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Congenital *cytomegalovirus* infection cases and follow-up findings in Antalya, Turkey

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ABSTRACT

Objective: To investigate the presence of congenital *cytomegalovirus* (cCMV) infection and the *CMV*-DNA virus in the newborns who applied for newborn hearing screening test (NHST) and *CMV*-DNA viruria with physical, mental-motor development and hearing status of c*CMV* cases in the second year of age.

Patients and Methods: *CMV*-DNA was investigated in 1150 newborns' oral swabs (0-21 days) by polymerase chain reaction kit and urine of patients with positive *CMV*-DNA in saliva. Transient Evoked Otoacoustic Emission test was performed for NHST.

Results: CMV-DNA was posititve in saliva of 38 (3.3%) newborns and urine of 10 out of 37 newborns. The prevalence of c*CMV* was 0.87% (95% CI=0.697-1.042). All newborns passed the NHST. In newborns with c*CMV*:jaundice in 60% (6/10), low birthweight in 40% (4/10), small for gestational age in 50% (5/10) of them. Jaundice was the most significant variable (P<0.001, OR:23.411, 95% CI=5.772-94.960) and bilirubin levels were slightly elevated. In the second year of 8 cases, *CMV*-DNA viruria was detected in all of them and sensorineural hearing loss was detected in one infant.

Conclusion: The c*CMV* infection rate is 0.87% in a population with high maternal seropositivity.When diagnosing c*CMV*, saliva may give false-positive results and urine should be tested. Bilirubin levels may not be as high as expected in c*CMV* cases in highly seroimmune populations and sequelaes may occur in the following years.

Keywords: Congenital CMV infection, High seroprevalence population, Urine, Saliva, CMV-DNA PCR

1. INTRODUCTION

Cytomegalovirus (*CMV*) is the most common cause of congenital infections worldwide [1]. To date, the factors associated with intrauterine transmission of *CMV* and the occurrence of disease in the fetal, neonatal or infantile period are not fully defined. However, in both the United States and other developed countries, congenital *CMV* (c*CMV*) is known to be a leading cause of neurologic disease and sensorineural hearing loss (SNHL) in children [1,2]. Furthermore, intrauterine *CMV* transmission to the fetus has been demonstrated in *CMV* seropositive pregnancies, and no difference has been found between primary and nonprimary maternal *CMV* infections in the severity and prognosis of symptomatic c*CMV* infection [3]. In recent years, the incidence of c*CMV* infection has been associated with the epidemiological characteristics of each community. It has been reported that c*CMV* infection rates were higher in populations with high seroprevalence [4].

Various data are available from developed countries on the prevalence of c*CMV* infection and its impact on newborns [4]. The prevalence of c*CMV* infection is approximately 0.6% – 0.7% in developed countries with low maternal seroprevalence [1]. However, these specific data are limited to developing countries [4]. Data from developed countries where *CMV* seroprevalence is low or moderate may not correlate with data from developing countries with high seroprevalence. Therefore, it seems necessary to investigate the health problems caused by c*CMV* infection in developing countries. When the data from different study groups are analyzed, Turkey is among the countries with

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a high seroprevalence of *CMV* that ranges between 93.6% and 100% [5-8]. Despite the high *CMV* seroprevalence, the prevalence of c*CMV* infection was reported to be 1.91% and 0.2% in two different studies from different geographical areas of Turkey [9,10].

The effects of c*CMV* infection in newborns are observed in a broad clinical spectrum; more than one symptom and/or anomalies affecting the central nervous system may occur during birth or lead to long term late-onset sequelae. The most common long-term sequela is SNHL [11]. In the long-term, hearing loss may follow a progressive or fluctuating course [12]. Psychomotor and cognitive impairments are observed in most symptomatic cases and visual impairments occur in almost half of the symptomatic cases [11].

This study aimed to investigate the prevalence of c*CMV* infection in newborns admitted for newborn hearing screening test (NHST) and the presence of *CMV*-DNA viruria with physical, mental-motor development and hearing status of c*CMV* cases in the second year of age.

2. PATIENTS and METHODS

Newborn period

Between January 2013 and May 2014, a total of 1150 (561 girls and 589 boys) newborns (age range: 0 – 21 days) were enrolled in the study who were admitted to Akdeniz University Medical Faculty, Otolaryngology Clinic for NHST. A NHST is mandatory during the newborn period (0 – 28 days) in Turkey. The order of admission was considered in the selection of the study group. In addition, including newborns into the study in the first three weeks of life was a criterion. Parents were informed about the study and written informed consent was obtained. Subsequently, saliva samples were collected from the newborns after NHST. The characteristics of the newborns (gestational week, birthweight, hyperbilirubinemia, NHST results, and cranial anomalies such as microcephaly/ hydrocephalus) were recorded.

Newborn Hearing Screening Test (NHST)

The NHST was performed in all newborns. For this purpose, a test of Transient Evoked Otoacoustic Emission (TEOAE) (Accuscreen, Madsen, Denmark) was performed [13]. In those who failed the TEOAE test, the Auditory Brainstem Response (ABR) test (Acuscreen, Madsen, Denmark) was performed [13]. The ABR test is only applied to newborns who failed the TEOAE test in our country. In addition, the ABR test was performed immediately based on TEOAE test results at the second-year admission of c*CMV* cases as part of the screening programme.

Laboratory tests

Saliva samples from the newborns were collected with sterile swabs (Copan Diagnostics, Italy), placed in sterile plastic tubes containing viral transport medium (Copan Diagnostics, Italy) and delivered to the laboratory. The median day of saliva collection was 1 day (range: 0 – 21 days) for all newborns. *CMV*-DNA extraction was done with a commercial kit (High Pure Viral Nucleic Acid Kit, Roche Diagnostics, Germany) and *CMV*-DNA was analyzed in the saliva samples using real-time quantitative polymerase chain reaction (RT-qPCR) method (LightMix Human *Cytomegalovirus*, TIB MOLBIOL, Germany). Subsequently, urine samples of newborns whose saliva was positive for *CMV*-DNA were tested for the presence of *CMV*-DNA using the same method. Urine samples were collected within the first three weeks of life. Data of total bilirubin, direct bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), complete blood count (CBC) and clinical findings of newborns diagnosed with c*CMV* infection were retrospectively analyzed.

Definitions of patients

The c*CMV* infection was diagnosed when *CMV*-DNA was detected in the newborn's urine. At least one of the following physical findings; cranial anomaly (microcephaly/ hydrocephaly), neurologic findings (lethargy/hypotonia, seizures) or radiologic findings such as chorioretinitis, intracranial calcification, intrauterine growth retardation, hepatomegaly, splenomegaly or laboratory findings of a direct bilirubin level higher than 2 mg/dL, ALT, AST or GGT levels at least twice the average and thrombocytopenia (<100000 mm^3/ mL) were considered evidence of symptomatic infection [14]. A birthweight of less than 2500 grams was diagnosed as low birthweight (LBW) irrespective of the week of gestation. Small for gestational age (SGA) was defined as birthweight below the 10^{th} percentile of the corresponding gestational week.

Evaluation of cCMV cases in their second year of life

The children diagnosed with c*CMV* infection were recalled in their second year of life to assess their physical, mental-motor development and hearing status. In our study, it was decided to follow-up on the c*CMV* cases at the age of two for research purposes. The complete audiological screening was performed and physical development was evaluated in c*CMV* cases at their admission in the second year of life following postpartum screening. *CMV*-DNA was measured by RT-qPCR (COBAS AmpliPrep/COBAS TaqMan, Roche Diagnostics) in urine samples. In addition, CBC and liver function tests were analyzed. Hearing status was determined in all infants using the TEOAE test, followed by an ABR test for those who failed the TEOAE test.

Statistical analysis

Data were analyzed in SPSS for Windows 23.0. Pearson chi-square test, Fisher's exact test, and Mann – Whitney *U* test were used to compare the results. Logistic regression was used to predict the relations between independent variables and binary logistic regression for dependent variables. Odds ratios (ORs) and 95% confidence intervals (CIs) were used to measure associations. $P < 0.05$ was considered significant.

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3. RESULTS

Newborn period

CMV-DNA was detected in the saliva samples of 38 (n = 1150, 3.31%) newborns. Urine samples were collected from 37 newborns and 10 were positive for *CMV*-DNA. The prevalence of c*CMV* infection was 0.87% (95% CI = 0.697 – 1.042) in our study group. The median day of saliva collection was 4 days (range: 0 – 18 days) in *CMV*-DNA detected newborns. The urine sample was collected within a few days after the saliva sample collection. The median day of urine collection was 6 days (range: 1 – 20 days) in newborns with c*CMV*. The characteristics of the newborns are summarized in Table I.

A total of 6 (60%) newborns were defined as symptomatic c*CMV* infected: Two with SGA, two with LBW and SGA, one with LBW, SGA and microcephaly, and one newborn with elevated GGT (three times higher than average), ALT/AST (two times higher than average) levels and thrombocytopenia (48000/mm3). Six infants had jaundice, but direct bilirubin levels were lower than 2 mg/dL. At birth, jaundice was 60% (6/10) and 5% (55/1140) (P < 0.001), LBW was 40% (4/10) and 12.5% (143/1140) (P = 0.029), and SGA was 50% (5/10) and 17% (194/1140) (P= 0.018) in newborns with and without c*CMV* infection, respectively. In multivariate analysis, the presence of jaundice was the most significant variable (P< 0.001, OR:23.411, 95% CI = 5.772 – 94.960) (Table II).

All newborns with c*CMV* infection passed the initial NHST. No significant relationship was found between sex, gestational week, presence of microcephaly/hydrocephalus, NHST results and c*CMV* infection. Two fetuses with c*CMV* infection were found to have oligohydramnios during pregnancy. Postnatal ophthalmologic examination of the children who participated in the study revealed no pathologic findings.

Main findings in children with cCMV

Only eight families agreed to be included in the screening program. Thus, 8 out of 10 c*CMV* cases were screened for c*CMV*-related complications in the second year of life. *CMV*-DNA was detected in the urine of eight c*CMV* cases. Case 9 had moderate mental-motor growth retardation and strabismus, enlargement of the lateral ventricles and mineralization of brain tissue on magnetic resonance imaging. Case 1 did not pass the TEOAE test in the left ear when she was admitted for screening at age two, although she had passed the initial hearing screening test with TEOAE for both ears in the newborn period. Then an ABR test was performed, which revealed a SNHL of 90 dB in the left ear. Laboratory screening revealed that four infants had slightly elevated AST levels (Table III).

Table I. *The characteristics of newborns with cCMV infection*

Case No	Sex	Birthweight (g)	Gestational week	LBW	SGA	Urine *CMV*-DNA	Jaundice	Total Bilirubin (mg/dL)[a]	Direct Bilirubin (mg/dL)[b]	ALT (U/L)[c]	AST (U/L)[d]	GGT (U/L)[e]	Microcephaly	Hearing Loss	Symptom
1	F	2100	40	+	+	+	+	4.61	0.26	N	N	N	-	-	+
2	F	3000	40	-	+	+	-	NA	NA	NA	NA	NA	-	-	+
3	F	3000	40	-	+	+	+	12.38	0.48	N	N	N	-	-	+
4	M	3580	39	-	-	+	+	13.94	0.39	N	N	N	-	-	-
5	M	3900	39	-	-	+	-	NA	NA	NA	NA	NA	-	-	-
6	M	4170	40	-	-	+	-	NA	NA	NA	NA	NA	-	-	-
7	M	1406	29	+	-	+	+	5.28	0.25	N	N	N	-	-	-
8	M	1744	34	+	+	+	+	13.90	0.39	N	N	N	-	-	+
9	M	1340	32	+	+	+	-	NA	NA	NA	NA	NA	+	-	+
10	F	3310	38	-	-	+	+	17.80	0.80	44	59	135	-	-	+[#]

cCMV: Congenital Cytomegalovirus, F: Female, LBW: Low birthweight, M: Male, N: Normal, NA: Not applied, SGA: Small for gestational age, aTotal bilirubin reference range: 0,1 – 1,2 mg/dL, bDirect bilirubin reference range: 0,0 – 0,2 mg/dL, cALT reference range: 0 – 33 U/L, dAST reference range: 0 – 32 U/L, eGGT reference range: 5 – 40 U/L, # with thrombocytopenia

Table II. *The relation of cCMV infection with presence of LBW, SGA, and jaundice*

	n (%)*	Odds Ratio and 95% Confidence Interval	Multivariate analysis	Univariate analysis, n(%)
LBW	4 (40)	OR: 0.880, 95% CI (0.118 – 6.554)	P = .901	P< .001, 143 (12.5)
SGA	5 (50)	OR: 3.295, 95% CI (0.669 – 16.219)	P = .143	P = .029, 194 (17)
Jaundice	6 (60)	OR: 23.411, 95% CI (5.772 – 94.960)	P < .001	P= .018, 55 (5)

cCMV: Congenital Cytomegalovirus, LBW: Low birthweight, SGA: Small for gestational age
** Distribution of cCMV cases according to symptoms.*

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Table III. *The characteristics of children aged two with cCMV infection*

Case No	Urine *CMV*-DNA		Hearing Loss		Mental Motor Retardation		Growth Retardation		ALT* (U/L)		AST# (U/L)	
	Newborn	2nd year	Newborn	2nd year	Newborn	2nd year	Newborn	2nd year	Newborn	2nd year	Newborn	2nd year
1	+	+	-	+	-	-	-	-	N	N	N	42
2	+	+	-	-	-	-	-	-	NA	N	NA	35
4	+	+	-	-	-	-	-	-	N	N	N	N
5	+	+	-	-	-	-	-	-	NA	N	NA	N
7	+	+	-	-	-	-	-	-	N	N	N	44
8	+	+	-	-	-	-	-	-	N	N	N	39
9	+	+	-	-	–	+	–	+	NA	N	NA	N
10	+	+	-	-	-	-	-	-	44	NA	59	NA

cCMV: Congenital Cytomegalovirus, N: Normal, NA: Not applied
*Case no 3 and 6 did not apply for 2nd year follow-up *ALT reference range: 0 – 33 U/L,*
#AST reference range: 0 – 32 U/L

4. DISCUSSION

The prevalence of c*CMV* infection was found to be 0.87% in our study group, which is the largest study group in Turkey to investigate c*CMV* prevalence. There are only two studies on the prevalence of c*CMV* infection in our country and very different results were found. In the first study, Şahiner et al., reported that the prevalence of c*CMV* infection in 944 newborns with *CMV*-DNA PCR in saliva was 1.91% [9]. This study reported that the *CMV*-DNA PCR test in urine was negative in five out of 18 infants who had positive *CMV*-DNA PCR test in saliva. In 2017, the European Expert Statement on Congenital *CMV* Diagnosis and Management reported that a single negative *CMV*-DNA result in urine is sufficient to rule out c*CMV* infection [14]. When the study of Şahiner et al., was evaluated according to this criterion, the prevalence of c*CMV* infection was 1.38% [9]. The rate was higher compared to our results. In the second study, Zeytinoglu et al., found that the prevalence of c*CMV* infection was 0.2% in 1000 newborns [10]. This rate seems low for a population with high seroprevalence and is lower than the rate we found. These differences could be due to the different methods used. To clarify, unlike the phenol-chloroform extraction and in-house PCR assay used by Şahiner et al.[9], standardized commercial kits were used in our study. The saliva collection method in Zeytinoglu et al.'s study differed from ours, and they did not use a standardized commercial kit for *CMV*-DNA isolation [10]. In addition, altering of the *CMV* seropositivity of mothers according to different ethnicities, races, and socioeconomic statuses in the same geography can also affect the epidemiology of c*CMV* infection [15]. In countries with high *CMV* seroprevalence, the prevalence of c*CMV* infection ranges from 0.65% to 5.4%; 0.89% in Mexico, 1.19% in Brazil, 0.7% in Israel, 0.7% in China, 5.4% in Gambia, 2.1% in India, and 0.65% in Iran [16-22].

In this study, *CMV*-DNA was not detected in the urine of 27 (73%) of the 37 newborns whose saliva was positive for *CMV*-DNA. The absence of *CMV* in the urine sample excludes the diagnosis of c*CMV* infection [14]. Previous studies have shown that *CMV*-DNA testing in saliva is as sensitive and specific as *CMV*-DNA testing in urine and that the saliva sample is easier to collect than the urine sample [23]. However, *CMV* also occurs in breast milk and causes postnatal *CMV* transmission [24]. In our country, breastfeeding rates are reported to be 96% – 97.4%[25,26]. Since, breastfeeding rates and early initiation of breastfeeding are high in our country, *CMV*-DNA can be detected in saliva without the presence of congenital infection. Koyano et al., collected urine and saliva samples from newborns whose mothers had positive *CMV*-DNA in their milk immediately before and within 30 minutes after breastfeeding [27]. *CMV*-DNA was found positive only in saliva samples collected after breastfeeding. In our study, breastfeeding status was not recorded when the saliva samples were collected. However, since 73% of the newborns with *CMV* positive saliva did not have *CMV* in their urine, it can be assumed that these positive saliva results are related to breastfeeding, considering the breastfeeding rates in our country. Therefore, urine should be tested for accurate diagnosis of c*CMV* infection in populations with high breastfeeding rates. Koyano et al., also recommended urine sample testing in populations where breastfeeding is common [27]. In addition to specimen selection, it has been reported that the collection of urine specimens on filter paper is sufficient to detect *CMV*-DNA [28].

Microcephaly, thrombocytopenia, SGA and jaundice (direct bilirubin> 2 mg/dL) are the symptoms described in association with symptomatic c*CMV* infection in a recent report [14]. In this study, the rate of symptomatic c*CMV* infection was 60% of newborns with c*CMV* infection. Jaundice in newborns was statistically associated with c*CMV* infection; however, the direct bilirubin level did not exceed 2 mg/dL in any of them. For this reason, jaundice was not considered as the sole symptom. It was reported that about 10% – 15% of infants with c*CMV* had very mild and nonspecific symptoms such as jaundice, hepatosplenomegaly, and LBW. Therefore, *CMV* has not been investigated in these groups of newborns [29]. Immunity in seroimmune mothers may alleviate symptoms in the baby. Fowler et al., reported that the presence of *CMV* antibodies

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before pregnancy protected the fetus from *CMV* infection and reduced the severity of symptoms and sequelae even if they did not protect it [30]. Lilleri et al., showed an early and robust neutralizing antibody response to the gH/gL/pUL128-130-131 pentameric complex in pregnant women who did not transmit *CMV* infection to their babies [31]. In addition, it was reported that the proliferative CD4 T-cell response to the pp65 antigen of the virus was high in pregnant women who did not transmit *CMV* infection to their babies [32]. According to these results, the degree of *CMV* specific maternal immunity may also play a role in the different c*CMV* prevalence rates in the same geography. In our study, maternal serology was not investigated. However, *CMV* seroprevalence is 93.6% in society and 97.4% in fertile women in our city, Antalya [5]. In studies conducted with pregnant women in different cities of our country, the rate of *CMV* seropositivity ranged from 98.3% to 100% [6-8]. Considering these data, all mothers of almost all babies with c*CMV* infection can be considered *CMV* seroimmune.

Although, eight newborns diagnosed with c*CMV* infection passed the NHST, left ear SNHL was detected in one child by ABR test during the screening at two years of age. In this case, symptoms at birth were nonspecific and at first glance did not suggest c*CMV* infection; however, hearing loss developed in the following years. In children, bilateral SNHL is caused by *CMV* in second place after genetic defects in the United States [2]. In our country, the prevalence of deafness in infants with probable c*CMV* infection is reported to be 4.08% [33]. Grosse et al., reported that about 14% of children with c*CMV* infection have permanent SNHL and about half of them are asymptomatic at birth and hearing loss develops later, so these children passed NHST after birth [2]. Fowler et al., reported that 43% of infants with c*CMV* infection who had hearing loss in infancy could not be [34] detected by the NHST. A systematic review by Fletcher et al., reported that 9% – 68% of babies with c*CMV* infection had late-onset hearing loss [35]. The results of these studies also support our findings. When hearing loss related to c*CMV* infection is diagnosed at an early stage, it is reported that the effect can be reduced by antiviral treatment, cochlear implantation and speech therapy [1]. Therefore, asymptomatic newborns with c*CMV* infection should be screened for late sequelae such as hearing loss. Currently, no country has a routine screening program for the diagnosis of c*CMV* infection. It is argued that screening should be targeted at all newborns or those who do not pass the NHST. According to the results of our study and the studies mentioned above, the selection of babies who do not pass the NHST is not sufficient for the diagnosis of c*CMV* infection because the NHST is not able to identify newborns with c*CMV* infection, as found in our study and screening should be applied for all newborns [2,34,35].

In c*CMV* infection, neurological sequelae such as microcephaly, intracranial calcifications, ventricular dilatation and cortical atrophy are encountered [11]. In our study, one patient who had microcephaly in the neonatal period was found to have mental-motor developmental retardation and growth retardation in the second year of follow-up (Case 9). In case of neurological involvement or severe regional organ damage in symptomatic c*CMV* cases, treatment protocols with antiviral agents should be considered, taking into account the possible side effects [14]. However, there was no case receiving antiviral treatment in our study. While it is known that antiviral treatment can improve hearing and neurologic development in the long-term in symptomatic cases, there is no evidence that it prevents the occurrence of late-onset disease in asymptomatic cases of c*CMV* [14].

The main limitation of our study is that the screening program covered only a single time point at two years of age and was not performed for all newborns with c*CMV*.

In children of pregnant women with nonprimary *CMV* infection, c*CMV* infection may present with nonspecific findings and therefore be overlooked after delivery. However, as observed in children of pregnant women with primary *CMV* infection, sequelae may occur in the following years. In our study, bilirubin levels were slightly elevated in c*CMV* cases. This finding may be overlooked in the diagnosis of c*CMV* infection. For this reason, it would be beneficial to implement screening programs in highly seroimmune populations.

Conclusion

This is the first study in our country in which infants with c*CMV* infection were followed-up and their laboratory and clinical findings were recorded in the following years and remarkable results were obtained. The prevalence of c*CMV* infection was 0.87% in a population with high maternal *CMV* seroprevalence. All newborns diagnosed with c*CMV* passed the NHST, and the target of c*CMV* screening should be all newborns. When diagnosing c*CMV* infection, the urine sample should be tested, as saliva samples may give false positive results. However, since it is difficult to collect a urine sample, saliva can be tested first and urine can be examined in those who are positive for *CMV*-DNA. Since, hearing loss develops after two years in a child with nonspecific symptoms at birth, it should be kept in mind that symptoms during birth may be mild in newborns of *CMV* seropositive mothers. More comprehensive and long-term studies can guide c*CMV* infection.

Compliance with Ethical Standards

Ethical approval

This study was approved by the Akdeniz University Ethics Committee (June 19, 2012, Approval Number 208) and conducted following the guidelines of the 1964 Helsinki Declaration. Parents were informed about the study and written informed consent was obtained.

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Conflict of interest

No potential conflict of interest was reported by the authors

Authors Contributions

ZES: Data curation, Formal analysis and Writing-Original draft preparation, BOP: Formal analysis, Visualization, Writing-Original draft preparation, DC: Conceptualization, Methodology, Data curation and Writing-Reviewing and Editing, MT, ABT, NO and ME: Investigation, Data curation, IS, RCS, DM and GO: Formal analysis, Writing – Reviewing and Editing.All authors approved the final version of the manuscript.

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