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# CYTOMEGALOVIRUS INFECTION IN MULTI-TRANSFUSED B-THALASSEMIA MAJOR PATIENTS IN BASRAH. SEROLOGY AND MOLECULAR DIAGNOSIS

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**Abstract:** The transfusion transmitted Cytomegalovirus (TTCMV) may complicate the frequent pRBC transfusion in βthalassemia major (Choobineh et al., 2009). The possibility of CMV transmission in these patients was looked up as the units of blood were neither screened for this infection nor leukoreduced. The aim of this study was to detect the seroprevalence of CMV infection in transfusion dependent thalassemia patients and its relation with the molecular study. A total of 123 children and adolescent; 50 normal and 73 were patients with β-TM on regular blood transfusion. A blood sample was taken from each individual, controls and pre-transfusion patients, and submitted to serological (ECLIA) and molecular (real tine PCR) study. The prevalence of CMV IgG specific antibody was 100% in both patients and control while no one of them showed reaction to CMV IgM specific antibody. The CMV IgG avidity index was high in all β-TM patients except for one patient (1.3%) showed low IgG avidity index, while all the control individuals (100%) have had high IgG avidity index. The viral DNA was not detected in the peripheral blood of the entire study sample detection,
**Keywords**: Cytomegalovirus, Multiple transfusions, Beta-Thalassemia major

## 1. Introduction

Beta-thalassemias are a group of hereditary blood disorders characterized by quantitative defect in the synthesis of the beta globin chains of hemoglobin resulting in variable phenotypes ranging from severe anemia to clinically asymptomatic individuals (Hoffbrand, 2016). Life-long red blood cell (RBC) transfusion remains the main treatment for severe thalassemia. It's improved their overall survival (Hoffman et al., 2013). However, the blood safety may be threatened by the transmission of CMV infection. Cytomegalovirus (TTCMV) transmission is a recognized complication of blood transfusions, uncommon in immunocompetent individual but carry a significant risk in immunocompromised patients (Kekre et al., 2013). Early studies established that the incidence of TT-CMV infection from untested nonleuko-reduced blood products can be as high as 37%. (Ljungman, 2004; Qu & Tran, 2007). Cytomegalovirus is a double-strand DNA enveloped Herpesviruses, highly ubiquitous throughout the world, the prevalence rate inversely correlated with the socioeconomic status of the human population (Firth, 2015), In developing countries the prevalence rate of CMV ranges from 85-100% (Cannon wt al., 2010). Primary infection with HCMV is usually asymptomatic or exhibits infectious mononucleosis-like symptoms in immunocompetent individuals (BAWA, 2014). However, in immunocompromised patients a life-threatening infection may be encountered (Ljungman, 2004). After primary infection, CMV establishes latency mainly in white blood cells, which are responsible for the transmission of CMV by transfusion (TT-CMV) of blood or blood products (Kekre et al., 2013). In vitro, study showed that reactivation of latent CMV in peripheral monocyte may be stimulated by transfusion of allogeneic blood or blood products, suggesting a possibility of inducing allogenic reactivation of latent CMV. (Söderberg-Nauclér et al., 1997). Exposure is the major determinant of CMV infection while the degree of immunosuppression is the major determinant for development of symptomatic CMV disease (Ariza-Heredia, Nesher, & Chemaly, 2014; Ramanan & Razonable, 2013).

After allogeneic BMT, CMV infection occurs in approximately 60% to 70% of patients who are CMV seropositive before transplant or who are seronegative but receive bone marrow from a seropositive donor, and is

the principal infectious cause of death (Reusser, Riddell, Meyers, & Greenberg, 1991). Studies have found that removal leukocyte from blood component or transfusion of CMV-seronegative blood may significantly reduce the risk of CMV infection (Bianchi et al., 2016; Blajchman, Goldman, Freedman, & Sher, 2001; Nichols, Price, Gooley, Corey, &

Boeckh, 2003) The most frequently used tests for screening recent and past CMV infection are by detecting antibodies (IgM and IgG) specific to HCMV respectively (Ross, Novak, Pati, & Boppana, 2011). A pre-transfusion sample is preferable for testing to avoid transient false-positive antibody with recent transfusion of blood products (Kotton et al., 2013; Preiksaitis, Sandhu, & Strautman, 2002). The quantitative DNA detection techniques has been increasingly used in recent years because they are highly sensitive and provide viral load measurements that can give important prognostic information. (Boeckh, 2011). In Iraq, thalassemia is a growing health problem. However, it is not mandatory to screen donated blood for CMV in blood banks and no regular leukoreduction of the transfused blood was adopted. The role of blood and blood products in acquiring cytomegalovirus (CMV) infections following transfusion was reviewed in this study.

## 2. Subjects and Methodology

### 2.1 Population and study design

This case control study was conducted on the 1st of April 2016 to the end of July 2017. The enrolled subjects were 123 children and adolescents, **73** were patients with transfusion dependent β-TM who have been attending the Center of Hereditary Blood Diseases (CHBD) in Basra, Iraq and **50** were apparently healthy individuals attending the Primary Health Care (PHC) Centers for minor health problems as a control group. Their ages ranged from 3 – 17 years

A blood sample was taken from each individual, controls and pre-transfusion patients prior to transfusion, 4 mL of venous blood was collected, the sample divided into two parts, 2ml was emptied into a disposable tube contain EDTA (Ethyline Diamine Tetra Acetic acid) as anticoagulant, for total DNA extraction and the rest was emptied into a disposable plain tube which was left stand for one hour at room temperature, centrifuged and the supernatent serum used for serological study of CMV

### 2.2 Serological detection

Determination of anti-CMV IgM and IgG specific Abs with CMV IgG avidity index in tested serum was done by elctro-chemiluminescence immunoassay (ECLIA) using cobas e411

### 2.3 Molecular detection

Total DNA was extracted using QIAamp® viral DNA Blood Mini Kit (Qiagen GmbH, Germany). The purified DNA was stored at -20°C in the deep freeze till real time PCR detection. Real-time PCR amplification was performed using artus® CMV RG PCR Kit (Qiagen GmbH, Hilden, Germany). Reaction PCR volume was 50 µl, real time PCR mixture contained 25µl of CMV RG Master, 5 µl of CMV Mg-Sol, 2 µl* of CMV RG internal control and 20 µl of the sample. The enclosed quantitation standards (CMV QS 1–4) were treated as samples to generate a standard curve. The amplification done with Rotor-Gene Q Instruments. (QIAGEN, GERMANY) with an initial hold step of 10 minutes to activate hot-start enzymes at 95 °C followed by 45 cycles of 15 seconds at 95°C denaturation, 60 seconds at 60 °C annealing and 15 seconds at 72°C extension.

### 2.4 Statistical analysis

Statistical Package for Social Science (SPSS) version 24 was used for statistical analysis of the data. Chi-square ($X^2$) and Fisher's Exact tests were to determine the difference between the study groups.

Student's t-test was used for comparing the means. Comparisons of proportions were performed by crosstab using the $\chi 2$ test to assess the significance of difference between groups. The significance level was set at $P < 0.05$, and the highly significance level was set at $P < 0.001$.

## 3. Results

### 3.1 The demographic characteristics of the study population:

The total number of subjects enrolled in the study was 123, their ages range from 3 -17 years. Of the total number, 73 were patients with β-TM and 50 were apparently healthy children and adolescents as a control group.

For descriptive purpose the subjects were divided into three groups in relation to their age and as showed in table -1. There is no significant difference in the age and gender distribution between patient and control groups, P value > 0.05.

**Table - 1 Distribution of case and control according to age groups and gender**

| Variables | Subject | | | | Total | | P-value |
|---|---|---|---|---|---|---|---|
| | Case N=73 | | Control N=50 | | | | |
| **Mean age ± SD** | | | | | | | |
| | 9.8 ± 4.4 | | 9.8 ± 4.0 | | 9.8 ± 4.2 | | .417 |
| **Age-group** | | | | | | | |
| < 5 | 12 | 16.4% | 6 | 12.0% | 18 | 14.6% | |
| 5-10 | 34 | 46.6% | 24 | 48.0% | 58 | 47.2% | .786 |
| > 10 | 27 | 37.0% | 20 | 40.0% | 47 | 38.2% | |
| **Gender** | | | | | | | |
| Female | 30 | 41.1% | 24 | 48.0% | 54 | 43.9% | .449 |
| Male | 43 | 58.9% | 26 | 52.0% | 69 | 56.1% | |

**1.2 Selected clinical variables for patients with β-TM**

The mean age of the patients with β-TM was 9.2 ± 4.2 years for male and 10.4 ± 4.6 years for female. the mean annual blood transfusion was 15.7 ± 4.9 with a rate of 12 – 14 units per year in 68(93.2%) of them. Nearly half (47.9%) of the patients received their blood transfusion without filter, the other half (47.9%) were infrequently transfused with filtered blood according to the availability of the bed side filters in the center, except for 3(4.1%) patients who always transfused with filtered blood products. Less than half 15 (20.5) of enrolled patients were splenectomised and 31 (72.1%) of them live in the rural area.

**Table - 2: Selected clinical variables for patients with β-TM**

| Variables | Males | Females | Total: n (%) |
|---|---|---|---|
| Number (%) | 43 (58.9) | 30 (41.1) | 73 |
| Mean age ± SD | 9.2 + 4.2 | 10.4 + 4.6 | |
| Mean annual blood transfusion | 15.3 ± 4.5 | 15.9 ± 5.3 | 15.7 ± 4.9 |
| | | | |
| **Rate of transfusion/ year** | | | |

| | | | | |
|---|---|---|---|---|
| **<12** | | **1 (2.3)** | **0** | **1 (1.4)** |
| **12-24** | | **42 (97.7)** | **26 (86.7)** | **68 (93.2)** |
| **>24** | | **0** | **4 (13.3)** | **4 (5.5)** |
| **Transfusion of filtered blood** | | | | |
| **Always** | | **2 (4.7)** | **1 (3.3)** | **3 (4.1)** |
| **Infrequent** | | **20 (46.5)** | **15 (50.0)** | **35 (47.9)** |
| **Never** | | **21** | **14 (46.7)** | **35 (47.9)** |
| **Splenectomy** | **Yes** | **6 (14.0)** | **9 (30.0)** | **15 (20.5)** |
| | **No** | **37 (86.0)** | **21 (70.0)** | **58 (79.5)** |
| **Residency** | **Rural** | **31 (72.1)** | **18 (60.0)** | **49 (67.1)** |
| | **Urban** | **12 (27.9)** | **12 (40.0)** | **24 (32.9)** |

### 3.3 Seroprevalence of CMV infection among the study population.

The sero-prevalence of HCMV infection among the study groups were analyzed and the figure-1 below showed two extreme of values. While the whole population enrolled in the study was positive for the IgG antibody specific to HCMV. The reverse is true for anti-CMV IgM specific antibody; where the whole population was negative.

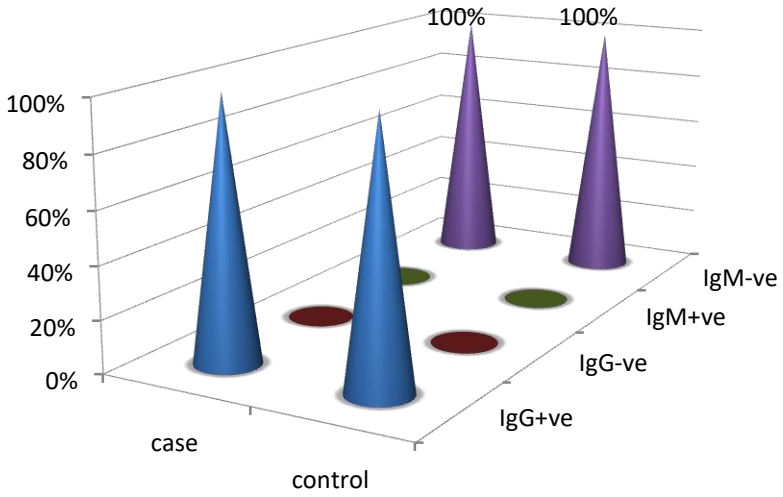


**Figure 1. Seroprevalence of CMV among the study groups**

### 3.4 HCMV-IgG avidity index in patients and control group:

Figure. -2 shows that all β-TM patients with positive HCMV IgG specific antibody, showed high IgG avidity index (98.7%) except for one patient (1.3%) showed low IgG avidity index. While no one of the control group had low IgG avidity index.

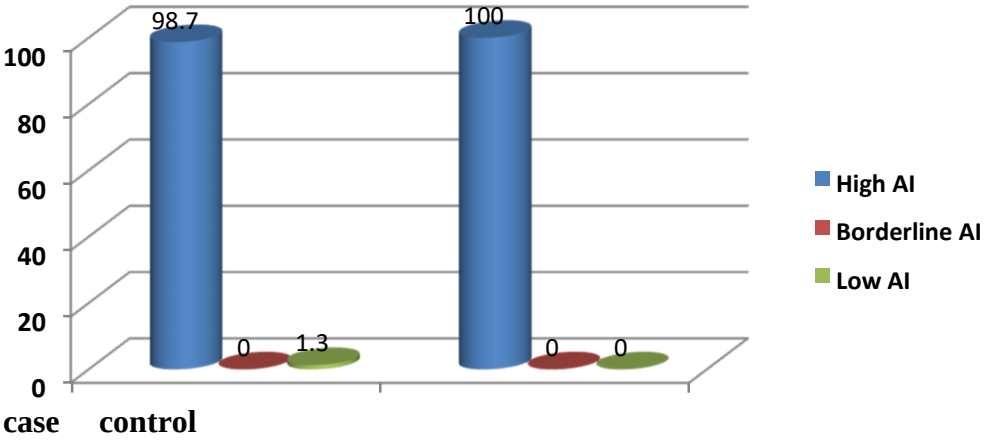


**Figure 2. IgG Avidity Index**

### 3.5 Molecular detection of CMV infection

Molecular analysis of CMV infection was done by real time PCR and the data was presented in a standard curve to qauntify a sample and an amplification plot as shown in fig -3 and fig -4 respectively. The amplification plot represents the plot of cycle numbers versus fluorescence signal which correlate with the initial amount of target nucleic acid during the exponential phase. The optimal threshould line was automatically selected.

There are five curves appearing in the plot. The first four curve (red, yellow, blue and purple) represents the 1st - 4th quantitation standards, their crossing points (CP) were in cycle 23.78, 27.05, 30.21 and 33.77 respectively, giving a concentration range from the highest (10,000) to the lowest (10) viral DNA copies.
The last (pink) curve that crosses the threshold line in cycle 43 represents one of the unknown samples, the concentration of viral DNA copies was beyond calculation limit (very low viral load if ever detectable). Whereas all other samples of the patients and the control groups were tested negative for viral DNA in the peripheral blood

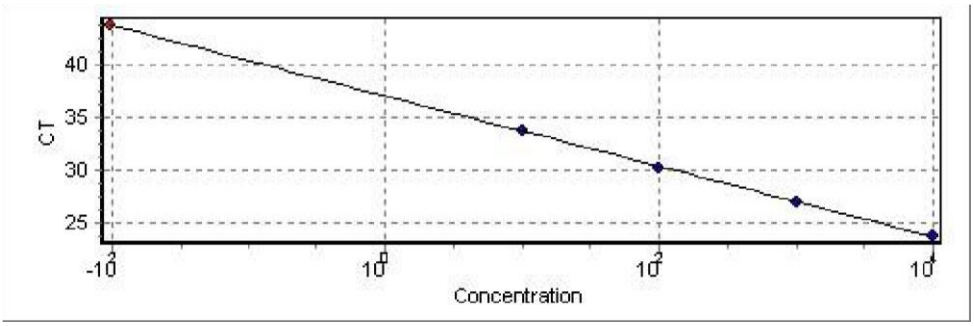


**Figure 3. Standard Curve of the artus® CMV RG real time PCR**

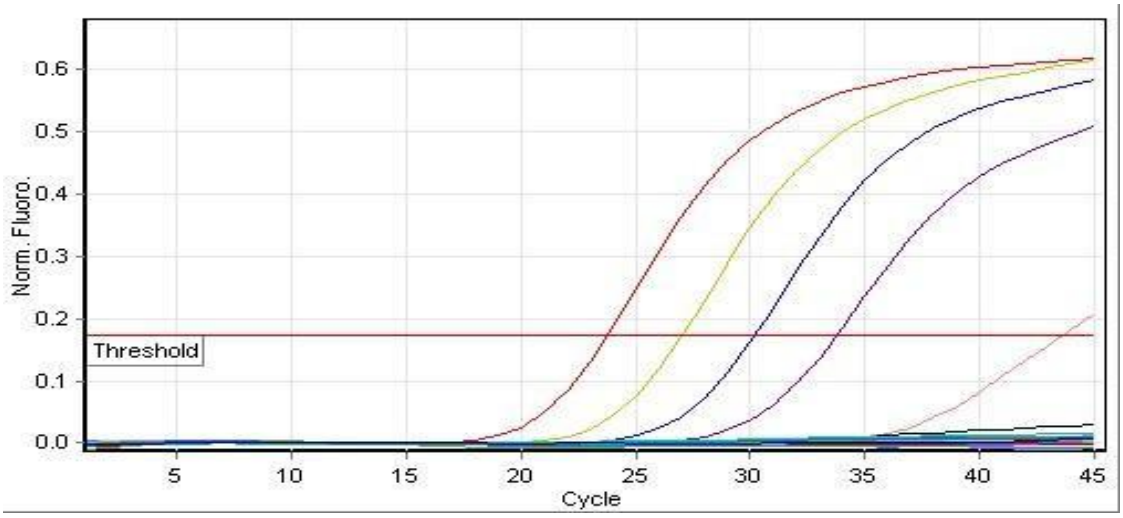


**Figure 4. The Amplification Plot;** *the first four curves (red, yellow, blue and purple) represents the 1st-4th quantitation standards, their crossing points (CP) were in cycle 23.78, 27.05, 30.21 and 33.77 respectively, with viral load range from the highest (10,000) to the lowest (10) viral DNA copies.. The last (pink) curve represents one of patients sample with CP in cycle 43; all other samples (case and control) were tested below the threshold line.*

## Discussion

### Seroprevalence

Cytomegalovirus infection is a matter of concern for blood transfusion recipients, particularly in cases of transfusions to immunocompromised patients (Eivazi-Ziaei et al., 2013), in whom TTCMV considered a causative agent of death (Ljungman et al., 2014).

In the current study, assessment of the serostatus of CMV in both patients and the control groups showed that all the study population (100%) were reactive to CMV IgG specific antibody, while none of them showed reactions to the CMV IgM specific antibody. These results are similar to the results of Delhi's study on the seroprevalence of CMV antibody on 200 healthy blood donors. They found that 95% were positive for CMV IgG antibody and none of them was positive to anti-CMV IgM antibody (Kothari et al., 2002). Similar results were reported in two studies carried out in Iran, during 2005 and 2013, when the latter detected the anti-CMV antibodies IgM and IgG in blood donors in a percentage of 1.6% and 99.2% respectively estimated by enzyme link immunosorbent assay (ELISA) (Aghaeipour et al., i 2005, Safabakhsh et al., 2013).

In the developed countries similar studies have been carried out decades ago. In 1989, a study done in Athena (Germenis et al., 1989) on 287 multitransfused thalassemia major patients aged 5–39 years.

They found that the mean prevalence of total Anti-CMV antibody (by ELISA) was 89.6% compared to 71.1% among 1,220 healthy controls. They observed a significantly higher prevalence of CMV antibodies in thalassemia patients of all age-groups compared to controls. The prevalence among splenectomized thalassemia patients was even higher than among non-splenectomized thalassemia patients (Germenis et al., 1989).

Another study done in Italy 1990 showed that the overall rate of CMV infection that confirmed serologically by ELISA was similar between the thalassemia patients and healthy individuals ($p > 0.05$), but the incidence of infection was higher in thalassemia patients than control ($P< 0.01$) (Nigro et al., 1990). The higher prevalence of anti-CMV IgG antibody in Iraq, Iran and India, compared to that reported in developed countries reflects the negative correlation between CMV infection and the socio-economic state. Studies showed that CMV IgM specific Ab can be detected during primary and recurrent infections in immune compromised patients, while in immunocompetent individuals IgM can be detected in primary infection but not upon recurrent infections (Reddehase 2013; Revello et al., 2002). This finding makes the total sero-negativity in CMV IgM specific Ab in previously infected patients and control individuals of the current study was not surprising.

**IgG Avidity Index (AI)**

In the current study, low avidity index, which is an accurate indicator of primary infection within the preceding 3 to 4 months, is reported in only one (1.3%) out of 73 of patients and was negative in all of the control individuals.

Many investigators that have tested the avidity for all samples with positive CMV IgG specific Ab, irrespective to the result of CMV IgM specific Ab, have constantly determined that 1-3% of the positive IgG, negative IgM samples showed low IgG AI (Dollard et al., 2011, BaAlawi et al., 2012, Prince et al., 2014). In a study done by Prince et al, 2014, reported that the vast majority of patients with primary CMV infection exhibit both low CMV IgG AI and detectable CMV IgM. And in rare cases, only one of these parameters existed. (Prince et al., 2014)

Others showed that a negative CMV IgM test result, in combination with a positive CMV IgG result, does not completely rule out the possibility of an acute infection with CMV as individuals at the early stage of acute infection may not exhibit detectable amounts of CMV IgM antibodies, as mentioned above (Revello et al., 2002). Therefore, low IgG AI represents the most reliable marker of recent infection with or without IgM.

**Molecular detection of CMV DNA by real time PCR**

The detection of CMV DNA in peripheral blood is influenced by the sampling interval. The highest incidence of CMV DNA detection in blood is achieved when the sample is taken in the period between the last seronegative and the early seropositive state (Ziemann et al., 2013, 2013). Studies showed that the concentration and prevalence of CMV DNA in the first seroconversion where higher than the last seronegative sample (Ziemann et al., 2010, 2013, 2013). As the sampling interval increased to weeks or months from the primary infection, the possibility of missed detection of the virus increased (Zhang et al., 2006).

In immunocompetent individuals, the duration of viremia tends to be short (Genser et al., 2001). The CMV DNA can be detected in a low concentration after detection of the antibody to viral antigen, and as the CMV avidity index is high no CMV DNA can be detected (Genser et al., 2001, Ziemann et al., 2013). In the current study viral DNA was not detected from the peripheral blood sample of the whole study group. This raised the possibility that all subjects were immunocompetent and/or with long term seropositivity, as the avidity index was high for almost all study population.

Our results are consistent with that of two previous studies done by Ziemann in 2007 and Drew in 2003 on the correlation between seroconversion and detection of CMV DNA. They found that the CMV DNA were negative

in all (1,086) their long term seropositive plasma samples (Drew et al., 2003, Ziemann et al., 2007). Another recent study done in 2013 by the author Ziemann who detected CMV DNA in only one (0.01%) out of 7,303 with long term seropositivity, weak antibody result and low concentration (<30 IU/ml) of DNA in plasma (Ziemann et al., 2013).

## Conclusion

1. CMV is highly prevalent viral infection in our community
2. Thalassemia patients included in this study showed adequate immune response to CMV presented by the high IgG avidity and negative viral DNA detection

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