) was appeared the frequency of GG genotypes in women patients with RPL and AHC groups which reached 9% and 6% ,respectively .It was significantly increased in women patients than control groups . While ,the frequency of CG genotypes in women patients with RPL and AHC groups which reached 18% and 8%, respectively. It was significantly increased in women patients than control study groups. On the other hand, the frequency of CC genotype in women patients with RPL and AHC groups was 5% and 4%, respectively, that increased in women patients compared with control groups. According to the results , CC, GG and CG were statistically higher than those of the control group according to the gene expression levels ($P<0.05$). These results agreement with Levkova *et al*.,(2020) who found individuals with a history of miscarriages had a significantly higher prevalence of 14 bp insertion

alleles compared with control patients (p=0.04).There was also a two times higher relative risk for miscarriage among carriers of this variant.

Also, Yazdani *et al*.,(2018) showed that −1573T>C and −1746C>A SNPs in the promoter of the HLA-G gene associated with RPL. The outcome of the haplotype analysis also showed that the association of two haplotypes, including H1 (ATCCAGGTACGCAA) and H2 (CTTCGAGAACGCAG) with RPL, is significant. The results showed that H1 is associated with a decreased and H2 is associated with an increased risk of RPL. These results agreement with our study.

In addition ,these findings correlate with another study, which concluded that the group of women with recurrent miscarriages showed a higher frequency of the ins allele in HLA-G, both in single and double copies(Amodio *et al*.,2016). Another research group found that the total number of ins alleles was higher among participants with fertility issues, but the number of heterozygotes was the highest(Arjmand *et al*.,2016).

However, the variant in the HLA-G gene will lead to alternative splicing and to the lack of 92 bps from the 3'untraslated region(Rizzo *et al*.,2012). Despite the fact that this ins increases the stability of the RNA transcript(Rousseau *et al*.,2012) in individuals who are homozygous for the 14 bp ins, there are lower concentrations of soluble HLA-G in serum compared with people who are homozygous for the 14 bp del(de Almeida *et al*.,2018). This might interfere with maintaining pregnancy because low serum concentrations of HLA-G are considered a prognostic marker for increased risk of miscarriage and poor possibility of successful implantation of the embryo after in vitro fertilization(Ferreira *et al*.,2017).

In a study conduncted in Denmark, there was a correlation between the 14 bp ins/del polymorphism and fetoplacental growth. The authors concluded that mothers who were homozygous for the 14 bp del gave birth to babies with higher birthweight compared with the children of mothers homozygous for the 14 bp ins(Hviid ,2004). However, the exact mechanisms of the protective effect of the HLA-G del remain to be determined.

One limitation of the study is that the sample size was small. If more people were included, a statistical difference might have been established because the p value was close to the level of significance for tests.

CONCLUSIONS

CONCLUSIONS & RECOMMENDATIONS

The following conclusions are obtained from the present study :

1. HHV6 might be one of the most recently identified uterus, cervical viruses in Iraqi female patients suffering from recurrent miscarriage and might play role in RPL in the Iraqi population.

2. Age factor have significant association with viral and non-viral infection; nor with the differences of mean ages of the HHV6.

3. TLR2 and HLA-G SNPs may be associated with the risk of RPL in the Iraqi women. While , IL-10 and IL-18 SNPs were identified as secondary factors that can also affect the risk of miscarriage.

4. Genetic polymorphisms in TLR2,IL10,IL18 and HLA-G genes might influence its function against cervical and uterus infection, thus could be involved in the pathogenicity of its infection.

5. The significant correlation of between the gene polymorphism (expression) of IL-18 (rs187238) with HHV-6 infection could indicate highly important role of these molecular factor in women patients suffering from recurrent miscarriage.

6. In contrast ,Less important role of both TLR-2(rs1898830) and HLA-G (rs1063320) with the HHV-6 infection in women patients suffering from recurrent miscarriage.

RECOMMENDATIONS

CONCLUSIONS & RECOMMENDATIONS

The following recommendations obtained from this study are dependent on current results :

1.iciHHV-6 could be added as another potential cause of recurrent spontaneous abortion.

2.Routine work use of viral detection such as (HHV-6, Rubella ,HCMV, HSV1&2 …etc) from blood ; tissues as well as vaginal swabs for Iraqi women in centrals health especially before married or in young age to prevent the pathogenesis; abortion ;inflammation or carcinogenesis.

3. Further prospective studies are required with a large number of HHV6 in Women patients with recurrent miscarriage cases that are needed to validate the result of currently study which may lead to a better understanding of the viral infection.

4. Studying the whole genome sequences of TLR-2(rs1898830) , IL10 (rs1518111) , IL-18 (rs187238) , and HLA-G(rs1063320) in Iraq and their differences from the global prevalence to explain their role in deregulation of cell cycle pathway in RPL.

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الخلاصة

ارتبطت العدوى الفيروسية أثناء الحمل بنتائج حمل سلبية وعيوب خلقية في النسل يوجد عدد محدود من الأدوات العلاجية أو الوقائية لحماية الأم والجنين أثناء الأوبئة. نادرًا ما تعبر الفيروسات حاجز المشيمة ، ولكن عندما يصل الفيروس إلى الجنين ، يمكن أن يؤدي إلى عيوب خلقية خطيرة مثل صغر الرأس أو حتى موت الجنين. يمكن أن يندمج فيروس الحلأ البشري ٦ في الكروموسومات البشرية ، مما يؤدي إلى انتقال جيني من الوالدين إلى الطفل. الأفراد من كلا الجنسين المصابين بفيروس الحلأ البشري الموروث والمتكامل كروموسوميًا 6 (iciHHV-6) يؤوي الفيروس في كل خلية. يمكن أن تحدث إعادة التنشيط الفيروسي من جينوم HHV-6 المدمج أثناء الحمل.

تلعب الحركيات الخلوية ، مستقبلات شبيه بالتول ، ومستقبلات البروجيسترون (هورمون الحمل) دور مهم وحرج في الزرع الجنيني وتطور الجنين حيث يتطلب في هذه المرحلة الحساسة من الحمل التوازن الخاص لهذه البروتينات لغرض انجاح الحمل.

أجريت هذه الدراسة لمعرفة تأثير فيروس الحلأ البشري -٦ على النساء الحوامل اللواتي يعانين من حالات الإجهاض. نوع الدراسة الحالية هي الحالات (المرضية) – والشواهد (سيطرة من نساء يبدين اصحاء ذوات ولادات طبيعية) حيث شملت الدراسة ١٠٠ مريضة من النساء اللاتي يعانين من الاجهاض والاجهاض المتكررو٥٠ من النساء اللاتي ليس لديهن اجهاض ; تراوحت اعمارهن من ١٧ إلى ٤٩ عامًا.

تم جمع العينات خلال الفترة من يناير ٢٠٢٠ إلى مايو ٢٠٢١.لقد شملت العينات مسحات من عنق الرحم وكذلك عينات الدم لاستخراج الجينوم الفيروسي لتشخيص فايروس الحلأ البشري نمط ٦ باستخدام تقنية تفاعل البلمرة المتسلسل في الوقت الحقيقي وجينوم الحمض النووي الكلي الدنا لتشخيص تعدد الأشكال للتغاير الوراثي ل مستقبل شبيه التول ٢ والحركيين الخلويين ١٠و ١٨ و مستضد التوافق النسيجي أ\د عن طريق التضخيم الحراري لنظام الطفرة. بالإضافة إلى ذلك ، تقديرمستوى تركيز مستقبل شبيه التول ٢ وانترلوكين١٠ و انترلوكين ١٨ و مستضد معقد التوافق النسيجي أ\د في المصل بوساطة مقايسة الامتزاز المناعي المرتبط بالإنزيم.

وبينت نتائج الدراسة الحالية يكون على النحو التالي:

- متوسط عمر النساء المريضات الذين يعانون من الاسقاط المتكرر (30.70 ± 12.41 سنة) أقل من متوسط عمر (28.67 ± 11.17) سنة النساء اللاتي لايعانين من الاجهاض المتكرر. لا توجد فروق ذات دلالة إحصائية (P = 0.47) بين RPL و AHC. بينما كانت الفئات العمرية (٣٠-٣٩) ، (١٧-٢٩)، (٤٠-٤٩) نسبة ٤٢٪ ، ٣٨٪ و ٢٠٪ على التوالي بالنسبة للنساء المجهضات . بينما أظهرت الطبقة العمرية ٣٠-٣٩ سنة ، ١٧-٢٩ ، ٤٠-٤٩ كانت ٤٠٪ و ٤٠٪ و ٢٠٪ على التوالي بالنسبة للنساء اللاتي ذات الولادات الطبيعية.
- لقد اوضح التشخيص الطبي وكذلك التشخيص المخبري للمريضات في هذه الدراسة وجد أن هناك ٣٢٪ تليها ٤٢٪ و ٢٦٪ فوق من النساء المريضات الذين يعانون من إجهاض واحد ومرتين وثلاثة مرات وما فوق على التوالي واوضحت الدراسة الحالية ان من بين ١٠٠ مريض ، وجد ٥٥٪ منهن أن لديهم جينوم فيروسي إيجابي كما أظهرت النتيجة الإيجابية وفقًا لـ تفاعل البلمرة التسلسلي أن ٤٦,٥٪ (٢٧ من ٥٨ حالة) كانت إيجابية بينما ٥٣,٥٪ (٣١ من ٥٨ حالة) سلبية.
- لقد كانت الفئة العمرية الأكثر إصابة بفيروس الحلأ البشري ٦ هي (٣٠-٣٩ سنة) بنسبة ٤٢٪ ، بينما شكلت الفئة العمرية (١٧-٢٩ سنة) ٣٨٪ ، تليها ٢٠٪ في الفئة العمرية (٤٠-٤٩) سنوات
- النتائج الإيجابية لاكتشاف الحلأ البشري بواسطة تقنية تفاعل البلمرة التسلسلي في النساء المصابات بالاجهاض المتكرر كانت ٤٥,٥٪ إيجابية و ٥٤,٥٪ سلبية.
- ظهر تضخيم حصيلة مثل المستقبلات نتيجة تضخيم أن تواتر الأنماط الجينية GG في النساء المصابات والطبيعيات قد وصلت إلى ١٧٪ و ٤٪ على التوالي. بينما بلغ معدل تكرار الأنماط الجينية AG في النساء المصابات والطبيعيات ٣٨٪ و ٨٪ على التوالي. لقد بينت الدراسة ان نسبة تواتر النمط الجيني AA في النساء المصابات والطبيعيات ١٦٪ و ٧ ٪ على التوالي ، وهو انخفاض في النساء مقارنة مع مجموعة التحكم. تم العثور على التركيب الوراثي AG الذي زاد بمعدل OR = 1.96 مقارنة مع التراكيب الوراثية GG و AA بين المجموعات المدروسة. كان كل من GG و AG أعلى من الناحية الإحصائية من تلك الخاصة بالمجموعة الضابطة وفقًا لمستويات التعبير الجيني

(P <٠,٠٥) ، ومع ذلك ، كان AA أقل إحصائيًا في النساء المريضات من المجموعة الضابطة.

- لقد اظهرت نتيجة تضخيم الانتلوكين ١٠) وجود شريطين (T Allele = 183 bp و C Allele = 133 نقطة أساس (بسبب وجود طفرة C> T. بينما تم تحديد النوع البري بواسطة جزء واحد من ٣١٦ زوج قاعدي. يمكن ملاحظة أن تواتر الأنماط الجينية CC في النساء المصابات والطبيعيات قد وصلت إلى ١١٪ و ٠٪ ، على التوالي ، والتي ازدادت بشكل ملحوظ في المريضات مقارنة بالضابطة.لقد كانت نسبة الطرز الوراثية لدى النساء المصابات والطبيعيات بلغت ١٧٪ و ١٦٪ على التوالي ، ولم يكن معنويا في النساء مقارنة مع مجموعة السيطرة. كانت ٩٪ و ٤٪ على التوالي ، والتي زادت في النساء مقارنة مع مجموعة السيطرة وجد أن النمط الجيني TC انخفض بمعدل OR = 1.7 مقارنة بالنمط الوراثي CC ومتساوي مع النمط الوراثي TT بين مجاميع الدراسة. كلا من TT و C كانت C أعلى إحصائيًا من تلك الخاصة بالمجموعة الضابطة وفقًا لمستويات التعبير الجيني (P <٠,٠٥)
- ظهرت نتيجة تضخيم الحركي الخلوي ١٨ وجود شريطين (G Allele = 178 نقطة أساس و A Allele = 210 نقطة أساس (بسبب وجود طفرة C> G. بينما تم تحديد النوع البري بجزء مفرد ٣٨٨ زوج قاعدي ويمكن ملاحظة أن تواتر التراكيب الجينية GG في النساء المصابات والطبيعيات قد بلغ ٤٪ في كل منهما. ولم يكن معنويا في النساء ومجموعات المراقبة. طرز CG الجينية لدى النساء المصابات والطبيعيات بلغت ١٥٪ و ٦٪ على التوالي ، وزادت بشكل ملحوظ في النساء مقارنة بمجموعات الدراسة الضابطة. كانت ١٦٪ و ٢٪ على التوالي والتي زادت في النساء مقارنة بمجموعات الضبط ،تم الكشف عن وجود النمط الجيني CG الذي زاد بمعدل OR = 1.9 مقارنة بالأنماط الوراثية GG و CC بين المجموعات المدروسة. و CG كانت إحصائيا أعلى من تلك الخاصة بالمجموعة الضابطة وفقًا لمستويات التعبير الجيني (P <٠,٠٥)
- بينت نتيجة تضخيم مستضد التوافق النسيجي وجود نطاقين (G Allele = 178 نقطة أساس و C Allele = 245 نقطة أساس (بسبب وجود طفرة C> G. في حين تم تحديد النوع البري بواسطة شظية مفردة ٤٢٣ زوج قاعدي ويمكن ملاحظة أن تواتر التراكيب الجينية GG في النساء المصابات والطبيعيات قد بلغ ٩٪ و ٦٪ على التوالي ، وزاد بشكل ملحوظ في النساء مقارنة بمجموعات الضبط. لقد كانت نسبة طرز CG الجينية لدى النساء المصابات والطبيعيات قد بلغت ١٨٪ و ٨٪ على التوالي ، وزادت بشكل

ملحوظ في النساء المصابات مقارنة بمجاميع الدراسة الضابطة. كانت ٥٪ و ٤٪ على التوالي والتي زادت في النساء مقارنة بمجموعات الضبط ، تم الكشف عن وجود النمط الجيني CG الذي زاد بمعدل OR = 1.8 مقارنة بالنمط الوراثي CC وانخفض المعدل مقارنة بالنمط الجيني GG بين المجموعات المدروسة. كانت نتائج CC و GG و CG أعلى إحصائيًا من تلك الخاصة بمجموعة التحكم وفقًا لمستويات التعبير الجيني (P <٠,٠٥)

ونستنتج من هذه الدراسة ان :

فيروس الحلأ البشري – ٦ فايروس مكتشف حديثا في اصابات الجهاز التناسلي الأنثوي للنساء العراقيات اللاتي يعانين من الإجهاض والاجهاض المتكرر. بالإضافة إلى ذلك ،من الممكن ان يرتبط تعدد الأشكال للتغاير الوراثي ل مستقبل شبيه التول-٢ و مستضد معقد التوافق النسيجي أ\د ارتباط وثيق مع حالات الاجهاض والاجهاض المتكرر في النساء ..بينما كان تعدد الأشكال للتغاير الوراثي للحركي الخلوي١٠ و الحركي الخلوي ١٨ كعاملان ثانويان مؤثران في حالات حدوث الاجهاض والاجهاض المتكرر في النساء العراقيات.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة بابل

كلية العلوم

قسم علوم الحياة

دراسة وراثية- مناعية لعدوى فيروس الحلأ البشري نمط ٦ في النساء المصابات بالإجهاض المتكرر

أطروحة مقدمة الى مجلس كلية العلوم في جامعة بابل، وهي جزء من متطلبات نيل درجة الدكتوراه فلسفة

في علوم الحياة / الآحياء المجهرية.

من قبل

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