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Urine qRT-PCR assay as a screening tool for the detection of congenital human cytomegalovirus infection of infants

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ABSTRACT

Congenital cytomegalovirus (CMV) infection is the most common infectious cause of birth defects. It may cause both, immediate and long term health problems in infants. These include variety of symptoms, such as hearing loss, microcephaly, jaundice, hepatosplenomegaly and seizures. In more severe cases CMV infection can cause the death of an unborn baby and loss of pregnancy. Despite being one of the most extensively studied vertically transmitted infections recently, the adverse effects of vertically transmitted CMV infection are still not well presented to the general public, resulting in a low awareness among potential expectant mothers in Bosnia and Herzegovina. This study aims to elucidate the sensitivity of urine samples for CMV detection in infants as well as to reflect the importance of quantitative real-time PCR (qRT-PCR) in diagnostics of CMV infection in infants. qRT-PCR was used in this study as a technique for the screening of CMV DNA in a cohort of patients based in Sarajevo Canton. These results have shown that urine samples are sufficiently sensitive for the detection of CMV DNA in infants. Furthermore, the simultaneous analysis of several patients has shown a higher number of CMV DNA copies amplified in urine compared to blood samples, derived from the same patient, thus proposing urine as a reliable sample of choice for congenital CMV diagnostics. These findings may propose a need to classify qRT-PCR CMV test among one of the recommended first-trimester pregnancy screening tests, which could help in early detection of CMV infection in Sarajevo Canton.

Keywords: Cytomegalovirus (CMV), real-time PCR, urine, infants

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1. Introduction

Human Herpes Virus 5 or Cytomegalovirus (CMV) is encapsulated, double-stranded DNA virus with a genome of 235 kb, which belongs to the β subfamily of herpesviruses [1], [2]. CMV is the most common cause of congenital infections and birth dysfunctions, known to affect 0.5-2% of all live births [3], [4]. CMV infects and replicates in several cell types, including epithelial glandular and mucosal cells, smooth muscle cells, fibroblasts, macrophages, dendritic cells, hepatocytes, and vascular endothelial cells [5]. Transmission of CMV occurs by direct contact with infected secretions (blood, saliva, breast milk, and urine) and through processes of inhalation, contact with wounds, blood transfusion, organ transplantation, and sexual activity [6], [7].

From October 2017 to November 2019, Eurofarm Center laboratory based in Sarajevo has recorded an increasing number of infants experiencing symptoms of congenital CMV infection. Similarly, the increscent incidence of congenitally infected newborns has been observed as a global problem [8]. Recent studies have shown improvements in the diagnostics, treatment, and prevention of CMV infection in pregnancy, ensuring better outcomes for the congenitally infected infants [9]–[11]. However, despite the tremendous research performed in the field of CMV pathogenesis, the consequences of CMV infection are still not familiar to the general public. Therefore, this study aims to elucidate the sensitivity and importance of urine samples for the detection of CMV DNA in infants as well as to reflect the importance of qRT-PCR as a molecular technique for non-invasive diagnostics of CMV infection of infants. This study demonstrates the fast and non-invasive testing of infants with accurate results, with the emphasis on the choice between urine and blood samples.

2. Materials and methods

Samples for this study were obtained from infants and expectant mothers inhabiting the Sarajevo Canton. Collected peripheral whole blood and urine samples were analyzed in the period from October 2017 to November 2019 in Eurofarm Center laboratory, Sarajevo.

The study was performed on a cohort of 72 patients aged from infancy up to 44 years of life. The descriptive analysis of patients is shown in Table 1. Among the 72 patients tested for the presence of CMV DNA, 41 are female patients (56.9%) and 31 are male patients (43.1%). Infants make 41.6% of total patients analyzed. Furthermore, the study included patients up to 13 years (19.4%), potential and expectant mothers (29.3%), while 9.7% of patients did not provide their birth year before the CMV DNA screening.

Table 1. Descriptive analysis of patients

Patient's characteristics	n	%	*P* value
Age range (years)			
0-2	30	41.6	
>2	35	48.6	>0.05
NA	7	9.7	
Gender			
Male	31	56.9	>0.05
Female	41	43.1	

2.1. Sample collection

Peripheral whole blood was drawn and collected into the BD Vacutainer® with EDTA anticoagulant. Following the collection of peripheral whole blood, a blood sample was left for 10 minutes to cool down and then stored in the fridge at 4°C until the DNA extraction was performed. Urine samples were collected into sterile plastic bags or the collection tubes. Following the collection, urine was transferred into sterile falcon tubes and centrifuged in a clinical centrifuge for 10 minutes at 45000 rpm. Similarly, urine was stored in the fridge at 4°C until the DNA extraction was performed. In total, 122 samples were analyzed. Patient's samples were analyzed according to the principles of the Declaration of Helsinki.

2.2. DNA extraction

Spin column-based DNA extraction from urine and peripheral whole blood was performed in Fume hood-BIOBASE (USA) by "Invitrogen - PureLink® Genomic DNA Purification Kit K1820-02" (InvitrogenTM, 2007). Genomic DNA was stored at +4°C prior to the qRT-PCR analysis. Following the qRT-PCR analysis, all DNA extracts were stored at -20°C.

2.3. DNA quantification

The concentration and purity of extracted genomic DNA were assessed by µDropTM plate (ThermoFisher Scientific, USA), MultiscanTM GO microplate spectrophotometer (ThermoFisher Scientific, USA) and the accompanying SkanIt Software. Samples were analyzed in triplicates, blanked against 10 mM Tris-HCl, and the mean values were calculated based on data obtained.

2.4. CMV qRT-PCR

For the purpose of CMV DNA detection, qRT-PCR analysis was performed by PrimerDesign Genesig Kit for Human Herpes Virus 5 (Primerdesign TM, 2018). The target sequence was glycoprotein B (gB). qRT-PCR reaction setup was performed in DNA free zone PCR workstation-UVC/T-AR (BioSan, Latvia). The following components were used in all qRT-PCR analyses: 10µl of PrecisionPLUS 2X qPCR Master Mix (containing Taq DNA polymerase, reaction buffer, MgCl2, and dNTPs), 4µl of RNase/DNase free water, and 1µl of CMV primer/probe mix. Herein, 5µl of extracted DNA was added to the test tubes, while 5µl of CMV DNA was added to the positive control tube (Primerdesign TM, 2018). Once set, 96-well plate containing test and control tubes were centrifuged at 900 rpm for 2 minutes. qRT-PCR analyses were run in the Thermal Cycler-Quant studio 5, (Thermo Fisher, Singapore) with following amplification conditions: hold stage at 95°C for 2 minutes, and PCR stage which includes DNA denaturation step at 95°C for 10 seconds and data collection step at 60°C for 1 minute.

PCR stage was repeated in 50 cycles. The collection of fluorogenic data through the FAM channel occurred during the data collection step. Each tested sample and positive control were assigned CMV target during the PCR analysis. Likewise, every analysis was performed without passive reference. Upon analysis completion, cycle threshold (CT) values of amplified samples were compared to the CT values of standards. Thus, the number of CMV DNA copies/ml of the sample was calculated.

2.5. Statistical analysis

Results were statistically evaluated using GraphPad Prism version 8.0.1 for Windows, GraphPad Software, La Jolla California USA. Results are considered significant when $P<0.05$.

3. Results and discussion

3.1. DNA quantification

Results demonstrated in this subsection correspond to 17 pooled patients screened positive for CMV DNA. Whole blood samples yielded more DNA (in µg/ml) compared to the urine sample, which was consistent with our expectations. As shown in Figure 1, the concentrations of DNA extracted from blood samples ranged from 12.8 to 53.6 µg/ml, with a mean value of 33.2 µg/ml. The concentrations of DNA extracted from urine samples ranged from 2.5 to 7.6 µg/ml, with a mean value of 5.05 µg/ml. Statistical significance of higher genomic DNA concentrations extracted from blood samples is shown by P=0.003.

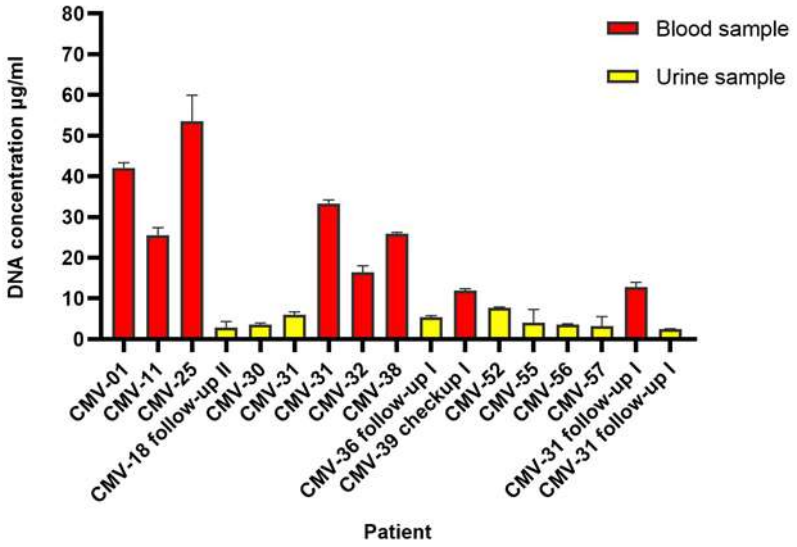

Figure 1. Measurement of the genomic DNA (µg/ml)

Results shown in Figure 2 demonstrate the analysis of DNA purity. Purities of extracted DNA from blood samples ranged from 1.7 to 2.7, with a mean value of 2.11, while purities of urine's DNA extract ranged from 1.2 to 3.3, with a mean value of 1.77. Regarding urine samples, results of quantification indicated DNA sufficiently pure for further testing, particularly considering the heterogeneity of urine samples. Statistical analysis of results related to the purity of extracted genomic DNA from blood and urine samples did not show statistical significance (P=0.211).

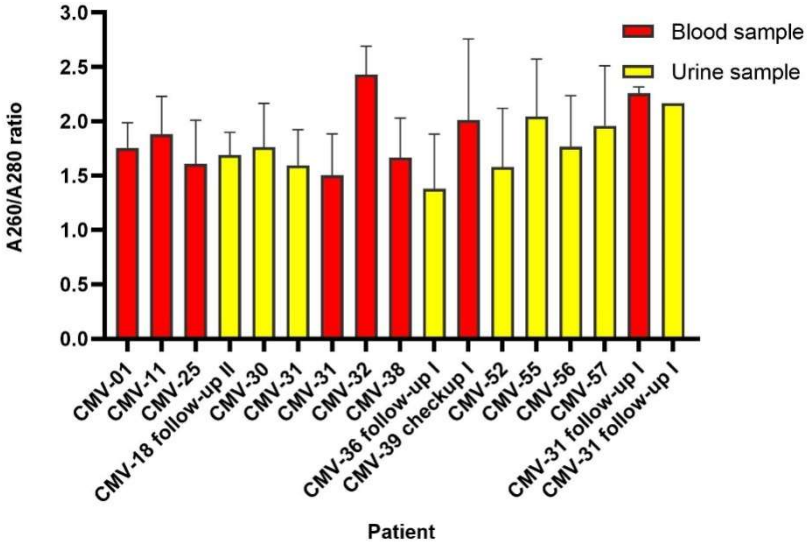

Figure 2. Measurement of the genomic DNA (260nm and 280nm)

3.2. qRT-PCR analysis

Results of qRT-PCR performed on blood and urine samples are shown in Figure 3. Among the 122 samples screened, results have shown 45 positive (36.8%) and 46 negative samples (37.7%), for the presence of CMV DNA. At the beginning of the study, 31 (25.4%) samples were analyzed but their sample type was not recorded and therefore not included in overall sample type distribution analysis.

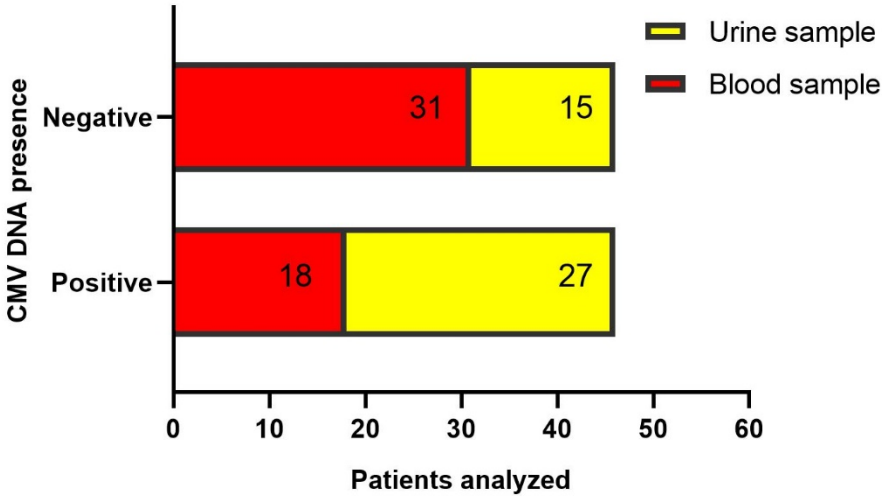

Figure 3. Number of CMV DNA copies/ml detected in blood and urine samples

All 45 samples screened as positive for CMV DNA were collected from infants. The age span of the patient's samples screened as negative vary, but women of child-bearing age predominate. When CMV DNA positive samples were classified by the source of extracted DNA, preferable body fluid for CMV shedding becomes clarified. Out of 45 positive samples, CMV DNA was found in 27 urine, and 18 blood samples.

Furthermore, the difference between the number of CMV DNA copies detected per milliliter of urine and blood samples are demonstrated in Figure 4. Analyzed samples are independent of each other. Data obtained may suggest that in infants CMV is rather shed in urine (Figure 4A) compared to blood (Figure 4B), as indicated by a higher mean of CMV DNA copies/ml of sample. Statistical analysis for 27 urine and 18 blood samples has shown *P*= 0.4544, meaning that a higher number of CMV DNA copies amplified from DNA extracted from urine samples is not statistically significant. Non-significant results may be explained by an unequal number of blood and urine samples included in statistical analysis. Thus, significant results may be obtained in a larger cohort of patients with two sets of equal sample numbers.

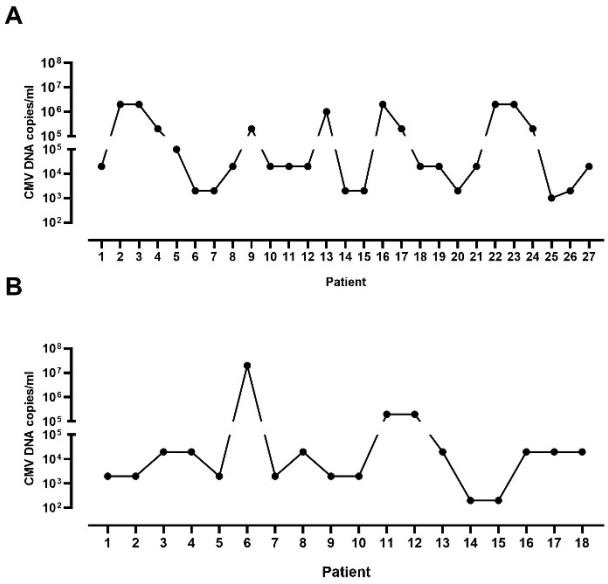

Figure 4. Number of CMV DNA copies detected in urine (A) and blood (B) samples

Results shown in Figure 5 demonstrate the difference between the detection of CMV DNA in urine and blood samples of infants that have performed simultaneous screening for CMV DNA. Patients CMV-31, CMV-32, and CMV-69 have been subjected to the multiple analyses throughout the study, primarily due to the lack of the therapy response. In all three patients, discrepancies between the number of CMV DNA copies/ml of blood and urine samples can be observed. In the case of CMV-31,a higher number of CMV DNA copies/ml was detected in urine when compared to the blood sample. However, for CMV-32 and CMV-69 qRT-PCR screening has detected no CMV DNA in blood, while in urine analysis has detected $2x10^6$and $2x10^3$CMV DNA copies/ml of sample, respectively. This analysis suggests that urine samplesmay be more sensitive for CMV DNA amplification in infants and as such should be suggested as a sample of choice for confirmation of CMV infection in infants. Likewise, results shown in Figure 5 indicate that solely blood or solely urine sample may not be sufficiently reliable for accurate diagnosis of CMV infection in infants. Thus, the evaluation of both urine and blood samples should be establishedas one of the golden standards for CMV DNA screening in infants.

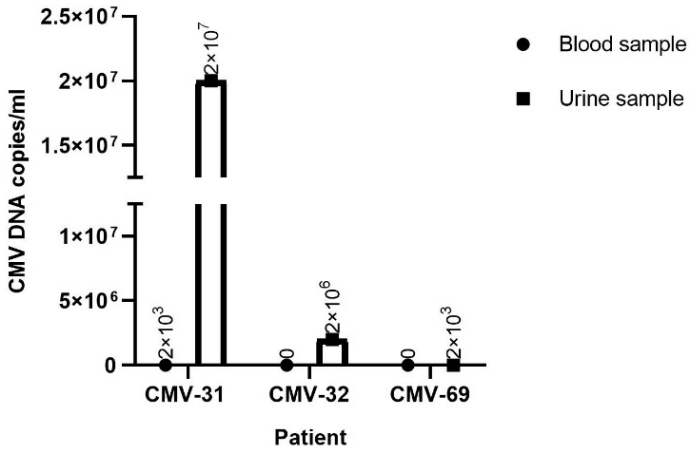

Figure 5. Comparison of CMV detection in urine and blood samples

Six infants were included in the follow-up study, and the results of their individual analyses are shown in Figure 6. Follow-up analyses were performed every three to four weeks. All patients included in the follow-up study were given oral antiviral therapy Valganciclovir. Valganciclovir efficiently inhibits CMV DNA polymerase [12], as shown by a gradual decrease of CMV DNA copies in patients CMV-32 and CMV-39. Such decreasing trend cannot be observed in patients CMV-18, CMV-31, CMV-36, and CMV-44 which have been associated with more prominent symptoms and multiple complications.
Interestingly, CMV DNA screening of the patient CMV-36 has shown complete drug unresponsiveness as indicated by more than $2x10^3$CMV DNA copies/ml of sample on last four follow-up analyses. Moreover, a higher number of CMV DNA copies can be observed in urine when compared to blood samples, as shown in Figure 4C-F.

Since this study was performed as a retrospective cohort study, there was no opportunity to select the type of patient sample for analysis. However, exactly this issue has clarified the differences between urine and blood samples regarding the amplification of CMV DNA.

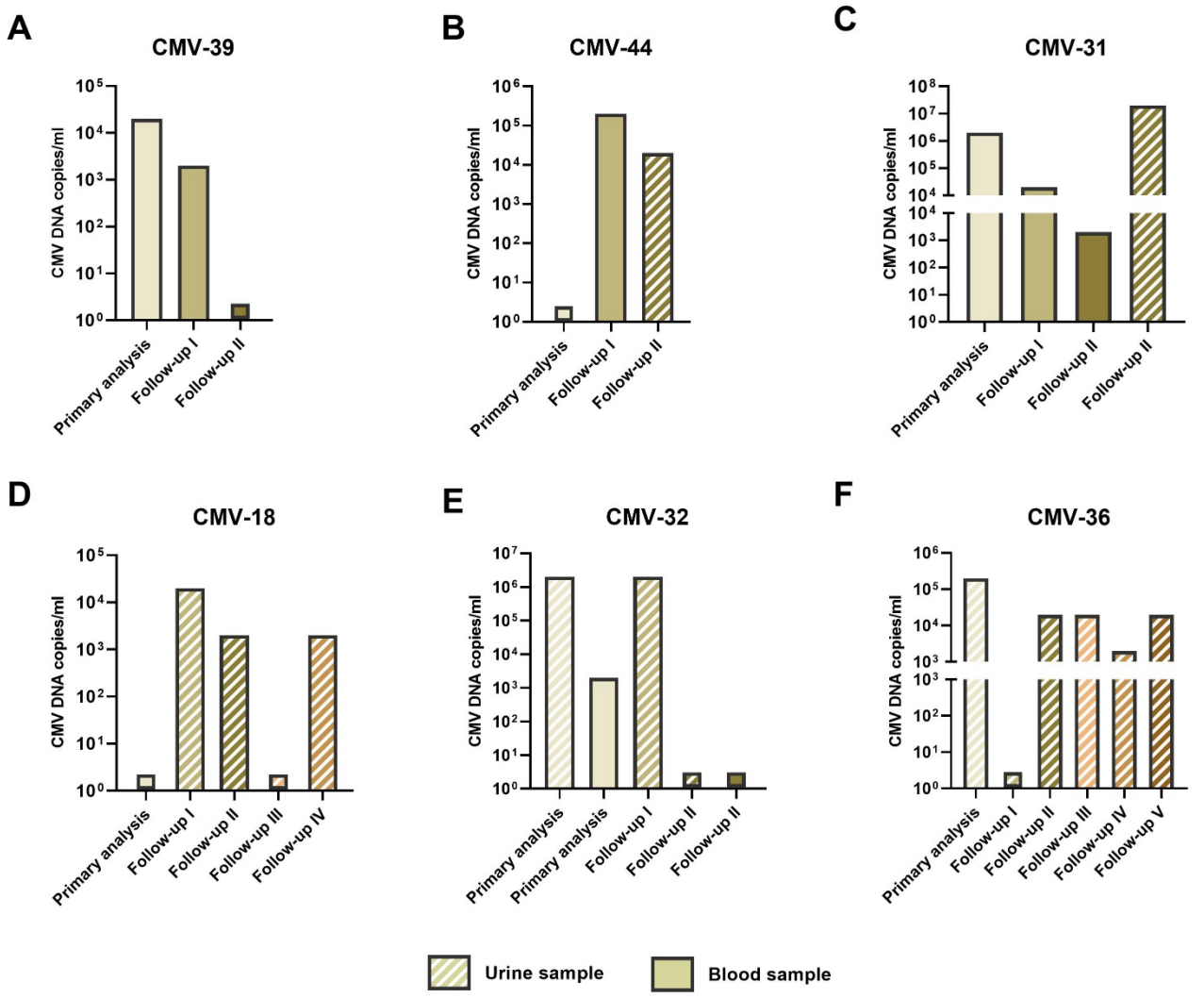

Figure 6. The number of CMV DNA copies detected in patients included in the follow-up study

4. Conclusion

This study aimed to elucidate the sensitivity and importance of urine samples for the detection of CMV DNA in infants. Quantification of extracted DNA has demonstrated that DNA yield from urine samples is sufficient for the qRT-PCR analysis (>1 μg/ml). Furthermore, the sensitivity of the urine sample for the detection of CMV DNA has been confirmed by qRT-PCR amplification. As demonstrated by simultaneous blood and urine analysis and follow-up study, urine samples have been confirmed as those with higher CMV DNA amplification. Therefore, great care should be taken when making a selection for an infant's sample type for CMV DNA screening. Having said this, one may propose that in infants, CMV-related genitourinary infections are more common, when compared to systemic infections. Also, the lower number of CMV DNA copies detected in blood samples may be explained by the assumption that patients did not develop viremia at the time of the sample collection.

Despite the limitations of sample type selection for qRT-PCR analysis, this retrospective cohort study has confirmed that urine is a sensitive and preferable sample for thefast, non-invasive, and accurate screening of

CMV DNA of infants. Demonstrated results may prompt the classification of CMV screening among one of the first-trimester pregnancy screening tests, which would serve as one of the means to reduce the incidence of CMV infection in Sarajevo Canton.

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