Cytomegalovirus frequency in neonatal intrahepatic cholestasis determined by serology, histology, immunohistochemistry and PCR

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INTRODUCTION
The frequency of cholestatic jaundice is difficult to determine, varying between 1:2500 and 1:5000...
newborns\textsuperscript{[3-5]. The initial objective in the management of neonatal cholestatic jaundice is to distinguish between intrahepatic and extrahepatic causes, as the latter requires urgent surgical intervention\textsuperscript{[4]. Neonatal intrahepatic cholestasis (NIHC) represents 2/3 of the cases of neonatal cholestasis\textsuperscript{[5-9]. The most common causes of the disease are infection, including cytomegalovirus (CMV)\textsuperscript{[10-12]. Data on the prevalence of CMV in NIHC vary greatly depending on the diagnostic method used (5\%-46\%)\textsuperscript{[5,12,14]. Congenital CMV infection is assessed by viral isolation detected within 2-3 wk after birth\textsuperscript{[13]. If CMV is detected three weeks after birth, diagnosis of congenital infection should be supported by clinical and epidemiologic features\textsuperscript{[12,14,16]. Congenital disease might occur due to maternal primary infection (where the vertical transmission rate ranges from 40\%-50\%), or as a recurrence (where the vertical transmission rate ranges from 0.5\%-2\%). The clinical manifestations are essentially limited to neonates of mothers presenting with a primary infection during pregnancy and include: purpura, intracerebral calcifications, retinitis, ventriculitis, hepatosplenomegaly, microcephaly, intrauterine growth retardation, and jaundice\textsuperscript{[15,17-22]. The general prevalence of congenital CMV infection in Brazil is similar to that reported in studies on highly immune populations\textsuperscript{[16,25-27]. There is a higher rate of congenital infection, but fewer clinical manifestations\textsuperscript{[28,29].

The purpose of the present study was to establish the frequency of CMV infection in patients with NIHC based on serology (IgM-ELISA), histological revision (searching for cytomegalic cells), immunohistochemistry (IHC), and polymerase chain reaction (PCR) and to verify the relationships among these methods.}

**MATERIALS AND METHODS**

Data from NIHC patients were evaluated at two tertiary centers between March 1982 and December 2005: the Pediatric Department of the State University of Campinas (Unicamp) Teaching Hospital and at the Children’s Institute of the Medical School of the University of São Paulo (USP). A uniform diagnostic approach was followed throughout the observation period.

Cholestasis was defined by laboratory criteria as suggested by Moyer et al\textsuperscript{[8]. Inclusion criteria were: jaundice appearing up to three months of age and hepatic biopsy performed during the investigation.

To establish the etiology of NIHC, one author (Bellomo-Brandao) collected the following data: neonate’s identification, symptoms, history, clinical findings, physical examination, and results of laboratory testing [alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AlkPhos), \( \gamma \)-glutamyltransferase (GGT), international normalized ratio (INR), direct bilirubin (DB), albumin, liver biopsy, serum \( \alpha \)-1-antitrypsin, sweat sodium and chloride, innate metabolism errors in urine, polymerase chain reaction (PCR), CMV antigenemia, and serology of: CMV, HIV, EBV, rubella, toxoplasmosis, and syphilis].

**CMV serology**

An Enzyme-linked immunosorbent assay (ELISA) was employed using commercial kits from Sorin Biomedica (Italy) and Vidas Biomerieux (France) at the Unicamp Teaching Hospital. Cobas Half Core, Roche (Germany) and MEIA, Abbott Axsym\textsuperscript{®} system (USA) kits were used at USP.

**Histological revision**

Histological revision was carried out by one hepatopathologist (CAFE). When necessary, new slides were created and examined. Among the 84 liver biopsies, five were surgical and 79 were percutaneous. The presence of at least one cytomegalic cell was considered suggestive of CMV.

**IHC**

Four-\textmu\textsuperscript{m} thick histological sections of the liver biopsies were placed on silanized slides, deparaffinized in xylene, and rehydrated. For antigen retrieval, the slides were immersed in 10 mmol/L citrate buffer (pH 6.0) in a steamer at 90\degree C for 30 min. A commercially available cocktail of two mouse monoclonal antibodies to CMV was applied to the sections (clones DDG9/CHH2 [pre diluted]; Cell Marque, Hot Springs, AR, USA) for 1 h at 37\degree C and subsequently overnight (18 h) at 4\degree C. The reaction was amplified using the peroxidase-conjugated NovoLink polymer (Novoceastra, Newcastle, UK). Staining was achieved using 3,3-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, USA) and counterstaining with Mayer’s hematoxylin. All reactions were performed with appropriate positive and negative controls. The positive control corresponded to a lung fragment from a patient who had died due to a generalized CMV infection. The negative control corresponded to the same specimen, in which a specific anti-CMV antibody was replaced by saline buffer. Positive results consisting of brown-colored nuclei were evaluated using a conventional optical microscope.

**PCR**

The recommendations of Kwok and Higuchi\textsuperscript{[30]} were followed to prevent sample contamination.

DNA was extracted according Saiki et al\textsuperscript{[31]}, Shibata et al\textsuperscript{[32]} and Demmler et al\textsuperscript{[33]} from formalin-fixed, paraffin-embedded fragments using a commercial kit (Dnasey, Qiagen, Germany). The reaction mixture consisted of 0.5-1 \textmu\textsuperscript{g} sample DNA in a total volume of 20 \textmu\textsuperscript{L}, containing 50 mmol/L KCl, 10 mmol/L Tris (pH 8.4), 2.5 mmol/L MgCl\textsubscript{2}, 0.1 mmol/L of each primer, 200 mmol/L of deoxyribonucleotide triphosphates (dATP, dCTP, dGTP, and dTTP) and 2 U of \( \text{Taq} \) DNA polymerase. Water was used to complete the total reaction volume. The mixture was covered with a drop of mineral oil.

Amplifications were carried out in a DNA thermocycler (PTC100; MJ Research, Inc., Watertown, MA, USA) using 30-35 cycles for each sample (94\degree C for 45 s, 55\degree C for 45 s, and 72\degree C for 1 min). The cycles were preceded by an initial denaturation at 94\degree C for 5 min and...
were followed by a final extension for 7 min at 72°C.

The human β-globin gene was amplified as an internal control for the reaction [36]. Primers for detection of the β-globin gene were as follows: PCO3 (5’ CTTCTGACAC AACTGTGTTCACTAGC 3’) and PCO4 (5’ TCACCA CACATTTCAAGG 3’).

CMV was detected by PCR and nested-PCR[32-35]. External primers were as follows: MIE4 (CCAGGGCC CTCTGATAACCAAGCC) and MIE5 (CAGCACCA TCCTCCTCTCCTCTG); internal primers were as follows: IE1 (CCACCCGTGGTGCCAGCTCC) and IE2 (CCCGCTCCTCCTGAGCCACC). AD169 DNA strain was used as a positive control and water was used as a negative control.

Both β-globin and nested PCR products were visualized on ethidium bromide-stained, 2% agarose gels, after electrophoresis.

**Ethical aspects**

The present research study was approved by the Medical Research Ethics Committees of both institutions. Informed consent was not needed, because serologies and liver biopsies had been done during the investigation process.

**Statistical analysis**

The frequencies of CMV positive results of each test were calculated. Sensitivity, specificity, positive predictive value, negative predictive value, and accuracy were calculated between the results obtained with PCR and serology[36].

**RESULTS**

One hundred one non-consecutive patients with NIHC were included (84 patients from Unicamp and 17 patients from USP). Sixty-nine patients were males and 32 patients were females. The median age at the time of the biopsy was two months and 14 d.

The etiologies of NIHC are presented in Table 1. Most of them had an idiopathic etiology (58%). Five patients were previously diagnosed with CMV infection based on serology, PCR (plasma) and antigenemia.

This was a retrospective study and there was a paucity of liver biopsy fragments (most of the biopsies were obtained percutaneously); therefore, it was not possible to perform all four tests in all patients. In 17 patients, histological revision was not done because a shortage of paraffinized sample material did not permit making new slides. In the remaining 84 cases, the pathologist did not observe the presence of cytomegalic cells. Only one of 44 patients had positive IHC, 6/77 had positive PCR and 7/64 were IgM-ELISA positive. Table 2 shows the results of IgM-ELISA, histological revision, IHC, and PCR and the number of patients submitted to each diagnostic method.

Table 3 presents the clinical and laboratory data of PCR-positive patients. Five of six patients birth weights > 2500 g. Two of them had positive IgM-ELISA: one had a previous diagnosis of cystic fibrosis and the other had a previous diagnosis of cystic fibrosis and the other had

| Etiology                                         | Number of cases |
|-------------------------------------------------|-----------------|
| Neonatal sepsis                                 | 9               |
| Cytomegalovirus                                 | 5               |
| Urinary infection tract                         | 4               |
| Syphilis                                        | 2               |
| Toxoplasmosis                                   | 2               |
| α1-antitripsin deficiency                       | 5               |
| Other metabolic diseases                        | 5               |
| Galactosemia                                    | 4               |
| Allagie's syndrome                              | 2               |
| Byler’s disease                                 | 1               |
| Cystic fibrosis                                 | 1               |
| Secondary to use of parenteral nutrition        | 1               |
| Down’s syndrome                                 | 1               |
| Panhydropituitarism                             | 1               |
| Idiopathic                                     | 58              |
| Total                                          | 101             |

**DISCUSSION**

The ubiquitous nature of CMV makes for difficulties in establishing direct evidence of the actual role of CMV in neonatal cholestasis, as stated in the editorial of Persing and Rakela[15]: “When does the detection of an infectious agent become unequivocally the etiologic agent of a specific condition?” Detection of the virus from the liver provides strong evidence that neonatal cholestasis is caused by CMV infection[16].

Histological searching for cytomegalic cells was negative in 84 patients. Lurie et al[35], after studying four fatal cases of NIHC from CMV, described the presence of cytomegalic cells in the liver from two of them. None were detected in the present study or observed in cases of extrahepatic cholestasis (EHC)[36-38]. In CMV hepatitis following orthotopic liver transplantation

**Table 2 Serological results (IgM-ELISA), presence of cytomegalic cells (histological analysis), IHC, and PCR**

| Methods               | Positive | n (%) |
|-----------------------|----------|-------|
| IgM-ELISA             | 7        | 64 (11)|
| Histological analysis | 0        | 84 (0) |
| IHC                   | 1        | 44 (2) |
| PCR                   | 6        | 77 (8) |
(immunosuppressed patients), demonstration of cytomegalic inclusion bodies in hematoxylin and eosin sections is sufficient for a diagnosis of CMV hepatitis [39].

Only one patient had positive immunostaining and a positive PCR, despite encouraging data in immunosuppressed patients [40,41]. There are no reports in the medical literature in which the IHC method has been used to identify CMV in NIHC.

In the literature, the frequency of positive CMV in NIHC by PCR techniques varies between 5% and 46% [14,42,43]. Different techniques and samples make it difficult to establish a correlation between the data. In the present study, PCR was positive in 8% of the patients (6/77). Three patients had been previously diagnosed with other defined etiologies, indicating that concomitant infection by CMV and other agents is possible. This finding suggests that multiple agents must be investigated in the search for a diagnosis of NIHC. Four of these six cases presented with a negative IgM-ELISA, probably due to immaturity of the neonatal immune system [17,44].

Few studies have been carried out comparing the different diagnostic methods of CMV infection in neonatal cholestasis. Fischler et al [45] found positive serology for IgM-CMV in 32% of cases tested. In another study [6], the same author found IgM-CMV and/or the presence of positive CMV in the urinary samples of 19/54 (35%) patients with NIHC and positive results in 4/11 carriers of α1-antitrypsin deficiency. A retrospective analysis of 39 patients (who presented with CMV in blood or urine cultures, or positive PCR in liver biopsies collected between 30 and 220 d of life) showed the presence of CMV by PCR-CMV on filter papers collected during the first three days of life in two neonates, suggesting that congenital infection was not the cause of cholestasis [42].

Chang et al [14] studied 50 children diagnosed with neonatal hepatitis by PCR-CMV of liver samples. Twenty-three children had positive PCR, 13 of them had serology suggestive of an acute infection, and nine children had negative serologies; in one case serology was unknown. Twenty-one of the 27 patients with a negative PCR presented with negative serologies and six had positive serologies.

Shibata et al [43] evaluated 26 non-cholestatic infants with an unexplained hyperbilirubinemia and found a high frequency of positive serology for IgM-CMV in the maternal samples of 35% of positive PCR patients.

### Table 3
Clinical and laboratory data of CMV-positive patients by PCR: gender, diagnosis, weight at birth, serology (IgM-ELISA), IHC, and presence of cytomegalic cells

| Patients | Gender | Diagnosis         | Weight at birth (g) | IgM-ELISA | IHC     | Cytomegalic cell |
|----------|--------|-------------------|---------------------|-----------|---------|------------------|
| 1        | M      | Cystic fibrosis   | 3460                | Positive  | -       | Absent           |
| 2        | F      | Idiopathic        | 2500                | Negative  | Negative | Absent           |
| 3        | M      | Byler’s disease   | 3320                | Positive  | Positive | Absent           |
| 4        | M      | Idiopathic        | 2700                | Negative  | -       | Absent           |
| 5        | F      | Idiopathic        | 1500                | Negative  | Negative | Absent           |
| 6        | M      | Neonatal sepsis   | 2570                | Negative  | -       | Absent           |

-: Not performed.

### Table 4
Results of PCR and serology (IgM-ELISA) and number of patients tested

| IgM-ELISA | PCR | Total |
|-----------|-----|-------|
| +         | 2   | 5     | 7     |
| -         | 4   | 40    | 44    |
| Total     | 6   | 45    | 51    |

### Table 5
Results of positive predictive value (PV+), negative predictive value (PV-), accuracy, sensitivity, and specificity between PCR and serology with 95% confidence intervals (CI)

|                      | Percent | CI (95%)       |
|----------------------|---------|----------------|
|                      |         | Inferior limit |
|                      |         | Superior limit |
| Sensibility          | 33.3    | 6.00           |
| Specificity          | 88.89   | 75.15          |
| PV+                  | 28.57   | 5.11           |
| PV-                  | 90.91   | 77.42          |
| Accuracy             | 82.35   | 68.64          |

![Figure 1](image1.png)

**Figure 1** Immunohistochemistry of a positive case, consisting of brown colored nuclei (arrow), using a conventional optical microscope (× 640).

![Figure 2](image2.png)

**Figure 2** HCMV DNA results of 2% agarose gel electrophoresis under ultraviolet light (PCR). L: Ladder; C+: Positive control; C-: Negative control; 1, 4 and 5: Negative samples; 2 and 3: Positive samples.
(1-24 mo old), who presented with an increase in ALT, through a quantitative PCR technique on their plasma. CMV was positive in four patients (15.4%). Three patients who presented with positive IgM-CMV did not have a positive PCR.

These studies were conducted in areas that were considered to have a low prevalence of CMV\[15,16\]. In Brazil, infection due to CMV is highly prevalent in the population and occurs early in the first year of life\[17,18,19\]. In populations where the majority of women of reproductive age have antibodies against CMV, there is a higher rate of congenital infection (as compared to populations with a low prevalence of antibodies against CMV), but without clinical disease. Although the presence of maternal antibodies does not prevent CMV transmission, it appears to confer protection or may be a marker of another factor that protects the child\[20,21\].

In the present study, compared to PCR, the sensitivity and positive predictive value of IgM-ELISA serology were low, whereas specificity, negative predictive value and accuracy were high.

In conclusion, the frequencies of positive results for CMV varied from 0 (searching for cytomegalic cells) to 11% (IgM-ELISA). Disparities in serology and PCR were observed in the present study, as well as in other studies using different PCR techniques. Although our data are not encouraging, it will be necessary to conduct new studies to establish the role of IHC in the diagnosis of CMV in neonatal cholestasis. Even if there was a previous diagnosis, the involvement of CMV should be determined. All results should be interpreted considering the sum of clinical and epidemiologic features.

ACKNOWLEDGMENTS

The authors thank Biologist Marisa de Almeida Matsura for assistance with the Immunohistochemistry techniques. Jose Vassallo is researcher of the Conselho Nacional de Pesquisas Científicas (CNPq-Brazil).

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