

## Frequency of congenital cytomegalovirus infections in newborns in the Sao Paulo State, 2010-2018

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### ABSTRACT

Human cytomegalovirus (HCMV) infections remain a neglected public health issue. The aim of the present study was to evaluate the frequency of HCMV congenital infections in newborns up to 1 month in the Sao Paulo State, from 2010 to 2018. The molecular characterization of HCMV-positive samples was also undertaken. Urine samples from 275 potential congenital HCMV-infected patients were tested by real-time Polymerase Chain Reaction (qPCR). HCMV-positive samples were amplified by conventional PCR targeting the UL89 gene, sequenced and searched for mutations. A total of 32 (11.6%) positive-HCMV cases were detected (mean Ct 30.59); mean and median age of 10.3 and 6 days old, respectively. Children aged between 0-3 weeks had higher HCMV detection rates (84.4%; 27/32). UL89 gene was successfully sequenced in two samples, both classified as the human betaherpesvirus 5. No described resistance-associated mutations were identified. A routine screening in newborns coupled with the genetic characterization of key viral genes is vital to decrease sequels associated with congenital HCMV infections.

**KEYWORDS:** Congenital cytomegalovirus infection. Real-time PCR. Surveillance. DNA sequencing.

### INTRODUCTION

Human Cytomegalovirus (HCMV) is a ubiquitous human-specific DNA virus, belonging to the Herpesviridae family<sup>1</sup>. A key characteristic of HCMV is its latency capacity, and after the primary infection HCMV can exit latency and re-enter the lytic lifecycle any time during the host life<sup>2</sup>. HCMV infection can be acquired through contamination with different biological fluids, and intrauterine transmission can occur in pregnant women without pre-existing immunity who acquire the primary infection HCMV infection in pregnancy or in women with pre-existing antibodies to HCMV through the reactivation of the primary infection or by acquiring a different HCMV strain<sup>3</sup>.

HCMV seroprevalence in adults ranges between 60% in developed countries and 100% in developing countries<sup>4</sup>. HCMV seropositivity in the Brazilian population range from 70% to 97%<sup>5-8</sup>. Congenital HCMV infection is the most common congenital infection worldwide, affecting 0.2% to 6.1% of live newborns<sup>1</sup>. The prevalence of congenital HCMV infection in Brazil is 1-8%<sup>9,10</sup>.

The diagnosis of the congenital HCMV infection should be conducted before the 3<sup>rd</sup> week of life. After this, it is difficult to distinguish congenital infection from infection acquired in the postnatal period<sup>11</sup>. Traditionally, the virus isolation from

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**Received:** 13 May 2020

**Accepted:** 14 July 2020

urine or saliva samples in cell cultures has been the standard method for diagnosing the congenital HCMV infection. Recently, molecular techniques, as the polymerase chain reaction (PCR) and the real-time polymerase chain reaction (qPCR) has led to important advances in the congenital HCMV diagnosis<sup>12</sup>. The early diagnosis of congenital HCMV infection and the clinical follow-up are essential to detect and manage the disease and prevent sequels<sup>13</sup>. Despite its importance and the availability of rapid and specific molecular tests, numerous newborns suffering from congenital HCMV infection remain undiagnosed in Brazil as the public health system do not perform the screening of HCMV in regular bases<sup>10</sup>.

The purpose of the present study was to evaluate the frequency of HCMV congenital infections in newborns up to 1 month of age in the Sao Paulo State from 2010 to 2018. The qPCR technique was used to screen urine samples aiming to apply this approach in future screening programs for HCMV congenital infections in the Brazilian Health System (SUS). In addition, molecular characterization of HCMV positive samples was also undertaken.

## METHODS

This is a retrospective and descriptive study conducted with urine samples collected from newborns (< 1 month old). Urine samples were obtained from potential congenital HCMV-infected patients at Brazilian Unified Health System (SUS) units following medical request, and sent to the Respiratory Diseases Laboratory of Adolfo Lutz Institute, a reference center for HCMV surveillance in the Sao Paulo State. The present investigation was funded between collaborating institutes involved in public or non-public HCMV surveillance programs: (i) Regional Health Departments (DRS I to XVII), (ii) Clinical Hospital of Sao Paulo, (iii) Emilio Ribas Hospital, (iv) University Hospital of Sao Paulo and (v) Santa Casa Hospital of Sao Paulo. The routine of diagnostic tests conducted for HCMV at the Respiratory Diseases Laboratory includes more complex methodologies, such as viral isolation in cell cultures, conventional polymerase chain reaction (PCR), real-time PCR (qPCR), and eventually, DNA sequencing. Serological tests to detect IgM and/or IgG are conducted at the requesting units. From 2010 to 2018, a total of 1,279 urine samples were received at the Respiratory Diseases Laboratory and the original urine samples were preserved at -70 °C. A total of 275 samples were randomly selected based on IgM-positive results and radiological exams suggestive of HCMV infection according to data retrieved from the medical records; and preserved specimen's availability. All the 275 urine samples were tested for the presence of

HCMV exclusively by qPCR methodology as no other biological specimens belonging to the same selected patient were available for testing.

Viral DNA was extracted using the QIAamp® DNA Mini Kit (QIAGEN Valencia, CA, USA), according the manufacturer's protocol. The qPCR for the major-immediate early (MIE) gene was performed according to a previously described protocol<sup>14</sup>, generating amplicons of 76 bp. As a control to rule out the presence of PCR inhibitors, and to monitor the nucleic acid extraction efficiency, each sample was tested by qRT-PCR for the presence of the human ribonuclease (RNase) P gene following the protocol described by Emery *et al.*<sup>15</sup>. The threshold cycle (Ct) value from this qPCR was used as a proxy measure of the HCMV load. The Ct value is inversely proportional to the amount of virus present in the sample, so the lower the Ct value the higher the viral load<sup>16</sup>. Samples were considered positive when a Ct value  $\leq 39$  was obtained<sup>14</sup>. HCMV-positive samples were subsequently targeted for amplification by a conventional PCR with HCMV-specific primers 707s and 707as, amplifying a 519-nucleotide (nt) portion of the DNA packaging terminase subunit 1 (UL89), as described elsewhere<sup>17,18</sup>.

PCR amplicons were subjected to purification using the ExoSAP-IT™ Kit (Applied Biosystems, Foster City, CA, USA), and sequenced using the BigDye™ Kit v3.1 (Applied Biosystems, Foster City, CA, USA) with the same primer pairs used for PCR<sup>18</sup>. Dye-labeled products were sequenced using an ABI 3130 sequencer (Applied Biosystems, Inc., Foster city, CA, USA), following purification using Centri-Sep™ spin columns (Invitrogen, Carlsbad, CA, USA). Sequencing chromatograms were edited manually using the Sequencher 4.7 software (Gene Codes Corporation, Ann Arbor, MI, USA). Sequences were screened on the NCBI website using the Basic Local Alignment Search Tool (BLAST) to assign the types of the studied strains. The typing verification was conducted using the MEGA software version 6.0 (NCBI, Bethesda, MD, USA). Prototype HCMV sequences, available in GenBank were aligned together with the sequences generated here using the BioEdit software, version 7.0.5.2 (Ibis Therapeutics, USA). Neighbor Joining (NJ) trees were constructed based on a Kimura 2-parameter model determined by MEGA 6.0 with 1000 bootstrap replicates<sup>19</sup>. Nucleotide sequences determined in this study were deposited in GenBank under the accession N° MT345567 and MT447555. The publically available web-based search tool MRA-mutation resistance analyzer<sup>20</sup> was used to identify possible mutations associated with drug resistance.

The ethical approval was granted by Adolfo Lutz Institute (CEP-IAL 2.984.740, CAAE 96782618.5.0000.0059). This

was an anonymous unlinked study and informed consent was not required according to the Resolution N° 466/12 concerning research involving human beings (Conselho Nacional de Saude (CNS)/Ministerio da Saude (MS), Brasilia, 2012).

## RESULTS AND DISCUSSION

Of the 275 urine samples tested, 32 specimens (11.6%) were positive for HCMV by qPCR targeting the MIE gene (mean Ct value of 30.59). The mean age of the newborns was 10.3 days (range: 2 days to 30 days), while the median age was 6 days old. Newborns aged between 0 and 3 weeks had higher detection rates of HCMV (84.4%; 27/32; mean Ct = 30.62) than infants in the age group older than 3 weeks (15.6%; 5/32; mean Ct = 30.40), emphasizing the importance of the early specimen collection (under 3 weeks of life) for the accurate diagnosis<sup>21</sup>. After this period, the assessment of the viral infection origin is impaired as the contagion could have occurred throughout the birth canal, saliva or breast milk<sup>10</sup>. No significant differences were observed between HCMV Ct levels and the newborns age. HCMV infections were detected throughout the study period (from 2010 to 2018), suggesting that urine samples frozen at -70 °C were stable for a long-term storage (around 10 years) after collection. It is well known that viruses can be stored and preserved at low or ultra-low temperatures as an infectious material for long periods of time<sup>22</sup>.

The frequency of congenital HCMV infections detected here (11.6%) was similar to the one observed in other study carried out in Japan (10%) using conventional PCR in urine samples<sup>23</sup>. However, the infection rate was higher in comparison with Portugal (0.4%) and Belgium (0.5%) using viral isolation in cell cultures from urine samples<sup>24,25</sup>; the USA (0.5%) using qPCR in saliva specimens targeting the glycoprotein B (gB) and the immediate early gene 2 (IE-2) exon 5 region<sup>12</sup>; and with the sub-Saharan Africa (3.8%) using qPCR in saliva, serum and urine samples targeting the phosphoprotein pp65 (UL83)<sup>26</sup>. Lower prevalences have also been reported in previous studies conducted in Brazil: Sao Paulo (1%) (1980-1982) using viral isolation in cell culture from urine samples and specific immunofluorescence anti-HCMV IgM in umbilical-cord serum<sup>27</sup>, Belo Horizonte (6.8%) (1995-1998) and Porto Alegre (0.8%) (2003) using conventional PCR in urine samples<sup>28,29</sup>; Ribeirao Preto (1%) (2003-2009) and Ilheus (1.2%) (2010-2012) using conventional PCR in urine and/or saliva samples<sup>9,10</sup>. This observed disagreement may be mostly associated with differences in the diagnostic methods and clinical samples<sup>10,24</sup>. It is important to note that in-house qPCR is not a standardized methodology and

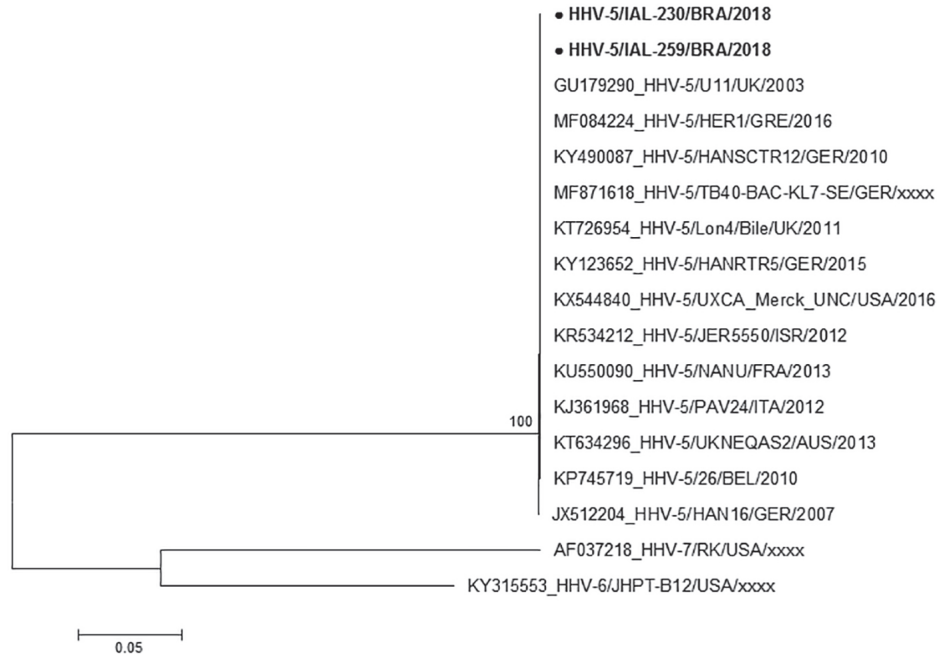
different primers, target genes and amplification cycles can be used for HCMV detection<sup>12,14,26</sup>, making comparison between studies rather complex. Divergence in prevalence rates might also be linked to the study design, recruitment criteria, settings, the number of specimens tested, and the socioeconomic status of the population<sup>10,24</sup>. An investigation conducted by Pannuti *et al.*<sup>27</sup> suggested that in populations with unfavorable socioeconomically conditions, congenital HCMV infection tends to be more prevalent. This 9-year retrospective study helped to clarify the HCMV burden in congenital infections in the Sao Paulo State.

Regarding the 32 HCMV-positive samples, the UL89 gene could be amplified in six samples (18.7%; 6/32), but only two were successfully sequenced (2/6 or 33.3%). It is well known that qPCR provide a rapid diagnosis with increased sensitivity for the HCMV detection<sup>14</sup>. However, the molecular characterization depends mainly on conventional PCR followed by Sanger sequencing, which clearly impaired our capacity in identifying all the HCMV-positive samples.

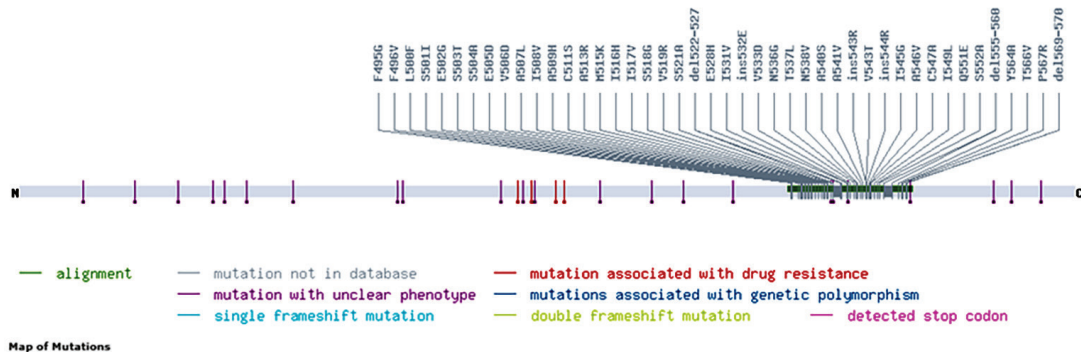
The typing verification and the phylogenetic analysis was performed with the 510 bp DNA packaging terminase subunit 1 (UL89) fragment. The genetic trees include the strains sequenced in this study (highlighted in bold) along with prototype sequences obtained from GenBank. Both samples (IAL-230 and IAL-259) were classified as Human betaherpesvirus 5 (HHV-5), forming a well-supported monophyletic group (Figure 1). The comparison of nucleotide sequences between the two Brazilian isolates showed a maximum identity of 100 % (100% aa), with similar high values also observed following the comparison with other HHV-5 strains (94.3% nt, 93.9-100% aa) identified worldwide.

Recent attempts to develop new anti-HCMV compounds have focused on the viral terminase complex involved in the viral DNA cleavage/packaging. The terminase complex is highly HCMV-specific, with no counterpart in mammalian cells, thus representing a target of choice for new antivirals<sup>30</sup>. The small subunit of the HCMV terminase complex is encoded by the Open Reading Frame - ORF UL89<sup>31</sup>, and multiple mutations have been detected in this ORF of drug-resistant isolates<sup>32</sup>. No described resistance-associated mutations within the UL89 gene were identified by the MRA-mutation resistance analyzer web tool in the Brazilian HCMV samples. Nevertheless, several mutations of yet unknown clinical significance were found (Figure 2). A large number of new mutations of undetermined medical implication has been identified in either clinical or laboratory isolates<sup>33</sup>; however their impact on the HCMV treatment remain to be evaluated.

Despite the recognized importance of the congenital



**Figure 1** - Nucleotide sequence-based similarity analysis of the Brazilian Human betaherpesvirus 5 (HHV-5) DNA packaging terminase subunit 1 (UL89) (indicated in bold) with other selected HCMV strains. A neighbor-joining tree of partial UL89 ORF sequences was generated using the MEGA 6.0 software. Reference HHV-5 to -7 strains were obtained from the GenBank database. The accession number, isolates, country and year of each strain are indicated. The scale indicates the number of divergent nucleotide residues. Bootstrap values are shown as percentages at the branch nodes.



**Figure 2** - Map of mutations (substitutions, insertions and deletions) identified in DNA packaging terminase subunit 1 (UL89) partial ORF gene sequences of the Brazilian Human betaherpesvirus 5 (HHV-5) IAL-230 and IAL-259 strains using the MRA-mutation resistance analyzer web tool<sup>20</sup>.

HCMV infection, it is still a wide neglected syndrome in Brazil. Significant advances in diagnostic techniques have occurred in the last years. Real time PCR is highly sensitive and could be applied in large scale. The implementation of a routine screening policy in newborns coupled with the genetic characterization of key viral genes is an important strategy focusing on decreasing the sequels associated with congenital HCMV infections.

**AUTHORS' CONTRIBUTIONS**

MIO conceived the study; ELD, DBLO, AMSA and MIO designed the study protocol.; CGF, AL, VBS, RMM

and MIO participated in the conduct of the study; CGF and MIO acquired the data and performed the PCR assays; CGF, AL and MIO conducted the sequencing assays; CGF, AL, ELD, DBLO, VBS, RMM and MIO analyzed and interpreted the data; AL and MIO drafted the manuscript; CGF, ELD, DBLO, AMSA, VBS and RMM critically revised the manuscript for intellectual content. All authors read and approved the final version. MIO is guarantors of the paper.

**CONFLICT OF INTERESTS**

The authors have no conflict of interests to disclose.

## FUNDING

This study was partially supported by Graduate Program in Science, Coordinator for Disease Control; Ministry of Health-PPG-CCD-SES/SP. Carla Grasso Figueiredo was supported by a fellowship of the Association for the Improvement of Higher Education Personnel (CAPES).

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