Human cytomegalovirus infection in neonates of a public hospital from Mérida, Yucatán

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Abstract

Introduction: Human cytomegalovirus (HCMV) is recognized as the most common cause of congenital viral infection, which can occur as a result of primary infection, reinfection or infection reactivation in the pregnant woman and be the cause of delay in neuronal development and sensorineural hearing loss in the neonate. **Objective:** To identify HCMV infection in newborns by real-time polymerase chain reaction (RT-PCR) and cell culture. **Method:** Observational, cross-sectional, retrospective study with oral swab samples from 362 neonates born within a 10-month period in a public hospital of Mérida, Yucatán. RT-PCR was carried out for the detection of HCMV. Fibroblast primary cell culture was obtained from human foreskin tissue to isolate the virus. Only positive cases were followed. **Results:** A prevalence of HCMV infection of 0.86 % was found by RTPCR. No virus was isolated with cell culture. In the follow-up visits, sensory health and neurodevelopment were adequate. **Conclusion:** The prevalence of HCMV infection is similar to that of worldwide reports, and only was detected by RT-PCR. Asymptomatic infection detected 12-14 h after birth had no long-term health consequences.

KEY WORDS: Cytomegalovirus. Newborns. Congenital infection. Real time PCR. Cell culture.

Introduction

Human cytomegalovirus (HCMV) is a virus that is widely distributed in the world and is the cause of several diseases in humans, which are related to immune response status. HCMV is the most representative member of the *Betaherpesvirinae* subfamily (herpesviridae family). These are enveloped viruses with icosahedral symmetry and a linear double stranded DNA genome with approximately 120 to 250 kilobase pairs (kbp).¹⁻³

HCMV is an opportunistic pathogen of wide global distribution.³⁻⁵ An increase in its prevalence has been reported in developing countries. In addition, prevalence increases with age and, in populations with poor socioeconomic conditions, the infection is acquired at early ages.^{3,6} HCMV infection is the most important cause of congenital infection in developed countries.⁷ Fetal infection rate is 0.15 to 2 % when there is

maternal primary infection; in that period, the rate of vertical transmission is 20 to 45 %, out of which 10 to 15 % will cause clinical infection with 90 % of sequelae.⁸⁻¹⁰ Latin American countries such as Chile, Ecuador and Mexico show that, by the end of childhood and adolescence, approximately 90 % of the population has already been infected with the virus and that almost all HCMV-negative patients at this age suffer their first infection during early adulthood.^{8,9}

It has been documented that 1 to 4 % of pregnant women will suffer from primary infection throughout their gestational period, 40 % will transmit the HCMV to the fetus, congenital infection will affect 1 % of newborns and infection acquired at the moment of delivery will take place in 2 to 6 % of newborns and will usually be asymptomatic.¹¹⁻¹³ The form acquired in the birth canal causes respiratory diseases such as bronchitis or interstitial pneumonitis, and it manifests itself after the third week of life. Between 10 and 15 %

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of children with congenital asymptomatic HCMV infections in the neonatal period will develop persistent problems of varying severity, which can be lethal; among them, congenital malformations such as intra-cardiac communication, atresia of the esophagus or bile ducts, congenital hip dislocation, cataracts, megacolon and sensorineural hearing loss have been described.^{12,13} On the other hand, in very low weight premature newborns, postnatal HCMV infection can occur with symptoms, sometimes serious.¹⁴

The recommended methods to diagnose HCMV infection include IgM antibody and low IgG avidity detection, PCR detection and viral culture from urine or saliva samples obtained within the first two weeks of the newborn's life.¹⁵ Cell culture is still considered the gold standard for the diagnosis of congenital infection in newborns,¹⁵ although detection in saliva has been shown to be a highly powerful and promising test.¹⁶

The purpose of this study was to determine the prevalence of HCMV infection in newborns from O'Horan Hospital (Mérida, Yucatán, Mexico) in saliva samples and describe existing epidemiological factors.

Method

Cross-sectional, retrospective, observational study, with a population of 362 samples from the virology laboratory repository. The samples consisted of frozen-preserved oral swabs, taken from infants of up to 24 hours of age during 2010 and 2011 at "Dr. Agustín O'Horan" General Hospital of Mérida, Yucatán, in a 10-month sampling period, as part of a project carried out with the approval of the Bioethics Committee of the hospital (registration CIE-032-3-09).

Samples of infants who had medical records and whose DNA was positive for the β -globin gene (indicative of DNA quality) were included. Samples without patient records or with insufficient volume were excluded. Those that were negative for the β -globin gene were eliminated.

From oral swabs, total DNA was extracted with the DNeasy Blood and Tissue[®] kit (Qiagen, Maryland, USA). For quality determination of the obtained DNA, the β -globin gene was detected by endpoint PCR, according to Saiki et al.¹⁷

Real-time PCR (RT-PCR)

To determine the presence of HCMV in the samples, real-time PCR (RT-PCR) was used. The reaction was

carried out using approximately 20 ng/ μ L of DNA, adjusting to the protocol described by Griscelli et al.¹⁸

- To the TaqMan Universal PCR Master Mixture[®] (Thermo Fisher Scientific, Foster City, CA, United States) reaction mixture, the CMVF-UL83 5'- and CMVR-UL83 oligonucleotides were added at a concentration of 0.4 μ M; as well as the TaqMan[®] probe labelled with FAM, at a concentration of 0.1 μ M. All assays were carried out using the 7500 Fast Real-Time PCR System. Evaluation of the amplification product was carried out based on the number of amplification cycles associated with fluorescence emitted by the TaqMan[®] probe (tC). As the positive control, a clone prepared in our laboratory of a plasmid vector containing as insert a fragment of the UL83 gene from HCMV AD169, which was previously verified by Sanger sequencing (data not shown). As negative control, a nucleic acid-free mixture was used.

Cell culture

A primary culture of human foreskin fibroblasts (HFF) was obtained from foreskin tissues of electively circumcised infants in a private clinic, voluntarily donated by their parents. The tissues were chopped into small fragments. The cells were obtained by incubation of the tissue with 0.05 % trypsin (Gibco[®], Thermo Fisher Scientific) and PBS 1:6. Scattered cells were filtered and incubated in Dulbecco's modified Eagle medium (DMEM), supplemented with 10 % fetal bovine serum, 1 % L-glutamine, gentamicin at 200 μ g/mL, streptomycin, penicillin and amphotericin B at 2.5 μ g/mL. Cells were grown at 37°C and 5 % CO₂. When the culture achieved confluence at between four and six weeks, the first passage was carried out and was maintained until passage number 26.

Infection of primary fibroblast culture cells

Once the fibroblast culture reached passages 15 to 18, HFF susceptibility to being infected with a reference virus was confirmed. Since the HCMV-AD169 reference strain was not available, other viruses of the same herpesviridae family were used: herpes simplex virus type 1 and 2 (HSV1 and HSV2, McIntyre and G reference strains). The cytopathic effect on the cells was taken as indicative of infection. To infect the cell cultures from HCMV-positive samples, they were passed through a 13-mm Swinnex Filter Holder[®] filter (Merck Millipore, Concord Road Massachusetts, USA)

and centrifuged at 20,000 rpm at 4 °C. The supernatant was resuspended in 200 µL of DMEM with 2 % fetal bovine serum, in order to inoculate the HFF cells in a confluence state. The cytopathic effect was assessed from the first four days of infection until 21 days post-infection.

Follow-up of positive cases

Only the cases that were HCMV-positive were followed-up at the domicile reported on the record. The children were assessed by rehabilitation undergraduate students according to the Motor and Physical Activity Development Evaluation Guidelines for Pediatric Patients, based on Pan American Health Organization general parameters, provided by the Rehabilitation Undergraduate Program Coordination of the Autonomous University of Yucatán. In addition, the mothers were interviewed regarding the age of onset of certain motor (such as walking, crawling, ability to grab objects, eating and scribbling) and speech milestones (onset of babbling, number of known words and ability to follow simple instructions), as well as general health indicators (whether the child had been hospitalized, had seizures, etc.).

Results

General characteristics of the study population

Three-hundred and sixty-two samples were selected for the study, with their respective records. Recorded birth characteristics included an average of 38.33 weeks of gestation (Table 1), 201 cesarean sections (55.50 %) and 161 (44.5 %) vaginal deliveries, out of which in 45 (12.4 %) premature rupture of membranes occurred.

Regarding the newborns' mothers, 22.9 % of them were between 16 and 18 years of age and 77 %, 19-year-old or older; 31.8 % had completed secondary school, 11.3 % had completed high school and 1.7 % had college education. In addition, 96.1 % had no history of sexually transmitted diseases.

HCMV detection by RT-PCR in newborns

Of the 362 samples that underwent RT-PCR, results could be obtained for 95.30 % (345/362). The samples came from 168 (46.40 %) female and 194 (53.60 %)

Table 1. Distribution of the number of weeks of gestation in the study population

Weeks of gestation	Frequency	%
30-34	14	3.88
35-39	248	68.50
40-43	100	27.62
Total	362	100.00

male neonates, with an average birth-weight of 2.99 kg and average length of 49.56 cm. Congenital malformations were found in five of the 345 infants: supernumerary toe or cleft lip and cleft palate. None had clinical data suggestive of acute HCMV infection.

RT-PCR yielded a result of three positive and 342 HCMV-negative samples, which corresponded to a frequency of 0.86 % in the study population. The characteristics of positive results are presented in Table 2.

Ct values for samples 52, 236 and 263 were 35, 33 and 29, respectively; values between 1 and 39 were taken as the criterion to consider a sample as HCMV infection-positive (Figure 1).

Cell culture establishment

The fibroblast primary culture was obtained from human foreskin tissues, until a keratinocyte-free monolayer of a single cell type was obtained. The fibroblasts were cultured until passage number 26; a confluent monolayer of the obtained primary culture is presented in Figure 2A. Fibroblasts were susceptible to HSV1 and HSV2 infection, since the cytopathic effect resulting from viral infection was observed from 24 to 48 hours and from 48 to 96 hours post-infection, respectively (Figures 2B and 2C). However, when the monolayer was inoculated with HCMV-positive samples, no viruses were recovered and the culture showed senescence before presenting any cytopathic effect identifiable with microscopy.

Follow-up of positive cases

Positive cases were visited at home between 18 and 20 months after birth. Case 236 was localized in the municipality of Halachó (western Yucatán urban area) and case 263 in Chacsinkin (rural Mayan area south of the state); case 52 could not be located in Playa del Carmen (Quintana Roo urban area), apparently due to change of domicile. In case 236, which was a twin pregnancy, both infants were assessed, although

Table 2. Positive neonates characteristics

Sample number	Gender	Weight (kg)	Length (cm)	Head circumference (cm)	Apgar	Weeks of gestation	Type of birth	Cephalic presentation
052	Male	2.96	49	33	8/9	39	C. section	Yes
236*	Female	2.06	45	32	8/9	36	Vaginal	Yes
263	Male	2.79	39	33	9/9	39	Vaginal	Yes

*Twin pregnancy.

Figure 1. Representative result of the RT-PCR assay for HCMV. Amplification of a positive clinical sample and positive and negative controls. The graph shows the amplification curves in fluorescence units, expressed as the difference in the reported fluorophore intensity minus background fluorescence (ΔRn) throughout the amplification cycles (horizontal axis).

Figure 2. Cell culture assays. **A)** Human foreskin fibroblasts (HFF) primary culture monolayer. **B)** Cytopathic effect on HFF infected with type 1 or 2 herpes simplex virus. **C)** Cells in passage 7. Inverted optical microscope white-light images at 10x (**A**) and 40x (**B** and **C**).

only one had been positive at HCMV detection. All had length, weight and neurodevelopment according to their age, with no hearing loss or decreased vision. In the interview with the mothers, they referred adequate motor and speech characteristics at previous ages. Therefore, infections at birth that had an asymptomatic and inapparent course did not affect the assessed areas in the long term.

Discussion

In this study, we were able to detect HCMV DNA in the saliva of three infants at the hospital under study, which corresponded to 0.86 % of prevalence.

Cytomegalovirus infection is highly prevalent worldwide.^{16,19} Vertical transmission occurs in 40 % of cases of infected mothers²⁰ and it can be transplacental, at the passage of the newborn through the birth canal or after birth by contact with maternal genital tract

secretions, by feeding on breast milk or through biological fluids.²¹ Since the samples of our project were taken between 12 and 24 hours after birth, it is suggested that the children could have been infected during pregnancy; congenital infection is unquestionable in the case of infants born by cesarean section; however, contamination during the passage through the birth canal cannot be excluded in both children who were vaginally delivered.

Case 236 was a twin pregnancy, where only one of the infants was positive; the mechanisms of transmission that may be participating in this type of cases are not yet known in detail.

In Mexico, HCMV infection is the main cause of hearing loss and sensorineural deafness in children, in addition to being a cause of cerebral palsy and cognitive disorders.²² In the follow-up assessments of the positive children, no long-term consequences of the infection detected at birth were identified.

The prevalence value found is consistent with the results of studies in other populations in the world, and it is even slightly higher: in an investigation carried out with neonates of seven USA hospitals between 2008 and 2012, HCMV assessment by RT-PCR showed a prevalence of infected neonates of 0.36 % (266 positive samples out of 23,239).²³ There are few reports analyzing HCMV in Mexico: in a nested endpoint PCR study using filter paper blood samples from neonatal screening, a prevalence of HCMV congenital infection of 0.68 % was obtained in the state of San Luis Potosí.⁷ The authors themselves refer that the type of sample used may have low diagnostic sensitivity, and thus they consider it to be a prevalence underestimate. In our case, we obtained a higher frequency (0.86 %) of positivity in oral swab samples and with a real-time PCR method with fluorescent probes.

In addition, we carried out the cell culture with the purpose to corroborate the result obtained by RT-PCR. Since in Yucatan this diagnostic system is not available, we decided to standardize the technique. The use of fibroblast cell lines to produce CMV infection has been reported;^{24,25} however, purchasing the cell lines and adapting a cryopreservation system to store them is necessary.

To avoid the above requirements, in this work we obtained fibroblasts by primary culture from samples of neonate elective circumcision tissues, yet we failed to observe HCMV infection using RT-PCR-positive samples, probably due to causes attributable to the samples, such as having worked with samples from a repository, since the freezing and thawing process can affect infectivity. In addition, low viral load in the samples can limit recovery from cultures, which became evident by the high Ct values obtained by RT-PCR.

Ct (or cycle threshold) indicates the PCR cycle at which the amplification curve crosses the threshold line and reaches exponential amplification. This value is directly related to the number of DNA copies, so that a lower Ct corresponds to a higher viral load than a higher Ct. Although the assay was not a quantitative PCR (it requires the use of a standard curve, which makes the test more expensive), we observed high Cts, which indicates low viral concentration. In our experience, a Ct of 34 represents a viral load of approximately 1500 copies/ μ L (unpublished data). As a reference, the Mexican Institute of Social Security guidelines²⁶ indicate a cutoff $> 10^5$ copies/mL of amniotic fluid as a predictor of symptomatic infection;

however, this value may not be comparable and there are no cutoff points for saliva.

RT-PCR is a valuable tool in epidemiology and in clinical settings. In addition, it is an advantage that a non-invasive sample such as oral swab is used, which is faster and unexpensive.²³

Given that vertically-transmitted HCMV infection in newborns is an underdiagnosed problem due to the wide range of symptoms patients can present with,²⁷ it is of utmost importance making an early and accurate diagnosis to treat the newborn and reduce long-term complications.

Real-time PCR offers benefits in terms of sensitivity and simplicity,²⁸⁻³⁰ and thus it enables detecting positive samples with low viral load.²⁶

One limitation of this work was that serological data of the studied neonates mothers were not available. However, we can assume that a high percentage of mothers were seropositive, based on a study of pregnant women at the same hospital, which indicated 97 % seroprevalence and low incidence of primary infection during pregnancy (1.6 %), which indicates that the risk of congenital infection is also low.³¹ This is consistent with our observations. It would be important to study the consistency between maternal serological and viral excretion in the newborn at birth.

Conclusion

The prevalence of HCMV infection was determined in newborns at O'Horan Hospital in saliva samples using RT-PCR, a test that demonstrated the advantages of molecular methods in terms of more rapidness, sensitivity and economy with regard to cell culture. Asymptomatic infections detected at 12 to 24 hours of birth did not have long-term repercussions. The results of the research generated evidence of RT-PCR usefulness as a HCMV detection method for contributing to the knowledge on the epidemiology of infection with this pathogen.

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