Phylogenetic Correlation between CMV, HHV-6, and EBV Infection of Unexplained Infertility Males and Females in Dyiala Province

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Abstract

Back ground: A reproductive system disorder known as infertility is the inability to produce a clinical pregnancy after at least a year (12month)of unprotected sexual activity. Medical conditions, genetics factors, hormonal imbalance, and infectious agents could be the main causes. Viruses, as well as many other microorganisms, are able to interfere with the reproductive function and suspected to be involved in development to infertility; However, the viral infection such as Cytomegalovirus (CMV), Epstein Barr virus (EBV) and Herpes virus -6 A or B (HHV-6) may be defined as a main reason of infertility cases. This study aims to understand the possibility of relationship between the viral infection and unexplained infertility. The evolutionary relationships among viruses and their ancestors were investigated too.
Materials and methods: Current study included 90 unexplained infertile cases in Diyala province \Iraq from November 2020 to April 2021. CMV, HHV-6, and EBV viruses were identified using multiplex real time PCR. SPSS version -26 and GraphPad Prism 3.06 were used for the statistical analysis, both of which were considered significant.
Results: In this study we observed 10 (40%) of males were infected by CMV (20±8 copy\reaction), while females were infected by 14(56%) with viral load (2±0.8 copy \reaction). HHV-6 infection in males were 5(20%), viral load (14 ±8 copy\reaction) with high significant result ($p<0.01$)), No women in present study were infected with appearance significant between male and females ($P\leq0.01$).On the other hand,the infection in males with EBV was distributed equally in both gender by 2(8%) with viral load (0.1±0.01), however, the differences was observed in the viral load only (1±0.7copy \reaction). A number of herpes virus infection was detected in infertile males 17(37%) more than females 16(35%). The samples were screened to amplify UL122 gene for Human betaherpesvirus 5(CMV) and showed about 99% sequences similarities with European origins. Furthermore, an amplifying tripartite terminase subunit 3 (TRM3) gene sequences of the Human betaherpesvirus -6(HHV-6) showed 100% sequences similarities with African and European sources. Finally, amplify the DNA pol B gene that codes for subunit UL15-like protein in the Human gammaherpesvirus 4(EBV) showed about 100% sequences similarities with an American strain of human gammaherpesvirus.

Keywords:Infertility, Multiplex Real-time PCR, CMV, IBV, HHV-6, iTOL.

1. Introduction

Infertile clinically defined as a reproductive system disease by inability to produce clinical pregnancy during a 12-month period or more of unprotected sexual activity. The duration of unwanted non-conception, the aging of the female partner, and disease-related infertility are three significant factors that influence the spontaneously probability of conception [1](Vander Borght;eatl;2018). Infertility is classified as primary for a couple with no children, and secondary after having one child [2]. Approximately 33% of infertility cases have both male and female factors, and up to 49.5 %, dependent on the fairness of the viral DNA diagnostic techniques used. [3]. Infectious agents could impair different critical activities; for example, viruses and other microbes can interfere with reproductive function in the both sexes [4]. Human herpes viruses (HHVs) may cause male infertility by generating viral toxicity in genital tract cells or by eliciting local and systemic infectious or immunological responses. Herpes viruses related to infertility [5]. Human herpes infections and sperm parameters were linked in male studies [6]. EBV, CMV, and HHV-6 are common in semen, but their effect on male fertility is unknown. Several studies have suggested the incidence of CMV in human sperm samples in various countries to be between (0 - 62.5 %). One other study revealed that EBV had a viral DNA incidence of 3.4(5%), CMV had a viral DNA incidence of (5.2) %, and HHV-6 had a viral DNA incidence of (6.5) %, with different value for viral co-infection among them [7]. In CMV and HHV6, it has been presented in vaginal secretions, in the uterine cervix, in placenta, ln tissues, and in endometrial epithelial cells, and as associated with primary idiopathic infertility. HHV-6 infection alters the immunologic profile of the endometrium, which includes the tolerogenic molecule human leukocyte antigen G, and can compromise embryo implantation [8]. CMV is often identified at the uterine-placental interface impairing cytotrophoblast differentiation and invasion, and degradation of extracellular matrix (ecm could make a contribution to the shallow invasion of the uterus and restraint of fetal growth in pregnancies affected by CMV infection during early pregnancy and may ends with fetal death and spontaneous abortion [4] Tamimi, Epstein-Barr

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infection is possibly associated with auto - immune ovarian failure and impact fetal growth [9]. Herpesviruses are big DNA viruses that are divided into three groups. α-herpesviridae, β-herpesviridae and γ-herpesviridae families. Herpesviruses cause lifelong infections in humans. In healthy people, these infections are asymptomatic. [10] & Human cytomegalovirus (HCMV), sometimes called human herpesvirus 5 (HHV5), infects 60-90% of individuals in underdeveloped nations. Serum positive for infection in the general population is 83% [12] [12]. Primary infection is transmitted through intrauterine breast milk and contaminated exposure pathways (saliva or vaginal fluids) [13] In healthy people, HCMV infection is frequently mild or asymptomatic. It causes CNS infections and hearing loss in congenitally infected newborns. It endangers fetal development and immunocompromised individuals, like transplant recipients [14].

Epstein–Barr virus (EBV) has double-stranded linear DNA in an icosahedral capsid with a tegument between the nucleocapsid and outer envelope. EBV has been linked to nasopharyngeal carcinomas, Burkitt's lymphoma, and Hodgkin's lymphoma, as well as autoimmune illnesses such lupus and multiple sclerosis [15] & [1] (Carbone & Gloghini, 2018)HHV-6's genome consists of a unique (U), 143- to 145-kb region flanked by identical terminal direct repeats (DRL and DRR) and is 159 to 162 kb long [16]. 1% of the world's population HHV-6 is inherited because the viral genome is incorporated in germline cells. A and B herpes viruses infect humans. [11].

Understanding how viral infection affects various levels of infertility is the goal of this investigation. To ascertain the frequency of the viral infection in the chosen samples, multiplex RT PCR will be used. Additionally, a specific set of comprehensive trees were built to assess the accurate genotyping of the detected variations and their phylogenetic distribution.

2. Materials and Methods

2.1.1. Participants in study

The current study took place in Diyala province from November 2020 to April 2021. A total of 90 clinical samples (referred as eligible couples) were collected from A-Batol teaching hospital (gyncology department). the collection information on age and gender, a customized questionnaire was created pre-constructed. Participants in the control group had no fertility problems. All participants were given a unique study number to label all samples and identify participants in all written and electronic databases. The participants were clinically diagnosed with idiopathic infertility; the selected cases were divided into two groups, first group consisted of 45 women (25 infertility women and 20 control women), and similar classification was used with male participants.

2.1.2. Seminal fluid of male

This study followed WHO guidelines (5th edition 2010), which included pH, concentration, morphology, and volume. sperm motility is characterised as follows: Type 1, Type 2 (fast sluggish moving sperms), type 3 (locally shaking sperms), and type 4 (immotile or non-motile sperms) are the four types of sperm Instructions on how to collect the seminal were given to the participants which were Absents for 3-7 days within an hour of receiving and delivering the sample to the laboratory. The rest of samples were transported using a cooled box to the hospital's blood bank department sample and stored at -80°C for molecular analysis [17].

2.1.3. Higher Vaginal Swab

Higher vaginal swabs were used to collect samples from the of genital tract by opening the vaginal with a disposable Casco tool on days12 or 13 of the menstrual cycle. The swab VIR-Swab (Heinz Herenz or Germany Copan / Italy)and transported by a cooled box to the hospital's blood bank department and stored at -80°C for molecular analysis [18].

3.2. Methods

3.2.1 Molecular detection and phylogenic sequences

3.2.1.1. DNA extraction

Following the instructions provided by the manufacturer of the Qiagen QIAamp®DNA mini kit (Germany), genomic DNA was extracted using the kit,;with minor modification. All obtained samples were raise at room temperature (25°C). Quantitative detection and differentiation of obtained viruses was made by Multiplex Real Time PCR used the CMV/EBV/HHV6 Quant Real-TM kite (Sacace Biotechnology, Italy, cat: TV48-100FRT), the amplification conditions are shown in Table (1).

3.2.1.2. Conventional PCR amplification

These primers were supplied by Macrogen Company (South Korea). The final volume of each PCR reaction was 25 µl (Master mix 12.5 µl, forward primer1 µM and Reverse primer 1 µM,DNA 4 ng\ µl,and complete by 6.5 µl Nuclease Free Water) used in this study. Prior to use, the primer solution was thoroughly mixed with a vortex for homogenization. After PCR amplification, gel electrophoresis was used using agarose gel (1.5%) to detect the presence of the PCR product

3.2.1.3. Standard Sequencing: ABI3730XL, an automated DNA sequences, was used by Macrogen Corporation in Korea to do Sanger sequencing on PCR products. The result was analyzed with the help of some genious software.

3.3. Phylogenic cooperatio

In this investigation, three specific PCR regions containing the coding areas of Tripartite terminase subunit UL15-like protein and cytoplasmic envelopment protein;2 – TRM3 of human gammaherpesvirus 4, human betaherpesvirus 5, and human betaherpesvirus 6 were chosen. Amplified fragments are directly sequenced to assess viral genetic polymorphism. Then, comprehensive trees were built to examine the variations' genotyping and phylogenetic distribution.

3.3.1. Nucleic acids sequencing of PCR amplicons

According to the instructions provided by the sequencing company, the resolved PCR amplicons are sequenced commercially in forward direction (Macrogen Inc. Geumchen, Seoul, South Korea). The sequencing results

of the PCR products of the target samples were edited, aligned, and analyzed using BioEdit Sequence Alignment Editor Software Version 7.1 as long as they matched the proper sequences in the reference database ((DNASTAR, Madison, WI, USA). SnapGene Viewer ver. 4.0.4 (https://www.snapg ene.com) was used to annotate each discovered variant within the viral sequences.

3.3.2. Translation of nucleic acid variations into anima acid residue:Internet access was used to retrieve the

amino acid sequences of the targeted proteins from the protein data bank (http://www.ncbi.nlm.nih.gov). The observed nucleic acid changes in the coding regions of the investigated genetic loci are translated into the a reading frame matching the relevant amino acid residues in the encoded protein using the Expasy online tool (http://web.expasy.org/translate/). Multiple amino acid sequence alignments between the reference amino acid sequences and their observed mutant counterparts were carried out using the "align" script on the BioEdit website.

3.3.3. Comprehensive phylogenetic tree construction

This work created a thorough tree using (Hashim et al; 2020) neighbour-joining.'s protocol. Observed variations were matched to homologous reference sequences using NCBI-BLASTn (Zhang et al. 2000). Next, a full inclusive tree, including observed variant, was created using the neighbour-joining method and visualized as a circular cladogram using the iTOL suit [19].

3.4. Ethic approves

This study was approved by the Diyala Health Department's Training and Human Development Center's Research and Knowledge Management Division (No. 48950, 12/17/2020) and the College for Pure Science/University of Diyala. All participants gave written consent prior.to registration.

3. Results

4.1. Results of multiplication

The positivity rate of molecular detection of CMV, HHV-6, and EBV among of un explained infertile male and females was summarized in Table 1 and Figure 1 &2. In this 40% of males were infected with CMV (20±8 copy\reaction), while 56% of females were infected with viral load (2±0.8 copy \reaction) with no significant differences (Figure 2 - a &b. Whereas HHV-6 infection in males was 20% including viral load by 14 ±8 copy\reaction, there is no women infection presented in present study (Figure 2 - c& d). On the other hand,the infection in males with EBV was 8% with viral load (0.1±0.01), infertile women that infected by EBV have different in viral load ((1±0.7copy \reaction) with no significant differences (Figure 2- e&f), a high prevalence of total number herpes viruses was indicated (37%) in infertile males and it was more than the other gender (35%). However, there is no viral infection was observed in control samples.

In the beginning Viruses percentage of CMV,HHV-6 and EBV in our investigation when compeer with previous studies was lower;because the sample number previous was higher like [20] from 113 sample found (56.6%) infected male and [21] found from 143 present (83%) samples were infected, This may explain their high viral prevalence. We agree that male sperm in the research population had 17.7% human herpes virus DNA. not agree with him in finding EBV, CMV, and HHV-6 prevalence were 3.7, 1.9, and 5.7% respectively, also he observed viral load was (copies \ ml) 500 EBV, 2250 CMV, 250 HHV-6 [6] Kapranos study identified EBV 19 (16.8%) and CMV in 8 (7.1%) semen samples, respectively. HHV-6 was observed in 48 (53.3%;1.260.51) then CMV 39 (43.3%;1.510.95) followed by EBV 34 (37.8%; 2.201.47) in idiopathic male fertility [20].A nested PCR investigation found 66.8% of fertility clinic men have HHV-6, although HHV-6A/B doesn't impact spermiogram (Muhsin et al., 2019). Another study discovered a significant association between CMV virus prevalence and HCMV DNA in semen samples, 23% in infertile men and 7% in fertile men. This could be beneficial considering HCMV positive cases in male infertility [17].Unlike our investigation, CMV DNA was found in 28 (18.6%) semen samples [22]. Also in Chinese study of male infertile 4% for HHV4, 22% for HHV5 and 2% for HHV6 [23], we didn't agree with Iranian findings showing no association between infertility & CMV virus. [24] and [25]. Bezold found CMV, and EBV in his investigated in male only (17.1%–18.7%) [26]. In Taiwan using PCR to detection CMV in Sixty nine (69) documented infertile men participated showed (37.7% male were positive for the CMV [27]. Twelve percent of males and 14 percent of females with unexplained infertility had HHV-6 DNA. 40% of infertile women's endometrial epithelial cells contained HHV-6 DNA. [28]. In contrary, other study found 43% of endometrial epithelial cells were positive for HHV-6A DNA [29].The different in results may be due to small size of sample by swab and may de mast take biopsies sample. Similarly to our results, A study found around 54 % of infertile women were infected with CMV [4]. Whereas, study in Samara city found the infection with CMV was only 17 % [30]. Contrasted to current study, EBV nfection in cervical was detected in 10% of infected women [31]. Similarly, other study on the cases of spontaneous miscarriage in Baghdad city presented higher rates of CMV and IBV -DNA infection by 37.5% and 22.5%, respectively [32].CMV in endo cervical material was associated by impairing cytotrophoblast differentiation and invasion of the uterus and restriction of fetal growth in pregnancies affected by CMV infection during early pregnancy results in seriousabnormalities to the fetus that may ends with fetal death and spontaneous abortion [4].Additionally, CMV replication may be suppressed which leads to a latent state with the virus during immunosuppression. Old previous study also were screened for EBV DNA in(91)_cervix of women attending the Sexual Transmitted Disease clinic by used PCR-technique, and their results appeared EBV DNA was demonstrated In 35 (38%), this result higher than our results therefore not agree with our results [33]. Also, a study suggests a probable sexual route of transmission for EBV and infertility by using Real-time polymerase chain reaction to quantify EBV DNA in Swedish women's cervical secretions. EBV DNA was identified in 10\112 cervical secretion samples, which is

not in agreement with us. [34].

Table 1 -Distribution of virus infection according to the Gender.. X2 was used to compare means between different groups and results are expressed as percentages.

Patients	CMV	Viral load (copy\reaction)	HHV-6	Viral load (copy\reaction)	IBV	Viral load (copy \reaction)
	Positive (%)	(mean± SEM)	Positive (%)	(mean± SEM)	Positive (%)	(mean± SEM)
Male	10(40 %)	(20±8)	5(20%)	(14 ±8)	2(8%)	(0.1±0.01)
Female	14(56 %)	(2±0.8)	0	0	2(8%)	(1±0.7)
X2	P<0.05		P< 0.01		NS	

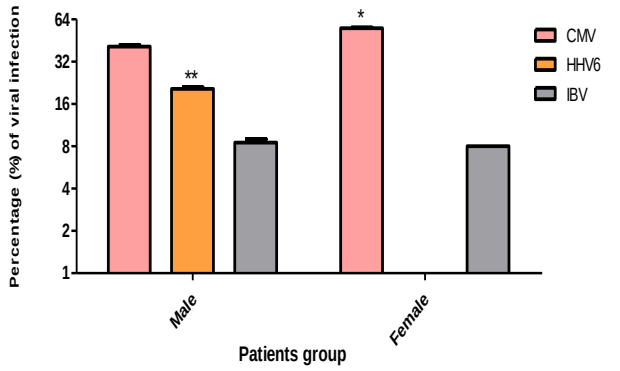
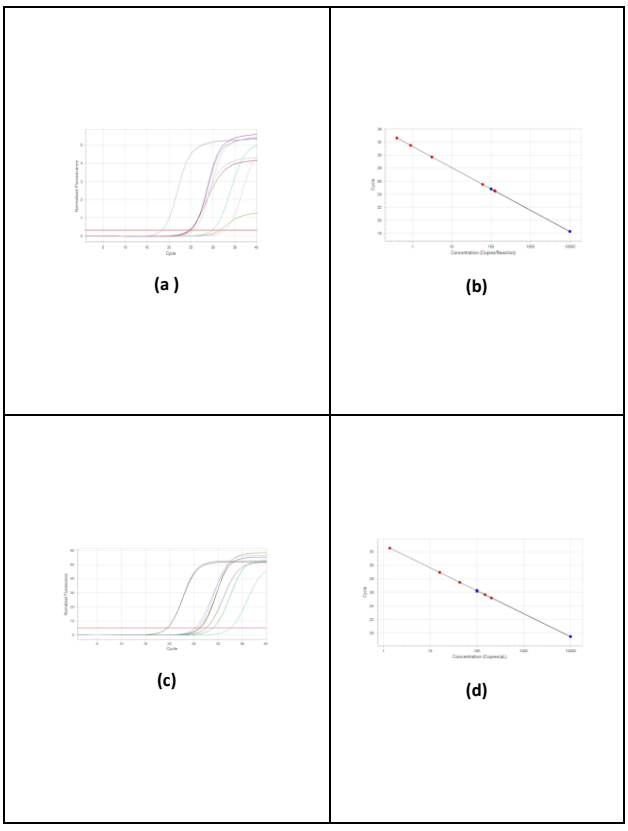


*Figure -1 A comparison of the virus infection (%) between two selected groups. X2 was used to compare means between different groups and results are expressed as mean ±SEM:** p<0.01; * p<0.05.*



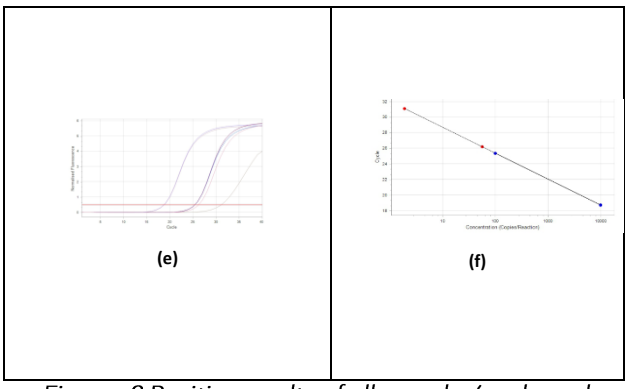


Figure -2 Positive results of all samples(male and female) detection the CMV,HHV-6 and EBV by Multiplex Real-time PCR. a) Total positive results of CMV by real-Time PCR, b) viral load of CMV (Copy \reaction, c) Total positive results of HHV-6 by real-Time, d) viral load of HHV-6 (Copy \reaction), e) Total positive results of EBV by real-Time,f) viral load of EBV (Copy \reaction).

4.2.1.Human betaherpesvirus 5 (human cytomegalovirus or HCMV)

This study amplified the UL122 gene for Human betaherpesvirus 5. NCBI BLASTn indicated 98% sequence similarity between sequenced samples and reference target sequences. The alignment results of the 167 bp samples indicated only one nucleic acid variation represented by one nucleic acid substitution in two of the tested samples (Figure -3). The variation of the UL122 gene can be used for Human betaherpesvirus-5 genotyping for its possible adaptability to variable genetic variety, such as that which was observed in different forms of viral infections. However, the viral sequences usually alter their sequences to adapt to the host environment in which they are living [35].

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10 20 30 40 50 60 70 80 90 100
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....
|....|....|....|....|
ref.
TCTGCCAGGACATCTTTTTCGGGGTTCTCGTTGCAATCCTCG
GTCACTTGTTCAAAGGTTTTGAGGGATTCTTCGGCCAATTCT
GGGAACAGCGGGTCTC
S2.....C.....
.....
S3.....
.....
S4.....
.....
S5.....C.....
.....
S6.....
.....

110 120 130 140 150 160
....|....|....|....|....|....|....|....|....|....|....|....|..
ref.
CCAGGCTCAGCTGACTGTTAACCTCCTTCCTTAACATGGTCT
GCAGGAACGTCGTGGCCTTGGTCAC
S2.....
S3.....
S4.....
S5.....
S6.....
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Figure-3 Nucleic acid sequences alignment of five samples with their corresponding reference sequences of the 167 bp amplicons of the UL122 genetic sequences. The symbol "ref" refers to the NCBI referring sequence, letter "S", followed by a number refers to the sample number.

The S2 to S6 samples generated for them a comprehensive phylogenetic tree., as well as other human betaherpesvirus 5 relative nucleic acid sequences Within the cladogram, current samples were combined with other relative sequences to form only one type of incorporated sequence. (Figure -4).

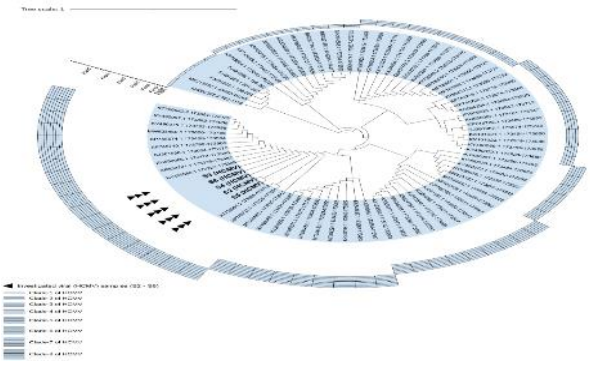

Figure -5 The comprehensive cladogram phylogenetic tree of genetic variants of the UL122 gene fragment of five human betaherpesvirus samples. The black-colored triangle refers to the analyzed viral variants. All the mentioned numbers referred to GenBank accession number of each referring species.

Current tree observations confirm sequencing reactions because they explain neighbor-joining-based positioning in observed variants. Our samples' European origins couldn't be ignored. Using UL122 gene sequences in this investigation has further indicated the genotype of this viral organism. The studied viral samples had close phylogenetic distances within the eighth clade of the cladogram. This showed that viral sequences within the clade have high genetic similarity. This UL122 gene-based comprehensive tree shows the high competency of such genetic fragments to efficiently identify viral genotypes. This shows that UL122 gene-specific primers may was as human betaherpesvirus 5 and its phylogenetic location. According to the detected nucleic acid substitutions, all these samples were positioned within two closely linked phylogenetic positions. These observations suggest the discovered mutation did not cause a noticeable evolutionary change in the human betaherpesvirus 5 genomes. Using UL122 gene-based fragments revealed the great ability of genetic fragments to identify viral sequences. This shows that UL122 gene-specific primers may accurately define human betaherpesvirus 5 and its phylogenetic location.

4.2.2. Human betaherpesvirus 6

The samples were screened to amplify tripartite terminase subunit 3 (TRM3) gene sequences of the Human betaherpesvirus -6. Thus, the TRM3 gene variant can be utilized for Human betaherpesvirus 6 genotyping because to its adaptability to diverse genetic variety. NCBI BLASTn found 100% sequence similarity between sequenced samples and reference target sequences. By comparing observable nucleic acid sequences with retrieved sequences (GenBank acc. MF994828.1)
NoteworthyTRM3 2 gene-based comprehensive tree shows how genetic elements can determine viral genotypes. This shows the capacity of the currently used TRM3 2 gene-specific primers to define human beta-herpesvirus 6A that our S7 to S10 viral samples belonged to and to discriminate from its close relative 6B. A thorough phylogenetic tree TRM3 2 was built in the present study based on nucleic acid differences detected in the amplification 176 bp of the TRM3 2 gene amplicons. The most notable feature of this tree is our samples' position in the human betaherpesvirus-6A clade.. This clade consisted of the majority of incorporated samples of human betaherpesvirus-6A sequences as 35 sequences of variable strains were incorporated within variable phylogenetic distances of this clade (Figure -6). However, the investigated samples (S7 – S10) were suited in the immediate vicinity to the GenBank acc. no. of MF994828.1. This strain of human betaherpesvirus-6A strain has recently been deposited from a country in the West Africa, Cote d'Ivoire. This data was attributed from the positioning of our investigated samples in the vicinity to other German strains (such as GenBank acc. no. of MF994815.1, MF994816.1, and MF994817.1, MF994818.1, MF994820.1, and MF994821.1). Thus, the African origin is not the only one for our investigated samples since the European sources were also observed

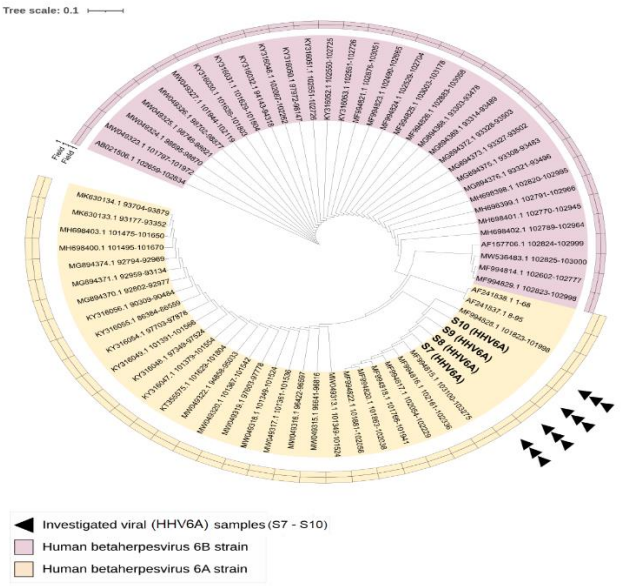

Figure -6 The comprehensive cladogram phylogenetic tree of genetic variants of the TRM3_2 gene fragment of four human betaherpesvirus 6 samples. The black-colored triangle refers to the analyzed viral variants. All the mentioned numbers referred to GenBank accession number of each referring species.

4.2.3. Human gammaherpesvirus 4 (Epstein-Barr Virus)

This study contained one sample from this locus. Human gammaherpesvirus 4's pol B gene codes for Tripartite terminase subunit UL15-like protein. Thus, the DNA pol B gene variation can be used to genotyping this virus known for its ability to respond to diverse genetic variety.

The concerning the 194 bp amplicons, NCBI BLASTn engine showed about 100% sequences similarities by comparing the investigated samples with the retrieved nucleic acid sequences (GenBank acc. MK540470.1), (Figure-7). To comprehend the phylogenetic distances of the S1 sample, a thorough phylogenetic tree was created using nucleic acid sequences. The phylogenetic tree includes S1 and other human gammaherpesvirus sequences. The S1 sample and relative sequences incorporate alongside the cladogram's sequences. This tree aligned 53 nucleic acid sequences. The most interesting fact about our viral isolates is their position within the Human gammaherpesvirus clade and their neighbor- sequences of the Human gammaherpesvirus within the major clade.

This clade consisted of the majority of incorporated samples of human gammaherpesvirus 4 sequences as 45 sequences of variable strains were incorporated within variable phylogenetic distances of this clade (figure -7). Within this major clade, the S1 sample was suited beside the GenBank acc. no. of LR813082.1 that belonged to a human gamma- herpesvirus 4 deposited from the USA. This style of positioning was due to the absence of any genetic variation in this sample. So, there is no deviation with respect to the original positioning these viral sequences occupied within the major clade of this cladogram. Because it explained the actual neighbour-joining-based positioning in such studied sequences, the current observation of this tree has confirmed sequencing reactions. However, the positioning of the S1 sample in the immediate vicinity to an American strain of human gamma -herpesvirus strain has been determined. Another origin of the S1 sample was also came from other incorporated sequences in the vicinity of this American strain. All these sequences were originated from China, namely GenBank acc. no. of MK540469.1, MK540464.1, MK540463.1, and MK540461.1). Interestingly, the Asian origins of our investigated samples could not be ignored. In the vicinity to the clade of human gammaherpesvirus 4 in which the S1 sample was positioned, it was found that the DNA pol B fragment has relatively close phylogenetic positions to the clade of human gamma-herpesvirus 8. Thus, both types of human gamma-herpesvirus share a high ratio of homology in this fragment.It is noteworthy that the study's use of DNA pol B gene sequences has provided additional evidence for the existence of the accurate identification of the viral organism's genuine genotype. However, this complete tree based on the DNA pol B gene has offered a tool that is all-inclusive about the high ability of such genetic fragment to effectively detect this viral genotype.

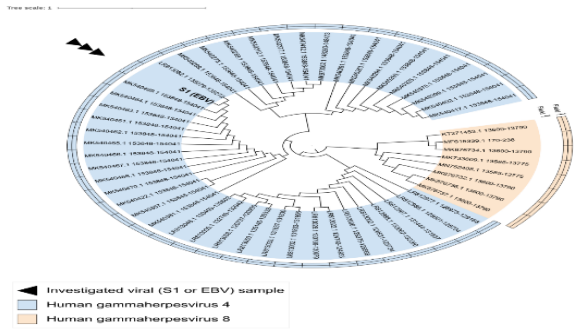

Figure-7 The comprehensive cladogram phylogenetic tree of genetic variants of the DNA pol B gene fragment of one samples of human-infecting human gammaherpesvirus 4. The black-colored triangle refers to the analyzed viral sequences. All the mentioned numbers referred to GenBank accession number of each referring species.

4. Conclusion

This study also reports for the first time, the prevalence of CMV, HHV-6, and EBV in Diyala province-Iraq. This study could be considered as continuous to previous studies. this study not found a significant differences between male and females who infected with CMV and EBV and while HHV-6 males infection showed a significant difference between male and females infection. Furthermore, the phylogenetic tree of CMV showed European origins, and HHV-6 belonged to African with European sources, while EBV related to an American strains.

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data collection.

7. Conflict of interest statement

The authors declare they have no conflicting interests. The study's design, data collection, analysis, or interpretation; the preparation of the paper; or the choice to publish the findings were all made independently of the funders.

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