Original Article

Detection and gB genotyping of CMV in Mexican preterm infants in the context of maternal seropositivity

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Abstract

Introduction: Congenital (CI) and perinatal cytomegalovirus (CMV) infections (PI) can be linked to maternal CMV seropositivity, with fatal consequences in preterm newborns. GB genotyping has been used to analyze genotypic similarity in mothers and infants. The frequency of CMV infection in the context of maternal seropositivity and the viral gB genotypes as well as the genotypic similarity in mothers and preterm infants were investigated.

Methodology: Saliva samples and dry blood spots (DBS) were taken weekly from preterm newborns from birth until the first month of life, and breast milk samples were taken from their mothers weekly during the first month of lactation. CMV IgG seroprevalence of the mothers and CI or PI in the infants were established. The gB status and genotypic similarities were established retrospectively in DBS and in the breast milk samples.

Results: In total, 387 neonates and 375 mothers were enrolled. The maternal CMV-positive IgG serology was 97.3% (365/375). Neonatal CMV was found in 5.1% (20/387) of newborns, and one infant presented with CMV-compatible symptoms. CI was 2.5% and PI in the first month after birth was 11.8%. GB2 was the most prevalent genotype and was also the genotype preferentially transmitted to newborns by mothers with mixed infections.

Conclusions: CMV PI and CI in preterm infants from highly seropositive mothers was high, but the rate of symptomatic infection was low. The prevalent genotype was gB2, and this genotype was preferentially transmitted to newborns by mothers with mixed infections.

Key words: seroprevalence; active CMV infection; congenital infection; perinatal infection; *gB* genotype

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Introduction

Human cytomegalovirus (CMV) is the virus most frequently identified in newborns and is associated with both congenital infections (CI) and perinatal infections (PI) [1,2]. The incidence of CI varies between 0.2% and 2.5% in all live births in both preterm and full-term newborns [3,4]. Several studies of PI, mainly in preterm infants, have shown a wide range of prevalence, from 5% to 50% [5,6]. Most newborns with CMV CI are asymptomatic, but 13%–15% of them are at risk of progressive hearing loss and neurological sequelae [2]. Symptomatic babies with CI (10%–15%) show slightly increased mortality. These infants will also develop disabling sequelae in the long term, which will affect the quality of their lives [6,7]. Although CMV PI should have no consequences in full-term newborns, it is particularly important in preterm newborns because symptomatic infections have been reported, with severe consequences. Breastfeeding continues to be the most frequent method of CMV transmission (5%–50%) [5,8].

Genotypic differences in the CMV envelope proteins, such as gB, have been proposed as markers of CMV pathogenicity; these can help to detect mixed infections, and to establish the genotypic similarities among viruses identified in different samples from a single patient [9,10].

CMV infections in neonates can be linked to maternal CMV seropositivity and the stage of prematurity, and the weight at delivery can have

important consequences for the clinical behaviour of the CMV infection in newborn infants [11,12]. Despite improvements in health services directed toward reducing neonatal mortality in Mexico, the occurrence of preterm births continues to be high, ranging between 4% and 12%, depending on several risk factors. Poverty is recognized as one of these factors [13]. The population of Mexico is economically heterogeneous, and CMV seropositivity can vary according to the population group analyzed. The frequency of CMV infection in preterm infants in the context of maternal seropositivity in a Mexican population from a third-level hospital attended specifically by women with low socio-economic resources was established. The risk factors evaluated were prematurity and weight at delivery. The distribution of the gB genotypes in the newborn infants and the genotypic similarities between the CMV identified in the blood of the newborns under intensive care in a neonatal intensive care unit (NICU) and that in the milk of their mothers was analyzed as well.

Methodology

Study and patients

Between December 2009 and August 2011, a group of preterm neonates admitted to the NICU of the Hospital de la Mujer, administered by the Ministry of Health of Mexico, in Mexico City, were evaluated to detect CMV CI or PI in weekly follow-up examinations within the first month of life. The inclusion criteria specified preterm newborns who were at risk of developing sepsis, respiratory distress syndrome, or perinatal asphyxia. All preterm infants born after gestation periods of less than 37 weeks were included. Preterm deliveries are those that occur at a gestational age of less than 37 weeks, and preterm births can be subdivided according to gestational age: born at less than 28 weeks (extreme prematurity), between 28 and 31 weeks (severe prematurity), between 32 and 33 weeks (moderate prematurity), and between 34 and 36 weeks (late prematurity). The neonates were also classified according their birth weights: low weight (< 2500 g), very low weight (< 1500 g), and extremely low weight (< 1000 g) [13,14]. Informed consent was obtained from the mother for the inclusion of each infant in the study. The study was approved by the Ethical Committee of the hospital (HIM/2009/040 and HIM/2009/01).

Serology

The presence of CMV-directed immunoglobulin G (IgG) antibodies was evaluated at the puerperal phase with the Platelia CMV Kit (cat. nos72680; BioRad Laboratories, Richmond, CA, USA), according to the instructions of the manufacturer.

Sampling

Saliva samples and dry blood spots (DBSs) were obtained in a weekly follow-up during the first month of life as described previously [15]. Briefly, saliva samples were obtained at least 2 hours before breastfeeding using a sterile cotton swab until the applicator was soaked, and were transported immediately to the laboratory in a transport medium. Additionally, a sample of breast milk (5 mL) was also collected weekly during the first month of lactation and stored at -70°C until it use. All newborns were evaluated by an expert group of pediatric infectious disease specialists and neonatologists, who applied the selection criteria to the patients.

Sample culture

The saliva samples were cultured in confluent monolayers of human foreskin fibroblast cells. The cultures were analyzed daily. When the cytopathic effect (CPE) was observed, the monolayer was scraped and tested for CMV PP65 antigen by immunofluorescence using the kit Light Diagnostics CMV pp65 Antigenemia (Millipore Cat #3247, Millipore, Watford, UK). The plates were discarded as negative after 30 days without a CPE.

DNA extraction

DNA was extracted from saliva and DBS samples from the newborn preterm infants; these samples and breast milk were processed as described previously, and DNA was extracted by the method of phenol-chloroform [16,17].

PCR assay

To confirm the results of the culture, the saliva samples were analyzed by qualitative PCR [17]. To corroborate the DNAemia in the neonatal DBS samples and DNAlactia in the maternal samples before genotyping, the DNA from each sample was analyzed with nested PCR using primers designed to amplify the fourth exon of the CMV *ie1* gene, as described previously [17].

GB genotyping

To determine the CMV genotypes in the newborns and to assess whether the virus identified in the blood of each neonate was genotypically similar to that of the mother, the DNA from the DBSs and the mothers' milk corresponding to all positive samples in saliva culture and confirmed by PCR were genotyped. The amplification reaction was performed in a semi-nested multiplex PCR system on a thermocycler (Maxygen, San Mateo, USA), as described previously, with 5 μL of DNA from either the DBS or the mother's milk, and sequenced using capillary electrophoresis [18]. CMV strain AD169 (*gB2* genotype) was used as the amplification control. The molecular methods (detection and genotyping) described have been standardized in the laboratory for patients undergoing allogeneic bone marrow transplantation and pediatric patients under critical care, as reported previously [19-21].

Analysis of the sequences

A multiple-sequence alignment was generated for the *gB* amplification products from symptomatic patients and well-characterized reference strains (strains AD169, Towne, Toledo, and Merlin; GenBank accession numbers X17403.1, FJ616285.1, GU937742.1, and AY446894.2, respectively) using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2) and edited with JalView (http://www.jalview.org). The DNA sequences of the genes encoding gB determined in this study were deposited in the GenBank database under accession numbers JX965942 and JX965943.

Definitions of variables

Active CMV infection was defined as the detection of the virus in the saliva by cell culture and confirmed by PCR. CI was defined as the detection of the virus in the saliva by cell culture during the first three weeks of life, whereas PI was defined as the first detection of the virus in saliva cultures and confirmed by PCR three weeks after birth [22]. Symptomatic CI was considered, in the clinical setting, to include a CMV-positive saliva culture during the first three weeks of life accompanied by CNS, retinal or auditory findings, microcephaly, growth restriction, hepatosplenomegaly, chorioretinitis, jaundice, petechiae, hearing impairment, thrombocytopenia, hyperbilirubinaemia, or/and anemia. Perinatal symptomatic infection was defined as a CMV-positive saliva cell culture from a sample taken three weeks after birth associated with symptoms related to pneumonia, hepatitis, sepsis, apnoea, bradycardia, hepatosplenomegaly, anemia, thrombocytopenia, and/or abnormal liver function [23].

Statistical analysis

Quantitative variables are presented as measures of central tendency, such as means or medians, for the population. The prevalence ratio was calculated by age group. The reference group was the group in the 39-43 year age range; 95% confidence intervals were obtained (95% CI). The analysis was done with EPIDAT (Pan American Health Organization, V3.0, 2003, Xunta de Galicia).

Results

Patients

During the study period at the Hospital de la Mujer of the Ministry of Health in Mexico City, a total of 12,500 births occurred. Of these, 1000 infants were preterm, and 387 neonates from 375 mothers were selected based on the inclusion criteria of the study (Table 1).

Maternal serology

In total, 365/375 (97.3%) of the mothers were CMV IgG positive. The maternal serological distribution is shown in Figure 1. The seropositivity rates differed between the whole group and the subgroups between 19 and 23 years of age (99.1%), between 29 and 33 years (96.6%), and between 34 and 38 years (95.7%) of age. The prevalence of CMV seropositivity in the group of adolescent mothers with a prevalence ratio of 0.92 (95% of confidence interval 0.86–0.99), between 14 and 18 years of age, differed most significantly from the prevalence in the total group.

Figure 1. Distribution of maternal IgG serology

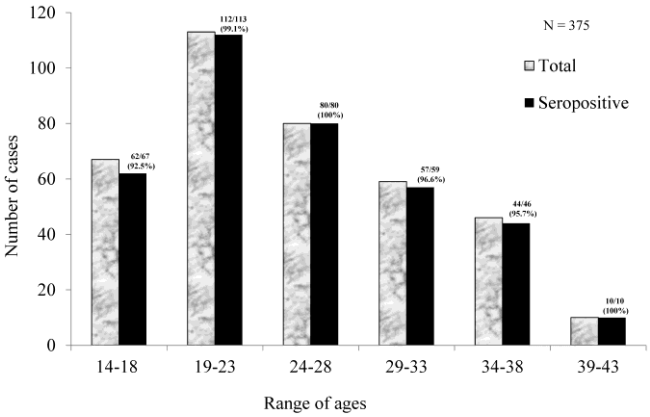


Table 1. Characteristics of the newborns included in this study

Characteristic	No.	Mean	Median	Maximum
Study Population	387			
Sex				
Male	186			
Female	201			
Weight (g)		1647.36	1620	2500
Gestational age (weeks)		33.26	33.5	37
Apgar score at 5 minutes		8	9	9
Head circumference (cm)		29.5	30	34.5
Age of the mother (years) (N = 375)		24.8	23	43

Table 2. Distribution of CMV-positive infants according to their prematurity (based on their gestational age) and weight at delivery

	No.	Percentage	CMV positive	Percentage
Prematurity				
Extreme	11	2.8	–	–
Severe	62	16	6	9.6
Moderate	120	31	9	7.5
Late	194	50.2	5	2.6
Weight at delivery				
Low	267	70.6	11	4.1
Very low	105	27.8	9	8.5
Extremely low	6	1.6	-	-

Table 3. Distribution of gB genotypes in preterm Mexican infants

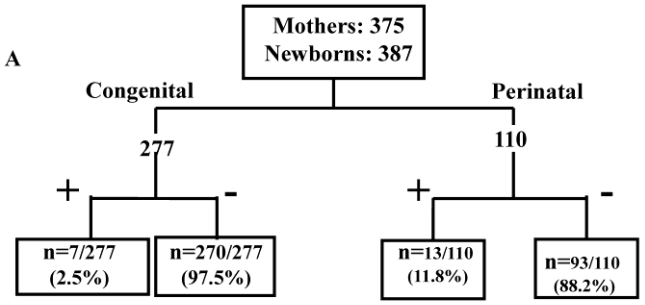
Infection*	gB1	gB2	gB3	gB2/gB4	Total
Congenital	1	4	–	–	5
Perinatal	–	9	2	1	12
Total	1	13	2	1	17

*χ^2 test for homogeneity, p = 0.05

Table 4. Comparison of the genotypes detected in the DBS samples of newborns and in the milk of their mothers

Sample	Infection	DBS (Newborn)	Milk (Mother)
1	CI	gB2	gB2/gB4
2	PI	gB3	gB3/gB4
32	PI	gB2	ND
173	CI	gB2	gB1/gB2
174	PI	gB2	gB2
252	CI	gB1	gB1/gB3
273	CI	gB2	gB2
274	PI	gB2	ND
286	CI	gB2	gB2
289	PI	gB2	gB2
291	PI	gB2/gB4	gB2/gB4
308	PI	gB2	gB2/gB3
321	CI	gB3	gB1/gB3
326	PI	gB2	gB2
327	PI	gB2	gB2/gB4
330	PI	gB2	gB2
331	PI	gB2	gB2

CI, congenital infection; PI, perinatal infection; ND, not determined



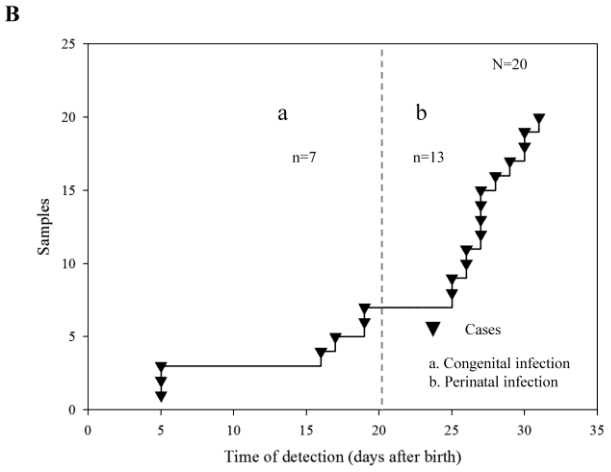
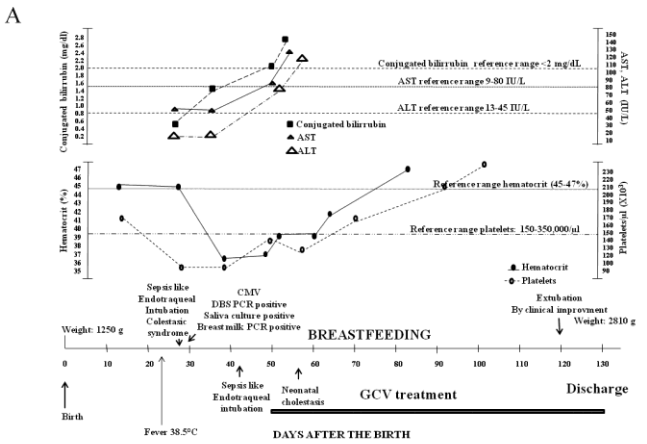


Figure 2. A) Analysis of CMV infection in Mexican preterm newborns. **B)** Accumulated frequency of cases of CMV infection in preterm infants



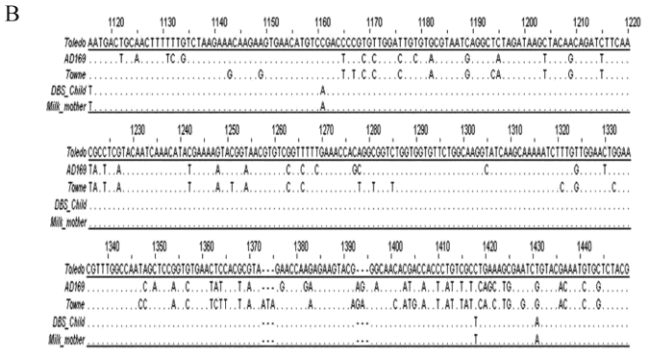


Figure 3. Follow-up of the one symptomatic patient identified. **A)** Clinical history of the patient and detection of CMV. **B)** Alignment of the gB3 genotypes identified in both the mother's milk and the DBS from the child showed 100% identity and 96.5% identity with the gB3 Toledo strain.

CMV IgG seropositivity was detected in 59/60 (98.3%) of mothers accorded medium socio-economic status, in 303/312 (97.1%) of those with low socio-economic status, and in 3/3 (100%) of those with very low socio-economic status, but the differences between these groups were not statistically significant (χ^2 test, p = 0.8).

Frequency of active CMV infections

Three hundred and eighty-seven preterm newborns from 375 mothers (366 single births, seven sets of twins, one set of triplets, and one set of quadruplets) were analyzed with a test for CMV in neonatal saliva samples within a 30-day follow-up period. CMV infections found within three weeks of birth were considered CIs; 7/277 (2.5%) infections were detected within the first three weeks of birth and were considered CIs, and 13/110 (11.8%) were detected at least three weeks after birth and were considered PIs (Figure 2A). In the group of patients considered to have PI, the average time to detection was 27.5 days, with a median of 27 days, a minimum of 25 days, and a maximum of 31 days. The distribution of PIs is shown in Figure 2B. Most of the neonates in this study experienced late prematurity (194/387, 50.2%), and five of them (5/194, 2.6%) were CMV positive. Of the newborns who experienced moderate prematurity (120/387, 31%), nine (9/120, 7.5%) were CMV positive, and of those who experienced severe prematurity (62/387, 16%), six (6/62, 9.7%) were CMV positive. Only 11 neonates (11/387, 2.8%) experienced extreme prematurity (Table 2). Most of the newborns were in the low weight class (273/387, 70.5%), and 11 of them (11/273, 4.1%) were CMV positive; of those in the very low weight class (108/387, 28%), nine (9/108, 8.5%) were CMV positive. Only 6/387 (1.5%) neonates were in the extremely low weight class, none of whom were CMV positive (Table 2).

Genotyping gB

Genotyping was performed in 17/20 DBS samples that were confirmed to be positive by PCR. The results were as follows: 1/17 (5.9%) was gB1, 13/17 (76.5%) were gB2, 2/17 (11.8%) were gB3, and 1/17 (5.9%) showed a mixture of gB2 and gB4 (Table 3). No patient with CI was symptomatic, whereas one patient with PI, typed as gB3, was symptomatic. The clinical characteristics of this patient are described below. Of the 17/20 DBS samples that were successfully typed, as previously described, the gB genotypes of 15/17 were compared with the results of the milk samples from their mothers. Two could not be compared because no product could be amplified from the maternal milk samples. All of these 15 infants harboured viruses (identified in DBSs) that were genotypically similar to the viruses detected in the maternal milk: 7/15 infants and their mothers carried the CMV gB2 genotype, whereas in 8/15 (53.3%) mothers, mixtures of genotypes were detected in their milk, although in only one case (number 291) both genotypes were detected in the blood of the neonate. The gB2 genotype was present in five mothers with mixed infections: two mothers with the gB2/gB4 genotype, one mother with the gB1/gB2 genotype, one mother with the gB2/gB3 genotype, and one mother with the gB2/gB4 genotype. The gB2 genotype was preferentially transmitted to their infants by all five mothers (Table 4).

Analysis of symptomatic infections

None of the neonates diagnosed within the first three weeks of birth were symptomatic, but 1/16 neonates diagnosed more than three weeks after birth was symptomatic. This patient was admitted to the NICU immediately after delivery, based on the inclusion criteria described above. The infant was classified with moderate prematurity and very low birth weight, and showed persistent unresponsive fever on day 25 of hospitalization and sepsis on day 30; cholestatic syndrome was diagnosed, accompanied by thrombocytopenia and hyperbilirubinaemia. On a liver test on day 50, hepatic aspartate and alanine aminotransferase were above the upper limits of the reference ranges. At this time, a saliva sample taken on day 28 and cultured was reported to be CMV positive, and intravenous ganciclovir therapy was commenced (12 mg/kg/d) on day 120. With clinical improvement in the infant's pneumonia and cholestasic syndrome, the patient was extubated and discharged on day 130 with significant weight gain (Figure 3A). The genotyping gB from the maternal samples showed two genotypes (gB1 and gB3), but the method that was used detected only gB3 in the neonate and the sequencing of the gB3 products. The alignment of the gB3 genotypes identified in both the mother's milk and the DBS from the child showed 100% identity and 96.5% identity with the gB3 Toledo strain.

Discussion

Newborn infants usually acquire active CMV infections from their mothers, as either CI or PI [23]. Other studies undertaken in other parts of the world have described the association between maternal seropositivity and CMV infection in newborns [24]. In Mexico, the population displays well-documented high CMV seropositivity. A previous study undertaken in the south of Mexico to determine the CMV IgG status of pregnant women reported an incidence of 97% [25]. The findings in this study were based on follow-ups from birth until the first month of life and during the first month of lactation of preterm newborns from a hospital in Mexico City that specializes in women's health, attended by poorer members of the population, and showed that CMV seropositivity was as high in puerperal women as that reported in other regions of Mexico. The distribution of CMV IgG seropositivity was similar in all age groups of mothers in this study, with no significant differences. Only 5 among the younger (14–18 years old) and 5 in the other age groups (19–43 years old) were seronegatives, which could have increased the risk of primary infection. Three hundred and eighty-seven preterm newborn infants were tested for CMV based on a saliva culture at the one-month follow-up. Most studies are performed using the reference method, in which the virus is isolated from the urine [26]. Although collection of urine samples is easy, we could not use urine samples because the sampling procedure could not be controlled at the hospital where the newborns were being treated, and the transport of such samples to our laboratory was complicated. Dried urine has recently been recommended for this purpose [27], but this study began before that method was reported. Therefore, we used cultured saliva samples, a method that is recommended for the detection of CMV with high reliability [15]. Recent studies have described the influence of breastfeeding on CMV infection, so we sampled the mothers' breast milk at least two hours before feeding [15,28]. The CMV CI rate was 2.5% in our sample. A previous study undertaken in San Luis Potosi, Mexico, described an incidence of 1.48% [29]. Another study performed in Brazil compared CMV infections in newborn term and preterm infants, with an overall rate of 2.1% in preterm infants and similar results for both populations, with 95% maternal CMV seropositivity [24]. As in that study, we believe that the high rate of maternal seropositivity in the population was the factor that most affected the results. Another study described a rate of 6.3% for CI in a group of preterm newborn infants from a population with 100% maternal seropositivity. In our study, the group of patients was small, so it was possible to show with one screening that the rate of CI among this type of patient was high, as has been observed previously [30].

All the newborns were breastfed from birth, which is a known mechanism of CMV PI acquisition. During the first 31 days of life, 11.8% of the babies were positive for CMV infection, whereas in another study, this rate was 11.1% in a group of newborns admitted to a NICU within a follow-up period of six months [29]. Our study differed from that study in that our patients were more specifically selected – only preterm infants at increased risk were included. A previous study also performed in a CMV-endemic area reported that 22.1% of preterm infants were infected within an average period of 75 days, although 10 of them excreted CMV within 60 days of birth [24]. Our results strongly suggest that the population analyzed in Mexico is highly CMV endemic and that a significant percentage of preterm infants acquire the infection perinatally, soon after birth. Further longitudinal studies in this type of Mexican population should be performed to determine the exact rate of infection, at least during the six-month period of breastfeeding. It has been proposed that low birth weight and preterm birth in infants with CI contributes to the outcome in CMV infection, whereas the preterm newborns most commonly affected with PI are those with extremely low birth weights and extreme prematurity [31,32]. Thus, the risk of symptomatic infection is greater in infants who have experienced extreme prematurity and extremely low birth weights [32]. In this study, most of the CMV-positive neonates had experienced moderate or severe prematurity and had low and very low birth weights. Most of the newborns with CI had low birth weights and late prematurity. However, most newborns with PI had very low birth weights and severe prematurity, and one infant was symptomatic, at least during the first month of life. We consider this one of several factors contributing to the lack of symptomatic CI in our sample and also the lack of symptoms in most cases of PI.

It has been reported that the method of DNA extraction can affect the sensitivity of CMV detection in DBS [33]. Therefore, we used phenol to extract the DNA, because it is one of the best methods available [34]. Using this method, CMV infections were confirmed in 17 DBS samples from 20 newborns who were diagnosed as positive with a saliva culture. The gB genotype was successfully identified in the clinical CMV strains in the 17 positive DBS samples, and gB2

was the significantly most frequent genotype. Only one infant had a mixture of genotypes. These results differ from those of studies undertaken in other parts of the world. For example, a study in the United States and another in Italy showed that the most frequent genotype was gB1, followed (in order) by gB3, gB2, and gB4 [35,36]. In a population in China, gB1 was the most frequent genotype, followed bygB2 and gB3 (which did not differ significantly), and more mixed infections were also observed [37]. In Brazil, gB1 and gB2 were the most frequent genotypes and did not differ significantly, followed by gB3, whereas no gB4 genotype was found [38]. Another study in India reported that gB2 was the most frequent genotype [39], which is more consistent with the results of this study. In a previous study conducted by our group in patients undergoing allogeneic bone marrow transplantation, gB1 and gB2 were the most frequently observed genotypes [20].

When we analyzed the CMV genotypes in the mothers' milk and those in the newborns, we observed genotypic similarities in all cases. Half of the mothers analyzed had mixed infections. However, both genotypes were transferred to the infant in only one case, and gB2 was the genotype preferentially transferred in most cases. Because we analyzed our patients for only one month, we cannot exclude the possibility that our neonatal population could have become infected in the following months of lactation even by several genotypes of virus. However, based in this result, we hypothesize that the immune response directed against one specific genotype plays an important role in the control of viral replication and dissemination, and that this influences the transfer of the virus from the mother to the newborn. One limitation of this study was that we could not analyze the newborns in a follow-up study after six months and we had only one month in which to define CI or PI in these infants. A case-control study of populations with high and low seropositivity should be performed during the full period of lactation (six months) to determine specifically whether the immune responses of the mothers in both groups control the transmission of different genotypes to their infants.

We observed no cases of CI compatible with CMV symptomatology. However, one infant in the PI group was symptomatic. We detected two genotypes in the milk of the mother of the symptomatic PI child, suggesting that the mother was infected with two genotypes but the child was infected with only one. This indicates that the infant developed a symptomatic infection with only a single strain from its mother. It is possible that the mother was previously infected with one strain that was controlled by her immune response, and it was therefore unable to infect her infant, whereas the other strain was probably a new infection, and the mother had no immune response to protect the infant. The transfer to twins of CMV from a mother infected with two strains has been previously reported. Each twin was infected with a different strain, and only one of them developed a symptomatic infection [40].

Most of the preterm infant patients who were positive for CMV in this study were asymptomatic, even though they were born to mothers who were highly seropositive. This might be attributable to the fact that the neonates acquired the virus from their mothers, who had already developed an immunological response. This response was transferred, together with the viral infection, to the newborns, affording them some protection. However, this does not exclude the possibility of long-term sequelae associated with early CMV infection.

In conclusion, the rates of CMV PI and CI in preterm infants within one month of birth from highly seropositive mothers were high, but the rate of symptomatic infection was low. The most prevalent genotype was gB2, and this genotype was preferentially transmitted from mothers with mixed infections to their newborn infants within the first 30 days of life.

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