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Congenital Cytomegalovirus Infection Screening in Newborns From Saliva Samples by Real-Time Polymerase Chain Reaction Analysis

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What is already known on this topic?

- *Early diagnosis and successful antiviral treatment of congenital CMV infection significantly reduce neurological sequelae.*
- *Although newborn screening of congenital CMV infection with PCR studies in blood, urine, and saliva samples has been recommended in recent years, it has not been routinely practiced yet.*
- *CMV-DNA analysis by polymerase chain reaction (PCR) method in saliva samples is reported as a sensitive method in the screening of congenital CMV infection.*

What this study adds on this topic?

- *Congenital CMV infection screening by saliva RT-PCR has high false-positive results.*
- *It is seen that the frequency of congenital CMV infection is lower than in most of the other studies, no case with a definitive diagnosis was found in 545 newborns.*
- *Results of our study will be a guide in deciding whether to screen or which screening method would be useful for congenital CMV infection in newborns in our country.*

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ABSTRACT

Objective: Congenital cytomegalovirus infection is the most common congenital infection. Although screening of congenital cytomegalovirus infection with polymerase chain reaction studies in blood, urine, and saliva samples has been developed in recent years, it is not yet in routine use in any country.

Materials and Methods: In our study, cytomegalovirus deoxyribonucleic acid analysis was performed by real-time polymerase chain reaction method in saliva samples taken before the first feeding during the first day following birth in neonates born in a university hospital between January 2021 and January 2022. To support the diagnosis, additionally, cytomegalovirus deoxyribonucleic acid positivity in urine and blood samples was investigated in newborns with cytomegalovirus deoxyribonucleic acid positivity in saliva.

Results: Cytomegalovirus deoxyribonucleic acid was investigated in saliva samples of 545 neonates by real-time polymerase chain reaction method in 1-year period and positivity was found in 6 neonates. Since cytomegalovirus deoxyribonucleic acid was found negative by the real-time polymerase chain reaction method in the urine and blood samples of 5 of these neonates, the positivity in the saliva sample was interpreted as false positivity. In 1 case, cytomegalovirus deoxyribonucleic acid positivity was detected in urine and blood samples 5 weeks later. As a result, definite congenital cytomegalovirus infection could not be diagnosed in 545 cases, while possible congenital cytomegalovirus infection was diagnosed in 1 case.

Conclusion: It has been concluded that the frequency of congenital cytomegalovirus infection is low in our study group and studying saliva samples showed high false-positive rates. It is seen that saliva is not a suitable sample for detecting cytomegalovirus deoxyribonucleic acid by real-time polymerase chain reaction method.

Keywords: Congenital CMV infection, CMV DNA, RT-PCR, saliva, neonatal screening

INTRODUCTION

Cytomegalovirus (CMV) is the most common congenital infection virus and is one of the leading causes of hearing loss, cognitive retardation, and visual impairment in children.[1] The frequency of congenital CMV infection varies in a wide range such as 0.2%–2.4% in developed countries and may increase up to 6% in some countries.[2] Considering the frequency of the disease, it seems to be an important public health problem.

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The definite diagnosis of congenital CMV infection is made by isolating the virus in saliva, urine, or blood samples within the first 2-3 weeks after birth.[1] Since routine CMV screening is not performed in newborns and the possibility of acquired CMV started to be considered in the differential diagnosis after the first 3 weeks in most of the cases, the diagnosis of congenital CMV infection cannot be confirmed and its true frequency is not known. Without routine screening testing, the diagnosis of congenital CMV infection remains only clinical suspicion. Only a small portion of symptomatic CMV infection cases can be diagnosed clinically, while most of the symptomatic cases and all of the asymptomatic cases remain undiagnosed.[3,4]

It has been reported that CMV deoxyribonucleic acid (DNA) analysis by polymerase chain reaction (PCR) method in saliva samples is a sensitive method and can be used as a screening test.[5] In this study, we aimed to determine the frequency of congenital CMV infection in children born in 1-year period in a university hospital and to evaluate the diagnostic power of using a saliva sample for the test to detect virus nucleic acid in the diagnosis of congenital CMV infection. We think that the results of our study will be a guide for routine screening for congenital CMV infection in newborns in our country.

MATERIALS AND METHODS

This study protocol was reviewed and approved by Ethical Review Board of Kocaeli University (GOKAEK-2019/13.08 2019/220). Written informed consent was obtained from the patients' parents who agreed to take part in the study.

Study Design

The study was carried out at Kocaeli University Faculty of Medicine, Turkey, between March 2021 and March 2022. In this period, all newborns born in Kocaeli University Faculty of Medicine, Department of Obstetrics and Gynecology were included in the study, regardless of delivery type, gestational age, and sex. Due to the limited study budget, the number of cases to be included in the study was planned to be limited to a maximum of 600. Saliva samples were taken with a sterile buccal swab (Omni swab, Whatman) before the first feeding within the first 24 hours following birth by a fellow of pediatrics and delivered to the Molecular Microbiology Laboratory of the Department of Medical Microbiology in 600 µL 1X phosphate-buffered saline (PBS) (Lonza, Accugene) by the cold chain.

Real-Time Polymerase Chain Reaction

To isolate DNA from saliva samples, buccal swab samples that were transported to the laboratory in 600 µL PBS were vortexed for 30 seconds, then with the QiaAmp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendation, nucleic acid isolation has been made with a final volume of 100 µL. The total nucleic acid samples taken into the microcentrifuge tube were stored at −80°C until the real-time PCR (RT-PCR) study.

Genesig Real-Time PCR kit (Primer Design Ltd TM, Advanced kit, United Kingdom) was used to detect CMV DNA in saliva samples. For RT-PCR, 5 µL of total nucleic acid was used and the PCR mix was prepared with a total volume of 20 µL. The reaction was performed on the Rotor-Gene Q (Qiagen) instrument with an initial denaturation of 2 minutes at 95°C, followed by 50 cycles of denaturation at 95°C for 10 seconds, and bond/extension at 60°C for 60 seconds. The mixture in the kit was used as a positive control, and distilled water was used as a negative control. Blood, saliva, and urine samples of the patients who were determined as positive were re-studied with the Artus CMV RGQ MDx Kit (Qiagen).

Procedures in Cases with Cytomegalovirus Deoxyribonucleic Acid Positivity or Negativity in Saliva Sample

The presence of CMV DNA was investigated in urine and blood samples in the first 15 days of newborns with CMV DNA positivity in their saliva sample, and serum CMV-immunoglobulin (Ig) M and CMV-IgG levels were also checked in these newborns. Studying CMV DNA in the urine sample is considered the gold standard in order to confirm the diagnosis of congenital CMV infection in patients with CMV DNA positivity in their saliva samples. Cases with CMV DNA positivity in their saliva but negative urine and blood samples were accepted as false positives. Detection of CMV DNA positivity in urine and/or blood samples in addition to the saliva sample was interpreted as true positivity. Urine and blood samples were not studied in infants with negative CMV DNA in their saliva RT-PCR sample. It was planned to record the existing neurological problems and make a neurological follow-up for a child with congenital CMV infection and for children with no neurological problems a hearing test every 6 months who passed the initial hearing test.

Statistics

Only descriptive analysis was used.

RESULTS

Number of Newborns Screened

During the 1-year study period, the presence of CMV DNA was studied in saliva samples from 545 infants by RT-PCR method (Figure 1).

Newborns with Cytomegalovirus Deoxyribonucleic Acid False Positive

The CMV DNA positivity was detected in 6 cases (6/545 = 1.1%). All 6 newborns passed the neonatal hearing test. In 5 of these cases, CMV DNA analysis was performed in urine and blood samples by RT-PCR method within the first 21 days after the saliva sample was collected, and it was found to be negative in all of them. Therefore, these cases were not considered congenital CMV infection and CMV DNA positivity in the saliva sample was interpreted as false positivity. In all of these cases, serum CMV-IgM was negative and CMV-IgG positive.

A Newborn with Possible Congenital Cytomegalovirus Infection

In 1 case, after CMV DNA positivity was detected in the saliva sample, urine and blood samples could only be studied after 5 weeks, and CMV DNA positivity was detected in both urine and blood samples. In addition, serum CMV-IgM was found positive and CMV-IgG negative. In this case, since urine and blood samples could not be obtained within the first 21 days, acquired CMV infection could not be excluded, so a definite diagnosis of congenital CMV infection could not be made, and considered possible congenital CMV infection.

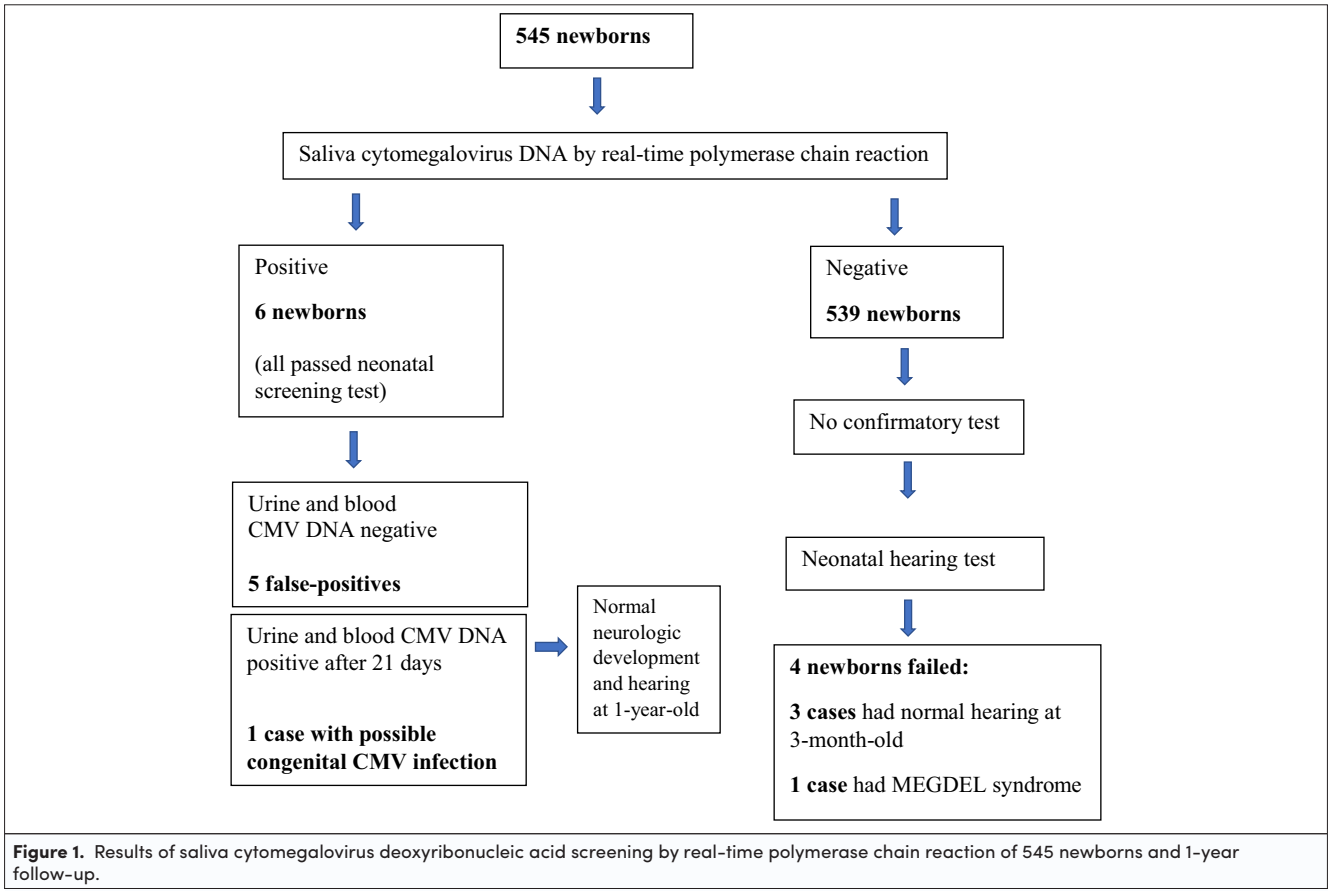

Figure 1. Results of saliva cytomegalovirus deoxyribonucleic acid screening by real-time polymerase chain reaction of 545 newborns and 1-year follow-up.

Evaluation of Salivary Cytomegalovirus Deoxyribonucleic Acid Positive Cases at 1 Year Old

Five cases with false positivity and 1 case with possible congenital CMV infection were evaluated when they were 1 year old. Parents did not have concerns about their children's neurologic development, vision, and hearing functions. Gross motor, fine motor, language, and social neurologic development of these cases were normal. Head circumference and other anthropometric measurements were within normal limits. In all cases, tympanogram, otoacoustic emission examination, and brainstem auditory evoked potential thresholds were evaluated as compatible with normal hearing. Neuroimaging was not performed in any of the cases.

Newborns with Cytomegalovirus Deoxyribonucleic Acid Negative

Of 539 newborns with negative screening for congenital CMV infection in their saliva sample, 4 failed the neonatal hearing screening. Detailed hearing analysis was performed on these children when they were 3 months old. The hearing was found to be normal in 3 of these children, and hearing loss continued in 1 child. The child whose hearing loss continued was diagnosed with MEGDEL syndrome in the follow-up and hearing loss was associated with this syndrome.

Patients with negative salivary CMV DNA screening who passed the neonatal hearing screening and were asymptomatic in the neonatal period were likely to be missed in our study design.

DISCUSSION

Congenital CMV infection is the most common congenital infection, but its true incidence is unknown. Although screening congenital CMV infection with PCR studies in blood, urine, and saliva samples has come to the fore in recent years, it has not been routinely practiced yet. Since routine CMV infection screening is not performed in newborns and the possibility of acquired CMV infection is started to be considered in the differential diagnosis after the first 3 weeks in most of the cases, the diagnosis of congenital CMV infection cannot be confirmed, and its actual frequency is not known.[6-8]

There are congenital CMV infection screening studies conducted with the PCR method in Turkey and other countries. Zeytinoğlu et al[9] performed a survey in 2019 in Turkey, and CMV DNA positivity was detected in 16 of 1000 newborns (1.6%) from saliva samples taken within the first half hour after birth. In 14 of these infants, CMV DNA was negative in urine and blood samples by PCR, and the saliva test was evaluated as false positive. In this study, PCR CMV DNA positivity was found to be significant in 2 out of 1000 newborns, and the rate of congenital CMV infection was reported as 0.2% (2/1000). In another study conducted in Turkey, CMV DNA was found positive in 18 of 944 newborns (1.91%) in saliva samples examined in the first 3 days by PCR. The CMV DNA was not detected in the urine and blood of 5 of 18 asymptomatic cases (3 twin pregnancies). Since the samples of these cases were taken in the first 3 days, it was emphasized that CMV DNAs might originate from breast milk

due to breastfeeding, and the rate of definite congenital CMV infection may be lower.[10] Eres et al[11] found CMV DNA positivity of 3.3% in saliva samples of 1147 newborns aged 0-20 days and evaluated 10 (0.87%) cases confirmed by urine samples as congenital CMV infection.

Extensive international studies on congenital CMV infection use the PCR screening test. In the study of Barkai et al[12] CMV DNA was found positive in 56 of 9845 (0.57%) newborn saliva samples, while CMV DNA was negative in urine in 7 of them. Negative CMV DNA in urine and positive in saliva was explained as a low amount of CMV DNA contamination originating from the birth environment or breast milk. Boppana et al[5] detected the sensitivity and specificity rate as >97.4%, positive predictive values as 91.4% and 90.2%, and negative predictive values as 100% and 99.9%, respectively, in liquid and dry saliva samples.[5] This study emphasized that the negative result detected in liquid and dry saliva samples excluded congenital CMV infection, and the overall false-positive rate was <0.003%. In the study of Ross et al[13] the false-positive rate of CMV DNA in saliva samples of newborns screened for congenital CMV infection was found to be between 0.03 and 0.14%. In a recent study, 21 (0.66%) of 3151 newborns had CMV DNA positivity in their saliva samples and the diagnosis of congenital CMV infection was confirmed by urine CMV DNA analysis performed within 21 days.[14] In the same study, false positivity was found in 54 newborns (1.7%).

In our study, there is a possibility of missed cases of congenital CMV infection due to false-negative CMV DNA in a saliva sample. However, none of the 539 patients with saliva CMV DNA negativity were found to have hearing loss, microcephaly, or neurodevelopmental disorder favoring congenital CMV infection. Excluding the children with asymptomatic congenital CMV infection, who may have been missed due to possible false negativity in the saliva for CMV DNA, the frequency of congenital CMV infection was found to be at most 0.18% (1/545; only 1 case with possible congenital CMV infection) in our study group. This value is lower than the ones reported for developing countries.[2]

Our study is the second study in Turkey in which PCR studied CMV DNA in saliva samples taken within the first half hour following the birth of congenital CMV infection. Although the number of cases in our study was less than in the study by Zeytinoğlu et al[9] the study method was very similar. Our study found CMV DNA positivity in 6 of 545 newborns with a saliva PCR study. Still, salivary PCR positivities were accepted as false positives due to urine and blood CMV DNA negativity in 5 newborns. Although meager false-positive rates were found in the PCR study of the saliva sample in the study of Ross et al[13] high false-positive rates were noted both in the study of Zeytinoğlu et al[9] and in our study. We could not measure viral load in saliva. In the study of Chiereghin et al[14] CMV virus load was studied in saliva sample screening and it was shown that false positivity was higher in those with very low/low viral load. In our study and in the study by Zeytinoğlu et al[9], the frequency of definite or possible congenital CMV infection was found to be very close, such as 0.18% and 0.2%, respectively. According to these results, it is seen that the frequency of congenital CMV is approximately 1 in 500 newborns in our country. It seems that studies with much higher numbers of cases are needed to determine the accurate frequency of congenital CMV infection.

Screening for neonatal congenital CMV infection is universal or targeted. The targeted screening is performed in children who fail the neonatal hearing screening.[15-17] This strategy risks missing a considerable subgroup of children with late-onset hearing loss due to congenital CMV infection. Universal screening is applied to all newborns. It aims to detect asymptomatic congenital CMV infection and follow up on screen-positive cases regarding hearing loss and neurodevelopmental disorders.[14,18,19] It is stated that both screening methods are cost-effective.[1,20] However, it still needs to be clarified to screen for neonatal congenital CMV infection or which process can be used for screening. It is seen that urine and saliva samples are used most frequently in screening neonatal congenital CMV infection, but urine sample is considered more accurate.[21] On the other hand, studies on saliva samples have gained momentum due to the difficulty of collecting urine in newborns. According to the results of our research, CMV DNA false positivity in saliva samples by RT-PCR method is an important problem for population-based screening. We collected saliva samples before the first feeding to avoid contamination from breast milk in saliva samples. Therefore, the cause of false positivity in our cases was not breasted milk-borne transmission.

Although our study also aimed to evaluate the neurological development and hearing functions of asymptomatic and symptomatic infants diagnosed with congenital CMV infection by PCR scanning in the saliva sample in the neonatal period, this phase of our study could not be realized since no infants diagnosed with definite congenital CMV infection were detected.

The most important limitation of our study is the limited number of cases. The limited number of cases was due to the constraints in the study budget. In addition, we did not perform CMV DNA analysis in urine and serum samples in cases where CMV DNA positivity was not detected in saliva samples. For this reason, some patients may have been missed in the screening due to false negativity of saliva CMV DNA analysis.

CONCLUSION

The absence of any newborn diagnosed with definite congenital CMV infection in our study indicates that our country's frequency of congenital CMV infection is relatively low. It has been observed that the saliva may not be a suitable sample for detecting CMV DNA by RT-PCR method due to high false-positive rates when applied alone and must be used together with other supportive diagnostic tests. The results of our study may be a router in deciding whether to screen or which screening method would be helpful for congenital CMV infection in newborns in our country.

Ethics Committee Approval: This study protocol was reviewed and approved by Ethical Review Board of Kocaeli University (GOKAEK-2019/13.08 2019/220).

Informed Consent: Written informed consent was obtained from the patients who agreed to take part in the study.

Peer-review: Externally peer-reviewed.

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Declaration of Interests: The authors have no conflict of interest to declare.

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