Newborn Screening for Congenital Cytomegalovirus Using Real-Time Polymerase Chain Reaction in Umbilical Cord Blood

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ABSTRACT: **Background:** Congenital cytomegalovirus (C-CMV) infection affects 0.4–2% of newborn infants in Israel, most of whom are asymptomatic. Of these, 10–20% will subsequently develop hearing impairment and may have benefitted from early detection by neonatal screening.

Objectives: To retrospectively analyze the results of a screening program for C-CMV performed at the Sheba Medical Center, Tel Hashomer, during a 1 year period, using real-time polymerase chain reaction (rt-PCR) from umbilical cord blood.

Methods: CMV DNA was detected by rt-PCR performed on infants' cord blood. C-CMV was confirmed by urine culture (Shell-vial). All confirmed cases were further investigated for C-CMV manifestations by head ultrasound, complete blood count, liver enzyme measurement, ophthalmology examination and hearing investigation.

Results: During the period 1 June 2009 to 31 May 2010, 11,022 infants were born at the Sheba Medical Center, of whom 8105 (74%) were screened. Twenty-three (0.28%) were positive for CMV and 22 of them (96%) were confirmed by urine culture. Two additional infants, who had not been screened, were detected after clinical suspicion. All 24 infants were further investigated, and 3 (12.5%) had central nervous system involvement (including hearing impairment) and were offered intravenous ganciclovir for 6 weeks. Eighteen infants (82%) would not otherwise have been diagnosed.

Conclusions: The relatively low incidence of C-CMV detected in our screening program probably reflects the low sensitivity of cord blood screening. Nevertheless, this screening program reliably detected a non-negligible number of infants who could benefit from early detection. Other screening methods using saliva should be investigated further.

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KEY WORDS: congenital cytomegalovirus (C-CMV), congenital infection, newborn screening, polymerase chain reaction (PCR), hearing loss

Cytomegalovirus infection is the most common cause of congenital infection in the developed world [1]. According to a previous report, 0.4–2% of Israeli infants excrete CMV in their urine [2]; yet CMV screening during pregnancy is not recommended by Israeli health authorities. Only approximately 10% of infected infants have a symptomatic infection at birth [3]. When symptoms of central nervous system are apparent, treatment with ganciclovir may prevent deterioration of hearing [4]. Yet most newborns infected with CMV are born without any apparent symptom of infection. Of these, 10–20% will subsequently develop hearing loss and they will not be detected through the in-hospital early universal hearing screening program (late-onset hearing impairment) [5]. Such children should undergo periodic hearing evaluation since speech prognosis is dependent on prompt diagnosis and adequate hearing habilitation [6].

The high infection rate of congenital CMV, the significant disability it can cause, the availability of treatment to prevent progression of hearing loss, and the fact that early detection of hearing loss can improve speech outcome make C-CMV a good candidate for infant screening. Virus isolation in cell culture from urine or saliva during the first 3–4 weeks of life is considered the gold standard test for diagnosing C-CMV. Urine collection from newborn infants and cell culture techniques are labor-consuming and are therefore not suitable for screening large populations. Real-time polymerase chain reaction technology is suitable for mass screening as it can be performed using automated techniques. Since dried blood spot specimens, used to screen for metabolic diseases, are available for almost all infants, several authors have attempted to use DBS samples to diagnose C-CMV with rt-PCR [7-11]. Detection of CMV DNA directly from umbilical venous blood using rt-PCR has been proposed as a simpler,

CMV = cytomegalovirus
C-CMV = congenital CMV
DBS = dried blood spot
rt-PCR = real-time polymerase chain reaction

non-invasive method for early and accurate diagnosis of C-CMV [12].

The aim of this study was to retrospectively analyze the results of a screening program for the detection of C-CMV in infants born in a large medical center in Israel during a 1 year period, using rt-PCR from umbilical venous cord blood.

PATIENTS AND METHODS

SAMPLE COLLECTION

From 1 June 2009 to 31 May 2010, venous umbilical cord blood samples were collected at birth from infants born at the Sheba Medical Center, Tel Hashomer. The samples were refrigerated at 4°C and transferred to the virology laboratory in EDTA-containing tubes within 48 hours. Mothers were informed of the screening project through a leaflet distributed on their arrival at the delivery room and were permitted to refuse the test.

VIROLOGICAL STUDIES

DNA was extracted from 200 µl umbilical blood using the MagNaPure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics GmbH, Germany), according to the manufacturer's instructions. The CMV viral genome was detected by rt-PCR. The ABI 7700 rt-PCR system (Applied Biosystems Inc., Israel) was utilized to detect the highly conserved region of the CMV major envelope glycoprotein B gene. All positive specimens were retested for confirmation, and specimens were considered positive if at least 10 or more genomic equivalents per reaction were detected on both PCR runs. C-CMV infection was confirmed by urine CMV culture (Shell-vial assay, using CMV Pre-CPE Culture Identification test, Light Diagnostics™, UK) obtained during the first 3 weeks of life. Infants with clinical suspicion of C-CMV, either due to maternal seroconversion during pregnancy or because of clinical symptoms suggesting congenital infection, were tested for CMV by urine culture, regardless of rt-PCR result. Infants with a positive Shell-vial test were referred to the pediatric infectious diseases clinic for workup and follow-up. DBS samples from infants with confirmed C-CMV taken at 48 hours for metabolic diseases were retrieved, and one complete blood spot was cut from the filter paper by scissors (a new pair of scissors was used for each specimen). The blood spot was then added to a tube containing 2 ml of NucliSENS Lysis Buffer and incubated in a horizontal position on a roller mixer for 30 minutes at room temperature. The fluid was then transferred to a new tube and extracted by the automatic system NucliSENS EasyMag (BioMerieux, France) according to the standard instructions. PCR was carried out by ABI rt-PCR system, as mentioned previously.

The workup for infants with confirmed C-CMV infection included maternal and fetal pregnancy history, physical and neurological examination, complete blood count, serum liver enzymes, head ultrasound and retinal examination.

HEARING EVALUATION

All infants were tested using transient evoked otoacoustic emissions (TEOAE) as part of a hearing screening test performed in all infants prior to hospital discharge. Furthermore, all infants underwent an auditory brainstem response within 10 days after discharge. Periodic audiological follow-ups were also carried out in accordance with the recommendations of the Joint Committee on Infant Hearing 2007 Position Statement [13], which requires frequent audiological assessment for infants with congenital CMV. Specifically, infants underwent follow-ups at regular intervals, including behavioral audiometry, TEOAE and when necessary repeated ABR at 3 month intervals until the age of 1 year and at 6 month intervals until the age of 2 years. Subsequently, they will continue to undergo hearing evaluations once a year until reaching the age of 6. Hearing impairment was defined as unilateral or bilateral sensory-neural hearing loss greater than 25 dBHL in the 500–4000 Hz frequency region. HI severity was determined based on the following classification: mild HI (26–40 dBHL), moderate HI (41–55 dBHL), moderately severe HI (56–70 dBHL), severe HI (71–90 dBHL), and profound HI (> 90 dBHL) [14].

In cases of symptomatic C-CMV involving the CNS, a 6 week course of intravenous ganciclovir 6 mg/kg twice daily was offered. Periodic follow-up visits at the pediatric infectious diseases clinic took place at the ages of 3, 6 and 12 months, or more often if needed. Approval by the local ethics committee was obtained for retrospective analysis of data collected during the screening period.

RESULTS

From 1 June 2009 to 31 May 2010, 11,022 live infants were born at the Sheba Medical Center [Figure 1]. In 8105 of these births (74%), a venous cord blood sample was obtained for rt-PCR. Twenty-three samples (0.28%) were found positive for CMV, and all except one (22, 0.27% of total samples) were confirmed by urine culture. In addition, during this period, two infants with clinical suspicion of C-CMV, for whom a cord blood sample was not available for rt-PCR, tested positive for CMV by urine culture. Altogether, 24 infants with confirmed C-CMV were referred for further workup and follow-up at the pediatric infectious diseases clinic and at the speech and hearing center. For 113 additional infants with clinical suspicion of C-CMV, both cord blood rt-PCR and urine culture were negative for CMV, and congenital infection was ruled out. For 20 of the 24 cases positive for C-CMV, DBS was available and tested positive by real-time PCR.

TEOAE = transient evoked otoacoustic emissions
ABR = auditory brainstem response
HI = hearing impairment
CNS = central nervous system

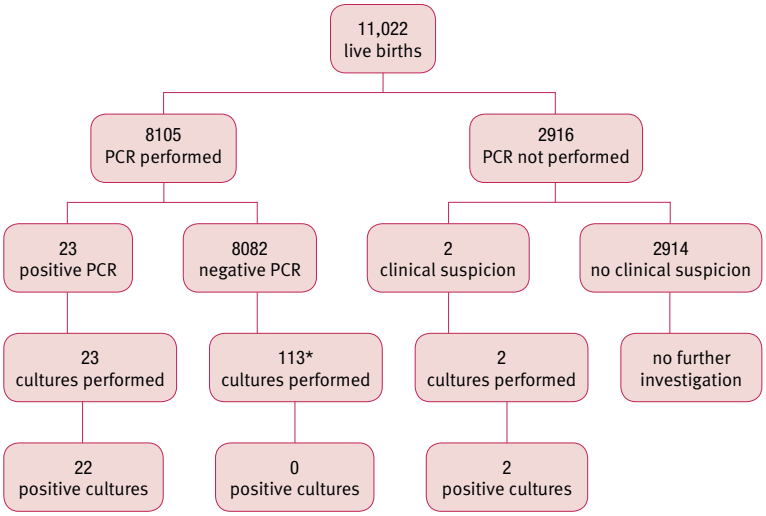
MATERNAL CHARACTERISTICS

Mean maternal age was 30 years + 2 months (± 4.7 years). In 5 cases (21%) this was a first pregnancy. In 23 cases (96%) CMV serology was performed during or before pregnancy. According to maternal serological tests in 13 cases (57%), transmission occurred during a primary infection, and in 16 cases (67%) the exact timing of seroconversion was not documented. In 10 cases (43%) the mothers were immune before pregnancy, suggesting transmission during reactivation or re-infection with another strain of CMV. In 18 (80%) of the pregnancies there was no suspicion of C-CMV and the infants would not have been diagnosed without the screen.

INFANT CHARACTERISTICS [TABLE 1]

Twelve infants (50%) were male, mean gestational age was 38 weeks + 5 days (± 1.4 weeks), mean birth weight was 3128 g (± 502 g), and mean head circumference was 33.8 cm (± 1.4 cm). All 24 infants completed the full workup for symptoms of C-CMV. For all infants, complete blood count and liver enzymes were within normal range. None of the infants had retinal findings. In 21/24 infants (87.5%) the workup did not detect any abnormal finding consistent with C-CMV. In three infants (12.5%) CNS symptoms were detected and ganciclovir treatment was offered. The findings for these three infants are summarized as follows: one infant had mild unilateral sensory-neural hearing loss (30 dB, left ear) on ABR and no other symptom. This child had passed the hearing screen. One infant had lenticulostriate vasculopathy on head ultrasound and high signal intensity lesions on magnetic resonance imaging, and normal hearing. One infant had multiple periventricular cysts on repeated ultrasounds, with normal MRI and normal hearing. In two of the three infants with symptomatic C-CMV (67%), maternal infection was primary. Two infants whose workups revealed only lenticulostriate vasculopathy with no other symptom (repeat ultrasound was normal in one and MRI was normal in the other) were not classified as symptomatic and were not offered ganciclovir.

Figure 1. Patient enrollment



* Cultures were performed upon clinical suspicion of congenital cytomegalovirus due to maternal seroconversion during pregnancy or due to a clinical symptom in the newborn.
PCR = polymerase chain reaction

DISCUSSION

This study presents the results of a novel newborn screening method for C-CMV. Only one previous pilot study used umbilical blood to screen infants for C-CMV [12] where 2 of the 433 infants tested (0.5%) were positive. However, in that study no information regarding the infants' clinical findings was available. Furthermore, urine cultures were not taken for comparison. Therefore, ours is the first report of a large-scale screening project using umbilical cord blood to detect C-CMV in which positive cases were confirmed by urine cultures and thorough clinical investigation of infected infants was performed. We screened 8105 neonates born at Sheba Medical Center during a 1 year period and detected 22 positive infants (0.27%), of whom 18 (82%) would not have been diagnosed otherwise.

The only previous report on rates of C-CMV in Israel used PCR to detect CMV DNA in urine samples of infants randomly selected at two hospitals serving different populations [2]. The results showed that at Shaare Zedek, a hospital in Jerusalem serving a primarily Jewish and urban population, rates of C-CMV were 1%. At HaEmek Hospital in Afula, where half the patients were Jewish and only 61% were urban, the rates were 0.4%. The combined incidence calculated in that report was 0.7%. Using rt-PCR from umbilical cord blood, we found lower rates (0.27%) of C-CMV. Several reasons can be speculated to explain this lower rate. One explanation could be a high rate of abortions in a population in which CMV screening during pregnancy is prevalent. Although no data exist regarding abortion rates due to CMV infection during pregnancy, the rates are presumed to be higher than at both described hospitals, since a large proportion of patients at Shaare Zedek Hospital are Orthodox Jews and most of the non-Jewish patients at HaEmek Hospital are Muslims. Both religions oppose abortion. The Sheba Medical Center is located in an urban area serving a population of which around two-thirds are secular Jews, among whom the abortion rates are probably higher.

Another explanation for this low rate could be low sensitivity of the screen. In a recent publication by Boppana et al. [15], rt-PCR was performed on DNA extracted from DBS samples and compared to rt-PCR from saliva specimens. The results showed that DBS was inferior to saliva in detecting CMV DNA, and when a single primer was used it detected only 28.3% of cases.

Table 1. Clinical characteristics of 24 infants with congenital CMV

Patient no.	Gender	Gestational age (wk)	Birth weight (g)	Head circumference (cm)	Head ultrasound	Head MRI	Retinal examination	ABR
1	F	39	3245	33	LSV	ND	Normal	Normal
2	F	40	3660	ND	Normal	ND	Normal	Normal
3	F	38	4075	35.5	Normal	ND	Normal	Normal
4*	M	40	3575	35	LSV	Multiple calcifications	Normal	Normal
5	M	40	3360	35	Normal	ND	Normal	Normal
6	M	37	2235	31	Normal	ND	Normal	Normal
7	M	39	3360	35	Normal	ND	Normal	Normal
8	F	39	3420	35.5	Normal	ND	Normal	Normal
9	F	41	3166	34	Normal	Normal	Normal	Normal
10	M	40	3250	34.5	Normal	ND	Normal	Normal
11	M	39	3590	35.5	Normal	ND	Normal	Normal
12*	M	38	3025	33	Normal	ND	Normal	Mild SNHL
13	M	40	3385	35	Normal	ND	Normal	Normal
14	F	36	2805	33	Normal	ND	Normal	Normal
15	F	40	3140	33.5	Normal	ND	Normal	Normal
16	F	38	3018	34	Normal	ND	Normal	Normal
17	F	38	2165	31.5	Mild LSV	Normal	Normal	Normal
18	F	38	2415	31.5	Suspected calcification	Normal	Normal	Normal
19	M	39	4055	35	Normal	ND	Normal	Normal
20	M	35	2595	33	Normal	ND	Normal	Normal
21*	M	38	2615	32	LSV, cysts	Normal	Normal	Normal
22	F	39	3045	33	Normal	ND	Normal	Normal
23	F	40	2750	33.5	Normal	ND	Normal	Normal
24	M	40	3130	34.7	Normal	ND	Normal	Normal
Mean (± SD)		38 + 5 days (± 1.4)	3128 (± 502)	33.8 (± 1.4)				

*Patients treated with ganciclovir
ABR = auditory brainstem response, ND = not done, LSV = lenticulostriate vasculopathy, SNHL = sensory neural hearing loss

This low sensitivity probably results from the fact that only a minority of infected infants are viremic at birth, whereas practically all of them excrete the virus in urine and saliva [16].

To determine the sensitivity of the screen, we should have taken urine cultures (gold standard) from randomly selected infants. Although this was not done and therefore sensitivity could not be calculated, urine cultures were taken from 113 infants who were clinically suspected of having C-CMV and were negative on rt-PCR. In all cases, the urine culture was negative as well. If we assume that rt-PCR from DBS has approximately the same sensitivity as that performed on umbilical blood samples, since both reflect the presence of viral DNA in blood, this would mean that with our screening method we detected only about one-third of the actual C-CMV cases [15].

As previously described regarding PCR-based screening for CMV using DBS specimens, specificity approaches 100% [17]. The fact that in 8105 tests performed there was only one false positive result not only confirms the high specificity of the screen but also implies that the umbilical venous blood was not contaminated by maternal blood, in which case maternal viremia during labor could result in a positive rt-PCR finding in umbilical cord blood.

We detected 18 infants who were not suspected of having acquired CMV during pregnancy and would not have otherwise been diagnosed. Of these, two infants were found to have a symptomatic infection at birth and were offered treatment with ganciclovir. The asymptomatic infants were offered long-term follow-up, and some will potentially benefit from early detection of late-onset sensory neural hearing loss.

When analyzing the impact of infant screening for C-CMV, one must also take into consideration the functional and emotional effects of revealing this diagnosis to families whose

infants will need to undergo a thorough evaluation and long-term follow-up and most will end up being asymptomatic. Even in the case of detected CNS symptoms or hearing impairment, the true impact of intervention is still unclear.

To complete the evaluation of morbidity related to CMV infection in our patients, a final evaluation should be performed at the age of at least 5 years, since a good developmental evaluation is impossible during the first year of life and because late hearing deterioration has been described in as many as 15% of children who were asymptomatic at birth [3].

Among 8105 who were tested, we detected only 2 symptomatic infants who would not have been diagnosed without the screen and to whom we could offer intervention with ganciclovir (in the third symptomatic case, CMV seroconversion was documented during pregnancy and the mother refused amniocentesis). This means that in order to find one infant who would potentially benefit from the screen directly after birth, we would need to perform approximately 4000 tests. Therefore, although cost-effectiveness was not calculated, a more sensitive screening method that can be performed in high-throughput is clearly needed. The saliva of infants infected with CMV contains large amounts of CMV DNA that could be detected by PCR and is easy to collect with oral swabs [15,17]. This method was recently proven highly sensitive and specific for the detection of C-CMV [18] and therefore seems to be a good candidate for C-CMV screening in the future.

CONCLUSIONS

The relatively low incidence of C-CMV detected in our screening program probably reflects the low sensitivity of cord blood screening. Nevertheless, this screening program detected a non-negligible number of infants who were offered a workup for CMV manifestations and long-term clinical and hearing follow-up. Other methods using saliva, which contains large amounts of CMV and is easy to collect, have the potential to be more sensitive and should be investigated further.

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"Too often we underestimate the power of a touch, a smile, a kind word, a listening ear, an honest compliment, or the smallest act of caring, all of which have the potential to turn a life around"

Leo Buscaglia (1924-1998), American author and motivational speaker, and a professor in the Department of Special Education at the University of Southern California. He was also known as "Dr. Love"

"Without the freedom to criticize, there is no true praise"

Pierre Beaumarchais (1732-1799), French playwright