# Diagnosis and Management of Human Cytomegalovirus Infection in Mothers and Newborn Infants based on Quantitative and Real-Time PCR

**Meguid N.A.[1], Housseiny L.[2], El Awady M.[2]**
Departments of Research on Children with Special Needs[1],
Biomedical Technology[2], National Research Center

## ABSTRACT

*Human cytomegalovirus (HCMV) is the leading cause of congenital viral infection and mental retardation. The virus can be transmitted to the fetus through primary (in 30 to 40%) or secondary maternal infection. Because there is currently no treatment for prenatal infection, early diagnosis of congenital infection during neonatal period is important. The aim of this study was the use of quantification and semi quantification techniques for CMV load in peripheral blood as a surrogate marker for disease severity and treatment follow up. Twenty HCMV infected infants and 50 women were included in this study. They were infected with CMV and positive PCR for CMV-DNA. HCMV-gpB-DNA load for all cases was determined by semi-quantitfication technique, where symptomatic infection is correlated with viral load above 1.6x 103 GE/ml, while asymptomatic infection was found below this level in infants (this correlation couldn't be applied to mothers). Ganciclovir as anti-HCMV was followed up in 12 mothers by semi-quantification technique, five of them were non responders. HCMV activity was detected by Real-Time-PCR of pp65 gene in 17 mothers and 3 children, only, 3 mothers and 2 children were found to have active HCMV infection whom are in urgent need for treatment. In conclusion, either HCMV semi-quantification and/or Real-time pp65-DNA are sensitive methods in detecting and follow up treatment in mothers prenataly as well as postnately with their children. Also, they are important markers in differentiation between latent and active infection.* **(Int. J. Ch. Neuropsychiatry, 2005, 2(2): 119-141)**

## INTRODUCTION

Cytomegalovirus (CMV) belongs to the family herpes viriolae. It is the most common vertically transmitted viral infection in the developed world, which may be transmitted pre-natally via transplacental or in-utero acquisition routes resulting in congenital CMV infection. The associated morbidity and sequelae of CMV vary depending on the route of acquisition of infection, and maternal immune status[1].

Neonatal morbidity associated with CMV most often results from congenital CMV infection, which occurs in 0.5–2.0% of all deliveries in the developed world[2]. Congenital CMV infection remains a major problem worldwide. Intrauterine infection occurs in up to 50% of pregnancies following primary maternal infection, and 10% of the infected infants are symptomatic at birth. In addition, 5 to 15% of asymptomatic (clinically 'silent') babies are at risk of developing neurological sequelae which may be progressive throughout early childhood. Reactivated maternal infections involve mostly minor consequences for the offspring (less than 2%)[3].

Cytomegalovirus genome is approximately 240 kilobases, includes more than 200 genes, encoding at least 35 structural proteins and a still undefined number of non structural proteins. The viral envelope is composed of at least eight glycoproteins; most of the neutralizing antibodies

are directed against glycoprotein B (gB) and glycoprotein H (gH)[4].

Although PCR for CMV DNA detection in peripheral blood leukocytes is the most sensitive procedure for detecting viral infection, when PCR is carried out in a non-quantitative (qualitative) way, it is of little clinical value since it lacks specificity for the diagnosis of CMV "disease"[5]. The measurement of viral load by quantitative PCR appears to be promising and important for the diagnosis of active infection, prediction of CMV disease, differentiation of latent from active infection, and monitoring of therapy[6]. Methods developed for DNA quantification by PCR may be classified into: End-point dilution method, competitive quantitative, non-competitive quantitative and real-time quantitative assays[7].

End-point dilution PCR methods provide only relative data. These procedures are designed to perform titer determinations of the target template or of an external control by end-point sample dilution prior to PCR or by coamplification of target and an endogenous cellular (e.g. β-globin) DNA sequence[8]. The introduction of Real-Time PCR has made it possible to accurately quantify starting amounts of nucleic acid during the PCR reaction without the need for post-PCR analyses[9]. Real-time PCR based on the TaqMan technology provides an accurate means to quantify viral DNA with the major advantage of avoiding post-PCR handling that can be the source of DNA carryover[10].

The aim of this study was to apply different quantitative PCR based assays capable of quantifying the CMV load in peripheral blood as a surrogate marker for disease severity and treatment follow up.

## SUBJECTS AND METHODS

Among infants with suspected congenital CMV infection and presenting by severe neurodevelopmental disorders, twenty infants were selected and included in the present study according to positive laboratory results for CMV (ELISA and PCR). These were 11 males and 9 females, aged between 6 months and 2 years. Moreover, fifty mothers presenting by repeated abortions (more than three times), repeated neonatal or infant deaths, repeated intra-uterine fetal deaths (IUFDs) (three or more) and those having affected children were also selected according to positive CMV IgM and/or high IgG antibodies and positive PCR for CMV-DNA. All selected mothers showed no gynecological or obstetrical problems. All cases were referred to the Outpatient Clinic of the Department of Research on Children with Special Needs, National Research Center.

**Cases were subjected to:**

1. **History:** Detailed history taking of the infants including pedigree analysis, developmental history, similarly affected family member and age of onset and presenting manifestation. As for the mothers: history of abnormal pregnancy, history of previous abnormal TORCH during pregnancy and history of any previous or current immuno-suppressant state were evaluated.
2. **Clinical Assessment:**
   a) *Evaluation of the infants included:*
      - Full general examination including all body systems with special emphasis on the neurological system.
      - Weight, height and head circumference were measured and compared to age and sex matched control. All measurements followed the recommendations of the International Biological Program (I.B.P.)[11].
      - Fundus examination, auditory brainstem evoked responses (ABR), electro-encephalogram (EEG) when indicated.
      - Brain neuroimaging (CT scan or MRI) for all cases.
      - Vineland Social Maturity Scale[12] was done for all infants. Mental subnormality was classified into mild (IQ 50-70), moderate (IQ 35-49) and severe (IQ 20-34) according to the Diagnostic and Statistical Manual of Mental Disorders, 4th edition[13].

   b) *Clinical evaluation of the mothers included assessment of all body systems.*
3. **Laboratory investigations:**

a) **Chromosomal complements** were normal in all cases.

b) **Enzyme-linked immunosorbent assay (ELISA) test:**
Antibodies against CMV (IgM and IgG) were tested in serum samples from all mothers and children (70 cases) using conventional enzyme immunoassay systems (Sorin Corporation, Italy).

c) **Polymerase Chain Reaction (PCR) to detect viral DNA**:
DNA extraction was carried out following the protocol of QIAGEN spin column of the QIAamp. blood mini kit (QIAGEN, Germany). Amplification of CMV DNA was based on gpB-DNA gene as template. Nested primer sets for amplifying fragments of gpB gene were designed with first-round primer 1: 5´-GAG GAC AAC GAA ATC CTG TTG GGC A-3´ & 2: 5´-GTC GAC GGT GGA GAT ACT GCT GAG G-3´and second-round primer 1: 5´- ACC ACC GCA CTG AGG AAT GTC AG-3´ & 2: 5´-TCA ATC ATG CGT TTG AAG AGG TA-3´. This primer set is providing an amplified product of 100 base pair (bp). These primers have been used extensively for analysis of CMV by different investigators[14]**.** Denaturation and primer annealing was performed and melting temperature (Tm) of primer hybridization was calculated. Primer extension and detection of PCR amplified products by agarose gel electrophoresis was done.

d) **Standardization of End-Point Dilution Nested PCR for CMV-gpB-DNA:**
End-point dilution nested PCR using defined volumes of DNA was performed to all cases (mothers and infants). Considering the different sample volumes and dilution factors during template preparation, nested DNA amplification and agarose gel electrophoresis, the resulting detection limit per assay, and the number of CMV/DNA genomic equivalents were calculated. Since the molecular weight of CMV DNA is 6.2x $10^6$ g/mol, one genome equivalent (GE) corresponded to 0.01fg CMV DNA. The resulting data readings were given in the form of GE/ml[15]**.**

A simple end-point dilution nested PCR in which the levels of viral CMV-DNA in the test blood sample was determined using a limited 10 folds serial dilution approach, in which, the end-point was read visually, following agarose gel electrophoresis (Fig. 2). Detection limit was about 0.5-5 fg CMV-DNA/5µl of the extracted DNA corresponding to 50-500 viral copies/ 5µl DNA following the method reported by Hamprecht et al.[16].

5µl (corresponding to 1ng of total genomic DNA) of serial 10 folds dilutions of purified DNA from whole blood were used as template DNA for the first round PCR. 5µl from the first PCR product were used as template for the second round (nested) PCR 14.

Then, the products of the nested PCR were electrophoresed in agarose gel and the end-point was read as the highest dilution of the starting DNA (lowest DNA concentration) which resulted in a visible band.

By this method, the smallest amount of the starting target DNA detected by ethidium bromide after the second round PCR was equivalent to 50 CMV copies. This volume will be multiplied by the dilution factor to obtain the original amount of DNA prior to dilution. Then referring this result to the original blood volume from which the DNA was extracted. Using the classical DNA phenol/chloroform extraction method, the lowest detection limit of CMV was achieved.

**e) Real-Time PCR Quantification of CMV-UL83 Gene:**

Twenty CMV-gpB-PCR positive blood samples from 17 women and 3 infants were included for detection of active CMV infection by detection and quantification using real-time PCR of CMV-UL83 gene. DNA was extracted from blood samples by QIAamp Blood mini-kit.

Real-Time PCR was performed according to a technique that was adapted to ABI Prism 7000 detection system with a sequence detection system based on an exonuclease assay using a Taqman probe. Taqman probes are oligouncleotides that contain a fluorescent dye, typically on the 5´ base, and a quenching dye typically located on the 3´ base. When irradiated, the excited fluorescent dye transfers energy to the nearby quenching dye molecule rather than fluorescing, resulting in a non-fluorescent substrate. Taqman probes are designed to hybridize to an internal region of a PCR product. These hybridization probes are relying on fluorescence resonance energy transfer (FRET) for quantification. Fluorescence increases in each cycle, proportional to the rate of probe cleavage. The sequence of the PCR primers and that of the probes used to quantify CMV were selected from the phosphorylated (pp65) matrix protein (UL83) gene with Primer Express software (Perkin-Elmer Biosystems, Foster City, California). The Taqman probe selected between the 2 primers was fluorescence labeled at the 5´ end with 6-carboxyfluorescein (FAM) dye, as the reporter dye and at the 3´ end with 6-carboxymethylrhodamine (TAMRA) as the quencher dye. PCR was performed with 25ul of Taqman Universal PCR Master Mixture (Perkin-Elmer Biosystems). The PCR product was detected as an increase in fluorescence with the ABI PRISM 7000 instrument (Perkin-Elmer Biosystems).

A known DNA concentration from CMV plasmid was used as a standard in this study. CMV was quantified using serially diluted CMV standards within the range of 10 and $10^7$ copies/well, and the number of CMV copies was calculated. The number of target copies in the reaction was deduced from the crossing point value, corresponding to the fractional cycle number (threshold cycle) at which the released fluorescence exceeded 10 times the standard deviation of the mean baseline emission using Sequence Detection System Software (ABI Prism 7000 SDS Software; Perkin Elmer Biosystems). To control against cross-contamination, a sample consisting of distilled water was utilized.

**4. Treatment:**

Oral ganciclovir (a specific FDA approved commercial anti-CMV medication) was administered to 12 mothers with positive CMV who were not pregnant at a dose of 1000mg tid for 14 days[17]. End-point dilution nested PCR amplification was done to these cases before and after treatment and the response to the antiviral therapy was evaluated.

## RESULTS

Among infants referred to the Clinic of Children with Special needs and presenting by severe neurodevelopmental disorders, and mothers presenting by repeated abortions, IUFDs, neonatal or infant deaths, or delivery of affected infants, we selected 20 infants diagnosed as congenital CMV and 50 mothers. All cases were tested positive for CMV IgM and/or high IgG antibodies and positive PCR for CMV-DNA. All infants and mothers had normal chromosomal studies.

The 20 infants studied had a gestational age between 34 and 41 weeks and. Neonatal manifestations in the form of low birth weight (< 2500 gm), neonatal sepsis and neonatal jaundice were represented in 85% of the affected infants. The most frequent clinical findings of the affected infants were developmental delay (90%), mental subnormality (75%), microcephaly (45%), and seizures (40%). Sensorineural hearing loss, chorioretinitis and hepatosplenomegaly accounted for 25%, 20% and 15% respectively (Table 1). There was a lack of association between severity of the disease and IQ scores, however, cases with chorioretinitis were associated the most devastating cognitive deficits, significant retardation and severe microcephaly (> -5 SD).

Brain neuroimaging findings in the presenting infants showed that cortical and central atrophic changes were the most predominant findings (60% of cases) followed by white matter hypomyelination (35%), intracerebral calcifications (20%) and cerebellar hypoplasia (10%) (Table 1) (Fig. 1). Strikingly, one case showed Dandy-Walker malformation and another case showed hypogenesis of corpus callosum. Moreover, normal brain neuroimaging findings were found in 3 cases (15%).

Regarding maternal clinical findings, delivery of an affected infant was the most frequent presenting complaint (40%) followed by repeated abortions (30%) then repeated neonatal deaths (20%) and repeated IUFDs (10%) (Table 1).

ELISA investigations of the 50 mothers and the 20 infants revealed CMV IgM positive results in 12 mothers and 9 infants (24% and 45% respectively). CMV IgG was highly positive in all mothers and infants (100%). PCR for CMV-DNA was positive in all cases (100%) (Table 2).

All infants and mothers were exposed to end-point dilution analysis of CMV-gbB-DNA. They were grouped into 5 groups according to the results of CMV-gpB-DNA load. Detailed results of CMV DNA levels in blood samples of the examined infants and women listed in Table (3). End-point dilution method was very accurate in detecting the viral load among infected mothers and infants, with viral quantity ranged from 1.6 $X10^3$ genome equivalents (GE)/ml to 3.3 $X10^6$ GE/ml (Table 3) (Fig. 2). Our results showed that 70% of examined women and 55% of infants had CMV viral load ranging from 3.3 $X10^3$ GE/ml to 3.3 $X10^4$ GE/ml, 22% of women and 20% of infants had a viral load more than 3.3 $X10^4$ GE/ml and 8% of women and 25% of infants had a viral load of 1.6 $X10^3$ GE/ml. These results indicate that viral load below the level of 1.6 $X10^3$ GE/ml is not accompanied by symptomatic CMV disease.

It is worth noting that levels of viral load did not correlate with the severity of the clinical picture of the mothers. On the other hand, in the affected infants, high viral load was associated with severe microcephaly (> -5 SD), severe brain neuroimaging findings and development of SNHL. Moreover, CMV IgM was negative in cases with very low viral load denoting that quantitative PCR is an accurate method in detecting the very low CMV quantity.

Twelve mothers receiving oral ganciclovir were followed. Viral load was very high in 6 cases (3.3x $10^5$ GE/ml to 3.3x $10^6$ GE/ml), moderate in 4 cases (3.3x $10^3$ GE/ml to 3.3x $10^4$) and mild in two cases (1.6x $10^3$). Efficacy of this medical intervention response was monitored by end-point dilution nested PCR amplification before and after treatment. Our results showed that only 6 cases showed decreased viral load, while one case showed complete recovery. The other 5 cases were not responding, where their CMV load was moderate to high and did not decrease after antiviral medication. It is worth mentioning that 2 cases who did not respond and had very high viral load get pregnant and delivered severely affected outcome with CMV infection, thus suggesting the presence of a significant correlation between CMV load in mothers and disease severity in their newborns.

Twenty cases (17 women and 3 infants) positive for CMV-gpB-DNA by PCR were exposed to further assessment of CMV infection by detection of UL83 gene quantification using real-time PCR. Positive real-time PCR results were found only in 5 patients (3 mothers and 2 infants) (25%) out of 20 (Fig. 3).

Comparison between levels of CMV-DNA using different genes (UL83 and gpB) and different methods in the five positive UL83 cases

by real-time PCR is presented in table (4) and (Fig. 4).

**Table 1.** Main clinical findings of affected mothers and infants.

| **Clinical Findings** | | |
|---|---|---|
| **Mothers:** | **No (50)** | **%** |
| • Birth of affected infant | 20 | 40 |
| • Repeated abortions | 15 | 30 |
| • Repeated neonatal deaths | 10 | 20 |
| • Repeated IUFD | 5 | 10 |
| **Infants:** | **No (20)** | **%** |
| • Low birth weight | 8 | 40 |
| • Neonatal septicemia | 6 | 30 |
| • Neonatal jaundice | 3 | 15 |
| • Developmental delay | 18 | 90 |
| • Microcephaly | 9 | 45 |
| • Growth retardation | 4 | 20 |
| • Severe hypotonia | 6 | 30 |
| • Spasticity | 2 | 10 |
| • Hemiparesis | 2 | 10 |
| • Seizures | 8 | 40 |
| • Hepatosplenomegaly | 3 | 15 |
| • Deafness (SNHL) | 5 | 25 |
| • Cataract | 1 | 5 |
| • Anophthalmia | 1 | 5 |
| • Chorioretinitis | 4 | 20 |
| • Iris coloboma | 1 | 5 |
| • Squint | 2 | 10 |
| • Mental Subnormality (Total) | (15) | (75) |
| - Mild mental subnormality (IQ: 50-70) | 3 | 15 |
| - Moderate mental subnormality (IQ: 35-49) | 6 | 30 |
| - Severe mental subnormality (IQ: 20-34) | 6 | 30 |
| • **Brain Neuroimaging Findings:** | | |
| - Basal ganglia calcifications | 1 | 5 |
| - Periventricular calcifications | 3 | 15 |
| - Cortical & central atrophic changes | 12 | 60 |
| - White matter hypomyelination | 7 | 35 |
| - Cerebellar hypoplasia | 2 | 10 |
| - Dandy-Walker malformation | 1 | 5 |
| - Hypogenesis of corpus callosum | 1 | 5 |
| - Normal findings | 3 | 15 |

**Table 2.** ELISA and PCR results among affected mothers and infants.

| Test | Affected mothers (no.=50) | | Affected infants (no.= 20) | | Total |
|---|---|---|---|---|---|
| | +ve | -ve | +ve | -ve | |
| IgM | 12<br>24% | 38<br>76% | 9<br>45% | 11<br>55% | 70 |
| IgG | 50<br>100% | 0<br>- | 20<br>100% | 0<br>- | 70 |
| PCR | 50<br>100% | - | 20<br>100% | - | 70 |

CMV IgM is considered positive if > 30 Eu/ml **(Nielsen et al., 1986).**
CMV IgG is considered positive if > 80 Eu/ml **(Nielsen et al., 1986).**

**Table 3.** End-point dilution method of CMV DNA among studied cases.

| No of CMV gpB DNA GE/ml whole blood | Affected women | | | | | Affected Infants |
|---|---|---|---|---|---|---|
| | Birth of affected infant | Repeated abortions | Repeated neonatal deaths | Repeated IUFD | Total | |
| $1.6X10^3$ GE/ml | 1 | 3 | - | - | 4 (8%) | 5 (25%) |
| $3.3X10^3$ GE/ml | 8 | 5 | 4 | 1 | 18 (36%) | 8 (40%) |
| $3.3X10^4$ GE/ml | 6 | 5 | 3 | 3 | 17 (34%) | 3 (15%) |
| $3.3X10^5$ GE/ml | 4 | 1 | 2 | 1 | 8 (16%) | 1 (5%) |
| $3.3X10^6$ GE/ml | 1 | 1 | 1 | - | 3 (6%) | 3 (15%) |
| **Total** | **20** | **15** | **10** | **5** | **50 (100%)** | **20 (100%)** |

**Table 4.** Comparison between UL83 and gpB genes levels.

| Cases | Level of HCMV-UL83-DNA copies/ml whole blood | Level of HCMV-gpB-DNA GE/ml whole blood |
|---|---|---|
| 1st Mother | $5 X10^2$ copies/ml | $3.3 X10^3$ GE/ml |
| 2nd Mother | 12 copies/ml | $3.3 X10^4$ GE/ml |
| 3rd Mother | $5.3 X10^2$ copies/ml | $3.3 X10^6$ GE/ml |
| 1st Infant | $3.5 X10^3$ copies/ml | $3.3 X10^6$ GE/ml |
| 2nd Infant | $1.5 X10^3$ copies/ml | $1.6 X10^3$ GE/ml |

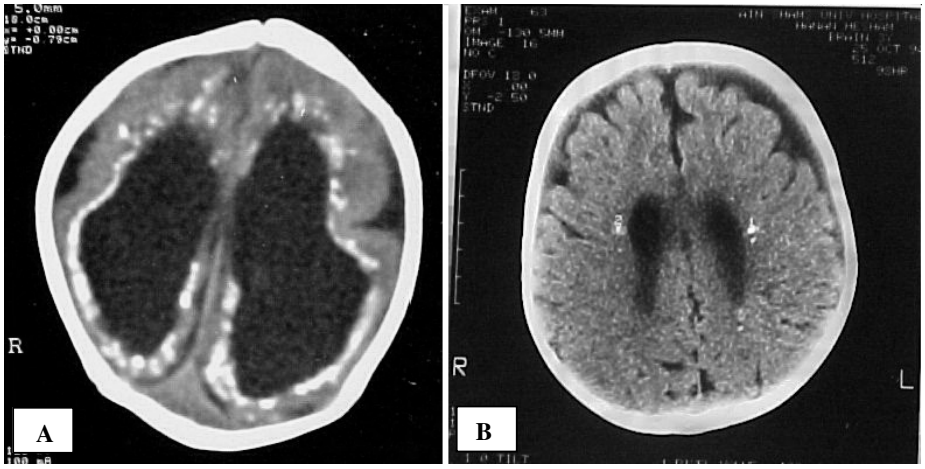



**Fig. (1):** CT brain scan: (A) Note ventricular dilatation and periventricular calcifications. (B) Note cortical atrophy and periventricular calcifications.

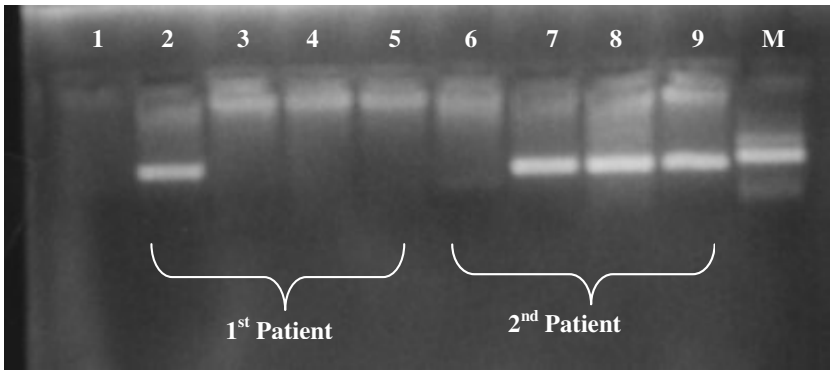
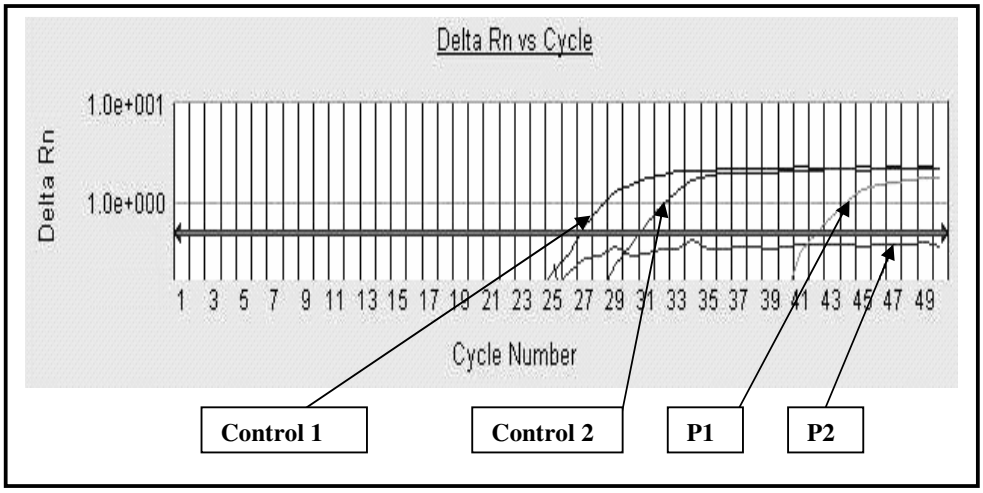



**Fig. (2):** Standardization results of end-point dilution method for CMV-gpB-DNA by nested PCR. 2nd PCR products was visualized by 3% agarose gel electrophoresis stained with ethedium bromide of 10 fold serial dilution of DNA samples from 2 patients.
***Lane 1:*** negative PCR assay.
***Lanes 2 to 5****:* 1st patient's sample, where in lane 2, positive blood samples to CMV, lanes 3 to 5 negative to CMV.
***Lane 6 to 9:*** 2nd patient's sample, gave positive results in spite of 10 fold serial dilution of DNA.

***Lane M***: 126 bp amplified DNA product of a known band, serves as a marker.




Control 1= 1000 copies/reaction
Control 2= 100 copies/reaction

**Fig. (3):** Real-time PCR of CMV-UL83 gene. A window graph showing 2 infants (P1 and P2) with congenital CMV. Viral load was high in P1 (cycle42) while it was very low in P2.

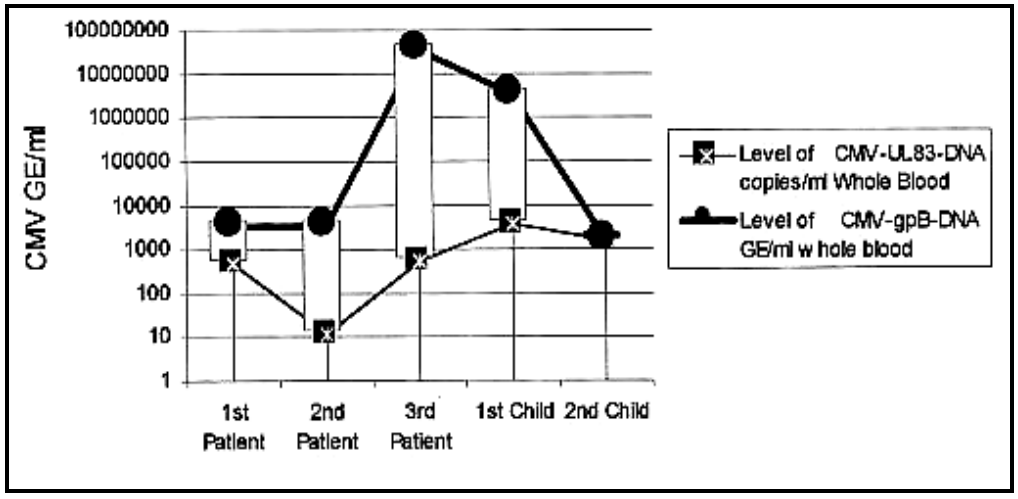



**Fig. (4):** Algorithmic graph, showing the comparison between the level of CMV-gpB-DNA and CMV-UL83-DNA in 5 patients. Only 2 patients almost have similar levels of infections.

## DISCUSSION

With regard to the severity of the symptoms and possible long-term consequences, the time of infection with CMV is of great importance[18]. Neonatal morbidity associated with CMV most often results from congenital CMV infection. Our study showed that neonatal manifestations in the form of low birth weight (<2500 gm), neonatal sepsis and neonatal jaundice were represented in 85% of the affected infants which is consistent with the recent study of de Vries et al.[19].

Mental sub normality (IQ ≤ 70) was noted in 75% of the presented infants, seizures in 40% and chorioretinitis in 20%. In the study done by Boppana et al.[20], mental subnormality was recorded in 47% of cases, seizures in 11% and chorioretinitis in 12%. Cases with chorioretinitis in the present study were associated the most devastating cognitive deficits, significant retardation and severe microcephaly (> -5 SD) which is consistent with Noyola et al.[21], who stated that central nervous system involvement in children with congenital CMV infection—as evidenced by the presence of microcephaly, seizures, abnormal tone, or chorioretinitis at birth—has been shown to predict the development of cognitive and motor deficits. It is generally accepted that symptoms of congenitally infected children are more severe after primary infection than after recurrent infection.

Twenty to 30% of all deafness cases are caused by CMV. Deafness represented 25% of infants in the present study which is less than that reported by Barbi et al.[22] (59%). This may be attributed to the younger age of our cases, as deafness experience continuous deterioration by age in cases with congenital CMV[23]. Evidence of disseminated infection (in the form of intrauterine growth retardation, petechiae, hepato-splenomegaly, hepatitis, thrombocytopenia, and intracerebral calcifications) with or without the presence of neurologic involvement at birth was predictive of the development of hearing loss in children with congenital CMV infection[24].

Congenital CMV infection can cause a wide range of brain anomalies. In the present study, white matter hypomyelination and intra cerebral calcifications represented 35% and 20% of affected infants respectively; compared to 100% and 66% respectively in the study done by Steinlin et al.[25]. Cerebellar hypoplasia accounted for 10% of the presented cases compared to 36% in the study of de Vries[19]. Strikingly, one case showed Dandy-Walker malformation and another case showed hypogenesis of corpus callosum in our study denoting that variable brain neuroimaging findings may be present in cases with CMV infection with no definite abnormality. Moreover, we found normal brain neuroimaging findings in 3 cases (15%).

Regarding maternal findings in the present study, 40% of cases presented by delivery of an affected infant followed by repeated abortions (30%) then repeated neonatal deaths (20%) and repeated IUFDs (10%). The above results indicate that repeated abortions is the most common antenatal presentation of CMV infection which is consistent with that stated by van Lijnschoten et al.[26].

Our results showed that ELISA investigations of the 50 mothers and the 20 infants revealed CMV IgM positive results in 12 mothers and 9 infants (24% and 45% respectively). CMV IgG was highly positive in all mothers and infants (70 cases). Definite diagnosis of CMV infection can't be based on ELISA results alone as, in adults, IgM antibody results should be interpreted along with the clinical findings as IgM antibodies may persist for months and even years and may be detected during reactivation of latent virus infections. Moreover, ELISA technique can't distinguish between CMV infection and CMV disease in the newborn[27].

All cases in the present study showed positive PCR for CMV-DNA. However, when PCR is carried out in non-quantitative way, it is of little clinical value to follow up patients during the

course of treatment, complementary tests are needed to help in the detection of the infection. Detection of CMV, differentiation between infection and disease, and monitoring of therapy have been recently sharpened by using molecular biology methods mainly based on measurement of viral load by quantitative PCR[28]. For that, we optimized two methods of CMV quantification. They are end-point dilution nested PCR amplification of CMV-gbB-DNA and real-time PCR amplification of UL-83 gene.

End-point dilution nested PCR methods are designed to perform titer determinations of the target template or of an external control by end-point sample dilution prior to PCR. Regarding CMV infection during pregnancy, the challenge is not only to detect infection, but also to determine whether the infection will have any clinical consequences. Concerning the need to distinguish CMV infection from CMV disease, quantification of CMV-DNA in whole blood has been proposed in this study to be more specifically associated with the disease in women during child bearing period.

All infants and mothers in the present study were included in end-point dilution technique for detection of CMV-gbB-DNA. This method was very accurate in detecting the viral load among infected mothers and infants, with viral quantity ranged from 1.6 $X10^3$ genome equivalents (GE)/ml to 3.3 $X10^6$ GE/ml. Our results showed that 70% of examined women and 55% of infants had CMV viral load ranging from 3.3 $X10^3$ GE/ml to 3.3 $X10^4$ GE/ml, 22% of women and 20% of infants had a viral load more than 3.3 $X10^4$ GE/ml and 8% of women and 25% of infants had a viral load of 1.6 $X10^3$ GE/ml. These results indicate that viral load below the level of $1.6X10^3$ GE/ml is not accompanied by symptomatic CMV disease. The amount of human cytomegalovirus (CMV) DNA in sera is considered to be a direct marker for CMV infection. However, elevated virus load continued to be the predominant risk factor for progression to CMV disease[29].

Viral load estimation using end-point dilution nested PCR method on serum samples positive by the qualitative method could be used to distinguish asymptomatic infection from CMV related disease with 100% specificity and sensitivity[30] had estimated CMV viral load in amniotic fluid of infected pregnant women and reported that viral load ranging from $10^3$ to $10^4$ GE/ml was found in 36% of the examined women, where 46% were above $10^4$ GE/ml. After birth, whole infants were found to have different CMV load infections. Marin et al.[31], studying 68 bone marrow and kidney transplanted patients, stated that patients having <867 CMV particles/µg DNA did not present symptoms suggestive of CMV disease while other patients with >867CMV particles/µg DNA presented these clinical symptoms. In another study on HIV infected patients, the diagnosis of active infection was based on the detection of the CMV replication in the blood. High CMV loads in the blood (8.000.000 copies/mg DNA) were important predictive factors for active infection and for the appearing of disease. Thus, end-point dilution nested PCR method for determination of CMV loads could be important tool for diagnosis of active infection and disease, as well as for CMV disease prediction in AIDS patients[32].

Our results indicated that the clinical manifestations of the mothers do not depend on viral quantity. On the other hand, in the affected infants, high viral load was associated with severe microcephaly (> -5 SD), severe brain neuroimaging findings and development of sensorineural hearing loss (SNHL). It was reported that infants with symptomatic congenital CMV infection excrete large amounts of virus in the first few months of life[33] and the children who developed hearing loss had higher CMV quantitative titers during infancy than those with normal hearing which is consistent with our results.

The response to oral ganciclovir in the present study was monitored in 12 mothers by the end-point dilution method before and after treatment. Five cases were not responding to therapy, 2 of them had very high viral load and delivered severely affected outcome with CMV

infection, thus suggesting the presence of a significant correlation between CMV load in mothers and disease severity in their newborns. These results may be also suggestive of reduced efficacy of ganciclovir, perhaps due to the appearance of drug-resistant CMV strains to this antiviral drug with specific mutations in the UL97 and UL54 genes of CMV[34]. The role of host factors should not be ruled out in making up the overall response to antiviral treatment.

Real-time PCR provides accurate and reproducible measures of gene copies. This method was designed for quantification of the UL83 gene copies that seems to be linked to viral replication. Therefore, the importance of this technique comes from its ability to determine viral activity which, when compared with results of end-point dilution method, helps in differentiation between latent from active infection**.** The most significant advance of the real-time systems comes from its rapid thermocycling and simultaneous detection characteristics.

The 20 patients who were assayed by real-time PCR for UL83 copy number in the present study had positive determinations only in 5 patients (3 mothers and 2 infants) (25%). Differences in CMV end-point dilution method for gpB gene in correlation with real-time PCR for UL83 gene were noticed. Others had reported similar differences between real-time UL83 DNA PCR and other quantitative PCR results, but the clinical significance of CMV-DNA load associated with low antigenemia (UL83 gene level) remains to be established.

In general, there is good evidence that high CMV load is associated with a higher risk of progression to CMV disease especially in immunocompromized patients, as well it can be used to track response to therapy. Moreover, a high viral load is correlated with symptomatic congenital CMV infections at birth[35].

We concluded that end-point dilution quantitative PCR is a sensitive method for detection of low CMV quantity that adds to our capability of diagnosis and management. The amount of CMV DNA in peripheral blood seems to be a direct marker for CMV infection. There was a direct correlation between the severity of the clinical picture in affected infants and the viral load. Very low viral load was not accompanied by symptomatic CMV disease. Real-time PCR is a promising method for detection of CMV and helps in discrimination of viral load from viral replication and differentiation between latent from active infection. The invention of new antiviral drugs capable of overcoming the drug-resistant CMV strains is also recommended. Moreover, prevention of CMV by developing a vaccine which can be administered to seronegative women of childbearing age to prevent the occurrence of primary CMV infection during pregnancy will be of great help in controlling this health problem.

## REFERENCES


1. Schleiss M.R.: "Vertically Transmitted Herpesvirus Infections". Herpes; 2003;10(1): 4-11.
2. Demmler GJ.: "Congenital cytomegaloviral infection and disease". Semin Pediatr Infect Dis; 1999;10:195–200.
3. Gaytant MA, Rours GI, Steegers EA, Galama JM, Semmekrot BA.: "Congenital cytomegalovirus infection after recurrent infection: case reports and review of the literature". Eur J Pediatr. 2003; 162(4): 248-53.
4. Marin LJ; Cunha AA; Aquino VH; and Figueiredo LTM.: "The use of qualitative and quantitative polymerase chain reactions for diagnosis of cytomegalovirus infections in bone marrow and kidney transplant recipients". Rev. Soc. Bras. Med. Trop; 2004: 37(2): 158-64.
5. Gault E, Michel Y, Dehée A, Belabani C, Nicolas J-C, and Garbarg-Chenon A.: "Quantification of Human Cytomegalovirus DNA by Real-Time PCR". Journal of Clinical Microbiology; 2001: 39(2): 772-75.
6. Li H, Dummer JS, Estes WR, Meng S, Wright PF, Tang YW.: "Measurement of human cytomegalovirus loads by quantitative real-time PCR for monitoring clinical intervention in transplant recipients". J Clin Microbiol.; 2003: 41(1):187-91.
7. Matchida U, Kami M, Fukui T, and Kazuyama Y.: "Real-time automared PCR for early diagnosis

and monitoring of CMV infection after bone marrow transplantation". J. of Clinical Microbiology, 2000: 38: 2536- 42.
8. Cunha Ade A, Marin LJ, Aquino VH, Figueiredo LT.: "Diagnosis of cytomegalovirus infections by qualitative and quantitative PCR in HIV infected patients". Rev Inst Med Trop Sao Paulo.; 2002: 44(3):127-32.
9. Mengelle C, Pasquier C, Rostaing L, Sandres-Saune K, Puel J, Berges L, Righi L, Bouquies C, Izopet J.: "Quantitation of human cytomegalovirus in recipients of solid organ transplants by real-time quantitative PCR and pp65 antigenemia". Med Virol.; 2003 69(2):225-31.
10. Tanaka N, Kimura H, Iida K, Saito Y, Tsuge I, Yoshimi A, Matsuyama T, and Morishima T.: "Quantitative analysis of cytomegalovirus load using a real-time PCR assay". J. Med. Virol. 2000: 60:455-62.
11. Tanner J.M., Hiernaux J . and Jarman S.: "Growth and physique studies". In: Human Biology: A Guide to Field Methods, I.B.P. Handbook No.9, edited by Weiner J.S. and Lourie J.A., Blackwell Scientific Publications, . 1969, Oxford and Edinburgh, pp. 1-29.
12. Raggio DJ, Massingale TW.: "Comparability of the Vineland Social Maturity Scale and the Vineland Adaptive Behavior Scale--survey form with infants evaluated for developmental delay". Percept Mot Skills. 1990; 71(2):415-8.
13. DSM-IV Diagnostic and Statistical Manual of Mental Disorders, 4th ed. 1994: Washington, DC, American Psychiatric Association.
14. Jones, R. N., Neale, M. L., Beattie, B., Westmoreland, D., and Fox, J.: Developmemt and application of a PCR-based method including an internal control for diagnosis of congenital cytomegalovirus infection". J. Clinical Microbiology, 2000: 38(1): 1-6.
15. Walker A., Petheram S., Ballard L., and Murph J.: "Characterization of HCMV by analysis of short repeat polymorphism". J. of Clinical Microbiology, 2001: 39: 2219- 26.
16. Hamprecht K., Mikeler E., and Jahn G.: "Semi-quantitative detection of cytomegalovirus DNA from native serum and plasma by nested PCR: influence of DNA extraction procedures". J. Virological Methods; 1997: 69: 125-135
17. Laurenti L, Piccioni P, Cattani P, Cingolani A, Efremov D, Chiusolo P, Tarnani M, Fadda G, Sica S, Leone G.: "Cytomegalovirus reactivation during alemtuzumab therapy for chronic lymphocytic leukemia: incidence and treatment with oral ganciclovir". Haematologica.; 2004: 89(10):1248-52.
18. Vochem M.: "CMV infections of the neonate: prevalence, diagnosis, therapy". Geburtshilfe Neonatol., 2003; 207(3): 114-8.
19. de Vries LS, Gunardi H, Barth PG, Bok LA, Verboon-Maciolek MA, Groenendaal F.: "The spectrum of cranial ultrasound and magnetic resonance imaging abnormalities in congenital cytomegalovirus infection". Neuropediatrics. 2004: 35(2): 113-9.
20. Boppana SB, Fowler KB, Vaid Y, Hedlund G, Stagno S, Britt WJ and Pass RF.: "Neuroradiographic findings in the newborn period and long-term outcome in children with symptomatic congenital cytomegalovirus infection". Pediatrics, 1997: 99(3): 409-14.
21. Noyola DE, Demmler GJ, Nelson CT.: "Early predictors of neurodevelopmental outcome in symptomatic congenital CMV infection". *J Pediatr.*; 2001: 138(3): 325– 31.
22. Barbi M, Binda S, Caroppo S, Ambrosetti U, Corbetta C, Sergi P.: "A wider role for congenital cytomegalovirus infection in sensorineural hearing loss". Pediatr Infect Dis J.; 2003: 22(1): 39-42.
23. Fowler KB, Dahle AJ, Boppana SB, Pass RF.: "Newborn hearing screening: will children with hearing loss due to congenital cytomegalovirus infection be missed?" J Pediatr.; 1999; 135: 60-64.
24. Rivera LB, Boppana SB, Fowler KB, Britt WJ, Stagno S and Pass RF.: "Predictors of Hearing Loss in Children with Symptomatic Congenital Cytomegalovirus Infection". Pediatrics, 2002: 110(4): 762-7.
25. Steinlin MI, Nadal D, Eich GF, Martin E, Boltshauser EJ.: "Late intrauterine Cytomegalovirus infection: clinical and neuroimaging findings". Pediatr. Neurol.; 1996; 15(3): 249-53.
26. van Lijnschoten G., Stals F., Evers J.L., Bruggeman C.A., Havenith M.H. and Geraedts J.P.: "The presence of cytomegalovirus antigens in karyotyped abortions". Am. J. Reprod. Immunol., 1994; 32(3): 211-20.
27. Maine GT, Lazzarotto T, Landini MP.: "New developments in the diagnosis of maternal and

congenital CMV infection". Expert Rev Mol Diagn.; 2001; 1(1): 19-29.
28. Grangeot-Keros L, Cointe D.: "Diagnosis and prognostic markers of HCMV infection". J Clin Virol.; 2001; 21(3): 213-21.
29. Emery VC, Cope AV, Sabin CA, Burroughs AK, Rolles K, Lazzarotto T, Landini MP, Brojanac S, Wise J, Maine GT.: "Relationship between IgM antibody to human cytomegalovirus, virus load, donor and recipient serostatus, and administration of methylprednisolone as risk factors for cytomegalovirus disease after liver transplantation". J Infect Dis., 2000; 182(6): 1610-5.
30. Gouarin S., Gault E., Vabret A., Cointe D., Rozenberg F., and Freymuth F.: "Real-Time PCR quantification of human cytomegalovirus DNA in amniotic fluid samples (mothers with primary infection). J. Clin. Microbiol., 2002; 40(5): 1767-72.
31. Marin LJ; Cunha AA; Aquino VH; and Figueiredo LTM.: "The use of qualitative and quantitative polymerase chain reactions for diagnosis of cytomegalovirus infections in bone marrow and kidney transplant recipients". Rev. Soc. Bras. Med. Trop,2004; 37(2): 158-64.
32. Cunha Ade A, Marin LJ, Aquino VH, Figueiredo LT.: "Diagnosis of cytomegalovirus infections by qualitative and quantitative PCR in HIV infected patients". Rev Inst Med Trop Sao Paulo., 2002; 44(3): 127-32.
33. Stagno S. Cytomegalovirus. In: Remington JS and Klein JO., ed. Infectious Diseases of the Fetus and the Newborn Infant. 1995: 4th ed. Philadelphia, WB Saunders.
34. Erice A.: "Resistance of human cytomegalovirus to antiviral drugs". Clin. Microbiol. Rev.; 1999; 12(2): 286-97.
35. Lazzarotto T, Gabrielli L, Foschini MP, Lanari M, Guerra B, Eusebi V, Landini MP.: "Congenital cytomegalovirus infection in twin pregnancies: viral load in the amniotic fluid and pregnancy outcome". Pediatrics, 2003: 112(2): e153-7.

## الملخص العربى

# التقدير الكمي للحامض النووي لفيروس السيتوميجالو باستخدام تفاعل البلمرة المتسلسل وتطبيقاته الإكلينيكية

تعتبر الإصابة بفيروس السيتوميجالو من أهم الأمراض التي تنتقل من الأم الي الجنين أثناء الحمل وتؤدي الي نتائج خطيرة جدا. وهذا المرض يحدث بنسبة تتراوح من 0.5–2% من جميع الولادات. والطرق التقليدية لتشخيص المرض من خلال اكتشاف وجود الأجسام المضادة لا تعتمد علي اكتشاف كمية أو نشاط الفيروس ولهذا لا يمكن استخدامها في متابعة المرضى أثناء العلاج أو قبل حدوثه.

وتهدف هذه الدراسة الي تطبيق طرق الهندسة الوراثية الحديثة لإكثار أكثر من جين خاص بهذا الفيروس وذلك للتقدير الدقيق لكمية الفيروس في المرضى بهدف التشخيص الدقيق للإصابة وتحديد مدي نشاط هذا الفيروس مما يؤدي الي المتابعة الدقيقة للمرضى أثناء العلاج وبعده.

وتشمل هذه الدراسة (50 سيدة و20 طفل) من المترددين علي عيادة الأطفال ذوي الاحتياجات الخاصة بالمركز القومي للبحوث وتم تشخيص الإصابة بهذا المرض في جميع الحالات من خلال اكتشاف وجود الأجسام المضادة بالدم وأيضا من خلال اكتشاف وجود الفيروس باستخدام تفاعل البلمرة المتسلسل غير الكمي.

وقد أظهرت النتائج أن التأخر في النمو التطوري للأطفال قد وجد بنسبة 90% من الأطفال المصابين، والتخلف العقلي قد وجد بنسبة 75%، وصغر حجم الرأس بنسبة 45% والتشنجات بنسبة 40% والصمم الحسي–عصبي بنسبة 25%. وقد ارتبط التهاب شبكية العين (20% من الحالات) بشدة التخلف العقلي وشدة صغر حجم الرأس. وأظهرت نتائج الرنين المغناطيسي والأشعة المقطعية علي المخ في الأطفال أن ضمور المخ (60% من الحالات) والنقص في نخاع المادة البيضاء (35%) والتكلسات بالمخ (20%) من أهم مظاهر الإصابة بهذا الفيروس . هذا وقد كانت نتائج الأشعة طبيعية في 15% من الحالات. أما بالنسبة للأمهات، فكانت الأعراض كما يلى: 40% من الحالات كانت تعانى من ولادة أطفال مصابين بتأخر في النمو وإصابات في الجهاز العصبي، 30% من الإجهاض المتكرر، 20% من الحالات كانت تعاني من وفاة مبكرة لأطفالهن أثناء الشهر الأول بعد الولادة و10% من الحالات تعانى من تكرار وفاة أطفالهن داخل الرحم أثناء الحمل.

وقد تم تطبيق تفاعل البلمرة المتسلسل الكمي لإكثار جين (جليكوبروتين–ب) الخاص بفيروس السيتوميجالو علي جميع الحالات (الأمهات والأطفال). وقد تم اعطاء العلاج (مادة جانسيكلوفير) لعدد 12 سيدة مصابة وتم تحديد مدي فاعلية العلاج وذلك بمتابعة التقدير الكمي للفيروس قبل العلاج وبعده. وكانت نتائج التقدير الكمي للفيروس لجين (جليكوبروتين–ب) متفاوتة في حالات الدراسة. ومن أهم النتائج أن نسبة الفيروس القليلة لم يصاحبها ظهور أعراض مرضية، وأن كمية الفيروس بالدم تناسبت تناسبا طرديا مع شدة أعراض المرض وظهور الأصابة بالصمم الحسي–عصبي عند الأطفال. ومن النتائج الهامة أيضا أن الأجسام المضادة للفيروس من نوع (IgM) لم تكن موجودة في الحالات ذات كمية الفيروس القليلة مما يؤكد مدي أهمية هذه الطريقة في تحديد الكميات الضئيلة للفيروس مما يساعد في التشخيص والعلاج الدقيق للمرض. وبمتابعة العلاج في 12 سيدة بقياس كمية الفيروس قبل العلاج وبعده، وجد أن 5 حالات لم تتم استجابتهم للعلاج بل وأن حالتان منهما تم ولادة طفلين مصابين بالفيروس لهما. ومن الجدير بالذكر أن كمية الفيروس في هاتين الحالتين كانتا كبيرة مما يشير إلى أن إصابة الأمهات بالفيروس بكميات كبيرة أثناء الحمل يسبق إصابة أطفالهن بهذا المرض.

وتم أيضا في هذه الدراسة الكشف عن وجود(جين يو.ال.83) الخاص بنشاط الفيروس بواسطة تفاعل البلمرة المتسلسل المعتمد على الزمن الحقيقي لإكثار الفيروس أثناء التفاعل وتحديد كميته في 20 حالة (17 سيدة، 3 أطفال). كانت النتائج ايجابية في 5 حالات فقط (20%)، مما يعكس نشاط الفيروس في هذه الحالات وحاجتهم الماسة للعلاج.

ونستخلص من هذه الدراسة أن طرق الهندسة الوراثية الحديثة لتقدير كمية فيروس سيتوميجالو في الدم وتحديد مدي نشاطه عن طريق الكشف عن جين (يو.ال.83) هي من أهم الطرق الدقيقة والحساسة لتحديد مدى شدة الإصابة بهذا المرض ومتابعة المرضى لمعرفة تطور المرض ومدي الاستجابة للعلاج.