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Novel emergency of mutant *ul*97 resistance gene among HCMV obtained from first trimester pregnant women in Iraq

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Abstract

Human cytomegalovirus (HCMV) is a member of the Beta-herpesviridae family, and represent the most common factor of intrauterine viral infections. Usually systemic treatment of CMV infection is mainly depend on many drugs including ganciclovir, valganciclovir beside cidofovir as they stopped DNA synthesis of the virus . Emergency of resistance is due to alteration in*ul*97 gene (tegument serine / threonine protein kinase) thus prevents initial phosphorylation of ganciclovir GCV. Two hindered blood samples were obtained from selected pregnant women with history of abortion. There aged ranged from 16 – 50 year and they were admitted to Baghdad hospitals during the period from February 2014 to July 2014 .The primary diagnosis of HCMV was carried out depending on the presence of antibodies against the virus .Enzyme-linked Immunosorbent Assay (ELISA) was used for this purpose. The results showed that 43/200(21.5%) were IgM &IgG seropositive, and about 26/43(60.5%) of them belong to recurrent aborted women group. Highest incidence of seropositive CMV-IgM was observed in the first trimester of pregnancy (41.9%) followed by second trimester (34.9%) then third one (18.6%).Molecular investigations for*ul*97gene was carried out using Nested PCR relying on cDNA as a template. The results showed that 4/43 (21.5 %) of the isolates were harbored *ul*97 resistance gene. Results of the DNA sequencing and phylogenic analysis for the sequenced *ul*97 gene revealed that two of them were mutated and diverted in their amino acid profile as compared with NCBI control. This might considered a sign for emergency of resistant Iraqi CMV isolates.

Keywords: human cytomegalovirus, *ul*79, HCMV diversity.

Introduction

Human cytomegalovirus (HCMV) is a double stranded DNA, ubiquitous member of the Beta-herpesviridae family(1).It is the most common factor of intrauterine viral infections, and usually excreted in body fluids including urine, blood and salivaor by breastfeeding, genitourinary tract secretions, feces, tears, and transplanted organs(2).Women with IgM seropositivity without being positive to IgG antibodies are considered as acutely infected with CMV(3). Meanwhile, there are some cross reactivity about 3.3% for IgM positivity with other viral infectious including Epistein Barr Virus (EBV), measles and herpes simplex varicella- zoster as reported by(4).At least 60-70% of the world's population carrying the virus and usually they enter a life-long latency especially in healthy individuals after a frequently asymptomatic primary infection (5).The virus is responsible for the most common congenital viral infection in developed countries as seven infants out of 1000 births might have congenital CMV infections, of whom 1 to 2 (0.1%) will have permanent clinical squeal and mental retardation in infants including neurosensory deficits, thrombocytopenia, hepato splenomegaly or death of newborns (6).

Cytomegalovirus infections being the most important cases during first trimester which might increase the risk of abortion also they can cause congenital birth defects(7).Both maternal primary and recurrent infection during pregnancy can occur, but the rate of transmission is far higher for mothers with primary infection(8). Molecular diagnosis such as nested PCR is highly specific and sensitive, in this method it can use two specific primer for the forward and the same for the reverse but in two pairs, one for external and the other are internal, the product of the first round with external primer used as a template for the second round with internal primer(9).The systemic treatment of CMV infection is by using many drugs including ganciclovir, valganciclovir (oral prodrug of ganciclovir), cidofovir and foscarnet (10).All these drugs inhibit DNA synthesis and termination of DNA elongation by inhibiting of DNA polymerase (11).The target genes involved in GCV ganciclovir anabolism is called *ul*97 which has an important roles in the initial phosphorylation of GCV that is essential for the antiviral activity on these drugs thus the resistant strains is emerged after a mutation took place in the *ul*97 gene which prevents initial phosphorylation of GCV (12). This mutation might be due to base pair deletion mutation in the open reading frame of the HCMV *ul*97geneleading to drug resistance(13).The aims of this study involved the primary diagnosis of CMV among pregnant women , detection of tegument serine/threonine protein kinase (*ul*97) gene then to point out any mutation occurred in its sequences by comparing with NCBI recorded data .

Materials and Methods

Subjective: A prospective study was carried out during the period from February 2014 to July 2014 on studied group. Samples were collected from AL-elwea hospital in Baghdad\ Iraq. Two hindered selected pregnant women and those with history of abortion (there aged ranged from 16 – 50 year) were involved in this study. Blood sample (5 ml) was drawn from each woman under sterile conditions, and then it was divided into two different containers: Two and half ml of obtained blood was placed in EDTA tube then later were divided into 0.5 ml aliquots and were used later for extraction DNA of HCMV. The remaining two and half were centrifuged to separate the serum, which was used for immunological assay.

Serological Assay for Primary Detection: Serum anti-HCMV IgG and IgM antibody levels were assayed using enzyme-linked immunosorbent assay (ELISA)tests(Cytomegalovirus(CMV) IgG/IgM Enzyme Linked Immuno Assay (ELISA)/BioCheck/German and seropositivity was determined by using the manufacturer's guidelines.

Samples which gave positive results in ELISA test for CMV IgM antibodies were depended for RNA extraction as 1 ml trizol was added to 0.5 ml blood (duplicate were done) according to(14) then stored at -20c º.

HCMV DNA and RNA extraction: DNA extraction was done in Molecular Oncology Unit in Guy's hospital–Kings college London/United Kingdom using Qiacube for automated DNA extraction instrument, according to the protocol provided with the kit. Total RNA extraction from blood with trizol was achieved according to Guy's protocol for total RNA isolation (14).The protocol was carried out at room temperature and converted to cDNA by using High Capacity cDNA Reverse Transcription Kit/ Applied Biosystem/USA, under condition listed in table(1).

Table (1): Thermal Cycling Condition Program for converted RNA to cDNA.

	Heated Lid	Step 1	Step 2	Step 3	Step 4	Step5
Temperature	111ºC	25ºC	37ºC	42ºC	75ºC	4 ºC
Tim		10 min	10 min	1.Hr	5 min	∞

Note: These conditions are optimized for use with High-Capacity cDNA Reverse Transcription Kit

Nested PCR: HCMV-specific external and internal primers were designed using NCBI programs (Primer 3 tool). Beside a specific tail was designed according to this study an added to the primer as clear in table (2). Usually nested PCR are programmed to two rounds:

1-The first round of nPCR was performed for amplification of external primers of the genes *ul*97 A3,*ul*97 B3, *ul*97 C3 with a total reaction 50 µl as follow: AmpliTag Gold®360 DNA polymerase enzyme(0.4µl) , AmpliTag Gold®360 Buffer 10×(5 µl),$MgCl_2$(4 µl),dNTPs(1.5µl), external forward and reverse Primers(1.5µl for each), Template DNA orcDNA(5µl), and 32.6 µlH_2O under thermocycling conditions according to this study consisted of: 95°for 10min, 94°for 30sec, 58°for 30sec. 72°for 1min and 72° for 7 min.(50 cycles).

2-The second round of nPCR: was done for amplification of the previous genes(*ul*97 A2, *ul*97 B2, *ul* 97 C2) but using specific internal primers. The PCR product from the first run was used as a template (5µl)in the second round with a total reaction volume 25µlas follow: AmpliTag Gold®360 DNA polymerase Enzyme (0.4µl) , AmpliTag Gold®360 Buffer 10×(2.5µl), $MgCl_2$(1.5µl), dNTPs (1µl), internal forward and reverse primers 1µl, and 13.6µl H_2O under thermocycling conditions according to this study consisted of:95°for 10min,94°for 30sec,55°for 30sec.72°for 1min and 72° for 7 min (50 cycles).

Table (2): The Primers Used in the Current Study

No	Primer	Primer sequence 5′----------→3′	Origen	Product size (bp)	References
1	UL97A3F (external)	ACGTTGGCAGGCGCTATCA	Eurogenetic	149	Designed
2	UL97A3R (external)	CTGAGGCTGTAATCGCACAG			
3	UL97A2F (internal)	gtctcagttcaggtgtccttcctgTTTCTCAATCAC CAGTGTCG	Eurogenetic	130	Designed
4	UL97A2R (internal)	agacgtcttgtgtcgtggctactgGATCTCGCTGG GGTTGTG			
5	UL97B3F (external)	CCTATCCGGATTACAACGAG	Eurogenetic	190	Designed
6	UL97B3R (external)	ATGCAATAACGCCGTAGG			
7	UL97B2F (internal)	gtctcagttcaggtgtccttcctgGCCGTCTTTCAG GGGAC	Eurogenetic	160	Designed
8	UL97B2R (internal)	agacgtcttgtgtcgtggctactgGGTCGCAGATGA GCAGCTT			
9	UL97C3F (external)	CTCATGCGGCTGTTGTAC	Eurogenetic	235	Designed
10	UL97C3R (external)	ACATCTTGGCCTCCACAAG			
11	UL97C2F (internal)	gtctcagttcaggtgtccttcctgACGGAGGCGTTGCTCTT A•	Eurogenetic	161	Designed
12	UL97C2R (internal)	agacgtcttgtgtcgtggctactgAGGCGCCGTACCTCAT TT			

- The capital letters refers to the primer sequence, the small letters refers to the tail.

DNA Sequencing: This part of the study was also done in Molecular Oncology Diagnostic Unit/ Guys and ST Thomas's Hospital /London/UK depending on(15).The data analysis achieved by Mutation Surveyor Software of reading sequencing Version 3.24.

Sequencing and Dendrogram Analyzing: The results were analyzed using Geneious software version NO.7.1.2R(2013) .Phylogenic analysis and Dendrogram for the sequenced data were also performed with same software using Tamura –Nei genetic destine model and UPGMA tree build method.

Results and Discussion

Human cytomegalovirus (HCMV) is a common agent of congenital infection and causes severe disease in immune compromised patient. In current study the serological test revealed that the percentage of infected pregnant women with of HCMV was43/200(21.5%) and IgM seropositivity represent an indicator for the acute infection. This results is closed to the results of (16) in Iraq, who found that the total HCMV-IgM seroprevalence rate was 44/210 (21%) among pregnant women. Also the results of the current study in agreement with another research in India whom found that 28.2%of women were positive for anti-CMV IgM, which it is considered high in relation to that reported in developed countries (3%-10%) (17). This incidence might be due to CMV infections and highly associated with poor hygienic conditions, communal life style, and close contact with day care units (18).CMV is the most common viral infection worldwide which affected different age groups (19). The results showed that the highest incidence of seropositive CMV-IgM was observed in the first trimester of pregnancy (41.9%) followed by second trimester (34.9%) then third one (18.6%) as in table(1).The diagnosis of active CMV infection was based on the detection of CMV replication in the blood. Therefore reactivation of CMV in the absence of an effective immune response is central to the pathogenesis of the disease (20).

Table (3): Distribution of Seropositive CMV-IgM Women in Correlation with Gestational Age

Gestational age	IgM+ cases	Percentage %
First- trimester	18	41.9
Second-trimester	15	34.9
Third- trimester	8	18.6
No pregnant	2	4.6
Total	43	100

Themolecular part of this study focused on detecting CMV in the DNA and cDNA obtained from patient blood. cDNA is a more convenient way to work with the coding sequence than mRNA because RNA is very easily degraded by RNases (21). Results of detecting *ul*97 gene (tegument serine/threonine protein kinase) showed that only 4/43 (9.3 %) were positive and all of them conducted from or proceed using cDNA as a template by nested PCR technique depending on [A3(ext), A2(int)], [B3(ext), B2(int)] and [C3(ext), C2(int)] primers table (1). All positive results appeared with *ul*97 (C3,C2) primer and all the internal primers designated with tail .These result was not agreed with (22) who found that real-time PCR was the best methods for diagnosis of HCMV *ul*97 and detected mutations later.

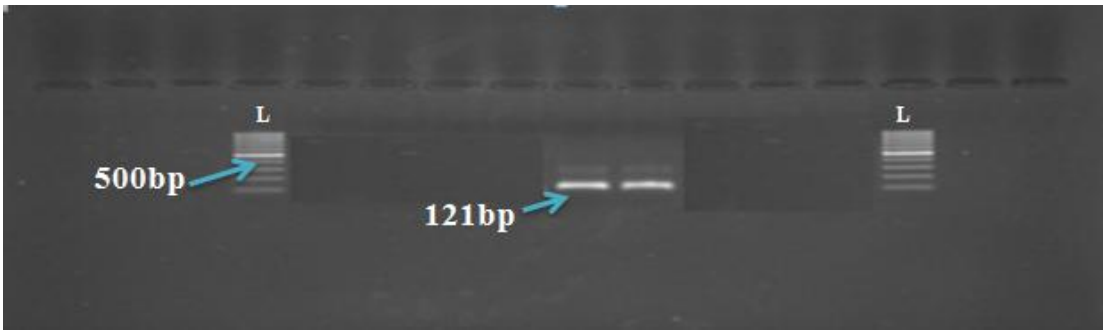

Figure (1): Gel Electrophoresis on (2% Agarose, 160V for 15 min) for*ul* 97Gene (the Template was cDNA) Isolated from Blood. Lane 5 and 6 Represent Positive Results

In Iraq, this is the first time to identify HCMV resistant gene. The PCR products related to the *ul*97gene were analyzed in the current study by comparing with NCBI (figure 1-A), the results showed that the amplified fragment was started from 1,763 to 1,858 bp in the original gene (2124bp). The amplified gene was compared with *ul*97 gene related to a strain control accession no. AF345353. The protein ID: GI: 15919299 Taxon: 10350. The sequence of amplified fragment or Query compared with the origin NCBI strain is illustrated in (figure 1-B) and as its clear the Pairwise identity was 100% in this isolate.

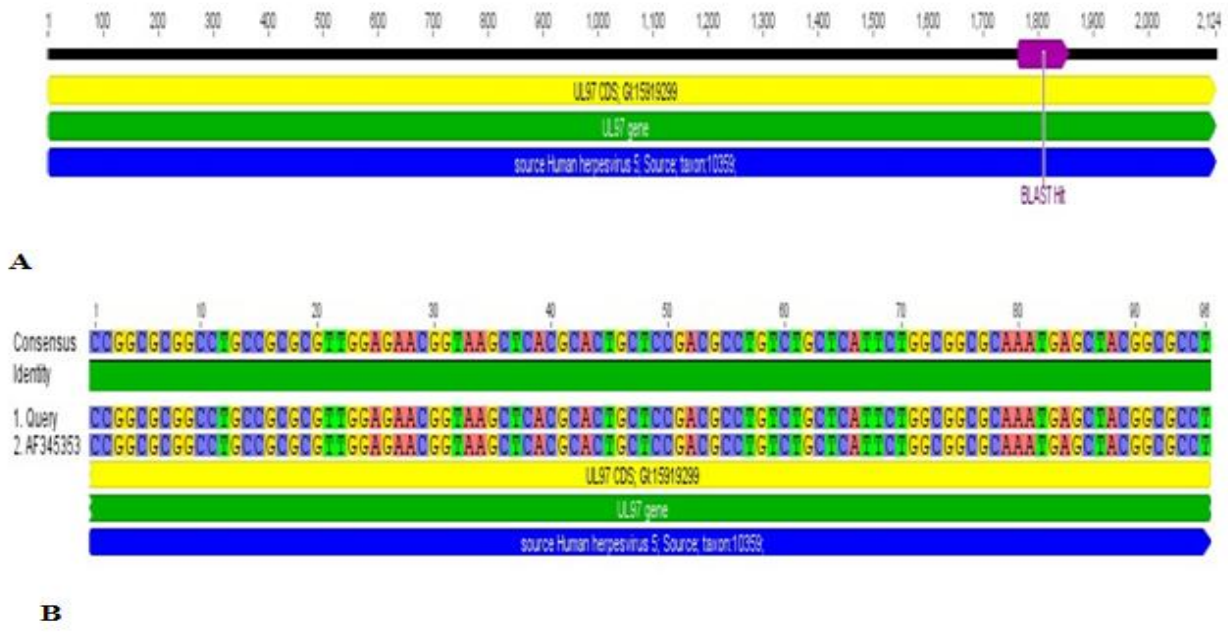

Figure (1): a- The Amplified *ul*97 Sequence of HCMV (Violet Color) as a Part of Total Origin Gene Bank ID: GI: 15919299, Reference Taxon: 10359. b- Pairwise Identity and the Complete Nucleotide Sequence for *ul*97 Gene Related to Human Herpes Virus-5

In order to compare *ul*97heterogeneity, all the sequenced genes were analyzed according to Pairwise identity over all pairs. Figure (2-A) represent sequences alignment for *ul*97 genes while figure (2-B) illustrates the phylogenetic tree obtained from this alignment. It could be predicted the diversity between isolates CMVcDNA11 and CMVcDNA26 as compared with the HCMV control obtained from NCBI (the last line). There were some changes in the nucleotide sequencing observed in some isolates that lead to mutations. Other studies mention that Ganciclovir (GCV) is the drug used as first choice therapy for HCMV and a GCV-resistant phenotype is mainly linked to mutations of the viral protein kinase *ul*97.GCV is pharmacologically a pro-drug that requires phosphorylation to the monophosphate by the HCMV *ul*97-encoded protein kinase to gain its antiviral activity(23)(24). The dendrogram for the isolates (figure 2-B) was divided into two groups; A and B. The first one(A) contain HCMV cDNA11 only which was more genetically distance among the rest as compared with the NCBI control strain then HCMV cDNA 26 from group B which contain two similar isolates also HCMVcD40 and HCMVcD 41. It could be said that the genetic diversity among HCMV isolates might related to some changing occurred in nucleotide sequence, and some of them happened under drug pressure or due to mixed virus populations exist in the patients as mentioned by (25)who noticed that over 80% of GCV-resistant isolates carried mutations in the *ul*97 gene. In this study, all the positive isolates for *ul*97 were related to isolates that obtained from pregnant aborted women in the first trimester of pregnancy and that might be due to the impairment of the immune system in this period. The last observation agreed with (26) who found that, immunity is one of the most crucial elements of the host environment for the virus, and *ul*97 gene is highly connected with the ability of virus reproduction therefore, the immunity of the host could have a great impact on the choice of polymorphism type of the *ul*97 gene. The result of translation of *ul*97 gene indicate that CMVcD11 and CMVcD26 were completely different comparing with the other isolates as well as the control (last line) for example in CMVcD11: G, T and A converted to T, A and C in bp no. 37, 38 and 39 ,respectively , as clear in the figure (3). Those differences might be due to long-term therapy and suboptimal drug concentrations which increase the risk of development of GCV resistance as illustrated by (27).

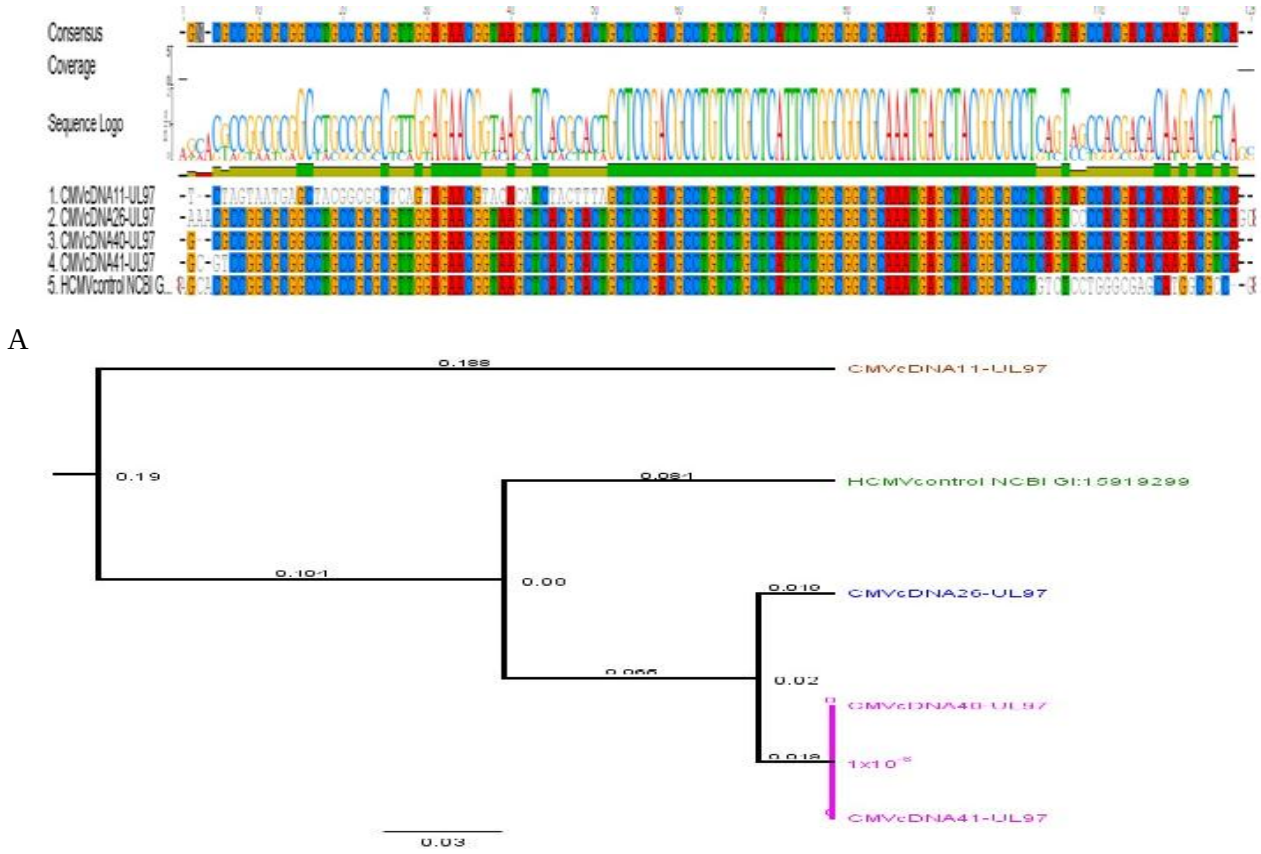

Figure (2): a) Alignment for ul97 Genes Sequences (cDNA as a Template) Showed some Point Mutation Take Place, b) Dendogram and Phylogenetic Tree to the Sequences of *ul* 97 Gene was Divided into 2 Groups A and B. The First One A Contains HCMV cDNA 11 only which was more Genetically Distance among the Rest as Compared with the NCBI Control Strain then HCMV cDNA 26 from Group B which Contains 2 Similar Isolates also HCMVcD40 and HCMVcD 41.

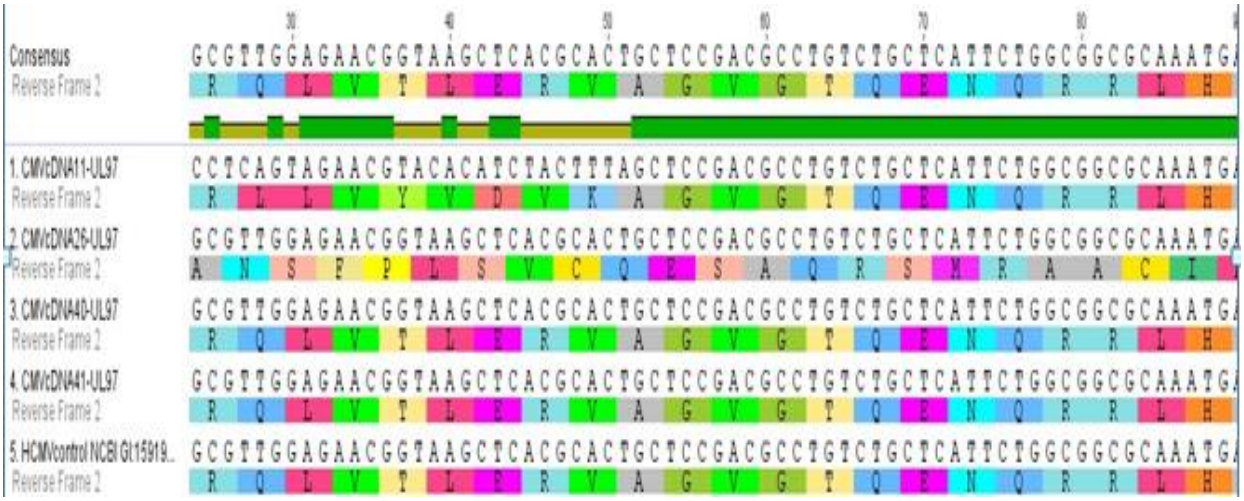

Figure (3): Protein Translation of *ul*97 Gene Sequences Gene Indicate that CMVcD11 and CMVcD26 were Completely Different Comparing with the other Isolates as well as the Control (Last Line) for Example in CMVcD11: GTA Converted to TAC in bp no. 37, 38, 39 respectively

The results of this study was very close to the results of another report who amplified the same fragment of the gene but by using DNA rather than cDNA, and they illustrated that automated sequencing represents the state of-the-art approach to genotypic detection of drug resistance (28). The results of the sequencing of the *ul*97 gene from GCV sensitive isolates demonstrate that the *ul*97 gene is highly conserved among clinical isolates, with an average 99% sequence identity. These base line sequences along with the well-defined sites for drug resistance mutations provide the foundation for a rapid genotypic drug resistance assay based on direct sequencing of PCR-amplified products containing these sites as mention by (29 In other study which pointed that the HCMV drug resistance may due to that all available anti-HCMV drugs share the same target so that one single mutation may lead to multidrug resistance (30). Therefore, it is imperative to find new drugs against HCMV having different targets: targeting viral entry, replication, or virion assembly and egress (24 In the study who has been done by using a combination treatment with ganciclovir (GCV) and tricin- after human cytomegalovirus (HCMV) infection which has synergistic effects on both infectious virus production and HCMV DNA synthesis In addition, reduced-dose combination therapy may provide a direction for treatment in patients with HCMV infection while reducing drug toxicity (31).

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