Cytomegalovirus Identification in Blood and Urine of Newborns by Nested Polymerase Chain Reaction

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ABSTRACT

Aim: *To study the frequency of congenital cytomegalovirus (CMV) infection in newborns admitted to the Division of Neonatology, using nested polymerase chain reaction (PCR) and DNA to detect differences in blood and urine specimens.*

Methods: *The study was carried out for eight months. Newborns (n = 520) hospitalized in five hospitals in Campo Grande, Mato Grosso do Sul, Brazil, were checked for CMV by analysing blood and urine samples.*

Results: *Cytomegalovirus was PCR positive in 13 urine and 10 blood samples. Of the 13 positive urine patients, three (23%) had no clinical signs suggestive of CMV, and another three (23%) patients admitted to the neonatal intensive care unit (NICU) had no definite findings of bacterial infection, with negative blood culture and some clinical signs consistent with CMV as cholestasis, hepatomegaly and eosinophilia. Three patients were on mechanical ventilation and showed improvement after prescription of ganciclovir. One CMV positive child progressed to death.*

Conclusion: *Cytomegalovirus detection in urine was slightly more efficient than in blood, and showed better sensitivity than in serological analysis (*$p < 0.01$*) therefore, boiled urine may be a better and easier specimen tool for CMV diagnosis in neonatal infection. The findings of the present research suggest that patients admitted to the NICU, especially premature infants, whose laboratory results are not compatible with bacterial infection, and exhibiting signs suggestive of CMV infection should have PCR done on urine for confirmation.*

Keywords: Cytomegalovirus, neonatal infection, newborn, nested polymerase chain reaction

Identificación del citomegalovirus en la sangre y la orina de los recién nacidos mediante la reacción en cadena de la polimerasa anidada

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RESUMEN

Objetivo. *Estudiar la frecuencia de la infección congénita por citomegalovirus (CMV) en los recién nacidos ingresados en la División de Neonatología, mediante la reacción en cadena de la polimerasa (PCR) anidada y el ADN para detectar diferencias en las muestras de sangre y orina.*

Métodos. *El estudio se llevó a cabo durante ocho meses. Los recién nacidos (n = 520) hospitalizados en cinco hospitales de Campo Grande, Mato Grosso do Sul, Brasil, fueron sometidos a chequeo de CMV mediante análisis de muestras de sangre y orina.*

Resultados*. El citomegalovirus fue positivo a la PCR en 13 muestras de orina y 10 de sangre. De los 13 pacientes de orina positiva, tres (23%) no tuvieron señales clínicas sugestivas de CMV. Otros tres pacientes (23%) ingresados en la unidad de cuidados intensivos neonatales (UCIN) no tuvieron hallazgos definitivos de infección bacteriana, siendo su hemocultivo negativo y presentando algunos signos clínicos consistentes con CMV, tales como colestasis, hepatomegalia y eosinofilia.*

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Tres pacientes fueron puestos en ventilación mecánica y mostraron una mejoría después de la prescripción del ganciclovir. Un niño positivo al CMV tuvo una evolución fatal.
Conclusión. La detección del citomegalovirus en la orina fue ligeramente más eficaz que en la sangre, y mostró mayor sensibilidad que en el análisis serológico (p < 0.01), por lo tanto, la orina hervida puede ser una muestra mejor y más fácil para el diagnóstico de CMV de la infección neonatal. Los resultados de la presente investigación sugieren que a los pacientes ingresados en la UCIN -- especialmente los bebés prematuros cuyos resultados de laboratorio no son compatibles con la infección bacteriana y presentan signos sugestivos de infección por CMV – se les debe realizar una prueba de PCR en orina para confirmación.

Palabras claves: citomegalovirus, infección neonatal, recién nacido, reacción en cadena de polimerasa anidada

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INTRODUCTION

Cytomegalovirus (CMV) is considered one of the main viruses responsible for congenital infections and shows infection rates ranging from 0.5% to 2.5%. Intrauterine definition transmission of infection from mother to fetus worsens prognosis and increases the chances of severe malformations. Transplacental infection (1) occurs between 3% and 5%, but can occur from organ transplants, blood transfusion, saliva, urine and genital secretions. In ninety per cent of asymptomatic newborns, delayed clinical manifestations such as deafness, mental retardation and chorioretinitis may occur over the first two years of life (2). Early diagnosis is important both for the symptomatic and asymptomatic patient. The viral DNA detection can be made by various molecular biology methods (1, 3). This research aims to study the frequency of congenital CMV infection in newborns admitted to neonatal units by molecular biological methods and to compare the use of samples of blood and urine.

SUBJECTS AND METHODS

A cross-sectional study was carried out from March 2010 to August 2012. Five hundred and twenty newborns who were admitted to the neonatal intensive care units (NICUs) in five hospitals were eligible for the study. The study was approved by the Ethics Committee. Blood and urine samples were collected appropriately. The collections of biological materials were made as the patients were admitted to the NICU. Medical records were reviewed to determine the variables related to prematurity, outcome and symptoms.

Urine samples were used directly for PCR, DNA extraction (GE Healthcare, UK) was performed on blood (4). The quantification of DNA was spectrophotometrically (BioPhotometer plus, Eppendorf, Hauppauge, NY, USA) standardized to a concentration of 10 ng/μL.

Polymerase chain reaction was carried out and DNA amplification was performed using nested PCR method, using the outside primers (5'-TG AGG AAT GTC TTC AGC-3'and 5'-TC TCC TCG AGG ATG AGA-3') in the first reaction, which yields an *amplicon* of 347 bp, serving as template for the second reaction which used the internal primers (5'-CCA ACT GCC AGA TCT TCA T-3' and 5'-TCG AGA TCC CCC AGG TTG TA-3') for an *amplicon* of 297 bp, according to Martiny *et al* (5).

The reactions were conducted in GeneAmp model 9600 thermal cycler (Applied Biosystems, Foster City, USA) in duplicates and the final products of the reactions were detected by agarose gel electrophoresis (Ludwig Biotec) at 1.8%, containing 2.5 μl/100 mL of dye SYBR SAFE (Invitrogen). The size of the DNA fragments was estimated based on the 100 bp DNA ladder marker (Invitrogen), photographed and computer documented. The positive control was analysed in a biological sample known to be positive, provided by the Molecular Biology Laboratory of the Hospital das Clinicas of Ribeirao Preto, SP. The negative control was set up with autoclaved ultrapure water and CMV negative DNA.

Statistical analysis was performed using the nonparametric paired Wilcoxon test, which used data collected from medical records tabulated using Epi Info 3.5.2.

RESULTS

Of 520 neonates analysed, 13 (2.5%) were positive for CMV in urine, while blood showed 10 (2%) positives (Figure). Among the 13 urine-positive patients, three (23%) had no clinical signs suggestive of CMV; three (23%) other patients admitted to the NICU had no significant clinical findings suggestive of bacterial infection; blood culture was negative and some clinical signs were consistent with CMV. The last three patients were severely ill on mechanical ventilation but showed improvement after starting ganciclovir treatment.

Among the clinical manifestations, prematurity was the most characteristic observed among symptomatic patients (100%), followed by cholestatic jaundice [70%] (Table).

In relation to the serology, only one (7.8%) had positive IgM and IgG. One patient (7.7%) died. Regarding sociodemographic characteristics, over 50% were female, 11 (84.5%) were Caucasian and 69.2% were delivered by Caesarean section. The average birthweight was 1.782.5 g and mean gestational age was 33.5 weeks.

DISCUSSION

Cytomegalovirus is an infection that affects 0.2 to 2.2% of live births worldwide, varying according to the country and

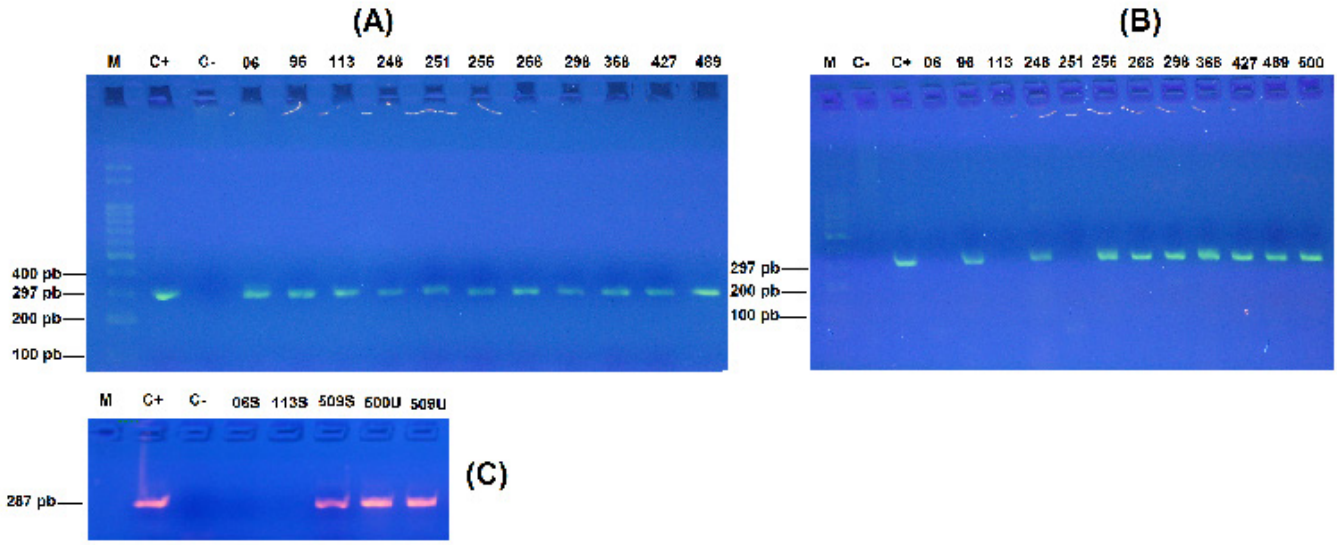

Figure: Agarose gel (1.8%) containing positive urine (n = 13) and blood (n = 10) samples. (A): 11 cytomegalovirus (CMV) positives in urine, (B): 9 positives in blood, (C): 2 CMV positives in urine (500U, 509U) and a positive in blood (509S). M = molecular weight marker; C+ = positive control; C- = negative control

Table: Clinical characteristics of symptomatic newborns cytomegalovirus polymerase chain reaction positive in Campo Grande-MS, 2010–2012

Symptoms	n	%
Prematurity*	10	(100.0)
Petechiae	2	(20.0)
Respiratory failure	5	(50.0)
Microcephaly	1	(10.0)
Lymphocytosis**	5	(50.0)
Eosinophilia***	4	(40.0)
Thrombocytopenia****	5	(50.0)
Transfusion	5	(50.0)
Hydrocephalus	1	(10.0)
Cholestatic jaundice$^{\varphi}$	7	(70.0)

* (< 37 weeks), ** > 50%, *** > 2%, **** < 140.000/µL, $^{\varphi}$ = direct bilirubin > 2 mg/dL

the different socio-economic classes. This infection may be clinically inapparent *ie* clinical signs are nonspecific and somewhat suggestive, overlapping with other viruses or infections. Cytomegalovirus infection can cause a disease with particularly severe clinical picture with severe sequelae.

Among the PCR results, we found that some positive samples in urine were negative in blood. This may be explained by the possibility of a patient not developing viraemia at the time of collection (7). Surveys report that venipuncture with the use of anticoagulant may also inhibit amplification of DNA from blood. However, the use of PCR instead of urine culture or serologic testing is becoming an important screening tool for congenital infections (8).

Polymerase chain reaction is a technique (1) that is being widely used in the detection of CMV genome. It has a higher sensitivity than other techniques, and can provide qualitative and quantitative results; other advantages include greater flexibility with the material being tested and the possibility of repeating the tests in case of doubtful results due to the use of small volumes of biological material.

Polymerase chain reaction technique offers speed and high sensitivity based on selective amplification of specific nucleic acid sequences from small amounts of biological specimens. Furthermore, the storage convenience of target specimens, such as its freezing at -20°C or -80°C, prior to test, gives PCR a larger advantage over any other technique, due to the stability of nucleic acids (5, 8).

Regarding clinical manifestations, they are almost exclusively in infants born to mothers with primary infection. The most frequent and major sequelae is early hearing loss (9, 10). Studies have reported the association of prematurity with congenital CMV infection, as well as an index of up to 30% mortality in symptomatic patients, because this infection is closely linked to the immune status of the patient (9, 10). Those who survive may exhibit neurologic sequelae or may be asymptomatic. Ten to fifteen per cent will show delayed changes (12, 13). Seventy per cent of children in this study had jaundice. Passos *et al* (14) reported jaundice in nine out of ten patients. Premature infants less than 32 weeks gestational age, and may have similar symptoms CMV sepsis. In addition, CMV infection in newborns can result in cholestasis (12). Studies also report high rates of hepatitis caused by CMV, around 7–17%. Hyperbilirubinaemia caused by CMV may be transient and liver injury in congenital cytomegalovirus infection is usually benign, but in some cases it can worsen, presenting pictures of chronic liver disease (12).

There was one death among the 13 positive cases, consistent with the results reported by other researchers. According to studies, newborns usually progress to death in the neonatal period as a result of severe infection that can develop (2, 7).

When relating the variables studied, Caesarean delivery theoretically would exclude contact with cervical secretions, however, the infant may acquire the infection transplacentally,

as the mother's antibodies do not protect the fetus (10). Other authors showed that females and Caucasians are more susceptible to CMV infection (1, 9). Nevertheless, it is here confirmed that the PCR test using specific gene target for identification of CMV in urine shows a slightly higher number of CMV positives than in blood and a greater sensitivity than serology, highlighting it as an important tool in the diagnosis of neonatal infection.

CONCLUSION

The detection of CMV in urine was slightly more efficient than in blood, and showed better sensitivity than in serological analysis; therefore, urine may be a better and easier specimen tool for CMV diagnosis in neonatal infection. Although CMV was highly correlated with prematurity, further studies must be done in order to clarify the role of other parameters such as mode of delivery, blood type, gender *etc*. The findings of the present research suggest that patients admitted to the NICU, especially premature infants, whose laboratory results are not consistent with bacterial infection, and presenting signs are suggestive of cytomegalovirus infection, should be referred for PCR on urine for confirmation.

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