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Cytomegalovirus UL83 Gene as Diagnostic Marker for Preliminary Infection in Pregnant Women in AL-Najaf City

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ABSTRACT
The case-control diagnostic study was developed to evaluate the prevalence of Human *cytomegalovirus* (*HCMV*) among the population of pregnant women (160 women of ages ranging from 15 -45 years old who were attended to get medical diagnosis at Al-Najaf public health laboratory, during a period extending from 15th January - 15th August 2019), and also 50 healthy pregnant women were included as a control group. Data about frequency of abortion, age, and residency were recorded. Diagnosis was firstly depending on Enzyme immunoassay (EIA) for the evaluation of the prevalence of anti- *HCMV* IgM and IgG antibodies in sera of pregnant women, then confirmed by the quantitative real-time PCR(qRT-PCR), using specific primers targeting of UL83 gene of HCMV. Results presented in this study showed that the overall prevalence of *HCMV* infection was 97:160 (60.63%) and among women of mean ages± Standard deviation (SD) equal to 26.243± 6.485. Out of 160 participant women; 95(59.38%) of cases had chronic *HCMV* infection while only 2 cases (1.25%) had acute *HCMV* infection. Results were revealing that 52:160 (32.5%) of *HCMV* infected cases were related to women of 16-25 years age group followed by those related to 26-35 years age group with a prevalence of 21.88%. Eight cases (5%) were related to 36-45 years age group, while only 2 cases (1.25%) had 15 years old age. Current results were also revealed that 35:59 (59.3%) from rural area, while 62:101 (61.4%) were from urban areas in Al-Najaf.
Recurrent abortion (two or more frequency) was recorded to 17:97 (17.53%) of *HCMV* seropositivity cases with significant correlation. Results of *HCMV* UL83 gene amplification by qRT-PCR were showing that the overall prevalence of *HCMV* UL83 gene was 85% (136:160) of the total diagnostic group, and also positive in 25:50 (50%) of healthy pregnant women, with significant statistically differences. The *HCMV* positive threshold cycle (**CT**) of UL83 gene was revealing mean ± SD of 19.0537±6.64501, and 4 fold ratio as compared with that detected in asymptomatic cases by the method of $2^{-\Delta\Delta CT}$. The interassay coefficient of variation (CV) of *HCMV* UL83 gene amplification **CT** was 34.88%. The qRT-PCR of *HCMV* UL83 amplification was revealing sensitivity, specificity and diagnostic accuracy of 100%, 96% and 99.4% respectively. While positive predictive and negative predictive values were equal to 99.3% and 100% respectively. We concluded that UL83 gene detection was successful, confirmatory to EIA and more précised for *HCMV* isolated from serum samples.

Keywords: *HCMV*, UL83, Abortion, qReal-time PCR
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INTRODUCTION
Human *cytomegalovirus* (*HCMV*) is also known as *human herpesvirus-5* or (*HHV-5*) a well-known threatening agent to human life in the world, especially for those who are immunologically inhibited or compromised. *HCMV* may cause a variable impacts ranging from opportunistic infections to congenital as well as central nervous system infections. *HCMV* is a species of the virus genus *Cytomegalovirus*, family Herpesviridae (Adams *et al*., 2013; Chen *et al*., 2020). The rate of susceptibility to *HCMV* during pregnancy is well established and among women of child-bearing age nearly ranging from 40% to 80% will be susceptible to *HCMV* at the beginning of pregnancy. The rate of susceptibility to *HCMV* infection varies by many factors including age, ethnic or racial, and socioeconomic factors (GAO *et al*. 2018; Grazia *et al*. 2015). Spontaneous or recurrent miscarriage is one of the most enormous pregnancy complications that is not merely related to morbidity or mortality, but also correlated with clear psychological and social impact on women. The spontaneous miscarriages incidence in pregnancy was reported to be about 15%, with a percentage of at least 80% of aborted cases were occurred in the 1st trimester of pregnancy (GAO *et al*. 2018; Slyker *et al*. 2009; Trinder *et al*. 2006). Spontaneous abortions may also cause by genetic factors and reproductive anatomical abnormalities. *HCMV* infection is usually mild or asymptomatic in children and healthy adults, vertical maternal viral transmission to her fetus or newborn is usually combined with significant visceral damage (Bopping *et al*. 2013). *Cytomegalovirus* is also cause of mental retardation, congenital abnormalities, and neonatal infections (Bostanabad *et al*. 2017). *Human cytomegalovirus* DNA may be present in human plasma in a variable biologic forms, including liberated naked DNA, DNA super coiled with chromatin or nucleosomal or inserted into vesicles (apoptotic bodies or exosomes), as well as encapsulated in intact infectious or noninfectious virions, and in plasma samples, about 98.8% to 100% of *HCMV* DNA was free DNA (Tong *et al*. 2017). UL83 gene is related to the *HCMV* (strain AD169), coding for 65 kDa phosphoprotein, or pp65 which is also referred as unique long 83 [UL83] (Chen *et al*., 2020). Other names of this protein are 65 kDa matrix phosphoprotein, Phosphoprotein UL83 and Tegument protein UL83. Rarely studies were concerning with *HCMV* UL83 gene detection in Al-Najaf area from Iraq, hence, the aim of this case control study was to determine the prevalence of *HCMV* by the detection of UL83 gene in serum by qRT-PCR method and to compare with serological detection by EIA.

MATERIALS AND METHODS
Patients and samples
Blood samples were collected aseptically through venous puncture using sterile 5 ml disposable syringes from 160 pregnant women of child bearing age (15-45) (160 women of ages ranging from 15 -45 years old who were attended to

get medical diagnosis at Al-Najaf public health laboratory, during a period extending from 15th January to 15th August 2019). Fifty healthy pregnant women were included as a control group. Data about frequency of abortion, age, and residency were recorded. Serum samples were then separated on to two aliquots one of them regarded to PCR assay and the other was specified for *HCMV* positivity testing and IgG or IgM titer evaluation. These vials had been stored at -70°C till used.

Ethical considerations

Information about all participants was recorded according to the questionnaire statement, with the demonstrations explaining the aims of this study; ethical approval was obtained.

Enzyme immunoassay (EIA)

This test was used to evaluate the presence of anti-*HCMV* IgM and IgG antibodies using EIA kit for *HCMV* IgG/IgM tests (Biomereux, France) according to the instructions of the manufacturer. In the double-sandwich IgM EIA, a result of ≥ 1.1 was interpreted as positive, a result of 1 to 0.9 was interpreted as equivocal, and a result of less than 0.9 was interpreted as negative, while for IgG a result of > 1.10 was interpreted as positive, a result of 0.9 to 1.194 was interpreted as equivocal, and a result of < 0.90 was interpreted as negative (Prince and Leber 2002).

DNA Extraction and PCR amplification

DNA was extracted and purified by using a Favor Prep ™ blood genomic- DNA extraction mini kit (Favorgen Co, Taiwan), according to the manufacturer's instructions. The purity of the extracted total DNA were determined by measuring the absorbance ratio at wavelength 260 nm /280 nm using Scan drop spectrophotometer (Biodrop Co, UK(. The estimation of the initial UL 83 gene concentration and DNA purity was also done according to Usman et al. (Usman *et al*. 2014). DNA sample was diluted with the elution buffer to 1:10, the optical density was read with spectrophotometer at wavelength 260nm, and the acceptable DNA concentration for PCR application was 50 ng/µl. The purity of DNA would be: Pure DNA: Absorbance (A) at 260nm/A280nm = 1.8-2.0. Analysis of the PCR products (10 µl) was undertaken by electrophoresis separation on an ethidium bromide-stained 2 % agarose gel. The purified *HCMV* amplicon was used for the application of standard curve and assessment of positive detection, while the purified genome of *HSV*-1 was used as a negative control and to ensure the absence of external cross reactivity or contamination.

Table 1: Forward, reverse primers and probe used for HCMV UL83 gene amplification

Primer	Primer Sequence (5` to 3`)	GC%	Tm(°C)	Product length(bp)
(pp549s) UL83 F	5`-GTCAGCGTTCGTGTTTCCCA-3`	52.63%	57.3°C	263bp (Gault *et al*., 2001)
(pp812as) UL83 R	5`-GGGACACAACACCGTAAAGC-3`	55%	60.5°C	
(pp770s) fluorogenic probe	5`-FAM-CCCGCAACCCGCAACCCTTCATG-3`-TAMRA	65.22%	64.4°C	
B-actin184F	5`-AGAGCTACGAGCTGCCTGAC-3`	60%	58.7°C	184bp (Kienka *et al*. 2019)
B-actin184R	5`-AGCACTGTGTTGGCGTACAG-3`	55%	58 °C	

Sample preparation for qRT-PCR

About 5 µl of internal control (β-actin gene) was added in order to control the isolation procedure and to check for possible PCR inhibition. This assay involved co amplification of *HCMV* DNA and an internal control in a single reaction using the overall sequence-specific primers. The target DNA amplification fluorescent signal was detected by a probe labeled at the 5' end with FAM, through the FAM channel. The fluorescent signal generated by the internal control amplification is detected by a different reporter molecule, Cy5 through the Cy5 channel (Bilenoğlu *et al*. 2015). The detection of UL83 gene of *HCMV* was done by qRT-PCR. The primer pairs (forward and reverse were manufactured by Humanizing Genomics Macrogen Company, (South Korea), shown in table (1) that mentioned by (Sowmya *et al*., 2006) for the amplification of a 263 bp DNA fragment of the UL83 gene for *HCMV*. The initial denaturation of 95ºC for 15 min, followed by 50 cycles of 95°C for 30 seconds, and 1:30 minutes at 54°C (Bilenoğlu *et al*. 2015). All the PCR samples were then kept at -20ºC until used. The fold change and variability of *HCMV* UL83 gene amplification was measured by qRT-PCR using the relative quantification method ($2^{-\Delta\Delta Ct}$ method). The fold-change in *HCMV* UL83 gene was normalized to a housekeeping gene β- actin and relative to a calibrator sample using specific equations (Gault *et al*. 2001; Livak KJ1 2001; Pfaffl 2004).

PCR product detection

The ascertained horizontal electrophoresis in 2 % agarose gel was done for PCR product by using Tris-boric acid-EDTA buffer (pH, 8.3) as a medium for gel preparation and running, with final concentration of 0.5 µg/ml Ethidium bromide staining. The visualization of bands was done by gel documentation system and ultra-violet light. The expected *HCMV* DNA fragment was 263 bp, positive and negative (containing only the reaction mixture) controls were tested with the samples each time. Procedures for avoiding contamination, DNA purification, preparation of

PCR composite and specific optimized amplification were strictly followed.

Statistical analysis

Results statistical analysis was performed using SPSS-18.0. Data were expressed as CT Mean ± SD, Chi-square test, Sensitivity and specificity as well as interassay coefficient of variation and diagnostic accuracy, positive predictive and negative predictive values was also applied. In all tests, P value of less than 0.05 was regarded statistically significant. Relative quantification of Livak method was used for the calculation for $2^{-\Delta\Delta CT}$ and gene fold ratio (Gault *et al*. 2001; Livak KJ1 2001)

RESULTS AND DISCUSSION

HCMV Seropositivity by EIA

Results presented in this study showed that overall the prevalence of *HCMV* was 97:160 (60.63%). Out of 160 women; 95(59.38%) of samples had chronic *HCMV* infection characterized by the presence of positive IgG antibodies while only 2 (1.25%) were had acute *HCMV* infection characterized by the presence of positive IgM antibodies, summery was shown in Table (2). Control group investigation showed that 12:50 (24%) cases were seropositivity for anti *HCMV* IgG, while all control cases revealed IgM seronegativity. These results were significant at $p < .05$ (The chi-square statistic was 20.4699, p-value < .00001), Table (3). Data of EIA were showed that *HCMV* positive samples were revealing OD of mean ±SD equal to 88.356± 87.682 and interassay coefficient variation of 99.236. The EIA was revealing sensitivity, specificity and diagnostic accuracy of 80.17%, 96% and 86.46% respectively. While positive predictive and negative predictive values were equal to 96.8% and 76.17% respectively. Variable results concerning abortion frequency were shown, out of 97 *HCMV* positive cases; no history of abortion was recorded in 32 (32.99%), One time abortion frequency in 48 cases (49.48%), two times abortion frequency in 12 cases (12.37%) and 3 or more abortion frequency times were recorded in 5 cases (5.16%) table (4).

Table 2: *HCMV* Seropositivity by EIA in symptomatic cases

Anti-*HCMV* EIA					
Total number of patients investigated	Anti- *HCMV* Antibody type	Positive		Negative	
		Number	%	Number	%
160	IgM	2	1.25	158	98.75
	IgG	95	59.38	65	40.62

Table 3: Control group results of *HCMV* Seropositivity by EIA

Anti-*HCMV* EIA					
Total control Number	Anti- *HCMV* Antibody type	Positive		Negative	
		Number	%	Number	%
50	IgM	0:50	0	50:50	100
	IgG	12:50	24	38:50	76

Table 4: Seroprevalence of *HCMV* according to the frequency of abortion

Abortion Cases	0 time (No history of previous abortion)	One time	Two times	≥ 3 times	Total *HCMV* positive cases
Number	32	48	12	5	97
%	32.99	49.48	12.37	5.16	100%

Age distribution

Results revealed that 52:160 (32.5%) of *HCMV* infected cases were related to women of 16-25 years age group followed by those related to 26-35 years age group with a prevalence of 21.88%. Eight cases (5%) were related to 36-45 years age group, while only 2 cases (1.25%) had 15 years old age, Table (5). Results were statistically significant at $p < .05$.

Demographic distribution

Out of the total *HCMV* positive samples, there were 35:59 (59.3%) from rural area, while 62:101 (61.4%) were from urban areas in Najaf. The chi-square statistic was 0.0665. The p-value was .796543. These results were not significant at $p < .05$, table (6).

Table 5: *HCMV* Seropositivity by age groups

Age group in years	*HCMV* positive cases	
	Number	%
< 15	2:160	1.25 %
16-25	52:160	32.5 %
26-35	35:160	21.88 %

36-45	8:160	5 %
Total	97:160	60.63

Table 6: *HCMV* total seropositivity according to the residence

Residence	Total investigated aborted women	*HCMV* positive cases	
		Number	%
Rural	59	35:59	59.3%
Urban	101	62:101	61.4%
Total	160	97:160	60.63%

Results of UL83 amplification

Results presented in this study showed that out of 160 women investigated, the overall prevalence of *HCMV* UL83 gene was (85%), as this test was positive in 136:160, while 24 samples (15%) were showing negative results. *Human Cytomegalovirus* UL83 gene was also positive in 25:50 (50%) of healthy pregnant women, with statistically significant differences at $p < .05$ (The chi-square statistic was 26.087, p-value was < .00001), as shown in Table (7), Figure (1&2). The *HCMV* UL83 gene positive CT values of diagnostic group (usually symptomatic) were revealing mean ± SD of 19.0537±6.64501, and 4 fold ratio of amplification as calculated by the method of $2^{-\Delta\Delta CT}$. The interassay coefficient of variation (CV) of *HCMV* UL83 gene amplification CT was 34.875%. While the *HCMV* UL83 gene positive CT values that detected in asymptomatic cases (healthy pregnant women) were revealing mean ± SD of 16.00264±1.16703. The qRT-PCR of *HCMV* UL83 amplification was reflecting sensitivity, specificity and diagnostic accuracy of 100%, 96% and 99.4% respectively. While positive predictive and negative predictive values were equal to 99.3% and 100% respectively.

Table 7: Distribution of *HCMV* UL83 gene in patients and control group

HCMV UL83gene qRT-PCR assay					
Investigated groups	Total Number investigated	Positive		Negative	
		Number	%	Number	%
Patients group	160	136	85	24	15
Control group	50	25	50	25	50

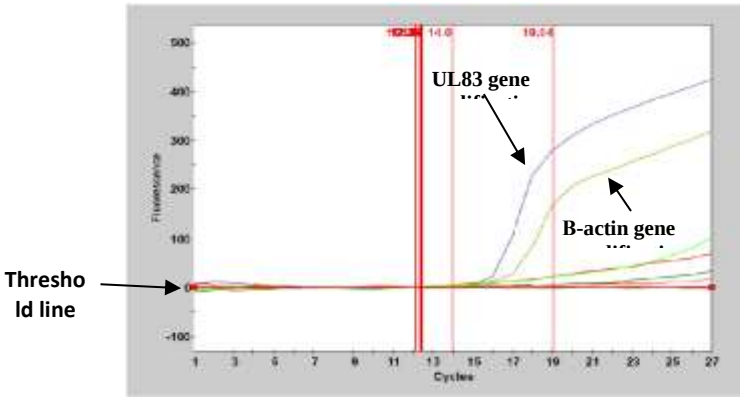

Figure 1: Real time polymerase chain reaction for *HCMV* UL83 gene amplification: florescence data (FAM) collection during the first 27 cycles, showing positive results (curves over the threshold line), as compared with negative control (curves with or under than threshold line)

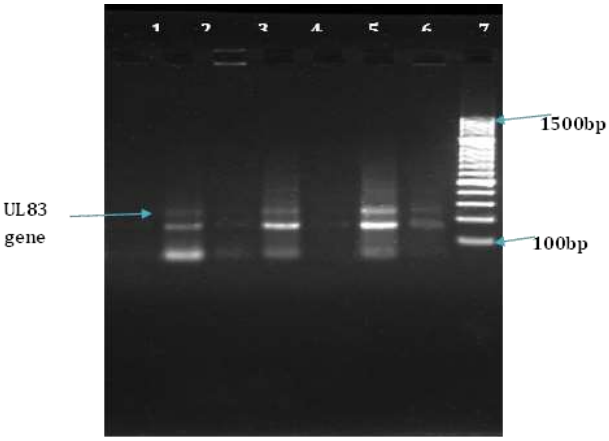

Figure 2: *HCMV* UL83 gene product (263bp) detection by 2% agarose gel electrophoresis profile ; Lane 1, 3 and 5 are UL83 Negative samples; Lanes 2,4 and 6 are UL83 gene positive samples- ; M: ladder (100-1500bp), Ethidium bromide (0.5 µg/ml) staining and at 80V for 1 hrs.

DISCUSSION

The present study carried out on 160 women of varying age from 15-45 from different areas in Al-Najaf city. This study was planned to elucidate *HCMV* UL83 gene as diagnostic marker for preliminary infection detection in serum of pregnant women in AL-Najaf city. The primary detection was done by Enzyme immunoassay (EIA) based on specific anti-*HCMV* IgM and IgG, and then qRT-PCR. In case of *HCMV* infection, early and accurate diagnosis remains crucial for susceptible hosts since the development of resistance could be avoided with early preventive antiviral therapy (Kotton and Nelson 2013). A TaqMan qRT-PCR was developed to measure *HCMV* UL83 gene specific sequence that code for *HCMV* tegument protein pp65, Primers and probe was tested for other human herpes viruses (*Herpes simplex virus types 1* and *Epstein-Barr virus*) and human fibroblast DNA (data not shown) and no cross-reactivity was observed. This UL83 gene detection was normalized by means of the quantification of β- actin gene as an internal control gene. The results of the real-time PCR assay were positively correlated with those of the *HCMV* infectivity as compared to non-infected healthy pregnant women (p-value was < .00001). The *HCMV* UL83 gene positive CT of diagnostic group (usually symptomatic) was reflecting 4 fold ratio of amplification as compared with that detected in asymptomatic cases (healthy pregnant women) by the method of $2^{-\Delta\Delta CT}$, the interassay coefficient of variation. Current results were revealing high sensitivity, specificity and diagnostic accuracy, at the same time with acceptable realistic PPV, NPV and less interassay CV concerning qRT-PCR as compared with EIA, The results were revealing that qRT-PCR was more sensitive than enzyme immunoassay suggesting that latter may be considered a useful alternative for diagnosing *HCMV* infection during pregnancy. The method of qRT-PCR has proven to be one of the major application protocols in the detection and quantification of different pathogens. This assay may introduce many advantages like high sensitivity, speed, suitable cost efficiency and practicality. However, the performance is highly depending on the specific target region (Bilenoğlu *et al*. 2015). The diagnosis of primary positive seroprevalence of maternal *HCMV* infection in pregnancy should be based on de-novo appearance of specific anti *HCMV* IgG in the sera of pregnant women who were previously seronegative, or on detection of specific anti *HCMV* IgM antibody associated with low IgG avidity. The diagnosis of secondary infection should be based on IgG antibody titer of significant rise with / without the presence of IgM and high IgG avidity. In case benefit ratio is variable depending on the low transmission rate (Yinon *et al*., 2018). Current results were came in agreement with that mentioned by Ligozzi *et al*. (Ligozzi *et al*. 2016) demonstrated that TaqMan qPCR was characterized with sensitivity and offer some advantages: including ease of management and execution, quantification of *HCMV*, rapid determination of positive and negative *HCMV* samples, possibility of semi-automation of protocol/assay or adaption of a molecular screening of *HCMV* congenital infection. Other reports mentioned that maternal plasma *HCMV* DNAemia identified a subgroup of Kenyan women and infants at high risk for death in the 2 years following delivery (Slyker *et al*. 2009). *Human Cytomegalovirus* DNA was detected in 136 serum samples from symptomatic cases and in 25 serum samples of healthy pregnant women. These results were come in agreement with that mentioned by Petrov and his colleagues in similar study that there was a high frequency of *HCMV* DNA in abortive samples from women with pregnancy risky or loss (Petrov *et al*. 2019). The majority of *herpes viruses* can persist in host cells indefinitely to have a latent infection pathway or persistency. Limited illness may be accompanied with the primary infection that extend for long-term latency and always were often asymptomatic. However, during latent period, a small number of viral genetic elements may transcribe latency- mRNA transcripts in variable host cells. In this squeal, viruses can maintain persistency in host cells for long term period, though limited clinical illness of primary infection, long-term latency may persist with no signs and symptoms (Y. Chen *et al*. 2018). During the latency period, *HCMV* may not cause any adverse reactions, but the transmission may be occurring to other hosts via direct contact. When *HCMV* are stimulated by any

enhancement factor, or during host immune system suppression, these dormant viruses can reactivate again to start generating large number of viral progeny with presence of symptoms and diseases initiating lytic life cycle. Routine serologic screening for *HCMV* during pregnancy will introduce understanding of *HCMV* infection and its prevention among pregnant women. The results of this assay will provide useful information about *HCMV* infection in the early stages of fetus development, and in combination with other diagnostic tools it will enable mothers as far as obstetricians to make informed decisions about the management of pregnancies complicated by *HCMV* infection (Jones *et al*. 2000). This study revealed that 17:97 (17.53%) of *HCMV* positive cases were significantly correlated with recurrent abortion of two or more frequency, this somewhat came in consistent with the findings of Khalf *et al*. (Khalf *et al*. 2012) who were mentioned that recurrent abortion correlate with 14% of pregnant women with *HCMV* positive results. *Human Cytomegalovirus* immunoglobulin M (*HCMV*-IgM) is initially secreted during early stage of *HCMV* infection, while the *HCMV*-IgM detection is indicative of acute, active, or recent infection. Weeks later to the primary viral infection, *HCMV*-IgG antibody is secreted and maintained for life. Hence, the detection of *HCMV*-IgG is an indication about past or previous infection (Fellous *et al*. 2013). Results were revealing that 52:160 (32.5%) of *HCMV* infected cases were related to women of 16-25 years age group followed by those related to 26-35 years age group with a prevalence of 21.88%. Eight cases (5%) were related to 36-45 years age group, while only 2 cases (1.25%) had 15 years old age. These results may came similar to that mentioned by many other studies (Baiati *et al*. 2014; Jihad 2015; Mahdi *et al*. 2011; Slyker *et al*. 2009), that the seropositivity of anti-*HCMV* IgG was higher in women of ages ranging from 20 to 34 years old. This is because the pregnancy chance is higher in women of younger ages (20-35) as compared with older age groups. Present results were also considered to be comparable with Mahdi et al., (Mahdi *et al*., 2011), who showed that an increase in seropositive *HCMV* IgG was positively correlated with abortion and infection, this might be due to the viral effect on cellular metabolism level and activation of other viruses that may co-infect the cells inducing subclinical illness. Our results were also come in agreement with the findings of Khalf *et al*. (Khalf *et al*., 2012) that there were non-significant differences among *HCMV* positive cases according to the residence. However, Seroprevalence rates may also vary with age, geographical area of residence (developing countries were usually with higher rates), and also socioeconomic status (economically depressed regions were usually with higher rates) (Bate *et al*. 2010; Razonable and Hayden 2013). Finally, there are several requirements for an optimized protocol for *HCMV* monitoring. These requirements include (1) a high sensitive assay that allows preliminary detection in pregnant women or individuals at high risk for disease, (2) use of quantification assay analysis to the results to increase the PPV and to measure viral load during the period of antiviral treatment, (3) Allowing early initiation or introducing an alternative treatment, and (4) a high level of reproducibility (Boeckh and Boivin 1998).

CONCLUSIONS

From current findings, we concluded that UL83 gene was universally detected in *HCMV* positive cases with four-fold amplification ratio in symptomatic cases that can be monitored by qRT-PCR. Hence, the latter may offer a new alternative diagnostic tool in methodology with improved diagnostic accuracy, acceptable sensitivity as well as specificity, so further future investigations should be manipulated to examine its expression variation according to the therapeutic field that may consequently become an object of choice in saving life for mothers and their fetuses during or after pregnancy period.

AUTHOR'S CONFLICT OF INTERESTS

The authors declare no conflict of interest, all authors approve of the final article.

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