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Association Between Human Herpes Virus 7 Infection and IL-33 Polymorphism in Aborted Women

A Thesis

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By

Ghufran Salam Hadi Wannas

B.Sc. Biology /University of Babylon (2010)

Supervised By

Prof. Shakir Hammad Mohammed Al.Alwany

Ph.D Molecular & Medical Virology

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

﴿قَالَ رَبِّ اشْرَحْ لِي صَدْرِي (٢٥) وَيَسِّرْ لِي أَمْرِي (٢٦) وَاحْلُلْ عُقْدَةً مِنْ لِسَانِي (٢٧) يَفْقَهُوا قَوْلِي (٢٨)﴾

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سورة طه

Certification

I certify that the thesis entitled (**Association Between Human Herpes Virus-7 Infection and IL-33 Polymorphism in Aborted Women)** was prepared under my supervision at the Department of Biology, College of Science, University of Babylon, in partial fulfillment of requirement for the degree of Master in Biology/ Microbiology.

Signature

Professor

Dr. Shakir Hammad Mohammed Al_Alwany

PH.D. (Ph.D. Molecular & Medical Virology)

Supervisor

In view of the available recommendation, I forward this thesis for debate by the examining committee.

Signature

Assistant Professor

Dr. Adi Jassim Abd Alrezzaq

Head of

Department of Biology

College of Science/University of Babylon

Dedication

To whom I carry his name with pride…To the one I miss since childhood… To whom my heart trembles by just remembering him… To whom I miss with all my heart…. Dad

To the source of tenderness…To the luminous candle of my life…And the delicate flower in my own spring… To the one who bow their stature out of respect for her presence and raise their heads in pride…To the one who was her name my joy and her tenderness was a balm for me ….my dear mother

To those who gave my life colors…And fill my soul with the sweetest melodies….And they were the sun that showed my desolation…And the healing balm to the wounds of my days…To my brother and sisters..

Ghufran

2022

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2022

SUMMARY

Summary

Viral infections during pregnancy are 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 fetal death. Human herpes virus 7 is thought to cause severe complications and spontaneous abortion. Normal pregnancy is related to the successful transition from type 1 cellular immunity to type 2 cellular immunity. Interleukin 33 , induced the production of type 2 cytokines . These experiments were conducted to study the effect of human herpes virus 7 on the pregnant women that cause abortion. This case control study was done for 100 patients including different ages that range from 18-47 years old that recurrent miscarriage. Also the study includes 50 apparently healthy control (normal pregnancy and delivery) , their age similar to the patients age. The specimens were collected during the period from October 2021 to February 2022. Endometrium; cervical swabs; fetal fluids swabs as well as blood specimens were collected and processed to extract viral genome and total DNA gene for screened human herpes virus-7 by using polymerase chain reaction and IL-33 rs1891385 polymorphism by polymerase chain reaction and sequencing. In addition, estimation serum IL-33 was concentrated by enzyme-linked immunosorbent assay.

The obtained results of this study are summarized as follows: The mean age of the patients with recurrent miscarriage was (32.70±12.41 years) was less than the mean age of the apparently healthy control (30.67 ± 11.17 years). There were non-significant statistical differences (p=0.47) between recurrent miscarriage and apparently healthy control. A strong positive relationship (with highly significant correlation) was found between number of participants; number abortion; week of

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

Out of 100 patients, 55 (55%) were found to have a viral infection whereas 45 % as negative with recurrent miscarriage. The positive result according to polymerase chain reaction shows 34.5 % (19 out of 55 cases) as positive while 65.5% (36 out of 55 cases) as negative. In women with recurrent miscarriage, the most commonly affected age stratum infected with DNA- HHV-7 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 – 47 years). The positive result, according to PCR amplification of a single band (422 bp) of *IL-33 rs1891385* gene in women patients with recurrent miscarriage and Apparently Healthy Control were 55% (55 of 100 cases) and 30% (15 of 50 cases), respectively. While, the negative results were in women patients with recurrent miscarriage and Apparently Healthy Control were 45% (45 of 100 cases) and 70% (35 of 50 cases), respectively. The results showed that DNA polymorphism for interleukin 33 distribution were DNA polymorphism distributions according genotype to A\C; A\T; A\G; T\G; T\A and T\C haplotypes were 9%; 7%; 9%; 6%; 9% and 5%, respectively in patient's female with Recurrent Pregnancy Loss and 4%; 0%; 8%; 2%; 3% and 2%, in Apparently Healthy Control group respectively. In addition, two types of mutation in *IL-33 rs1891385* gene transversion and transition were found. The frequency of transversion mutation (A\C; A\T; T\G; T\A and T\C) more than the transition mutation (A\G). We have recorded six new recordings in gene bank: ON564301; ON564302; ON564303; ON564304; ON564305; ON564306 .

The mean of serum interleukin 33 concentration in women patients with recurrent miscarriage and apparently healthy control were 10.66 ±0.95 pg./ml and 6.28 ±0.42 pg./ml, respectively.

The conclusions from these results of the current study: human herpes virus 7 is one of the most recently identified vaginal viruses in Iraqi women patients suffering from recurrent miscarriage. In addition, highly serum concentration of 33 interleukin level and IL-33 rs1891385 polymorphism in women patients with recurrent miscarriage could point that interleukin 33 acts as a risk factor in the pathogenesis of idiopathic recurrent miscarriage . The significant correlation of between the gene polymorphism of interleukin 33 with human herpes virus 7 infection could indicate highly important role of these genetic factor in women patients suffering from recurrent miscarriage .

List of Content

List of Figures

List of Tables

List of Abbreviations

Symbol	Description
AHC	Apparently healthy control
ARMS	Amplification refractory mutation system
bp	Base pair
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
C.I	Confidence interval
CMV	Cytomegalovirus
DNA	Deoxyribose nucleic acid
ECs	Endothelial cells
EDTA	Ethylene di amine tetra acetic acid
E genes	Early genes
ELISA	Enzyme linked immune sorbent assay
EPDS	Edinburgh postnatal depression scale
EVA	Electric vacuum aspiration
gB	Glycoprotein B
GAS	Gamma interferon activation sites
gM	Glycoprotein M
HESCs	Human endometrial stromal cells

HCMV	Human cytomegalovirus
HHV-6	Human Herpes Virus 6
HHV-7	Human Herpes virus 7
HLA	Human leukocyte antigen
iciHHV-6	Inherited chromosomally integrated HHV-6
iciHHV-7	Inherited chromosomally integrated HHV-7
ICTV	International committee on taxonomy of viruses
IE	Immediate early
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IFA	Immunofluorescence assay
IL1RL1	Interleukin 1 receptor like 1
IL1RAP	Interleukin 1 receptor accessory protein
IL-33	Interleukin 33
IFN-ɣ	Interferon-gamma
ISRE	Interferon stimulated response element
L genes	Late genes
L.S.D	Least of standard deviation
MHC	Major histocompatibility complex
mRNA	Messenger Ribose nucleic acid
NF-KB	Nuclear factor-kappa beta
NK cell	Natural killer cell
MVA	Manual vacuum aspiration
MW	Molecular weight
OBP	Origin binding protein
PBMCs	Peripheral blood mononuclear cells
O.D	Optical density
O.R	Odds ratio
oriLyt	Origin of lytic replication
ORF	Open reading frame
PCR	Polymerase chain reaction
Pg./ml	Picogram per milliliter
RFLP	Restriction fragment length polymorphism

RM	Recurrent Miscarriage
RNA	Ribose nucleic acid
RPL	Recurrent pregnancy Loss
RT PCR/ qPCR	Real Time Polymerase chain reaction/quantitative
S.D	Standard deviation
S.E	Standard error
S.E.M	Standard error mean
SNP	Single nucleotide polymorphism
SPSS	Statistical package for the social sciences
SSCP	Single strand conformation polymorphism
TBE	Tris-borate-ethylene di amine tetra acetic acid
Th2	T-helper cell 2
TNF-α	Tumor necrosis factor alpha
Tregs	T-regulatory cells
uRM	Unexplained recurrent miscarriage
VTM	Viral transport media
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 miscarriage (RM), is 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 RM (Carlo *et al*.,2020).

Indeed, there is a considerable discrepancy between the reported birth rates and the rates of gestational complications of the successive pregnancy in women with RM . The likelihood of a live birth in the successive pregnancy in untreated women with RM 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 RM ; 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, only around 50% of RM cases can defined as causes/risk factors and can be found, including advanced maternal age, genetic abnormalities, selected maternal autoantibodies, endocrine dysfunctions, and uterine abnormalities.

The remaining RM cases currently are unexplained recurrent miscarriage (Ticconi *et al*.,2019).

Recent studies have shown that the etiology of RM is complex and varied, including advanced age, genetic abnormalities, infections, immune disorders, coagulation abnormalities, and endocrine dysfunction (Ticconi *et al*.,2019; Guo *et al*., 2020).

Human Herpes virus 7 (HHV-7) is one of nine known members of the Herpesviridae family that infects humans. HHV-7 is a member of Betaherpesvirinae, a subfamily of the Herpesviridae that also includes Human Herpes Virus 6 (HHV-6) and Human Cytomegalovirus (HCMV). HHV-7 often acts together with HHV-6, and the viruses together are sometimes referred to by their genus, Roseolovirus (Jéssica *et al*.,2020).

The complete HHV-6 and HHV-7 genome is integrated into every nucleated cell of an individual with inherited chromosomally integrated human herpes virus 6 (iciHHV-6). Extremely high copy numbers of HHV-6 and HHV-7 DNA can be detected in clinical specimens containing nucleated cells, which can lead to a misdiagnosis of active viral infection. Theoretically, a parent with HHV-7 has a 50% chance of transmitting the integrated HHV-6 and HHV-7 genome to the next generation (Gaccioli *et al*.,2020).

HHV-6 and HHV-7 can integrate into human chromosomes, resulting in genetic transmission from parent to child. Individuals of either sex with inherited chromosomally integrated human herpes virus 7 (iciHHV-7) harbor the virus in every cell. Viral reactivation from the integrated HHV-6 and HHV-7 genome can occur in pregnancy (Miura *et al.*,2020).

The exact mechanism by which abnormal immune factors because 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).

The success of pregnancy is based on the completion of an accurate switch of immune defense mechanisms during the early stages of embryo colonization. The embryo is a semi-allogeneic graft to the mother in early gestation and is therefore antigenic in its initial stages of development. The uterus, as the site of embryo implantation and development, is non-specific in its initial immune response to the embryo. The endometrial stroma contains a large number of immune cells, including natural killer cells (NK), innate lymphocytes, macrophages, decidual dendritic cells and T cells(Wang *et al*., 2020).

Interleukin 33 (*IL-33*) is an important ligand for growth stimulation expressed gene 2 (*ST2*). *IL-33* is a dual-function cytokine whose full-length form acts as a transcription factor in the nucleus and inhibits the expression of *NF-κB*, a gene that regulates pro-inflammatory signaling. When cells are subjected to external stimuli or cellular necrosis, the precursor of *IL-33* can be processed by neutrophil-derived proteases into the mature *IL-33* form, which is then released as a cytokine into the extracellular compartment (Boga *et al*., 2016).

Mature *IL-33* can bind to *ST2L* and trigger an inflammatory cascade response. However, *sST2* binding to *IL-33* prevents its binding to *ST2L* in immune cells, which in turn inhibits the activation of *Th2* cells and the release of anti-inflammatory cytokines (*IL-4, IL-5, IL-10, IL-13*), and enhances the activation of *Th1* cells and the release of pro-inflammatory cytokines (*TNF-α, etc*.).Thus, *IL-33/ST2* has an immunomodulatory role and this signaling axis may play an important role in a variety of diseases associated with major *Th2* immune

responses, such as rheumatoid arthritis, sepsis, and ulcerative colitis (Zhao *et al*., 2022).

Several Iraqi studies have conducted in different cities and reported the relation between viral infections and abortion such as HCMV, HSV-1&2, Parvovirus B19, HHV-6&7 among aborted women. To our knowledge, there is no Iraqi study done about HHV-7.

1.2 Aim of Study

In view of the afore introduction, we designed this study to determine the percentage of Human Herpes Virus 7 and IL-33rs1891385 polymorphism in women suffering from abortion as well as recurrent miscarriage, **through achieving the following objectives:**

1. To determine the percentage of HHV-7 in Endometrium; cervical swabs; fetal fluids swabs as well as blood specimens that range from normal pregnancy and delivery women to patients suffering from abortion as well as RM by PCR technique.
2. Estimation of the genetic polymorphism of IL-33rs1891385 gene in study groups.
3. Evaluation of IL-33 level concentration in study groups by ELISA.
4. Find the association between these HHV-7 and IL-33 in women suffering from recurrent miscarriage and control groups.

CHAPTER TWO

LITERATURE REVIEW

2 Literature Review

2.1 Abortion

Abortion is the termination 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).

2.1.1 Types of Abortion

2.1.1.1 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).

2.1.1.2 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. About 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 Methods of abortion

2.1.2.1 Medical

The most common early first-trimester medical abortion regimens use mifepristone in combination with misoprostol (or sometimes another prostaglandin analog, gemeprost) up to 10 weeks (70 days) gestational age, methotrexate in combination with a prostaglandin analog up to 7 weeks gestation, or a prostaglandin analog alone. Mifepristone–misoprostol combination regimens work faster and are more effective at later gestational ages than methotrexate–misoprostol combination regimens, and combination regimens are more effective than misoprostol alone. This regimen is effective in the second trimester (Kulier *et al*., 2011).

Medical abortion regimens involving mifepristone followed by misoprostol in the cheek between 24 and 48 hours later are effective when performed before 70 days' gestation (Chen and Creinin, 2015).

In very early abortions, up to 7 weeks gestation, medical abortion using a mifepristone–misoprostol combination regimen is considered to be more effective than surgical abortion (vacuum aspiration), especially when clinical practice does not include detailed inspection of aspirated tissue. Early medical abortion regimens using mifepristone, followed 24–48 hours later by buccal or vaginal misoprostol are 98% effective up to 9 weeks' gestational age; from 9 to 10 weeks' efficacy decreases modestly to 94%. If medical abortion fails, surgical abortion must be used to complete the procedure (Jones *et al*., 2019).

Medical abortion regimens using mifepristone in combination with a prostaglandin analog are the most common methods used for second-trimester abortions in Canada, most of Europe, China and India, in contrast to the United

States where 96% of second-trimester abortions are performed surgically by dilation and evacuation (Jones *et al*.,2019;Gambir *et al*.,2020).

A 2020 Cochrane Systematic Review concluded that providing women with medications to take home to complete the second stage of the procedure for an early medical abortion results in an effective abortion. Further research is required to determine if self-administered medical abortion is as safe as provider-administered medical abortion, where a health care professional is present to help manage the medical abortion. Safely permitting women to self-administer abortion medication has the potential to improve access to abortion. Other research gaps that were identified include how to best support women who choose to take the medication home for a self-administered abortion (Gambir *et al*., 2020).

2.1.2.2 Surgical

Up to 15 weeks' gestation, suction-aspiration or vacuum aspiration are the most common surgical methods of induced abortion. Manual vacuum aspiration (MVA) consists of removing the fetus or embryo, placenta, and membranes by suction using a manual syringe, while electric vacuum aspiration (EVA) uses an electric pump. These techniques can both be used very early in pregnancy. MVA can be used up to 14 weeks but is more often used earlier in the U.S. EVA can be used later (WHO, 2017).

Manual vacuum aspiration, also known as "mini-suction" and "menstrual extraction" or EVA can be used in very early pregnancy when cervical dilation may not be required. Dilation and curettage (D&C) refers to opening the cervix (dilation) and removing tissue (curettage) via suction or sharp instruments. D&C is a standard gynecological procedure performed for a variety of reasons, including examination of the uterine lining for possible malignancy, investigation of

abnormal bleeding, and abortion. The World Health Organization recommends sharp curettage only when suction aspiration is unavailable (WHO, 2017).

Dilation and evacuation (D&E), used after 12 to 16 weeks, consists of opening the cervix and emptying the uterus using surgical instruments and suction. D&E is performed vaginally and does not require an incision. Intact dilation and extraction (D&X) refers to a variant of D&E sometimes used after 18 to 20 weeks when removal of an intact fetus improves surgical safety or for other reasons (Hammond and Chasen, 2009).

A First-trimester procedures can generally be performed using local anesthesia, while second-trimester methods may require deep sedation or general anesthesia (Templeton, Grimes, 2011).

2.1.2.3 Labor Induction Abortion

In places lacking the necessary medical skill for dilation and extraction, or where preferred by practitioners, an abortion can be induced by first inducing labor and then inducing fetal demise if necessary. This is sometimes called "induced miscarriage". This procedure may be performed from 13 weeks' gestation to the third trimester. Although it is very uncommon in the United States, more than 80% of induced abortions throughout the second trimester are labor-induced abortions in Sweden and other nearby countries (Borgatta,2014).

2.1.2.4 Other Methods

Historically, a number of herbs reputed to possess abortifacient properties have been used in folk medicine. Among these are: tansy, pennyroyal, black cohosh, and the now-extinct silphium (Cunningham *et al*.,2014).

Abortion is sometimes attempted by causing trauma to the abdomen. The degree of force, if severe, can cause serious internal injuries without necessarily succeeding in inducing miscarriage. In Southeast Asia, there is an ancient tradition of attempting abortion through forceful abdominal massage. One of the bas reliefs decorating the temple of Angkor Wat in Cambodia depicts a demon performing such an abortion upon a woman who has been sent to the underworld (Potts *et al*., 2007).

2.2 Human Herpes Virus-7

2.2.1 Historical Preview

Human herpesvirus 7 was discovered by Frenkel and colleagues in 1990 from CD4+ T cells taken from peripheral blood lymphocytes of a healthy donor and was shown to be a cause of exanthem subitum (Mandell *et al*.,2015).

Human herpesvirus 7 is shed in the saliva of healthy subjects. It establishes latent infection in CD4+lymphocytes and salivary glands. Human herpesvirus 7 is ubiquitous worldwide, and approximately 70% of children by the age of 4 years' contract infection via droplets or breast milk (Joseph *et al*., 2014).

2.2.2 Taxonomy and Classification of HHV-7

Herpesvirus was established as a genus in 1971 in the first report of the International Committee on Taxonomy of Viruses (ICTV). HHV-7 belongs to the β Herpesvirinae subfamily. HHV-7 was recognized by the ICTV as distinct species in 2012. β herpesviruses: consist the Human Cytomegalovirus, and human herpesviruses 6 and 7, with a long replicative cycle and restricted host range (ICTV, 2020).

Unranked: *Virus*

Relam: *Duplodnaviria*

Kingdom: *Heunggongvirae*

Phylum: *Peploviricota*

Class: *Herviviricetes*

Order: *Herpesvirales*

Family: *Herpesviridae*

Subfamily: *Betaherpesvirinae*

Human herpesvirus-6 and HHV-7, which share high genome similarity but differ for some biologic properties, epidemiology, and disease association. Although they are classified as lymphotropic viruses, there in vivo tropism is considerably broader, including T-lymphocytes, macrophages, endothelial cells (ECs), salivary glands, and brain, thyroid epithelial cells, natural killer cells, and endometrial cells (Caselli *et al*., 2017).

2.2.3 Morphology and Structure of HHV-7

2.2.3.1 HHV-7 Particles

The diameter of an HHV-7 virion is about 150-200 nm. The size of the HHV-7 virion increases from 120 nm to approximately 300 nm after the inclusion of the tegument and envelope .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, spherical to pleomorphic, and round geometries, and T=16 symmetry composed of 162 capsomeres (Kawabata *et al*.,2009).

2.2.3.2 HHV-7 Genome Organization

The genome of HHV-7, similar to those of other herpesviruses, consists of a large linear double stranded DNA molecules, 160 kb in length, containing a unique segment flanked by direct repeats (Heming *et al*., 2017; Finkel *et al*.,2020).

Complete genome sequences are available for HHV-7. The genomes of three different strains of HHV-7(JI, RK, and UCL-1), have been sequenced using viral DNA isolated from peripheral blood mononuclear cells (PBMCs) (JI and RK) or saliva (UCL-1) (Staheli *et al*., 2016; Heming *et al*., 2017).

The HHV-7 origin of lytic replication (oriLyt) is located uapstream of the major DNA-binding protein gene U41, similar to its location in other Betaherpesvirinae subfamily genomes. The oriLyt sequence contains two binding sites, origin-binding protein 1 (OBP-1) and OBP-2, both of which are recognized by an origin-binding protein (OBP) encoded by the viral open reading frame (ORF) U73.Interaction between OBP and the binding sites is required to initiate viral DNA replication. The HHV-7 unique region also contains two repetitive sequence motifs, R1 and R2 (Arbuckle *et al*., 2011).

The R1 segment is located between ORFs U86 and U89 and, in the RK strain, is composed of two 84-bp repeats plus two partial 67-bp repeats, with the nucleotide sequences being conserved among HHV-7 strains. The second segment, R2, is located downstream of R1 between ORFs U91 and U95 and is composed of 105-bp motifs repeated 16 times. The coding potential of the R1 and R2 repeat regions for either short proteins or noncoding RNAs is uncertain, and both regions have been shown to vary in size and sequence among different strains of HHV-7 (Tang *et al*., 2010; Arbuckle *et al*., 2011).

The HHV-7 genome is predicted to encode 82 different proteins in the unique region, with their ORFs being annotated U2 to U100. In addition, two protein-coding ORFs (DR1 and DR6) have been identified in each of the terminal repeated regions. All but one of the ORFs in the HHV-7 genome has counterparts among the other roseolovirus genomes. The exception is U55B, which is found only in different HHV-7 strains and not in either HHV-6A or HHV- 6B. Conversely, the HHV-7 genome lacks ORFs U22 and U94, which are present in both HHV-6 species. Some additional small expressed ORFs are present in the HHV-6B DR region but not in HHV-7(Figure 2.1) (Staheli *et al*., 2016).

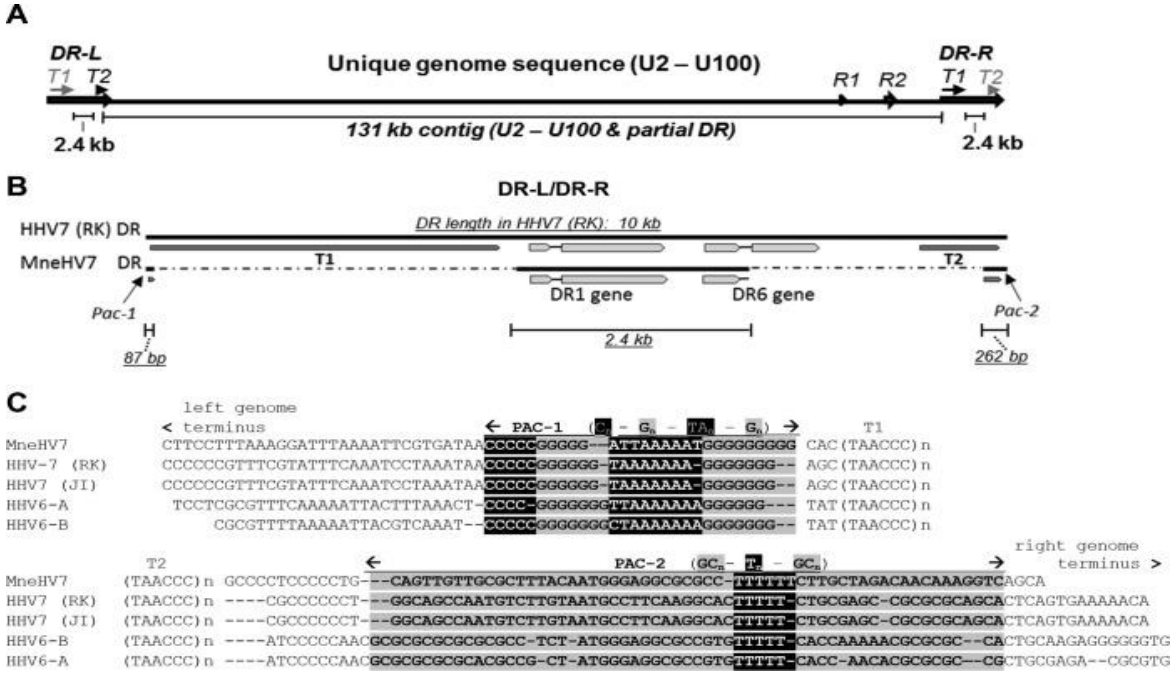


Figure 2.1: Schematic overview of MneHV7 genomic sequences obtained by de novo assembly (Staheli *et al*., 2016).

2.2.3.3 Cellular Effects

Human herpes virus 7 resides mostly in CD4+ T cells, albeit only in certain strains of them. To enter CD4+ T cells, HHV-7, unlike HHV-6, uses CD4 and possibly some cell-surface glyoproteins to enter CD4+ T cells. About a week after HHV-7 has infected a cell, it begins to downregulate CD4 transcription, which

interferes with HIV-1 infection but may reactivate HHV-6 infection. It is however unclear exactly what effect HHV-7 has on HIV infection. HHV-7 also has a number of other effects on cells. Among these include membrane leaking, the presence of lytic syncytia, occasional apoptosis, the supporting of latent infection, and increases and decreases in levels of certain cytokines (Tremblay *et al*.,2020).

2.2.3.4 HHV7 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-7 and HHV-6 are associated with the unique glycoproteins gQ1 and gQ2 encoded in the open reading frame 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-7 and 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-7/HHV-6 into the telomeres — highly expressed during latency (Arbuckle *et al*.,2011).

An 85-kDa phosphoprotein (pp85) represents an immunodominant protein specific for HHV-7and is localized to the tegument substructure of the HHV-7 virion. In addition, an 89-kDa protein was identified as a HHV-7–specific serologic marker by immunoblot assay. HHV-7 gB is involved in the early steps of infection by interacting with specific cell surface molecules. HHV-7 gB consists of 822 amino acids (aa) and shows features characteristic of type I integral membrane proteins. Computer analysis predicts that this protein has a signal peptide at the amino terminus, a large external domain which contains 11 potential N-glycosylation sites, and a transmembrane domain followed by a shorter cytoplasmic tail (Kempf,2002).

2.2.3.5 Replication Cycle of HHV-7

The HHV6 and HHV-7 have a similar replication cycle takes 72 hours to complete and consists of four general steps as shown in (Figure 2.2):

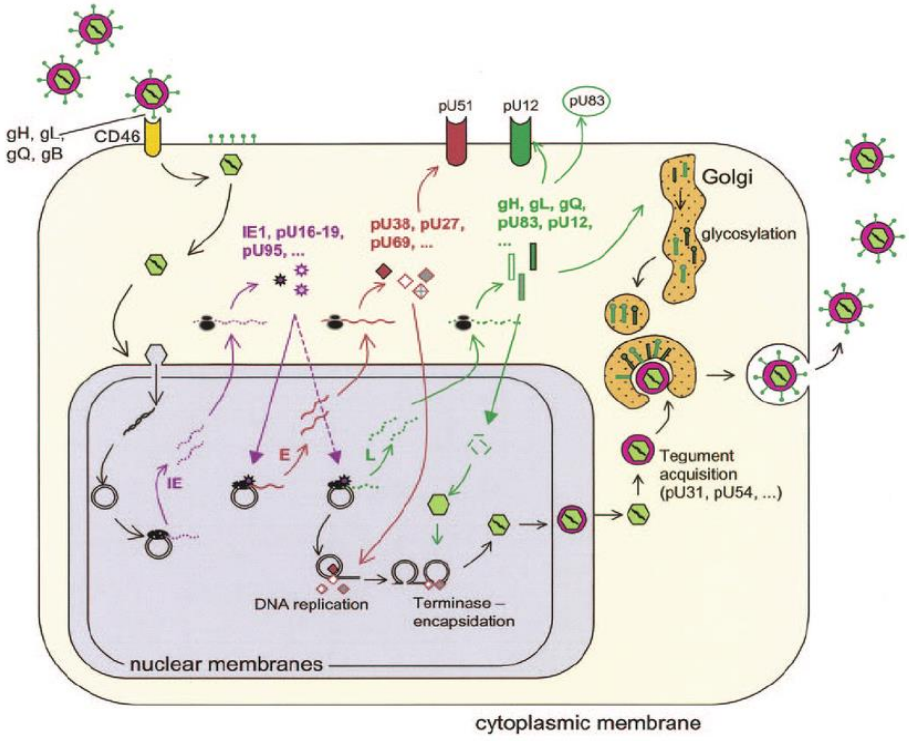


Figure 2.2: Schematic Representation of HHV-7 Replication Cycle (Leen *et al*., 2005).

2.2.3.5.1 HHV-7 Receptor and Host Tropism

Human herpesvirus 7 infects a wide range of human cells in vitro, but it preferentially replicates in activated CD4+ T lymphocytes. Human herpesvirus 7 uses the CD4 molecule as a critical component of the cellular membrane receptor. Synthesis and expression of cell surface CD4 becomes dramatically down-regulated in HHV-7–infected cells. Apart from CD4+ T cells, macrophages have been identified as target cells in vivo and in vitro. Moreover, simultaneous replication of HHV-7 was found in CD68+ positive cells of monocytic lineage in Kaposi's sarcoma (Hanson *et al*.,2018).

During primary infection, HHV-7 DNA sequences can be detected in peripheral blood mononuclear cells (PBMC) at acute and convalescent stages. HHV-7 DNA is excreted into saliva and transiently into stool at an early convalescent stage, after primary infection, HHV-7 is able to establish a latent infection in PBMC, from which it can be reactivated. In addition, the presence of viral antigens indicative of viral replication has been demonstrated in salivary glands and in a wide variety of normal tissues, indicating chronic persistent HHV-7 infection (Yoshikawa *et al*., 2004).

2.2.3.5.2 HHV-7 Attachment

Human herpesvirus 7 gB binds to heparin and cell surface heparin sulfate gp65. In fact, it has been shown that gB homolog glycoproteins play an essential role in virus attachment to and penetration of the host membrane for several herpesviruses. The gB homolog of HHV-7 is able to bind cell surface HS, which likely functions as an essential co-receptor for a productive infection of CD41 T-lymphoid cells, the major target of this virus (Akhtar *et al*.,2009).

2.2.3.5.3 Entry and Un-coating

Following attachment, HHV-7 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-7 DNA is released into the nucleoplasm (Henri *et al*.,2015).

2.2.3.5.4 Impacts of HHV-7 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 DRL and DRR regions (Tsao *et al*., 2009; Yamanishi *et al*.,2013).

In addition, independently of any complete virus-producing process, the expression of certain HHV-7 genes might occur from persisting episomal or iciHHV-7 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 (Yamanishi *et al*.,2013).

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-7 genome have immunomodulatory functions. Taken together, all these features provide molecular bases for understanding the pathological processes associated with acute and chronic HHV-7 infections (Caruso *et al*.,2009).

2.2.3.5.5 HHV-7 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).

2.2.3.6 Latency and Reactivation

Like other human herpesviruses, HHV-7 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-7, 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).

2.2.3.7 Pathophysiology of the Infection

Following penetration of the virus into the target cells, the genomic DNA migrates to the cell nucleus where key steps of viral replication take place. Viral genes go through three stages to be transcribed and expressed: immediate early genes, early genes, and late genes. The nucleocapsid is ultimately formed and binds to the proteins of the tegument in the nucleus cell before acquiring the envelope in cytoplasmic vacuoles derived from the Golgi apparatus. These vacuoles then release virions into the cellular membrane by exocytosis. This replication cycle corresponds to the lytic (or productive) cycle that leads to the death of host cells and to virus production that will disseminate through the organism at the time of primary infection or during reactivations. Following

primary infection, both species of HHV-6 and HHV-7 latently persist in various sites and cells of the organism, especially in monocytes-macrophages (Agut *et al*.,2016).

Various hypotheses have been put forward to explain this latency mechanism; all of these mechanisms might be present among various cell: presence of the sole viral genome in its episomal form with a potentially limited expression of some genes, replication cycle blocked at the intermediate stage or replication cycle completed but controlled and 5 compatible with the prolonged survival of the host cell. From this latent state, a productive cycle may be reactivated and may lead to a new production of infectious viruses in the blood and in other body compartments such as saliva. However, the selective expression of some viral genes may be enough to induce modifications of cell functions, even in the absence of a complete replication cycle as demonstrated in an experimental study. Serum antibodies react to various viral proteins, and some of these proteins seem to be dominant antigens in serological tests. Cellular immune response may be detected with the proliferation of CD4+ and CD8+ T cells following exposure to viral antigens (Agut *et al*.,2015).

2.2.4 Transmission of HHV-7

Transmission is believed to occur most frequently through the shedding of viral particles into saliva. HHV-7 found in human saliva, the former being at a lower frequency. Over 95% of adults have been infected and are immune to HHV-7, and over three quarters of those were infected before the age of six. Primary infection of HHV-7 among children generally occurs between the ages of 2 and 5, which means it occurs after primary infection of HHV-6 (Araujo *et al*.,2011).

A 2014 Washington University School of Medicine's analysis of 102 healthy adults sampled at as many as five major body habitats found that HHV-7 was present in 98% of them, especially in the mouth (Sodergren *et al*., 2014).

Other study looking at the human blood virome in 8,240 humans between the ages of 2 months to 102 years found that 20.37% of them were positive for HHV-7 (Moustafa *et al*., 2017).

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-7 and Diseases Association

Several diseases have been associated with HHV-7 reactivation in adults, although the causal correlations are still unproven. Most studies on pathogenic association do not specify the HHV-7 virus species, which, however, can be inferred by the reference strains used in the methods:

2.2.5.1 HHV-7 Associated with Autoimmune Diseases

HHV-7 is an opportunistic viral agent that is reactivated from persistence in the salivary glands in the body of an immunocompromised individual and is knows as a trigger of a number of human autoimmune disorders such as autoimmune thyroid disease, multiple sclerosis, and autoimmune anti-NMDA encephalitis (Dyachenko *et al*., 2018).

2.2.5.2 HHV-7 Associated with Neurological Diseases

Human herpesvirus-7 is proven to be a neurotropic virus, causing various neurological complications mostly in the adult population. We present the case of a 10-year-old girl, with confirmed active HHV-7 infection of the CNS, who

developed acute seronegative autoimmune encephalitis. Limited pediatric cases of autoimmune encephalitis following HHV-7 infection (Christou *et al*., 2022).

2.2.5.3 HHV-7 Associated with Acute Leukemia

The co-infection with HHVs, especially HCMV, HHV-6, and HHV7 may contribute to the development of serious clinical manifestations with profound lymphopenia, pneumonia rash and increased risk for bacterial and fungal co-infections. These findings may suggest the synergistic effect of HHVs associated infection (Handous *et al*., 2020).

2.2.5.4 HHV-7 Correlated to Multiple Sclerosis

Human herpesvirus 7 is known to play roles in the pathogenesis of MS. HHV-7 can remain in neural and oligodendroglia cells in latent phases after primary infection and can be reactivated later (Saberi *et al*.,2018).

2.2.5.5 HHV-7 Correlated to Epilepsy

Human herpesvirus 7 is usually associated with febrile seizures. Later onset and higher frequency of seizures are characteristic of pediatric HHV-7, compared with HHV-6 infection. The HHV-7-related severe neurological disorders are predominantly observed in immunocompromised individuals. the first reported case of epilepsy associated with HHV-7 in a previously healthy individual (Divakova *et al*.,2020).

2.2.5.6 HHV-7 Associated with Encephalitis

HHV-7 infection typically occurs in childhood and is frequently asymptomatic. Some clinical manifestations include fever and exanthem subitum. Human herpesvirus 7 is infrequently associated with encephalitis (Parente *et al*.,2021).

2.2.5.7 HHV-7 Associated with Roseola

Human herpes virus 6 and 7 infections typically are silent or manifested as mild febrile illnesses including classic roseola (Leach,2000)

2.2.5.8 HHV-7 Associated with Rheumatoid Arthritis

Involvement of latent/persistent Parvovirus B-19, HHV-6 and HHV-7 infections in ethiopathogenesis of rheumatoid arthritis and relationship with clinical and radiological findings (Kadisa, 2018)

2.2.5.9 HHV-7 and Covid-19 Patients

Several observational studies showed that patients with herpes zoster and pityriasis rosea, which is associated with reactivation of HHV-6/7, increased in number during the COVID-19 pandemic (Dursun *et al*.,2020; Maia *et al*.,2021).

2.2.5.10 HHV7 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).

Reactivation of Betaherpesvirinae HHV-6 and HHV-7 may be associated with mental illness and host fatigue. In both viruses, a significant correlation was observed between detection frequency and viral DNA load in saliva. In the low-shedding group, HHV-7 was at the time of delivery ($p = 0.0277$) and 1 month after birth ($p = 0.0235$). Viral DNA loads were significantly lower ($p = 0.0044$) in the subjects with abnormal Edinburgh Postnatal Depression Scale (EPDS) scores. The detection rate and viral DNA load of virus in saliva increased after the third trimester. Salivary virus DNA shedding was significantly lower in subjects with an abnormal EPDS score (Suzuki *et al*.,2022).

2.2.6 Laboratory Diagnostic of HHV-7

2.2.6.1 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).

2.2.6.2 Tissue Culture and Cell Lines

2.2.6.2.1 Cell Lines and HHV-7

The HHV-7 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).

2.2.6.2.2 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).

2.2.6.3 Antigen Detection

Uses conventional equipment, gives evidence of virus gene expression, discrimination between HHV-7 and HHV-6 (Agut *et al*., 2015).

2.2.6.4 Qualitative Viral DNA PCR

High sensitivity and specificity, discrimination between HHV-7 and HHV-6 (Agut *et al*., 2015).

2.2.6.5 Quantitative Viral DNA Real-Time PCR

High sensitivity and specificity, discrimination between HHV-7 and HHV-6, longitudinal follow-up studies, comparison of viral loads in blood versus organs (Agut *et al*., 2015).

2.2.7 Immune Response to HHV-7

2.2.7.1 Innate Immune Responses to HHV-7 Infection

Both HHV-7 and -6 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-7/HHV-6 interferes 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-7/HHV-6 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-7/HHV-6

was reported to establish a productive infection in CD3-negative NK cell clones, leading to the denovo expression of CD4 on the NK cell surface , and HHV-7/HHV-6 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-7/HHV-6 infection, NK cell function and autoimmunity (Rizzo *et al*., 2017).

2.2.7.2 Adaptive Immune Responses to HHV-7/HHV-6 Infection

2.2.7.2.1 Cellular Response to HHV-7

Information on HHV-7/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-7/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).

Both CD4+ and CD8+ HHV-7/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 IFN-γ and TNFα, while also performing cytolytic functions mediated by the secretion of perforin (CD4+) or granzyme B (CD8+) (Martin *et al*.,2018).

2.2.7.2.2 Antibody Response to HHV-7

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 , 2005).

Interestingly, 57% of individuals with HHV-7/HHV-6 had antibodies to the IE-A antigen present in the serum, while IE-A antibodies were undetectable in non-HHV-7/HHV-6 individuals. On the other hand, 14% of HHV-7/HHV-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 anti-gB titers may reduce the ability of HHV-7/HHV-6 individuals to mount an immune response to an exogenous HHV-7 infection (Agut *et al*.,2015).

2.3 Interleukin-33

2.3.1 Definition

Interleukin-33 is a member of the IL-1 cytokine family that includes IL-1α, IL-1β, and IL-18, IL-33 lacks a secretory signal peptide encoded by the IL1RL1 gene. IL-33 is a key cytokine involved in type 2 immunity and allergic airway diseases. Abundantly expressed in lung epithelial cells, IL-33 plays critical roles in both innate and adaptive immune responses in mucosal organs (Drake *et al*.,2017).

2.3.2 Location of IL-33

It is encoded by the *IL33* gene located on the human chromosome 9 (9p24.1) (Sneha *et al*.,2018).

2.3.3 Structure of IL-33

A determination of IL-33 based in part on the molecules β-trefoil structure, a conserved structure type described in other IL-1 cytokines, including IL-1α, IL-1β, IL-1Ra and IL-18. In this structure, the 12 β-strands of the β-trefoil are arranged in three pseudo repeats of four β-strand units, of which the first and last β-strands are antiparallel staves in a six-stranded β-barrel, while the second and third β-strands of each repeat form a β-hairpin sitting atop the β-barrel. IL-33 is a ligand that binds to a high-affinity receptor family member ST2. The complex of these two molecules with IL-1RAcP indicates a ternary complex formation. The binding area appears to be a mix of polar and non-polar regions that create a specific binding between ligand and receptor. The interface between the molecules has been shown to be extensive. Structural data on the IL-33 molecule was determined by solution NMR and small angle X-ray scattering (Figure 2.3) (Lingel *et al*.,2009; Chan *et al*.,2019).

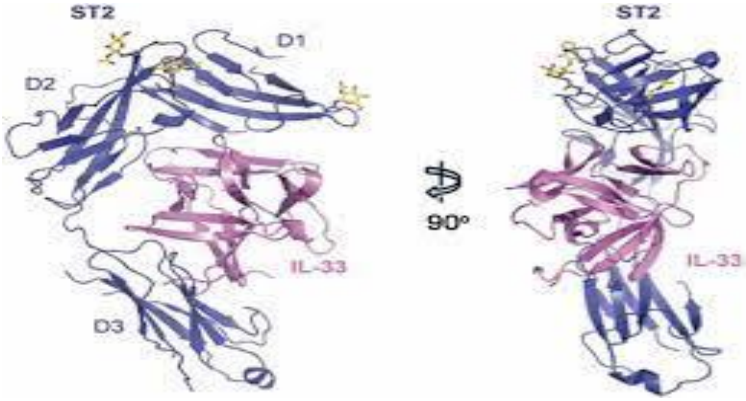


Figure 2.3: Structural Insights into the Interaction of IL-33 with its Receptors (Lingel *et al*.,2009).

2.3.4 Molecular Characteristics of *IL-33* Gene and Protein

2.3.4.1 IL-33 gene

The gene encoding IL-33 (Figure 2-4 A) comprises eight exons both in humans and mice. The promoter for the major human IL-33 mRNA form (NM_033439;

2718 nucleotides) is located upstream of an untranslated exon (exon 1 or 1a), more than 20 kb upstream of exon 2 that contains the AUG initiation codon (Liew *et al*.,2016; Tsuda *et al*.,2017).

It is active in normal human keratinocytes, endothelial cells and fibroblasts, and it contains an interferon stimulated response element (ISRE) and several gamma interferon activation sites (GAS) (Tsuda *et al*.,2017).

An alternative exon 1 sequence (exon 1b), located 4.6 kb upstream of exon 2, corresponding to the use of an alternative promoter has been described both in humans and mice (Tsuda *et al*.,2017).

Transcription starting sites are located downstream of consensus TATA box sequences in both promoters (Talabot-Ayer *et al*.,2012).

Interestingly, many of the single nucleotide polymorphisms (SNPs) in the human IL-33 gene associated with asthma are located in the promoter and intron, they could thus influence the activity of the two promoters of the IL-33 gene. SNPs in IL-33 are generally associated with increased asthma susceptibility (Grotenboer *et al*.,2013).

However, a rare loss of function mutation in IL-33 that disrupts a canonical splice acceptor site before exon 8 and is predicted to result in the production of a truncated IL-33 protein, has been found to cause reduced numbers of eosinophils in blood and to protect against asthma. Eosinophils counts are also reduced in IL-33-deficient mice. However, they have only been detected after amplification by qPCR and they appear to be very minor components in primary cells. At this point, it is therefore unclear whether alternative splicing is a relevant mechanism of IL-33 regulation (Gordon *et al*., 2016; Smith *et al*., 2017).

2.3.4.2 IL-33 Protein

Interleukin-33 protein is composed of two evolutionary conserved domains, the N-terminal nuclear domain and the C-terminal IL-1-like cytokine domain, separated by a divergent central part (Figure 2-4 B). Human IL-33 exhibits 58% and 52% identity over 270 residues with its canine and murine orthologues, respectively. The theoretical molecular weight (MW) of the human IL-33 precursor is 30759 Da and its isoelectric point (pI) is 8.89. The cytokine domain is highly acidic (aa 112-270, MW = 17 994 Da, pI = 4.80) similar to mature IL-1α (pI = 5.3), whereas the N-terminal part is highly basic (aa 1-111, MW = 12783 Da, pI = 10.28) (Cayrol and Girard, 2018).

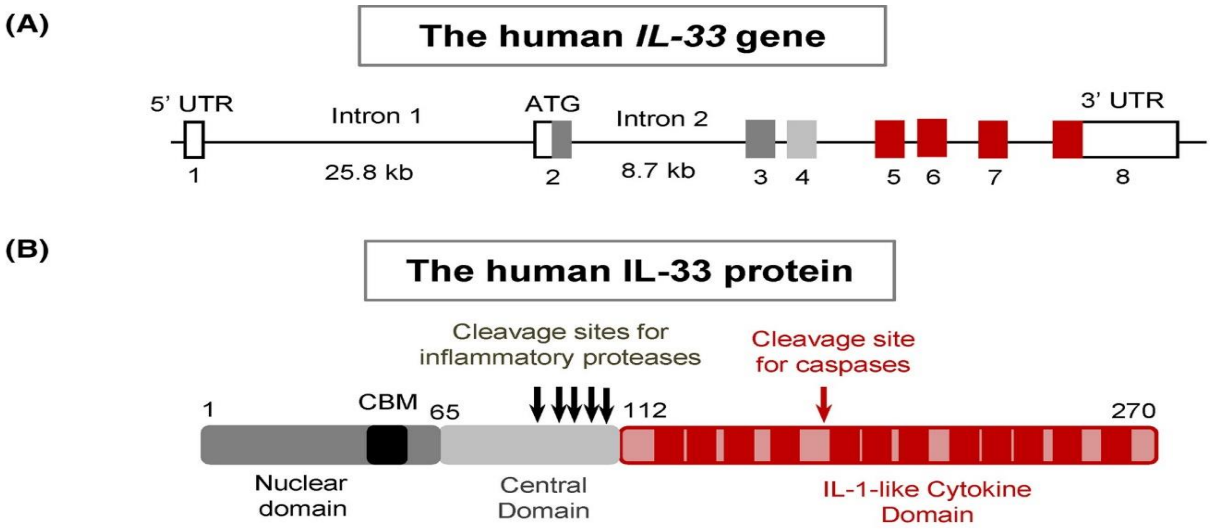


Figure 2.4: The human IL-33 gene and protein. (A) Structure of the human IL-33 gene. (B) Primary structure of the human IL-33 protein (Cayrol and Girard,.2018).

2.3.4.3 N-terminal Nuclear Domain

The N-terminal part of IL-33 contains a chromatin-binding motif (CBM, aa 40-58), and a predicted bipartite nuclear localization sequence (NLS, aa 61-78). The N-terminal domain (aa 1-65), that contains the IL-33 CBM, has been shown to be necessary and sufficient for nuclear targeting of epitope-tagged (GFP-fused) IL-33 in human cells but the possibility that the bipartite NLS may be required for

nuclear -IL-33 containing the CBM and NLS (aa 1-112) has been shown to be necessary and sufficient for targeting of a dsRed reporter to the nuclei of producing cells in vivo in a genetically engineered mouse model (Xi *et al*., 2016).

2.3.5 Function of IL-33

Interleukin-33 is a cytokine belonging to the IL-1 superfamily. Interleukin-33 induces helper T cells, mast cells, eosinophils and basophils to produce type 2 cytokines. IL-33 acts intracellularly as a nuclear factor and extracellularly as a cytokine (Shao *et al*.,2014).

2.3.5.1 Nuclear Role

Interleukin-33 is constitutively located in the nucleus of structural cells of humans and mice (Pichery *et al*.,2012). And has a helix-turn-helix domain presumably allowing it to bind to DNA. There is a paucity of research into the nuclear role of IL-33 but amino acids 40-58 in human IL-33 are sufficient for nuclear localization and histone binding .IL-33 also interacts with the histone methyl transferase SUV39H1, and murine appears to IL-33 interact to NF-κB (Ali *et al*.,2011; Shao *et al*.,2014).

2.3.5.2 Cytokine Role

As a cytokine, IL-33 interacts with the receptors ST2 (also known as IL1RL1) and IL-1 Receptor Accessory Protein (IL1RAP), activating intracellular molecules in the NF-κB and MAP kinase signaling pathways that drive production of type 2 cytokines (e.g. IL-5 and IL-13) from polarized Th2 cells. The induction of type 2 cytokines by IL-33 in vivo is believed to induce the severe pathological changes observed in mucosal organs following administration of IL-33. Interleukin 33 is also effective in reversing Alzheimer-like symptoms in APP/PS1 mice, by

reversing the buildup and preventing the new formation of amyloid plaques (Fu *et al*.,2016).

2.3.5.3 Regulation

Extracellularly, IL-33 is rapidly oxidized. The oxidation process results in the formation of two disulphide bridges and a change in the conformation of the molecule, which prevents it from binding to its receptor, ST2. This is believed to limit the range and duration of the action of IL-33 (Cohen *et al*., 2015).

2.3.5.4 Diagnostic of IL-33 polymorphism

2.3.5.4.1 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).

2.3.5.4.2 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).

2.3.5.4.3 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).

2.3.5.4.4 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 was 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 Study Design

This case control study was done for a one hundred-fifty specimens collected from female patients subjected to recurrent miscarriage and control group (normal pregnancy and delivery) from general hospitals as well as many private clinical in several cities (Babylon, Baghdad and Diyala) of Iraq. The age range of the study population was 18 years to 47 years. The specimens were collected during period from October 2021 to February 2022.

3.1.2 Clinical Samples Collection

Endometrium; Cervical swabs; fetal fluids swabs as well as blood from each study group of female patients suffering from recurrent miscarriage should be enrolled, that classified into: -

1. One hundred endometria; cervical swabs; fetal fluids swab as well as blood specimens from women suffering from abortion as well as recurrent miscarriage.
2. Fifty cervical swabs and blood specimens of control group.

All those specimens were submitted for genetic part for screening human Herpes virus 7 in patients and control group by polymerase chain reaction. However, the second part for detection SNPs of *IL-33* gene polymorphism by sequencing as shown in (Figure 3-1).

The first is the flocked swab regular for endometrium ; cervical swabs collection, according to (Cat. No. 21031 Heinz, Herenz; Germany). The second one is the flocked swab for fetal fluids swabs collection, according to (Cat. No. 80503CS Copan, Italy).

The two swabs were taken and mixed together in a (3ml) universal transport medium (VTM) tube, which provided with the flocked swab regular.

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

3.1.2.1 Inclusion criteria

Women aged from 18 to 47 years with unexplained miscarriage until 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.

3.1.2.2 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, rubella, human cytomegalovirus, herpes simplex 1 and 2 and human herpes virus 6.

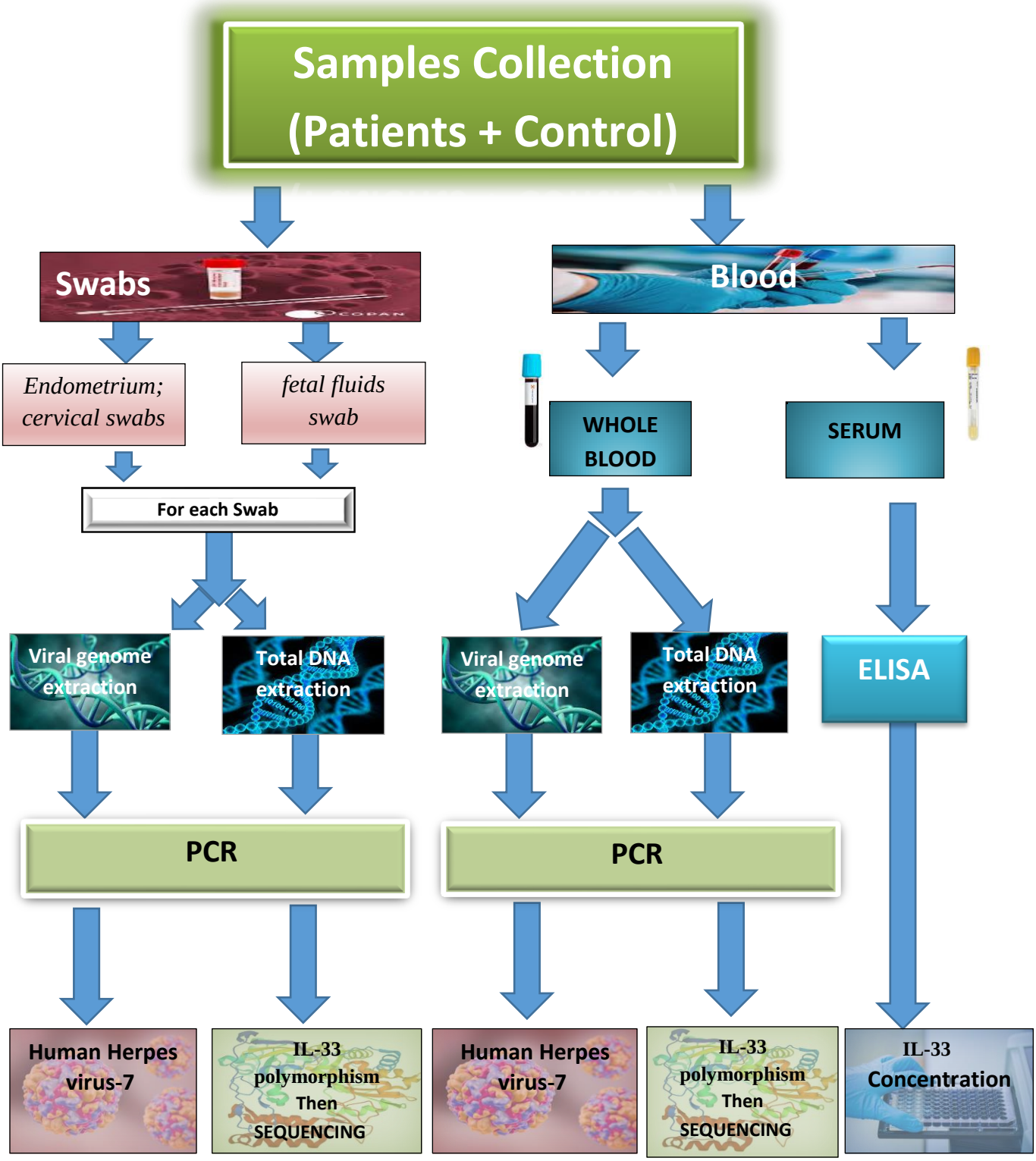


Figure 3-1: study design

3.2 Materials

3.2.1 Instruments and Equipments

Instruments and equipments used in this study are listed in (Tables 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	MSE / England
Centrifuge Eppendorf; Vortex	Eppendorf / Germany
Micro centrifuge	Hettich centrifuge, Sigma / Germany
Deep freeze (-70 C); Electric microwave	Sanio electric / Japan
Electrophoresis system	Fisher Scientific / USA
ELISA reader	Bio Tech / USA
Hood	Bio Lab / Korea
Electrical Oven; Incubator	Memmert / Germany
Nano drop	Optizen / Korea
PCR device	Biometra / Germany
Refrigerator	Arcelik / Turkey
Sensitive balance	Sartoris / Germany
Timer with alarm	Junghans / Germany
Gel documentation system	Cleaver Scientific / UK

Table 3-1-B: Equipment used in this Study with their Manufacturer Company and Origin.

Equipment	Manufactured Company (Origin)
Eppendorf tubes with different size (2ml, 1.5ml)	Extra gene / Taiwan
Disposable tips	Extra gene / Taiwan
PCR tubes	Extra gene / Taiwan
Baker	China
Inserted cylinder	China
Disposable gloves	China
Disposable syringes	China
Gel tubes	China
EDTA tubes	China
Micropipettes various sizes (0.5-10 , 20-200 , 100-1000) µl	Extra gene/Taiwan
VTM swabs	Heinz Herenz / Germany
Ice-box	Heinz Herenz / Germany

3.2.2 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	Intron /S. Korea
Absolute Ethanol alcohol	Merck - Germany
Agarose E	Conda / Spain
DNA Ladder (1000-100bp)	Intron /S. Korea
Proteinase K	Intron /S. Korea
RNase A	Intron /S. Korea
De-ionized sterile distilled water	BioNeer / Korea
Master mix	Promega / USA
Nuclease free water	Promega / USA
DNA loading dye	Intron / Korea
Safe stain	Intron / Korea

3.2.3 Kits and Markers

Kits and marker used in this study with their Manufacturer Company and origin as followings.

3.2.3.1 Contents of Patho G-spin DNA\RNA Extraction Kit

The contents of Patho G-Spin total DNA\RNA Extraction Kit (Cat. No. 17151, Intron / Korea) are listed in (Table 3-3).

Table 3-3: Contents of Patho G-spin DNA\RNA Extraction Kit.

Label	Contents 50 Columns
Lysis Buffer	25 ml
Binding Buffer	25 ml
Washing Buffer A	40 ml
Washing Buffer B	14 ml
Elution Buffer	20 ml
Spin Column / Collection Tube	50
Instruction Manual	1 sheet

3.2.3.2 Contents of G-Spin Total DNA Extraction Kit

The contents of G-Spin total DNA Extraction Kit Cat. No. 14001, Intron / Korea) are listed in (Table 3-4).

Table 3-4: List of Reagents and Buffers of G-Spin Total DNA Extraction Kit used in this Study.

Label	Contents 50 Columns	Contents 200 Columns
CL Buffer	25 ml	90 ml
BL Buffer	25 ml	90 ml
W A Buffer	40 ml	160 ml
W B Buffer	14 ml	56 ml
CE Buffer	20 ml	40 ml
Spin Column / Collection Tube	50	200
RNase A (Lyophilized powder)	3 mg x 1 vial	3 mg x 4 vials
Proteinase K (Lyophilized powder)	22 mg x 1 vial	22 mg x 4 vials

3.2.3.3 Reagent Provided of ELISA Kits to Evaluate IL-33 Concentration

Reagent provided of ELISA kits (E-EL-H2402, E lab Science Biotechnology Inc.\USA) to evaluate IL-33 concentration levels in serum women patients as well as control groups are listed in (Table 3-5).

Table 3-5: Reagent Provided of ELISA Kits to Evaluate IL-33.

Components	Quantity 96T	Quantity 48T
Pre-coated ELISA Plate	12 * 8 well strips x1	12 * 4 well strips x1
Standard Solution (1600pg/ml)	0.5ml x1	0.5ml 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 Human IL-33	1ml x1	1ml x1
User Instruction	1	1
Plate Sealer	2 pics	2 pics
Zipper bag	1 pic	1 pic

3.2.4 Primes of Human Herpes Virus-7 and IL-33 rs1891385, SNP

Primers sets used in this study to detect the HHV-7 and SNP of *IL-33 rs1891385* polymorphism with their product size and source as well as origin are listed in (Table 3-6).

Table 3-6: Primers sets that used for Detection of Human Herpes Virus-7 and *(IL-33 rs1891385*) Gene Polymorphism.

Gene	Sequence (5′-3′)	Product size (bp)	Source/origin
HHV-7 (IF)	5-CGCATACACCAACCCTACTG-3	300 bp	IDT / USA
HHV-7 (IR)	5-GACTCATTATGGGGATCGAC-3		
IL-33rs1891385 (IF)	GGAATAGTAAAATAAATCCAGAGAAAGC	422 bp	IDT / USA
IL-33rs1891385 (IR)	ATTCTTATATTATTATTTCCCCTGTGCC		

3.3 Methods

3.3.1 Extraction and Detection of HHV-7 by using Polymerase Chain Reaction

3.3.1.1 Principles of Assay

By using specific viral DNA/RNA extraction kit (Intron/Korea); the viral genome was extracted, purified and migrated using agarose gel from the endometrium; cervical swabs and fetal fluid as a first step to amplify the target Human Herpes virus-7 DNA.

3.3.1.2 Principle of Viral Genome Extraction

Patho Gene-spinTM DNA/RNA extraction kit is specifically designed to isolate high-quality nucleic acids from a variety of pathogen and specimen using low elution volumes that allow sensitive downstream analysis. The purified RNA/DNA is free of proteins and nucleases, and is suitable for use in downstream applications that allow pathogen detection.

Patho G-spin™ DNA/RNA extraction kit uses the chaotropic salt in lysis buffer inactivates immediately DNase/RNase to ensure isolation of intact DNA/RNA. Patho Gene- spin™ DNA/RNA Extraction Kit uses advanced silica-gel membrane technology for rapid and effective purification of DNA or RNA without organic extraction or ethanol precipitation. Furthermore, the buffering conditions are finely adjusted to provide optimum binding of the DNA/RNA to the column.

The protocol is based on the lysis buffer that effectively dissolves and denatures virus envelope and capsid protein without additional enzyme treatment to easily elute viral gene. The binding buffer added after the elution helps to attach genes only to silica-gel membrane, and two different washing buffers efficiently remove proteins and other contaminants to get high purity viral gene. Swab and blood specimens were used same protocol.

3.3.1.3 Specimens preparation

Endometrium and\or cervical swabs; fetal fluids swabs were mixed together in 3 ml liquid viral transport media tube, each specimen was aliquot into three cryotube containing 1000µl of the specimen which stored at (-70ºc) until genome extraction. After that, required part of specimens were taken and centrifuged at 10000 g/min for 5 minutes, discarded the supernatant except 100µl of the solution was left to be used in re-suspension of the pellet for RNA/DNA extraction.

3.3.1.4 Assay Procedure

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

1. Three hundred microliters from cell-culture supernatant or blood plasma was transferred into 1.5ml micro centrifuge tube.
2. Six hundred microliters lysis buffer was added, then the lysate mixed by vortex for (25sec). Mixture was incubated at room temperature for 15 min.
3. Six hundred microliters from binding buffer was added, and completely mix well by gently vortex. This step is conducive to efficient passage of cell lysates through a column and to increase binding onto column resins and it is important for effective deproteinization.
4. The lysates of cell were placed in a spin column that provided 2ml collection tube.
5. Loaded lysates on the column and centrifuged for two min at 13,000 rpm.
6. Discarded solution in collection tube and place the spin column back in the same 2ml collection tube.
7. Five hundred microliters of washing buffer A was added to spin column and centrifuged for two min. at 13,000 rpm.
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 microliters of washing buffer B was added to the spin column and centrifuged for one min. at 13,000 rpm .
10. The solution was discarded in the collection tube and places the spin column back in the same 2ml collection tube. Centrifuged for 1min at 13,000 rpm. It is

important to dry the membrane since residual ethanol may interfere with downstream reactions.

11. Placing spin column in an RNase-free 1.5ml micro- centrifuge tube; then 50µl of elution buffer was added directly onto the membrane and was incubated at room temperature for two min, then centrifuged for two min. at 13,000 rpm.
12. At this stage, the supernatant was containing viral genome DNA, then stored at -20°C after measuring the purity.

3.3.1.5 Estimation of the Extracted DNA Concentration and Purity

After extraction of viral DNA from specimens; the concentration of DNA yield and purity were measured by using Nano drop (Korea), by applying 5µl of the extracted DNA in the instrument curette. Extracted with purity in between (1.8-2) at absorption wavelength (260/280) was included in this study, otherwise; DNA extraction of the sample was repeated.

3.3.2 Extraction and Detection of *IL-33rs1891385* gene Polymorphism by Sequencing

Total DNA for SNPs of *IL-33rs1891385* polymorphism were extracted from peripheral blood and swabs of female patients using sequencing technique.

3.3.2.1 Principle of DND Extraction from Clinical Samples

The G-spin™ Total DNA extraction 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 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).

3.3.2.2 Procedure for Total DNA Extraction from Swabs

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

1. Three hundred microliters of swab sample was placed into a 1.5ml micro-centrifuge tube.
2. Adding of 400µl of CL Buffer, 20µl of proteinase K solution and 40µl of RNase A into sample tube and mixed well using vortexing vigorously. Then incubated the lysate at 56°C for 30 min.
3. A 1.5ml tube was centrifuged briefly (to remove drops from the inside the lid.
4. Adding 400µl of BL Buffer into the lysate, and mixed well by gently inverting (5- 6) times. After mixing, was incubated the lysate at 70°C for five min.
5. A 1.5 ml tube was centrifuged briefly to remove drops from the inside the lid.
6. Four hundred microliters 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 the lid.
7. Eight hundred microliters of the mixture 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 rpm for one min. Discarded the filtrate and placed the spin column in a 2 ml collection tube (reused).

8. Repeating step eight by applying up to 600µl of the remaining mixture from step seven to the spin column. Discarded the filtrate and placed the spin column in a new 2 ml collection tube.
9. Carefully applying the mixture from step seven to the spin column in a 2 ml collection tube without wetting the rim, then closing the cap, centrifuged at 13,000 rpm for one min. Discarded the filtrate and placed the spin column in a new 2 ml collection tube (additionally supplied).
10. Seven hundreds microleters of Buffer WA was added to the spin column without wetting the rim, and centrifuged for one min. at 13,000 rpm. Discarded the flow-through and reused the collection tube.
11. After then 700µl of Buffer WB was added to the spin column without wetting the rim, and centrifuged for one min. at 13,000 rpm. discarded the flow-through and placed the column into a new 2 ml collection tube (additionally supplied), then centrifuged for additional one min. to dry the membrane, discarded the flow-through and collection tube altogether.
12. Placing the spin column into a new 1.5 ml tube (not supplied), and adding 50µl of buffer CE directly onto the membrane, incubated for one min at room temperature and centrifuged for one min. at 13,000 rpm to elute, then stored at -20°C.

3.3.2.3 Extraction of Total DNA from Blood Samples

1. Two hundred microliters of whole blood was pipetted into a 1.5ml micro-centrifuge tube.
2. Fourty microliters of proteinase K Solution and 5µl of RNase A solution was added into sample tube and gently mixed.
3. Two hundred microliters of Buffer BL was added into upper specimen 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. The mixture was placed at room temperature for two min.
5. The lysate was incubated at 56°C for 10 min. For complete lysis, mix 3 or 4 times during incubation by inverting tube. If it breaks down perfectly, the red color of lysate became dark green.
6. The 1.5 ml tube was centrifuged briefly to remove drops from inside the lid.
7. Two hundred microliters of absolute ethanol was added into the lysate, and then mixed well by pulse vortex. After mixing, briefly centrifuged the 1.5ml tube to remove drops from inside of the lid. This step is an equilibration step for binding genomic DNA to column membrane. In addition, this step conduces to pass efficiently cell lysate through a column.
8. The mixture from step seven 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 rpm for one min. Discarded the filtrate and placed the spin column in a new 2 ml collection tube (additionally supplied).
9. Seven hundred microliters of Buffer WA was added to the spin column without wetting the rim, and centrifuged for one min. at 13,000 rpm . Discarded the flow-through and reused the collection tube.
10. Seven hundred microliters of Buffer WB was added to the spin column without wetting the rim, and centrifuged for one min. at 13,000 rpm . Discarded the flow-through and placed the column into a new 2ml collection tube (additionally supplied), then it was centrifuged again for additional 1 min to dry the membrane and discarded the flow-through and collection tube altogether.
11. The spin column was placed into a new 1.5ml tube (not supplied), and adding 50µl of Buffer CE directly onto the membrane, then incubated for one min.

at room temperature and then centrifuged for one min at 13,000 rpm to elute, then stored at -20°C.

3.3.2.4 Measurement of Concentration and Purity of Extracted DNA

The DNA quantity and purity was determined using a spectrophotometer (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 1.8-2.

3.4 Detection of HHV-7 and *IL-33rs1891385* Gene Polymorphism by Polymerase Chain Reaction

3.4.1 Primers Pairs Dilution

The primers' source was from IDT / USA. 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 (100 mol/µl) that will be used again to obtain working solution. As following: The tube was spin down before opening the cap, then the desired amount of water was added according to the oligoes manufacturer to obtain a (100 pmol/µl master stock). Vortex properly for re-suspend the primers evenly. Then 10µl of the master stock was transferred to a 2ml Eppendorf tube that contains 90µl of sterile, nuclease-free H_2O (Working Solution 10 pmol/µl). The master stock is stored at -20 C^o and the working solution is stored at -20 C^o. Finally, the working solution was thawed on ice and vortex before using in PCR and then stored at -20 C^o.

3.4.2 PCR Experiments

Polymerase chain reaction amplification was done using conventional thermal cycler (Biometra - Germany) as follows: Sample DNA about 4µl was added into PCR master mix tubes. Forward and reverse primers were added 2µl into PCR master mix tubes (for each one). Distilled nuclease free water was added 5µl into PCR master mix tubes to a total volume of 25µl as the (Table 3-7).

Table 3-7: Recommended Volumes and Concentration for Applying PCR.

No.	Content of PCR Reaction Mixture	Volume/ µl
1	Master mix	12 µl
2	Forward primer	2 µl
3	Reverse primer	2 µl
4	Sample DNA	4 µl
5	Nuclease free water	5 µl
Total		25 µl

3.4.3 Thermal Cycles Conditions

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 HHV-7 and *IL-33rs1891385* polymorphism were amplified using specific primers according to the mentioned conditions in (Table 3-8).

Table 3-8: Amplification Conditions of HHV-7 and *IL-33rs1891385* Genes in Patients with Recurrent Miscarriage.

Gene	Initial denaturation	Denaturation	Annealing	Extension	Final extension	No. of cycles
HHV-7	95C^0 /5 min	95C^0 / 30 sec	58 C^0 /30 sec	72 C^0 /30 sec	72 C^0 /10min	40
IL-33 rs1891385	95C^0 /5 min	95C^0 / 30 sec	63 C^0 /30 sec	72 C^0 /30 sec	72 C^0 /10min	40

PCR products of target regions HHV-7 and *IL-33rs1891385* polymorphism were electrophoresed on 1.5 % agarose at 75 V for 1h and visualized by safe stain. Photos were taken using gel documentation system.

3.5 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 genome; total DNA extracts, and PCR products.

3.5.1 Preparation of Solutions and Buffers

3.5.1.1 Loading Buffer

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

3.5.1.2 TBE Buffer (1X)

To prepare 500 ml of 1X Tris-Borate-EDTA (TBE) buffer, 50 ml of TBE (10X) stock solution was mixed with 450 ml of dH2O. Then the volume was completed to 500ml with dH2O.

3.5.1.3 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, 1.0mm above the base of gel casting tray so that the sample wells are near the cathode.
2. Gel dissolving: 1g of agarose was dissolved in 100ml of 1X TBE solution by melting to 100°C to prepare 1% agarose gel for migrated genomic DNA extracts, also 1% or/and 2% agarose gel was prepared in 1X TBE buffer for

migrated PCR products or digested DNA by restriction enzymes respectively.

3. Gel casting: After agarose gel dissolving completely, it is let to cooling to approximately 60°C and 5µl 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 1.5cm 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 5V/cm 75V for (60-120) min until bromophenol blue dye reached at the end edge of the gel.
6. The gel was photographed using gel documentation system (Cleaver Scientific - UK).

3.6 Sequencing

3.6.1 Nucleic Acids Sequencing of PCR Amplicons

The resolved PCR amplicons were commercially sequenced from both directions, forward and reverse directions, following the instruction manual of the sequencing company (Macrogen Inc. Geumchen, Seoul, South Korea). Only clear chromatographs obtained from ABI (Applied Bio system) sequence files were

further analyzed, ensuring that the annotation and variations are not because of PCR or sequencing artifacts. By comparing the observed nucleic acid sequences of local samples with the retrieved nucleic acid sequences, the virtual positions, and other details of the retrieved PCR fragments were identified.

3.6.2 Interpretation of Sequencing Data

The sequencing results of the PCR products of the targeted samples were edited, aligned, and analyzed as long as with the respective sequences in the reference database using Bio Edit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA). The observed variations in each sequenced sample were numbered in PCR amplicons as well as in their corresponding position within the referring genome. The observed nucleic acids were numbered in PCR amplicons as well as in their corresponding positions within the referring genome. Each detected variant within the genome sequences was annotated by Snap Gene Viewer ver. 4.0.4 (https://www.snapgene.com).

3.7 Evaluation of *IL-33* Concentration in Blood Serum of Patients and AHC

The concentration of *IL-33* in the serum of female patients with recurrent miscarriage was evaluated by enzyme linked immunosorbent assay.

3.7.1 Assay Principle

This kit is an Enzyme-Linked Immunosorbent Assay. The plate has been pre-coated with Human *IL-33* antibody. *IL-33* present in the sample is added and binds to antibodies coated on the wells, then biotinylated Human *IL-33* Antibody is added and binds to *IL-33* in the sample. Then Streptavidin-Horse Radish Protein is added and binds to the Biotinylated *IL-33* 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 *IL-33*.

The reaction is terminated by the addition of acidic stop solution and absorbance is measured at 450 nm.

3.7.2 Assay Procedure

1. All reagents was prepared, standard solutions and samples as instructed and Bringing all reagents to room temperature before use. The assay is performed at room temperature.
2. Determined the number of strips required for the assay and inserting the strips in the frames for use. The unused strips should be stored at 2-8°C.
3. Fifty microliters of standard was added to standard well. Note: Do not add antibody to standard well because the standard solution contains biotinylated antibody.
4. Fourty microliters of sample was added to sample wells and then add 10µl anti-IL-33 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. The sealer was removed and wash the plate five times with wash buffer. Soak wells with at least 0.35 ml wash buffer for 30 seconds to one min for each wash. For automated washing, aspirate or decant each well and wash 5 times with wash buffer. Blotting the plate onto paper towels or other absorbent material.
6. Fifty microliters of substrate solution A was added 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. Fifty microliters of stop solution was added to each well, the blue color will change into yellow immediately.
8. The optical density (OD value) of each well immediately was done using a

micro-plate reader set to 450 nm within 10 minutes after adding the stop solution.

3.7.3 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.

3.7.4 Typical Data

This standard curve is only for demonstration purposes.

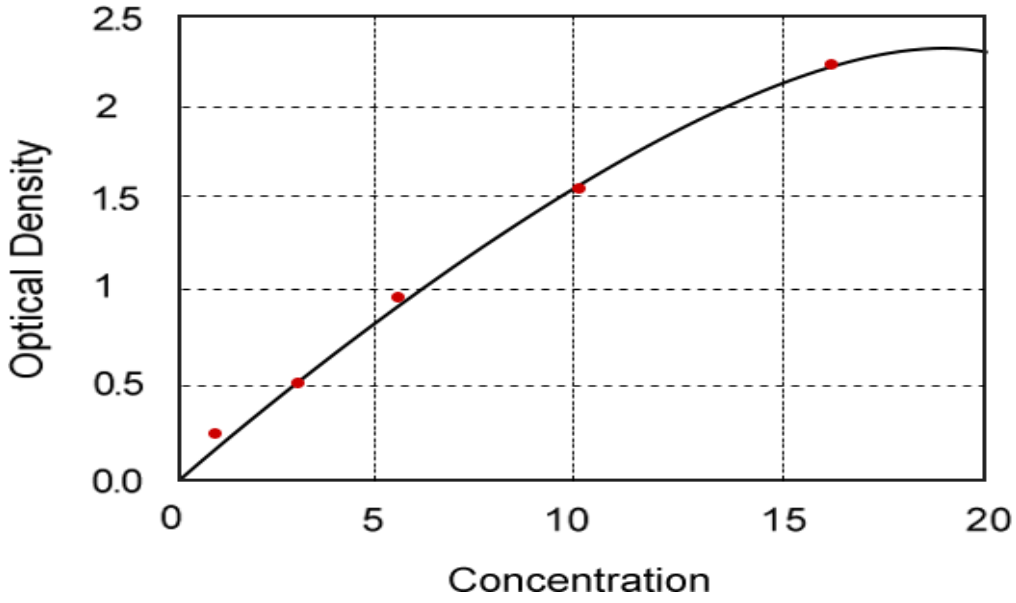


Figure 3-2: A Standard Curve of Human IL-33 Antibody.

3.8 Statistical Analysis

Statistical analyses were performed using Statistical Package of the Social Sciences (SPSS) version 24.0. 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-7 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 SPSS version 24.0. Asterisk (*) indicates that the differences were 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

RESULTS

4. The Results

4.1 Study groups according to their age

Table (4-1) shows the mean age groups of the study population. The mean age of the patients with recurrent miscarriage was 32.70±12.41 years was more than the mean age of the apparently healthy control 30.67 ± 11.17 years. There are non-significant statistical differences (p=0.3) between study population

Table 4-1: Distribution of Women Patients with RM and AHC According to their Age

Study groups	No.	Mean of age (years)	S. D	S. E	Range(years)	
					Minimum	Maximum
RM	100	32.70	12.41	1.979	18	47
AHC	50	30.67	11.17	2.403	18	47
Total	150	P-value = 0.3 No. sign. (P<0.05)				

4.2 A Comparison of the Pattern of Miscarriage between Women with and without Clinical Spontaneous Abortion

A strong positive relationship (with highly significant correlation) was found between number of participants; number of abortion; week of abortion and Maternal age ($P < 0.001$). However, there is 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)			Study groups (control)		
Maternal age (Y*)	18-29 No.(42)	30-39 No. (38)	40-47 No.(20)	20-29 No.(20)	30-39 No.(20)	40-45 No.(10)
Number of participants Means (SEM)	23(±0.7)	33(±0.3)	42(±0.6)	25(±0.7)	32(±0.5)	42(±0.8)
Number abortion Means (SEM)	1.6(±0.4)***	2.5(±0.1)**	4.5(±0.9)**	NF	NF	NF
Week of abortion Means (SEM)	12(±0.9)	13(±0.6)	14(±0.7)	NF	NF	NF

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

4.3 Detection of Human Herpes Virus -7 by conventional polymerase chain reaction technique

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

Out of 100 Endometrium; Cervical swabs; fetal fluids swab as well as Blood specimens involved in this study 55 (55%) were found to have a viral infection with RM 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) as the control group. There were statistically highly significant differences (P= 0.01) between women patients with recurrent miscarriage with viral genome and those without the viral genome as seen in table (4-3).

Table 4-3: Percentage of Viral Genome Extraction of Women Patients with RM and AHC Groups

Viral Genome		Study Groups		Pearson Chi-Square (P-value)
		RM 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-7 DNA by PCR

The positive result according to PCR shows 34.5 % (19 out of 55 cases) as positive while 65.5% (36 out of 55 cases) as negative, as shown in Table (4-4) as well as Figure (4-2). Statistically significant differences (p = 0.04) among patients group.

Table 4-4: Percentage of HHV-7 Positive Signals in Women Patients with RM using PCR Technique

Total Viral genome	No.	%	Chi-Square (P-value)
Positive	19	34.5	*P=0.04 sign. (P>0.05)*
Negative	36	65.5	
Total	55	100	

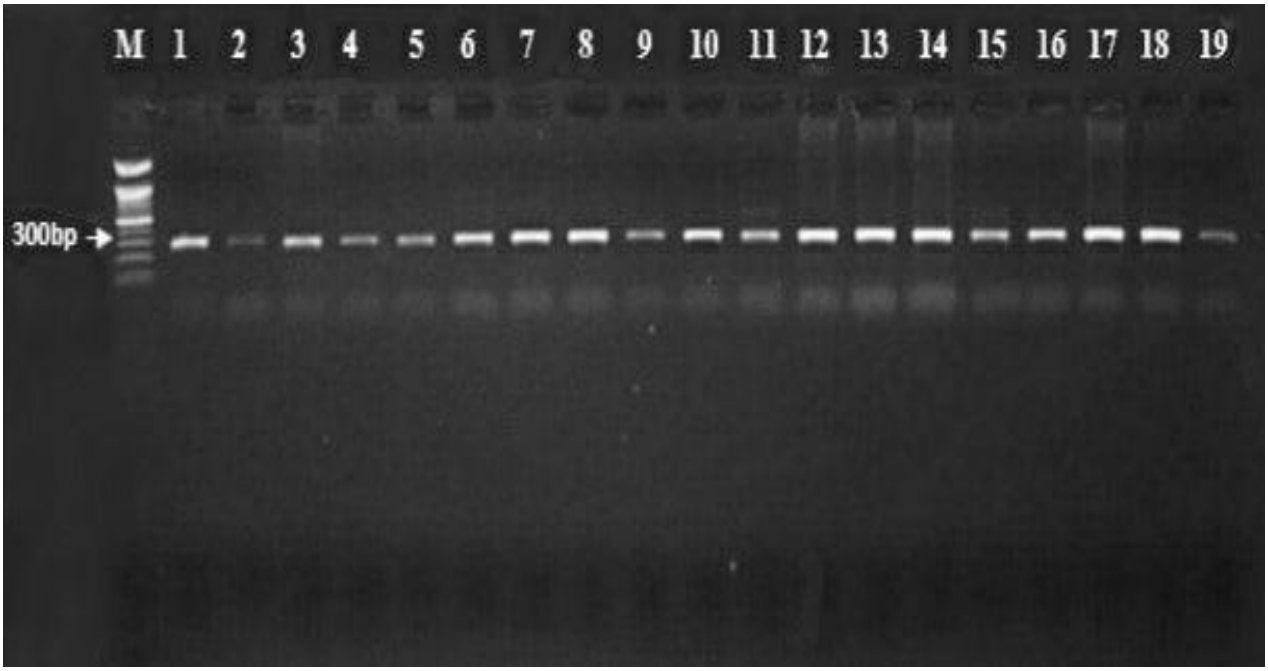


Figure 4-1: The electrophoresis pattern of HHV-7 DNA (300bp) detection in Endometrium ; Cervical swabs ; fetal fluids swabs of RM patients. Lanes (1 -19) refers to HHV-7 DNA specimens; Electrophoresis conditions, 1.5% agarose, 75 V, for 1h .

4.3.3 The Results of HHV-7 in the Women Patients with RM According to the Age Stratum.

In women with RM, the most commonly affected age stratum infected with HHV-7 DNA was (30-39 years) which constituted 45.5% (25 out of 55 cases), while the age stratum (18-29 years) was constituted 32.7% (18 out of 55 cases), followed by 21.8% (12 out of 55) in age stratum (40 – 47 years).

Statistical comparison of these HHV-7 in the patients with women with RM according to age stratum revealed significant differences ($p< 0.05$) Table (4-5).

Table 4. 5: Frequency of HHV-7 PCR Signal among the Women Patients with RM According to the Age Stratum

Age Stratum	Years	HHV-7			P value
		No. %	Positive	Negative	
	18-29	18	6	12	Anova test P=0.04 S. (P<0.05)
		32.7%	10.9%	21.8%	
	30-39	25	10	15	
		45.5%	18.2%	27.3%	
	40-47	12	3	9	
		21.8%	5.4%	16.4%	
Total		55	19	36	
		100%	34.5%	65.5%	

4.4 The results of gene polymorphism of *IL-33 rs1891385* SNP

4.4.1 Extraction total genome DNA from the endometrium; cervical swabs; fetal fluids swabs as well as blood specimens

By using specific Total genome DNA extraction kit (G-Spin total DNA Extraction kit, Intron / Korea) the genomic DNA Figure (4-3) was extracted, purifying and migrated using agarose gel from the Endometrium; Cervical swabs; fetal fluids swab as well as blood specimens of women patients with RM as well as apparently healthy control groups as a first step to amplify the target region of *IL-33 rs1891385* gene.

Figure 4-2: The electrophoresis pattern of gnomic DNA extracted from endometrium; cervical swabs; fetal fluids swabs as well as blood specimens of RM patients and healthy control groups. Lane 1 - lane 20 refers to genomic DNA specimens (1-10 patients with RM & 11-20control); Electrophoresis conditions 1% agarose , 75 volts, for 1h .

4.4.2 Genotyping of *IL-33 rs1891385* gene in RM and AHC

For *IL-33rs1891385* genotyping, the genomic DNA was amplified using specific primers and accomplished by the Thermo-cycler apparatus under the optimal condition as mentioned in the (table 3-7). The results revealed that the presence of a single band (422 bp) of the target sequence of *IL-33rs1891385* gene in agarose gel as shown in (figure 4-4).

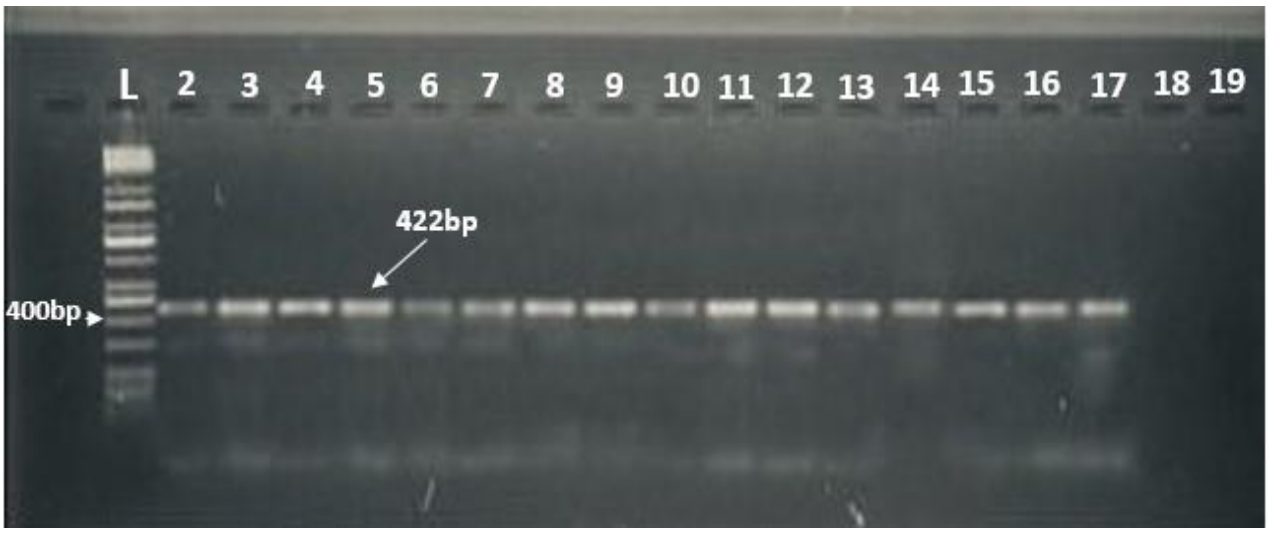


Figure 4-3: Agarose gel electrophoresis of an amplified product patterns of *IL-33 rs1891385* exon-5 region.

Table 4-6 shows the percentage of a single band (422 bp) of the target sequence of *IL-33rs1891385* gene. The positive result, according to PCR amplification of a single band (422 bp) of *IL-33 rs1891385* gene in women patients with RM and AHC were 55% (55 of 100 cases) and 30% (15 of 50 cases), respectively. While, the negative results were in women patients with RM and AHC were 45% (45 of 100 cases) and 70% (35 of 50 cases), respectively as shown the Table (4-6).

Table 4-6: Percentage of *IL-33 rs1891385* Signals in Patient's Women with RM and AHC groups by PCR Technique

***IL-33 rs1891385* gene band**	**RM No.(%)**	**AHC No.(%)**
Positive	55(55%)	15 (30%)
Negative	45 (45%)	35 (70%)
Total	100 (100%)	50 (100%)

4.4.3 Genotyping of *IL-33 rs1891385* among Study groups

The results showed that DNA polymorphism distribution were DNA polymorphism distributions according to A\C; A\T; A\G; T\G; T\A and T\C haplotypes were 9%; 7%; 9%; 6%; 9% and 5%, respectively in patient's women with RM and 4%; 0%; 8%; 2%; 3% and 2%, respectively in AHC group. In addition, was found two types of mutation in *IL-33 rs1891385* gene transversion and transition. The frequency of transversion mutation (A\C; A\T; T\G; T\A and T\C) more than the transition mutation (A\G) (Table 4-7).

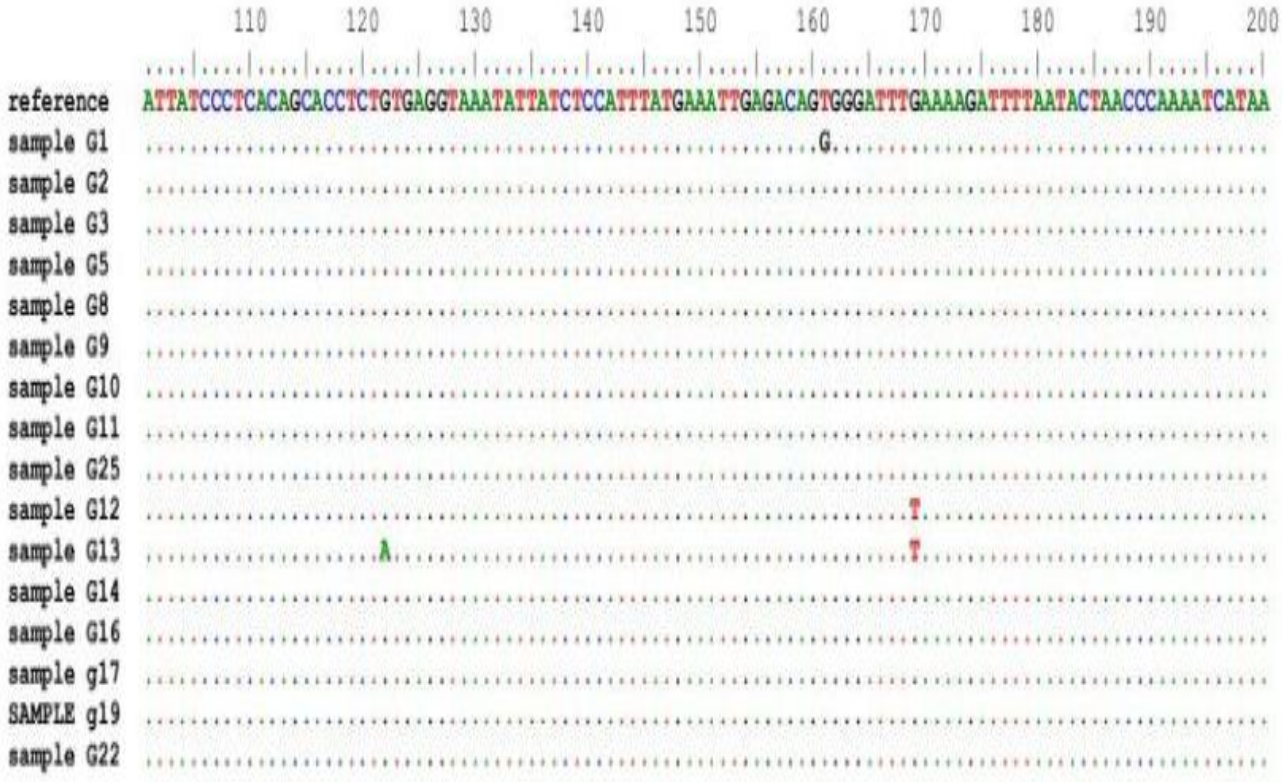
Table 4-7: Comparison between Women with and without Clinical Spontaneous Abortion based on Percentages of *IL-33 rs1891385* Expressed Gene Polymorphism.

Conformational Polymorphism of *IL-33 rs1891385* gene	Type of Mutation	Study group		OR Patients	OR Control	*P value*	95% C.I for OR Patients	
		RM NO.(100)	AHC NO.(50)				*lower*	*Upper*
A\C	Transvertion	9%	4%	1.7	1.9	0.08	1.7	1.95
A\T	Transvertion	7%	0	1.8	1.9	0.001	1.8	2
A\G	Transition	9%	8%	1.7	1.8	0.01	1.5	1.9
T\G	Transvertion	6%	2%	1.6	1.6	0.01	1.6	1.9
T\A	Transvertion	9%	3%	1.9	1.8	0.09	1.7	1.7
T\C	Transvertion	5%	2%	1.6	1.6	0.01	1.5	1.9

4.4.4 Sequences Alignment Fragment Results of *IL-33rs1891385* Gene, Exon-5 Region by Bio Edit Program Version 7.2.5

The sequencing results observed that many SNPs between the one resolved haplotypes and between the IL-33 rs1891385, exon5 for Primer3 plus reference sequences ID:NG-047209.1. The results appeared in the presence of fifteen SNPs Figure (4-5). Which revealed that which located at position 79 a substitution mutation (A→C) , position 80 substitution (A→ T) , position 88 substitution (A→ G) , position 122 substitution (G→ A) , position 161 substitution (T→ G) , position 169 substitution (G→T) , position 217 substitution (T→ A) , position 225 substitution (T→ A) , position 269 substitution (A→T) , position 290 substitution (T→ C) , and last one at position 326 substitution (T→ C) Figure(4-5) according to the reference sequence alignment of the human *IL-33 rs1891385* gene ID:NG-047209.1 (https://www.ncbi.nlm.nih.gov/Genbank/update.html).

We recorded the new recordings in the Japanese gene bank and the American gene bank (NCPI), as shown in the Appendix at the end of the thesis



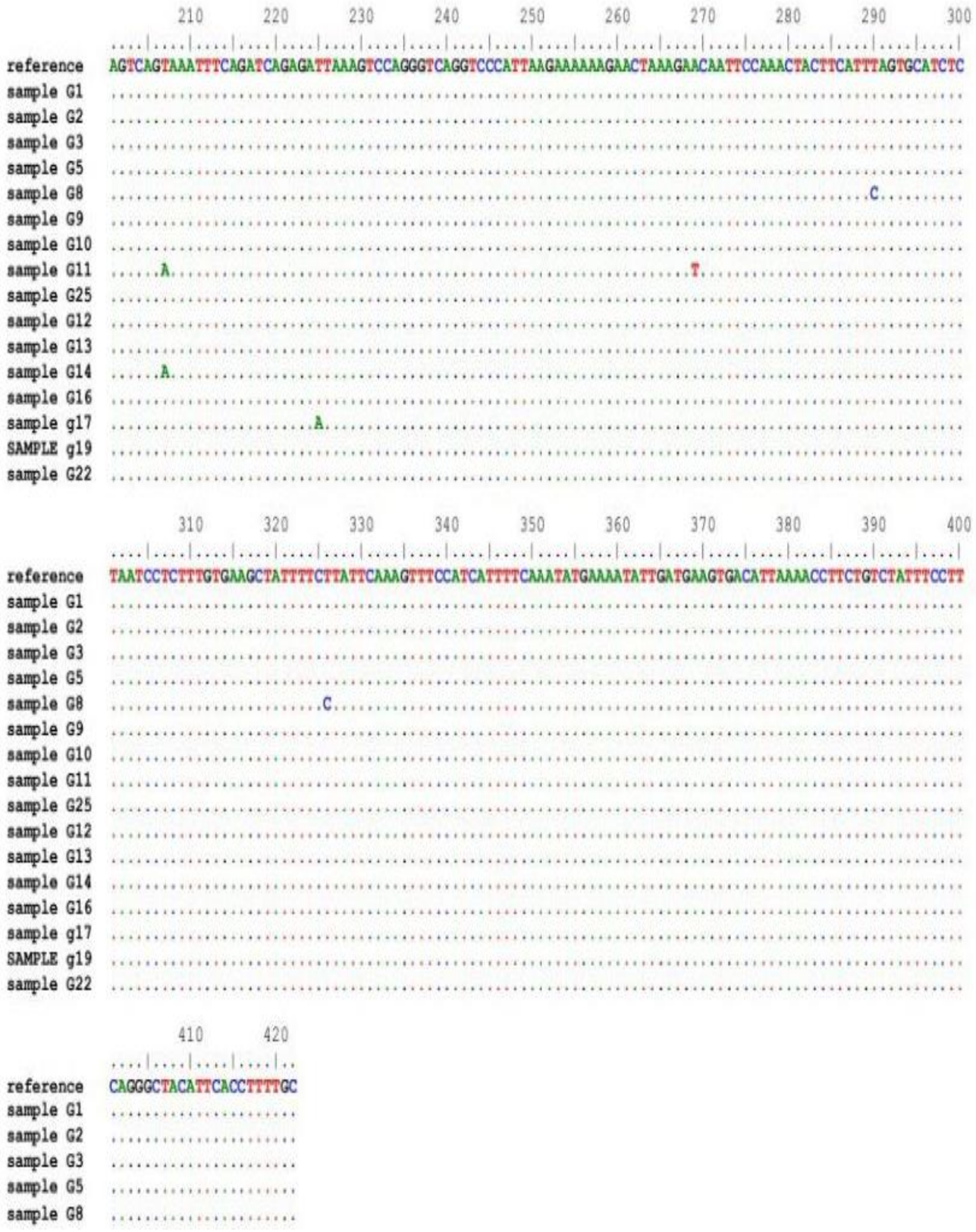


Figure 4-4: Sequences Alignment Fragment Results of *IL-33 rs1891385* Gene, Exon-5 Region by Bio Edit Program Version 7.2.5.

4.5 Evaluation of Serum *IL-33* Concentration by ELISA among Study Groups

Table (4-8) showed the concentration of serum *IL-33* that was detected by ELISA technique. The mean of serum *IL-33* concentration in women patients with RM and control groups were 10.66 ±0.95 pg./ml and 6.28 ±0.42 pg./ml, respectively.

A significant difference (p=0.002) was found by comparing the mean of serum IL-33 concentration among AHC and women with RM groups as shown in table (4-10).

Table 4-8: Results of Serum *IL-33* Concentration by ELISA for AHC and Women Patients with RM

Groups	*IL-33* Concentration Mean (S.E)	T-test	P= value
RM	10.66 ±0.95	3.79	0.002*
AHC	6.28 ±0.42		

***P ≤ 0.05**

4.6 Correlation between *IL-33* and HHV-7 Infection among Study Groups according to Age Stratum

There are highly significant correlations (p=0.001) between mean of *IL-33* concentration according to the age stratum (18-29 years; 30-39 years; 40-47 years) of women with a clinical spontaneous abortion and healthy control groups as shown in Table (4-9) and Figure (4-6).

Table 4-9: The Correlation of *IL-33* in Women with a Clinical Spontaneous Abortion and AHC Groups according to Age Stratum

Groups	Age groups		
IL-33	**18-29 Years**	**30-39 Years**	**40-47 Years**
RM (Mean ± SE)	10.00 ±0.69	9.42 ±0.48	9.14 ±0.82
AHC (Mean ± SE)	5.80 ±1.01	6.80±0.73	6.00 ±0.70
LSD	6.11		
P-value	0.001*		

***P-value <0.05**

4.7 Spearman's rho Statistical Testing to Evaluate Studied Molecular Markers in Relation with *HHV-7* Infections in Women with Clinical Spontaneous Abortion

There is a strong positive relationship (with highly significant correlation) between number of abortion and IL-33concentration {r = 0. 422, P = 0.007 (p <0.01)}. Also, there is a significant correlation between HHV-7 and IL-33 concentration {r = 0. 323, P = 0.042 (p <0.05)} (Table 4-10). In addition, there is significant correlation between IL-33 concentration and *IL-33 rs1891385* (p <0.01) (Table 4-10).

However, there are no significant correlations among HHV-7; number of abortion and *IL-33 rs1891385*{r = 0. 267, P = 0.182 (p <0.05)} and {r = 0. 067, P = 0.684 (p <0.05)}, respectively (Table 4.10).

Table 4-10: Spearman's rho Statistical Testing to Evaluate Studied Molecular Markers in Relation with *HHV-7* Infections in Women with Clinical Spontaneous Abortion

Spearman's rho		*IL-33 rs1891385*	*IL-33* concentration	HHV-7	No.Of Abortion
IL-33 rs1891385	r		.323*		
	P-value		.042		
HHV-7	r	.267			
	P-value	.182			
Number of Abortion	r	.067			
	P-value	.684			
IL-33 concentration	r			.323*	.422**
	P-value			.042	.007

*Correlation is significant (P<0.05). **Correlation is highly significant (P<0.01).

CHAPTER FIVE
DISCUSSION

5. Discussion

5.1 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 (with highly significant correlation) was found between number of participants, number of abortion, week of abortion and Maternal age ($P < 0.001$). However, there are no significant correlations between number of participants with control maternal age (and as illustrated in Table 4-2). Although the total burden related to maternal abortion and recurrent 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 of abortion in women with the first marriage and pregnancy at age over 40 years is 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 an old mother 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 old pregnant women.

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 (10 percent) lower in individuals aging 25 to 29 years and rose to 57 percent in people aging ≥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, some of the more common conditions that increase the risk of pregnancy loss are discussed below (Magnus *et al.*,2021).

After controlling maternal age and medical comorbidities, preconception paternal medical conditions may modestly increase the risk of pregnancy loss. In a retrospective cohort study of an insurance database covering 958,804 pregnancies in the United States, compared with men with no components of metabolic syndrome (MetS), the risk of pregnancy loss increased for men with one (relative risk [RR] 1.10, 95% CI 1.09-1.12), two (RR 1.15, 95% CI 1.13-1.17), or three or more (RR 1.19, 95% CI 1.14-1.24) MetS components after stratifying for maternal and paternal age (Kasman *et al*.,2021).

Bacterial, protozoan, and viral infectious agents have been associated with increased risk of miscarriage; the exact mechanisms are not fully known. Untreated syphilis leads to 21 percent increased risk of fetal loss and stillbirth (Giakoumelou

et al.,2016). As compared with uninfected pregnant individuals, maternal viral infections are associated with fetal loss rates nearly 8 percent for parvovirus B19, nearly 6 percent for Zika virus, and 2.5 percent 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 18-47 years old who have participated in the first cohort study among Iraqi female patients.

5.2 Detection of HHV-7 in women patients with RM by PCR

Although causal relationships between abortion and infections are difficult to establish, the detection rate of several viruses such as HCMV, HSV; B19V, HHV-6, and HHV-7 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-7 is fetal swab; cervical swab as well as blood specimens, although this may not be true for all HHV-6 & HHV-7 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 swab as well as blood specimens involved in this study, 55 (55%) were found to have a viral infection with RM. They were discovered to have viral infection by using PCR technique. A study done by Sayyadi-Dehno *et al*., (2019) and Miura *et al*., (2021) an aborted

woman, which revealed (38.2%) and (34.7%) positively viral infected women, respectively that support current study in the total of viral infection.

Furthermore , this results are consistent with previously published studies that investigated the etiology of RM, especially those focused on viral infection associated with recurrent miscarriage (Van der Eijk *et al.*, 2016; Bhatnagar *et al*., 2017).

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 PCR or RT-PCR.

HHV-7 is an etiological agent or a risk factor in a portion of pregnancy loss. A previous report has tied HHV-7 infection to spontaneous abortion and neonatal hypotonia . Accordingly, to these investigators have attempted to clarify the relationship between HHV-7 infections during pregnancy (Ohashi *et al*.,2002; Caserta *et al*.,2007; Suzuki. et al.,2022).

The concept of the relationship between HHV-7 and spontaneous abortion is based on the identification of HHV-7 genome sequences in fetal tissues, umbilical cord blood, and villous tissue and antigen has also been found in villous tissue (Aoki *et al*.,2016).

As this is up to what we know the first study in several cities of Iraq (Babylon; Baghdad; Diyala) for detection of HHV-7 DNA in aborted women, it's reasonable to compare the present result with relatively similar world-wide studies done for

similar purposes of detection of HHV-7 DNA in the endometrium swab, fetal swab, cervical swab as well as blood specimens by PCR.

In the current study, the positive result according to PCR technique shows 34.5 % (19 out of 55 cases) as positive while 65.5% (36 out of 55 cases) as negative, as shown in Table (4-4). A follow up study revealed similar results for women who developed RM in the first 15 weeks of pregnancy, with 39% of pregnancies ending in miscarriage (Giulia *et al* ., 2017).

In addition, the present result of HHV-7 is compatible with Al-Buhtori *et al*.,2011 and Luis *et al*., (2011) who found HHV-7 in 34% and 66.9% in spontaneous aborted women, respectively. Furthermore, a recent study found HHV-7, only one pregnant woman did not shed viral DNA at all, and most subjects (46/54, 85.2%) shed viral DNA in all five samples (Suzuki. *et al*.,2022).

Relatively high frequency of HHV-6 and HHV-7 reactivation or reinfection during pregnancy (17%–44%) has been inferred based on the detection of viral DNA in peripheral blood mononuclear cells. However, these studies used qualitative PCR analysis of peripheral blood mononuclear cells, which may detect latently infected HHV-7 (Ohashi *et al*.,2002; Caserta *et al*.,2007; Suzuki *et al*.,2022). Therefore, the high frequency of HHV-7 reactivation or reinfection observed may actually have been caused by the detection of latent viral infection.

Active infection can be identified by the detection of HHV-7 DNA or suggested by the detection of HHV-7 at multiple times in some specimens (e.g., plasma), or these tests can be combined with the determination of viral loads or serologic studies (Handous *et al*.,2020).

The effect of the virus on pregnancy outcome is linked to other factors such as hormonal effects (Marci *et al*.,2016), individual immunological stats (Ozkan *et*

al.,2014;Seshadri *et al*.,2014), which cause the virus to reactivate and inhibit the creation of an appropriate uterine environment for implantation and fetal growth, resulting in miscarriage (Eliassen *et al.*,2017).The presence of viral DNA in vaginal swabs after a 10-day incubation period suggests that horizontal transmission from mothers to newborns is possible (Pass,2004). Similarly, a team from the United Kingdom looked into the possibility of viral infection in fetal death and found viral DNA in 34% of tissue samples, with HHV-6 and HHV-7 discovered in 5 of them (Al-Buhtori *et al*.,2011).

Regarding RM etiology, the authors stated that HHV- 6 and HHV-7 have been studied as precipitating factors and that their DNA has been found in 17% and 39% respectively of RM plasmas (Gupta *et al.*,2017). They would like to remark that other recent studies established a causal role for HHV-6 and HHV-7 endogenous systemic active infection in the pathogenesis of RM. Indeed, HHV-6 and HHV-7 DNA were found not only in plasma but also in RM skin lesions by quantitative real time polymerase chain reaction. In addition, HHV6 mRNA expression and specific antigens have been found by immunohistochemistry in RM skin lesions (Broccolo *et al*.,2013; Gupta *et al*.,2017).

States of immunosuppression can favor HHV-6 or HHV-7 reactivation. Because pregnancy is a state of altered immune response and, as such, is a risk for viral reactivation, they studied the intrauterine transmission of HHV-6/7 in 38 women in whom RM developed during pregnancy. Sixty-two percent of women in whom RM developed within 15 weeks' gestation miscarried (D e Francesco *et al*.,2014).

By analogy, 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. Endometrial cells infected with HHV-6A 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 HHV-6 persistence.

The opinion of authors of current study, the differences in percentages of HHV-7 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 and 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 HHV-7 in spontaneous aborted women, this may need enrollment of large case-control studies . Exogenous hormones may result in a complete suppression of endogenous progesterone cycling, which leads to increase the susceptibility of women to HHV-7 infection.

5.3 Genotyping of *IL-33 rs1891385* Gene Polymorphisms in RM and AHC

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. Due to the

multifactorial nature of the RM, various factors investigated to identify cause of this condition, such as coagulation factors, infection factors, immunological factors, anatomical problems, and chromosomal abnormality (Soheilyfar *et al*.,2019; Kamrani *et al*.,2020).

The role of IL-33 polymorphisms in RM pathogenesis remains unknown. So far, very limited studies have reported to correlation between *IL-33* gene polymorphisms and RPL (Isazadeh *et al*.,2017).

The current results of of *IL-33rs1891385* amplified found two types of mutation in *IL-33rs1891385* gene transversion and transition. The frequency of transversion mutation (A\C; A\T; T\G; T\A and T\C) more than the transition mutation (A\G) as in table (4-7). The current result agrees with study by Yue et al., (2016) on Chinese women with RM that showed the variant exhibits significant association with RM in additive and recessive genetic model (additive model P = 0.015, recessive model P = 0.007). But they were in disagreement with the study of Liu *et al*., (2015).

Kamrani *et al*., (2020) showed an association between *IL-33rs16924159* gene polymorphism and RM in Iranian Azeri women. Also, they demonstrated for the first time that heterozygous genotype (GA) of *rs16924159* polymorphism is a risk factor in RM in Iranian Azeri women. In addtition, Soheilyfar *et al*., (2019) reported there were significant differences in the frequencies of GA genotype in *IL-33rs1929992* gene polymorphism between patients and the control groups (p = .001; OR =0.955; 95% CI: 0.239–9.807). Also, showed the *IL-33rs1929992* polymorphism was associated with RM in Iranian women.

Differences in the results of mentioned studies can be due to other involved genes, and differences in geographic area, sample size and selection bias, ethnicity and race heterogeneity, and environmental factors (Maroufi *et al*., 2019).

Effects of SNPs in *IL-33* was identified as statistically significant by multiple logistic regression.*IL-33* 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.

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.

5.4 Evaluation of Serum *IL-33* Concentration by ELISA among Study Groups

In the current study, the mean of serum *IL-33* concentration in women patients with RM was (10.66 ±0.95 pg./ml) higher than AHC (6.28 ±0.42 pg./ml), Table (4-8). These results inconsistent with result study by Yue et al., (2016) who found that serum *IL-33* levels are significantly lower in idiopathic RM cases than in control.

Activation of the *IL-33*/ST2 pathway in human endometrial stromal cells (HESCs) is critical for a successful pregnancy. Autocrine *IL-33* signaling in HESCs facilitates embryo implantation in mice. However, *IL-33* knockdown in decidualizing HESCs impairs embryo implantation (Salker *et al*., 2012). Women experiencing with RM have deficit in Treg cell number and function, compared with normal pregnant women (La Rocca *et al*.,2014). These studies suggest that high *IL-33* levels are critical in early pregnancy and impact the outcome of subsequent pregnancies.

However, compared with normal control pregnancies, there is a significant increase in serum *IL-33* levels at six weeks' gestation in patients who destined to abort. The biological significance of a rise of serum *IL-33* levels is uncertain in pregnant woman destined to miscarry. As *IL-33* is a crucial role in promoting

Tregs cells differentiation and adaptation to the inflammatory environment. One possible explanation is that the pregnancy is failing due to sensitization of the maternal immune system, and that the rise is a compensatory response to attempting to rescue the pregnancy. Although *IL-33* plays an exact role in embryo-maternal interactions, especially during early stages of implantation, our data support the notion that miscarriage may be attributed, at least in part, to low level of *IL-33* expression and secretion. In this study, serum IL-33 measurements were performed in non-pregnant women (Kaitu'u-Lino *et al*.,2012).

The results of Yue *et al*., (2016) also indicate that there are lower levels of serum *IL-33* in homozygous mutant (AA) than homozygous wild-type (GG) in this study, population is including cases and control groups. Moreover, there is a progressive decline in serum *IL-33* levels according to genotypes in RM cohorts. variant, located in the regulatory region of *IL-33*, was associated with reduced serum *IL-33* levels. This may be linked with defective transcriptional processing of *IL-33* mRNA.

A successful pregnancy depends on immune balance, including immune tolerance, 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 RM, intrauterine growth restriction, preeclampsia, etc. These leukocytes also secrete interleukins that act in placental immune regulation (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.

5.5 Correlation between number of abortion; age stratum and HHV-7 infection among study groups

In the current results, a strong positive relationship (with highly significant correlation) between number of miscarriage; age stratum in women with RM according to the HHV-7 infection. These results consistent with Gupta *et al*.,2017and Miura *et al*., (2021a) who found the spontaneous abortion rate was significantly higher in women with HHV-6 and HHV-7 than in women without iciHHV-6 but whose offspring carried paternally inherited iciHHV-6 and HHV-7 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).

By analogy, 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*., 2021b).

On other hand, results of the current study differed from these of Cusini and Ghislanzoni, (2001); Mohammad and Salman (2014), and Ben 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 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 and HHV-7 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 Suzuki *et al*.,2022).

There are many reasons that may explain the high incidence of HHV6 and HHV-7 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 (Caserta *et al*.,2007; Miura *et al*.,2021).

Finally, occurrence of primary or recurrent Herpes virus's infection reaches to the peak at this age period (Duran.,2004).

CONCLUSIONS AND RECOMMENDATIONS

The following conclusions are obtained from the present study:

1. HHV-7 might be one of the most recently identified uterus, cervical viruses in Iraqi female patients suffering from recurrent miscarriage in the Iraqi population. The positive results of HHV-7 DNA PCR in endometrium, cervical and fetal fluids swabs of female patients group, these findings lead to the proposal that HHV-7 acts as cofactor in the process of pregnancy loss as well as in recurrent miscarriage in female patients suffering from abortion.
2. It is observed that HHV-7 can be detected in women patients by taking samples from both the cervical and fetal fluids swabs and whole blood specimens.
3. Age factor has a significant association with viral and non-viral infection, rather than with the differences of mean ages of the HHV7.
4. Our study indicated that *IL-33rs1891385* polymorphism may be associated with RPL risk in the Iraqi women. But, the exact role and effects of *rs1891385* polymorphism in RM is not fully identified.
5. The serum levels and production rate of *IL-33* significantly increase in women with RM in compare women with successful pregnancy. It may be concluded that *IL-33* acts as a risk factor in the pathogenesis of idiopathic RM.
6. The significant correlation between the gene polymorphism of *IL-33* with HHV-7 infection could indicate highly important role of these molecular factors in women patients suffering from recurrent miscarriage.

The recommendations of these study dependent on current results are:

1. HHV-7 could be added as another potential cause of recurrent spontaneous abortion.
2. Routine work use of viral detection such as (HHV-7and 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 HHV7 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. However, the exact role and effects of *IL-33rs1891385* polymorphism in RM is not fully identified. Therefore, for better understanding of the association of this polymorphism with RM, further studies on different races and geographic areas with larger sample sizes are recommended to identify the effects of rs1891385 polymorphism on RM.
5. Studying the whole genome sequences of *IL-33rs1891385* in Iraq and their differences from the global prevalence to explain their role in deregulation of cell cycle pathway in RM.
6. The addition of highly sensitive RT-PCR test beside ELISA and PCR techniques are important to confirm the real HHV-7 infection especially in health centers in order to early detection of viral infection.
7. To study the sequence and the strains prevalent in Iraq and their differences from the global prevalence and importance, preparing traits against prevalence of HHV-7 in our country via proposing a vaccine which fits the Iraqi isolates is important.

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Appendix

Appendix 1

Facilitate the Task of a Researcher

جمهورية العراق

Ministry Of Health Babylon Health Directorate Email:- Babel_Healthmoh@yahoo.com Tel:282628 or 282621		وزارة الصحة والبيئة دائرة صحة محافظة بابـــل المديـــر العـــام مركـــز التدريب والتنمية البشرية وحدة أدارة البحوث العدد : ١١١٧ التاريخ: ٢٤/ ١١ /٢٠٢١

وزارة الصحة
دائرة صحة بابل
مركز التدريب والتنمية البشرية

إلى / مستشفى الأمام الصادق (ع)
مستشفى بابل للنسائية والأطفال
مستشفى الزهراء للولادة
م/ تسهيل مهمة

تحية طيبة ...

أشارة إلى كتاب جامعة بابل / كلية الدراسات العليا / شؤون الطلبة / العلوم الطبيعية ذي العدد ٤٣٢٤ في ٢٠٢١/١١/٢٣

نـــرفق لكم ربطا استمارات الموافقة المبدئية لمشروع البحث العائد للباحثة طالبة الدراسات العليا ماجستير (غفران سلام هادي).

للتفضل بالاطلاع وتسهيل مهمة المومأ أليه من خلال توقيع وختم استمارات اجراء البحث المرفقة في مؤسساتكم وحسب الضوابط والإمكانات لاستحصال الموافقة المبدئية ليتسنى لنا أجراء اللازم على أن لا تتحمل مؤسساتكم أية تبعات مادية وقانونية .... مع الاحترام

المرفقات :
استمارة عدد /٢

الدكتور
محمد عبد الله عجرش
مدير مركز التدريب والتنمية البشرية
/ / ٢٠٢١

نسخة منه إلى :
- مركز التدريب والتنمية البشرية / وحدة أدارة البحوث مع الأوليات ...

سوزان ١١/٢٤

Appendix 2

The Questionnaire Sheet for Each Patient in this Study

اسم المريضة	
العمر	
عنوان السكن	
عدد الولادات الطبيعية	
عدد الاجهاضات السابقة	
عمر الحمل الحالي بالأسابيع	
هل المريضة تستخدم علاجات اثناء الحمل -اسبرين -بريدنيزولون -ابر تحت الجلد -علاجات اخرى	
هل تعاني المريضة من امراض معينة ضغط الدم السكري الروماتيزم الربو داء الذئبة داء القطط	

Appendix 3

Sequence of PCR Product were Generated from 6 Isolates

Japan / Genes Bank:

2022/ 6/ 22 LC715211

LOCUS LC715211 422 bp DNA linear HUM 22-JUN-2022
DEFINITION Homo sapiens G1 IL33 gene for interleukin 33, intron 1, partial sequence.
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VERSION LC715211.1
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ORGANISM Homo sapiens
Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini; Catarrhini; Hominidae; Homo.
REFERENCE 1 (bases 1 to 422)
AUTHORS Jameel, Z.I. and Mohammed Al Alwany, S.H.
TITLE Direct Submission
JOURNAL Submitted (09-JUN-2022) to the DDBJ/EMBL/GenBank databases.
Contact: Zahraa Isam Jameel
Babylon University, Biotechnology; 40 Street, Babylon, Hilla 51001, Iraq
REFERENCE 2
AUTHORS Hadi, G.S. and Mohammed Al Alwany, S.H.
TITLE IL33 Gene Polymorphism
JOURNAL Unpublished (2022)
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 241 ggtcccatta agaaaaaaga actaaagaac aattccaaac tacttctttt agtgcatctc
 301 taatcctctt tgtgaagcta ttttcttatt caaagtttcc atcattttca aatatgaaaa
 361 tattgatgaa gtgacattaa aaccttctgt ctatttcctt cagggctaca ttcacctttt
 421 gc
//
```

NCPI / Genes Bank :

LOCUS G1 422 bp DNA linear PRI 19-MAY-2022
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VERSION
KEYWORDS UNVERIFIED.
SOURCE Homo sapiens (human)
ORGANISM Homo sapiens
Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini; Catarrhini; Hominidae; Homo.
REFERENCE 1 (bases 1 to 422)
AUTHORS Hadi Al_hujazi,G.S. and Mohammed Al.Alwany,S.H.
TITLE Direct Submission
JOURNAL Submitted (19-MAY-2022) biotechnology, Babylon university, 40 street, Babylon, Hilla 51001, iraq
COMMENT GenBank staff is unable to verify sequence and/or annotation provided by the submitter.
Bankit Comment: TOTAL # OF SEQS:6

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 181 atacgaaccc aaaatcataa agtcagtaaa tttcagatca gagattaaag tccagggtca
 241 ggtcccatta agaaaaaaga actaaagaac aattccaaac tacttctttt agtgcatctc
 301 taatcctctt tgtgaagcta ttttcttatt caaagtttcc atcattttca aatatgaaaa
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 421 gc
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DEFINITION UNVERIFIED: Homo sapiens.
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VERSION
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ORGANISM Homo sapiens
Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini; Catarrhini; Hominidae; Homo.
REFERENCE 1 (bases 1 to 422)
AUTHORS Hadi Al_hujazi,G.S. and Mohammed Al.Alwany,S.H.
TITLE Direct Submission
JOURNAL Submitted (19-MAY-2022) biotechnology, Babylon university, 40 street, Babylon, Hilla 51001, iraq
COMMENT GenBank staff is unable to verify sequence and/or annotation provided by the submitter.
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Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini; Catarrhini; Hominidae; Homo.
REFERENCE 1 (bases 1 to 422)
AUTHORS Hadi Al_hujazi,G.S. and Mohammed Al.Alwany,S.H.
TITLE Direct Submission
JOURNAL Submitted (19-MAY-2022) biotechnology, Babylon university, 40 street, Babylon, Hilla 51001, iraq
COMMENT GenBank staff is unable to verify sequence and/or annotation provided by the submitter.
Bankit Comment: TOTAL # OF SEQS:6

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//
```

الخلاصة

ارتبطت العدوى الفيروسية أثناء الحمل بنتائج سلبية للحمل وعيوب خلقية في النسل. نادرًا ما تعبر الفيروسات حاجز المشيمة ، ولكن عندما يصل الفيروس إلى الجنين ، يمكن أن يؤدي إلى عيوب خلقية شديدة مثل صغر الرأس أو حتى موت الجنين. يعتقد ان فايروس الحلا البشري نمط 7 له مضاعفات خطيرة مثل الاجهاض التلقائي. يرتبط الحمل الطبيعي بالانتقال الناجح من المناعة الخلوية من النوع الاول الى المناعة الخلوية من النوع الثاني. يسبب الحركي الخلوي الانترلوكين 33 في انتاج الحركيات الخلوية من النوع الثاني . اجريت هذه التجربة لدراسة تأثير فايروس الحلا البشري نمط 7 على النساء الحوامل اللواتي يعانين من الاجهاض والاجهاض المتكرر. تم إجراء دراسة الحالة والسيطرة على 100 مريضة و50 من النساء اللواتي يبدون انهن سليمات(حمل طبيعي و ولادة) ذوات اعمار مختلفة تتراوح من 18 الى 47 سنة. تم جمع العينات خلال المدة من اكتوبر 2021 الى فبراير 2022 . تم اخذ مسحات بطانة الرحم، عنق الرحم ، سوائل الجنين وكذلك عينات الدم ومعالجتها لاستخراج الجينوم الفيروسي وجين الحمض النووي الكلي لتشخيص فيروس الحلا البشري نمط 7 وتحديد حزم الانترلوكين 33 باستخدام تقنية تفاعل التضخيم المتسلسل ،على التوالي. بينما تم تحديد التغاير الوراثي للانترلوكين 33 عن طريق تقنية التسلسل لشركة ماكروجين \ كوريا الجنوبية. بالإضافة الى ذلك، تم تقدير تركيز الانترلوكين 33 في المصل بواسطة مقايسة الممتز المناعي المرتبط بالأنزيم . تتلخص النتائج التي تم الحصول عليها من هذه الدراسة على النحو التالي : كان متوسط عمر المريضات اللاتي يعانين من الاجهاض المتكرر (32.70±12.41 سنة) اقل من متوسط عمر النساء اللاتي يتمتعن بصحة جيدة ويبدو انهن سليمات (30.67 ± 11.17 سنة) .لا توجد فروق ذات دلالة احصائية عند مستوى احتمالية اقل من 5% (p=0.47) . تم العثور على علاقة ايجابية قوية (مع وجود ارتباط عالي) بين عدد المشاركات ;عدد اسابيع الاجهاض وعمر الام. مع ذلك، لا توجد هناك علاقة ارتباط ذات دلالة احصائية بين عدد المشاركات مع عمر الام من مجموعة السيطرة . من بين 100 مريضة، وجد ان 55% مصابات بعدوى فايروسيه بينما 45% سلبية من النساء اللواتي يعانين من الاجهاض المتكرر. تظهر النتيجة الايجابية لفيروس الحلا البشري نمط-7 وفقا لتفاعل البلمرة المتسلسل 34.5% (19 من 55 حالة)، بينما 65.5% (36 من 55 حالة) سلبية. في النساء اللاتي يعانين من الاجهاض المتكرر، كانت الفئة العمرية الاكثر اصابة بفايروس الحلا البشري نمط 7 هي (30 – 39 سنة) والتي شكلت 48% (12 من أصل 25 حالة)، بينما شكلت الفئة العمرية (17 – 29 سنة) 32% (8 من أصل 25 حالة)، تليها 20% (5 من أصل 25

حالة) في الفئة العمرية (40 – 49 سنة) . كانت النتيجة الايجابية وفقا لتضخيم تفاعل البلمرة المتسلسل لحزمة واحدة (422 زوج قاعدي) من جين الحركي الخلوي الانترلوكين 33 في النساء اللاتي يعانين من الاجهاض المتكرر و55% نساء سليمات (55 من أصل 100 حالة) و30% (15 من أصل 50 حالة) ،على التوالي. بينما كانت النتائج السلبية لدى النساء المصابات بالإجهاض المتكرر والنساء السليمات 45% (45 من أصل 100 حالة) و70% (35 من أصل 50 حالة) ،على التوالي . اظهرت النتائج ان توزيع تعدد الاشكال للحمض النووي كان عبارة عن توزيعات متعددة الاشكال للحمض النووي طبقا لادينين\سايتوسين ، في ثايمين\جوانين . كانت الانماط الفردية ثايمين\ادينين و ثايمين\سايتوسين 9% ، 7% ، 9% ، 6% ، 9% ، 5% على التوالي لدى النساء المصابات بالإجهاض المتكرر و4% ، 0% ، 8% ، 2% ، 3% ، 2% على التوالي في مجموعة السيطرة.بالإضافة الى ذلك، تم العثور على نوعين من الطفرات في الانترلوكين 33 هي التحويل الجيني والانتقال. تواتر طفرة التحويل (C \ A ؛ T \ A ؛ G \ T ؛ A \ T و C \ T) أكثر من طفرة الانتقال (G \ A) . متوسط تركيز الانترلوكين- 33 في النساء المصابات بالإجهاض المتكرر والنساء السليمات كان (10.66 ±0.95 pg./ml) من هذه الدراسة، و(6.28 ±0.42 pg./ml) على التوالي . تم تسجيل ستة طفرات جديدة لتسلسلات الانترلوكين -33 في بنك المعلومات الوراثية العالمي ذات تسلسلات موضحه في ادناه :

ON564301 ; ON564302 ; ON564303 ; ON564304 ; ON564305 ; ON564306

الاستنتاج من هذه الدراسة الحالية: ان فايروس الحلا البشري نمط 7 هو واحد من أحدث فايروسات المهبل التي تم تحديدها في النساء العراقيات اللاتي يعانين من الاجهاض المتكرر. بالإضافة الى ذلك، يمكن ان يشير تركيز المصل العالي لمستوى الانترلوكين 33 في النساء المصابات بالإجهاض المتكرر الى ان الانترلوكين 33 يعمل كعامل خطورة ممكن ان يتسبب بزيادة تعرض المرأة الحامل الى الإجهاض المتكرر المجهول السبب. ويمكن ان يشير الارتباط الكبير بين تعدد الاشكال الجيني للانترلوكين 33 مع الاصابة بفايروس الحلأ البشري نمط 7 الى إلى دور مهم للغاية لهذه العوامل الجزيئية في النساء اللاتي يعانين من الإجهاض المتكرر.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة بابل
كلية العلوم
قسم علوم الحياة

الترابط ما بين عدوى فيروس الحلأ البشري نمط ٧ والتغاير الوراثي للحركي الخلوي الانترلوكين ٣٣ في النساء المجهضات

رسالة مقدمة الى مجلس كلية العلوم في جامعة بابل، وهي جزء من متطلبات نيل درجة الماجستير في علوم الحياة

من قبل
غفران سلام هادي وناس
بكلوريوس علوم الحياة / جامعة بابل (٢٠١٠)

بأشراف
الأستاذ الدكتور
شاكر حماد محمد العلواني
دكتوراه فايروسات طبية وجزيئية
جامعة بابل/كلية العلوم

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