Accuracy of CMV-DNA detection by PCR in amniotic fluid samples according to gestational age

A influência da idade gestacional na acurácia da reação em cadeia da polimerase (PCR) na detecção do citomegalovírus no líquido amniótico

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ABSTRACT

Objectives: To evaluate the efficacy of the polymerase chain reaction in amniotic fluid for the detection of fetal cytomegalovirus and assess whether it was influenced by the gestational age at the time of the amniocentesis. Methods: The clinical charts of 65 patients referred to a fetal medicine reference center in the city of São Paulo over an 8-year period (1997 to 2004) were analyzed by identification of maternal serum IgM positive for cytomegalovirus or by fetal ultrasound signs suggestive of the infection. Amniocentesis was carried out to determine the PCR specific for viral DNA detection. Results of this test and of ultrasound monitoring were confronted with the immediate perinatal result, and viral cultures in newborn urine were performed using human fibroblasts, the gold standard for diagnosis of congenital infection. Results: With no restriction as to gestational age to the time of amniocentesis, results showed 30.8% sensitivity, 100.0% sensitivity, 100.0% positive predictive value, and 68.4% negative predictive value. There was a statistically significant difference between the negative result (median of 16 weeks) versus positive result (median of 18 weeks) groups, p value = 0.016. Considering the tests carried out as of the 21st week of gestation, the test shows the following performance: 87.5% sensitivity, 100.0% specificity, 100.0% positive predictive value, 75% negative predictive value. Conclusion: The efficacy of amniotic fluid PCR is influenced by the gestational age at the time of the amniocentesis, and should be performed only after 21 weeks of gestation.

Keywords: Cytomegalovirus; Amniocentesis; Infection; Pregnancy complications; Polymerase chain reaction

RESUMO

Objetivos: Avaliar a eficácia da reação em cadeia da polimerase no líquido amniótico para detecção do citomegalovírus fetal e se esta foi influenciada pela idade gestacional em que foi realizada. Métodos: Foram analisados os prontuários de 65 pacientes encaminhadas a um serviço de referência em medicina fetal da cidade de São Paulo, num período de oito anos, de 1997 a 2004, por identificação da IgM positiva no soro materno para citomegalovírus ou por sinais ultra-sonográficos fetais sugestivos de tal infecção. A amniocentese foi realizada para determinação da PCR específica para detecção do DNA viral. Os resultados deste exame e do acompanhamento ultra-sonográfico foram confrontados com o resultado perinatal imediato, sendo a cultura de vírus da urina dos recém-nascidos realizada em fibroblastos humanos, o padrão ouro para o diagnóstico da infecção congênita. Resultados: Sem restrição para idade gestacional da realização da punção, foi obtida sensibilidade de 30,8%, especificidade de 100,0%, valor preditivo positivo de 100,0%, valor preditivo negativo de 68,4%. Houve diferença com significância estatística entre os grupos com resultado negativo (mediana 16 semanas) versus positivo (mediana 18 semanas), p = 0,016. Se forem considerados os exames realizados a partir da 21ª semana de gestação, o exame apresentaria a seguinte performance: sensibilidade de 87,5%, especificidade de 100,0%, VPP de 100,0%, VPN de 75%. Conclusão: O exame da PCR no LA tem eficácia influenciada pela idade gestacional da realização da punção, devendo ser oferecido somente após 21 semanas de gestação.

Descritores: Citomegalovírus; Amniocentese; Infecção; Complicações na gravidez; Reação em cadeia da polimerase

INTRODUCTION

In many parts of the world, cytomegalovirus (CMV) is one of the most common etiological agents of congenital and perinatal infections. It occurs in 0.2 to 2.2% of newborns, with a greater incidence in populations with a low socioeconomic level¹.
Approximately 1% of the fetuses born in the USA present with congenital CMV infections, which corresponds to 40,000 affected newborns. Of these, between 3,000 and 4,000 are symptomatic at birth, and another 4,000 to 6,000 experience abnormal neurological or auditory development during the first years of life. CMV infection is the most common cause of hearing loss in childhood, and is considered a public health problem in the USA. The annual costs of caring for children with congenital CMV infections in that country are estimated at about 1.86 billion dollars\(^{(2)}\).

In a review of the last 15 years in different geographical areas and socioeconomic levels, the highest (100%) and the lowest (40%) seroprevalence rates for CMV antibodies were noted. In terms of congenital infection, the greatest percentage observed was around 2.2% and the lowest was 0.3%\(^{(3)}\).

Of the fetuses with congenital infection resulting from primary maternal infection, 10 to 15% displayed symptoms at birth. The most common clinical manifestations are hepatosplenomegaly, intracranial calcifications, jaundice, symmetrical growth restrictions, microcephaly, chorioretinitis, and hearing loss. The most frequent laboratory abnormalities are thrombocytopenia, hyperbilirubinemia, and elevated hepatic transaminases. Approximately 30% of the seriously infected newborns progress to death, and 80% of those who survive will experience serious neurological sequelae. About 85 to 90% of infected fetuses are asymptomatic at birth. Of these, 10 to 15% present one or more sequelae up to two years of age: neurosensory hearing loss, chorioretinitis, dental defects, mental retardation, and optic nerve atrophy\(^{(4)}\).

In Brazil, however, routine prenatal diagnosis of this disease is not sought, precluding early intervention with therapeutic resources that could minimize the severity of the cases and decrease intensity of the sequelae, especially as to early treatment of neurosensory hearing loss. This would allow the use of hearing aids for the correct development of language, maximizing the patient’s ability to interact with society.

Recently, at a neonatal intensive care unit in the city of Belo Horizonte, Minas Gerais, 6.8% of newborns were found infected by CMV\(^{(5)}\). This high rate of neonatal viral involvement, according to the authors themselves, is due to the low socioeconomic level of the study subjects and the more serious clinical characteristics of this population.

In our area, at UNIFESP, a survey carried out in 850 lower middle class pregnant women with a low risk of infection by CMV showed a 0.80% rate of acute infection during pregnancy in the first trimester and a 91.83% rate of IgG anti-CMV presence at the first prenatal visit\(^{(6)}\).

Considering the epidemiologic importance of this disease, a highly precise tool is necessary in order to provide correct genetic counseling in cases of maternal CMV infections or ultrasound results suggestive of fetal infection by this pathogen.

There are many studies seeking the earliest possible diagnosis of fetal infection, which along with the evolution of diagnostic methods, went from cordocentesis, with a nonspecific and specific assessment of fetal blood, to the more recent use of the polymerase chain reaction (PCR) technique carried out in fetal fluids, especially amniotic fluid.

This progression enabled the true role of the fetologist to be fulfilled, i.e., of not only “looking” by means of ultrasound at the natural progression of the disease, but of also selecting infected fetuses with the help of current technology and treatment arsenal. The goal is to afford a specialized postnatal follow-up and minimize sequelae, so that the affected patient is able to fight, at the same level as his peer, for his space in society.

In light of the aspects presented, associated with the fact that an infected fetus is a potentially high-risk patient who deserves specialized postnatal care, we feel motivated to analyze the accuracy of the main diagnostic instrument for fetal infection - polymerase chain reaction (PCR) - in samples of amniotic fluid for detection of viral DNA.

The first reference in literature as to the use of PCR for detection of CMV in the perinatal period dates back to 1988, when Demmler et al.\(^{(7)}\), described the use of this technique for diagnosing congenital CMV using the urine of 44 neonates with infections confirmed by urine culture. Compared to culture, the test under study presented a 93% sensitivity rate, and a 100% specificity rate.

In 1993, a comparison was made between the use of PCR and histopathological diagnosis of CMV in placental biopsies\(^{(8)}\). From 2,073 placentas, the diagnosis of chorionic villi inflammation was made in 44 (2.12%). Of these, the presence of CMV was detected in 4 placentas by means of PCR. In only one case was the diagnosis possible by means of microscopy (identification of cytomegalic inclusion). The authors concluded that PCR should be used for the diagnosis of CMV as an etiological agent of placental inflammations.

However, it was only in 1992 that fetal diagnosis through molecular biology was suggested at the University of Frankfurt, Germany\(^{(9)}\), where the use of the PCR technique in amniotic fluid for diagnosis CMV-DNA was reported in a woman referred due to her husband’s diagnosis of acute cytomegalovirus infection. Since her serum tests were yet inconclusive, this alternative diagnostic method was proposed for the couple. The test result was positive, the infection was confirmed in the
neonatal period, and the newborn did not present any sequelae up until the 6th week of life.

In 1993, on the other hand, the first protocol of the study technique was reported using a sample collected from the chorionic villus. The first report included 8 patients. The authors merely established the possibility of performing the technique that in some cases confirmed positivity compared to the viral culture in amniotic fluid.\(^\text{(10)}\)

The first papers describing the use of PCR in amniotic fluid were not very successful, as was demonstrated by Donner et al.\(^\text{(11)}\), who in 36 amniocentesis cases obtained 45% sensitivity, but 100% specificity. It is interesting to note that the material was collected between the 14th and 21st weeks of gestation. The gold standard established was virus culture in neonate urine. In 1995, the molecular biology technique was confronted with the amniotic fluid virus culture for diagnostic purposes.\(^\text{(12)}\) Twenty-six pregnant women were included in this study, and amniocentesis was carried out between 14 and 36 weeks of gestation. PCR showed a better performance than the culture, with 76.9% versus 69.2% sensitivity, respectively. The specificity was 100% with both techniques.

In order to compare the PCR performed in the amniotic fluid with PCR carried out in fetal blood,\(^\text{(13)}\) 82 pregnant women diagnosed with acute cytomegalovirus were referred to the Hospital Policlinico [Polyclinical Hospital] in Bologna, Italy. Amniocentesis was performed in order to obtain amniotic fluid (20 ml) and cordocentesis was done for the acquisition of fetal blood (2 ml) for the study. The gold standard was the virus culture in human fibroblasts in neonate urine. The test performed using the amniotic fluid showed greater sensitivity relative to the fetal blood: 100% versus 66.6%, respectively. This study had as a conflicting result a specificity of around 85% for both modalities, an isolated fact in medical literature that shows 97-100% of specificity in almost all studies. The authors suggest that this was caused by the high sensitivity of the method, allowing a diagnosis of the virus that was exterminated during pregnancy by the body’s natural defenses.

Bodeus et al.\(^\text{(14)}\) were the first to observe that when the puncture was performed later in pregnancy, sensitivity of the method improved, from 70% to 95.5% according to the authors, when carried out only after the 23rd week of gestation.

One study of this diagnostic tool was performed in 1999\(^\text{(15)}\) with postnatal follow-up until 2 years of age. Two hundred and ten patients diagnosed with acute CMV infection were studied, and 55 of these transmitted the virus to the fetus (26%). Approximately 18% of the neonates diagnosed with the congenital disease by means of this method presented serious neurological sequelae. The study of PCR in amniotic fluid allowed a global sensitivity of 89%, but the authors observed that when the test was performed after 21 weeks, or if a 7-week interval was kept between the time of the maternal diagnosis and the amniocentesis, the precision increased.

The observation of optimization of PCR use in amniotic fluid after 21 weeks was then proved statistically\(^\text{(16)}\) in a study that divided the population of 189 expectant women into two groups, 94 of them underwent the procedure at 21 weeks and 95 of them at > 21 weeks. There was a sensitivity difference of 66% to 96%, respectively, with a p < 0.05 using Pearson’s x² test.

In the previous study, when the use of fetal blood with PCR and IgM specific for CMV was associated to the use of PCR in amniotic fluid, there was 100% of sensitivity and specificity.

Other authors\(^\text{(17)}\) who did not discriminate gestational age for the amniotic fluid puncture, obtained lower rates of sensitivity and specificity, 77% and 97%, respectively.

From 2001 until today, no references in literature are cited using qualitative identification of PCR in amniotic fluid, as the use of quantitative PCR testing, which is not the focus of this study, was given preference.

**OBJECTIVES**

To evaluate, in our area, the diagnostic accuracy of PCR as an instrument in detecting CMV in amniotic fluid and the influence of the patient’s gestational age on the results.

**METHODS**

This clinical study is a cross-section retrospective analysis of the medical files of patients with acute CMV infections, as per inclusion criteria, seen by the Fetal Medicine departments of the Hospital São Paulo (HSP) and the Centro Paulista de Medicina Fetal (CPMF); all patients were under the coordination of Prof. Dr. Antônio Fernandes Moron, which assured uniform management. As inclusion criteria, the clinical records of these pregnant women were retrospectively analyzed over a period of eight years, from October 1997 to December 2004. Included were those patients who presented with positive or reactive serum IgM tests specific for CMV, or with values above the “cut-off” level, ultrasound signs suggestive of congenital infection, and who underwent outpatient investigations resulting in a diagnosis of acute or recurring CMV infection.

Exclusion criteria were twin pregnancy, follow-up failure during the prenatal or postnatal period, fetal
death, and a desire to interrupt the pregnancy manifested by the couple during any phase of the diagnostic investigation.

The initial series was comprised by 87 patients; 22 (25.3%) patients were excluded, 4 (4.6%) of them because of fetal death, 11 (12.6%) cases because of prenatal monitoring drop-out or loss of postnatal data, and seven (8%) cases due to the parent’s desire to interrupt the pregnancy.

The data from the cases included in the study (65 cases) were stored in an electronic chart.

During the genetic counseling for all pregnant women positive for cytomegalovirus-specific IgM or with fetal ultrasound abnormalities suggestive of congenital infection posteriorly confirmed by laboratory assessments, the following data were collected: identification of the parents, personal and family medical histories, history of the current pregnancy (gestational age, maternal serum tests, use of medication, prior and current ultrasound findings, and amniocentesis data). After the interview, the amniocentesis technique was explained in detail to the parents, discussing its risks and benefits.

Next, the patient was referred to an ultrasound examination for verification of the criteria for a diagnostic eligibility (gestational age greater than 15 weeks, absence of vaginal bleeding, absence of cramping pain or premature labor, and absence of signs indicating any abnormality of the amniotic membrane and placenta site).

Amniocentesis was performed on an outpatient basis after the ultrasound test in order to evaluate the best puncture site. Asepsis of the abdomen was performed with chlorhexidine and the area was delimited with surgical drapes. Local anesthesia was given using 2% lidocaine with no vasoconstrictor. A 20- or 22-gauge spinal needle was used, approximately 9 cm in length. A sterile 20 ml syringe extracted the sample. The puncture was monitored by ultrasound(18). After the puncture, Anti-D immunoglobulin was prescribed for non-sensitized RH- patients whose husbands were RH+.

The material was appropriately labeled and was sent to the laboratory in an insulated container (Clinical/Experimental Pediatrias Laboratory Department of Pediatrias, Medical School, Universidade de São Paulo) along with the completed form on the same day it was collected, or within five days, at the most, as long as properly refrigerated at the center where the test was performed. After sample collection, the patient was released and instructed to rest for 24 hours and seek emergency medical care in case of bleeding, loss of fluid, or abdominal cramps.

Primers for CMV were described by Stenberg et al.(19), chosen from a fragment of the gene that encodes the most abundant CMV surface antigen, called the “major immediate early antigen.” The primers are called MIE-4 (sense): 5’-CCAAGCGGCTCTGATAACCAAGGC-3’ and MIE-5 (anti-sense): 5’-CAGCACCTCCTCCTTT CCTCTGG-3’. These primers amplify a fragment of 435 bp. The primers for the second PCR were MIE-6 (sense): 5’-AGTGTGGATGACCTACGGGCCATCG-3’ and MIE-7 (anti-sense): 5’-GGTGACACAGAGATCA GAGGAGC-3’, and they amplify a fragment of 110 bp.

The PCR results obtained with the sample collected were delivered at the patient’s next visit, when guidance was given according to each result:

- Patients referred for IgM positive for CMV, with no ultrasound abnormalities.
  - Positive Result: Sonogram monitoring every two weeks + echocardiogram. Rigorous investigation of the newborn in order to detect neurosensory hearing loss, and further specialized pediatric follow-up.
  - Negative Result: Routine follow-up.

- Patients referred for fetal abnormalities suggestive of congenital infection (e.g., non-immune hydropsy). In addition to the amniocentesis, cordocentesis was also performed for determination of complete blood count, hepatic enzymes, fetal protein, and specific serology, in order to clarify the diagnosis and indicate fetal transfusion in cases of anemia.
  - Positive Result: Weekly ultrasound monitoring + echocardiogram + fetal treatment when necessary to improve intrauterine conditions.
  - Negative Result: Continue investigation of the cause of ultrasound abnormalities.

Obstetric options are chosen by the patient’s treating physician after the results of the work-up. Patients at the Hospital São Paulo are under the care of the Obstetrics Department of UNIFESP.

The virus culture in human fibroblasts obtained from neonate urine analysis was considered the gold standard.

Gestational age at diagnosis, gestational age at the time of amniocentesis, and perinatal results (positive or negative culture on the newborn) were assessed.

Initially, the results obtained in each group were allocated on contingency tables, and later these tables were reconstructed according to the gestational age at puncture. Comparisons were made by means of appropriate statistical tests and construction of 95% confidence intervals (95% CI), Sensitivity, specificity, and positive and negative predictive values were calculated using classic formulas(20). Additionally, the 95% CI was calculated for each of the abovementioned parameters.

Homogeneity between the groups formed according to the neonatal results was evaluated as per type of
variable. For quantitative variables, the comparison was made using Student’s t test, and in cases where the assumption of normality was not accepted, Mann-Whitney’s test was used. For qualitative variables with two categories, the study of possible associations was evaluated using Fisher’s exact test, and for variables with more than two categories, generalization of Fisher’s exact test was used.

For all statistical analyses, a 5% significance level ($\alpha$) was adopted, i.e., results were considered significant when $p$ was lower than 5% ($p < 0.05$).

- Statistical analysis was carried out using the following software:
  - STATA 7.0 for calculations of sensitivity, specificity, positive predictive value, negative predictive value, and ROC curve;
  - CIA version 2.0.0 (confidence interval analysis) for confidence interval calculations;
  - SPSS 12.0 for Windows for the study of homogeneity among the groups.

**RESULTS**

Patients were classified according to the presence of cytomegalovirus in urine cultures performed in newborns, thus characterizing congenital infection: negative urine - 39 cases and positive urine - 26 cases.

Therefore, we observed a 40% estimated perinatal transmission rate (95% CI [28.0%; 52.9%]).

Gestational age at the time of maternal diagnosis of acute CMV infection, characterized by the identification of CMV-specific IgM in maternal blood, varied from 9 to 26 weeks.

According to figure 1, the group with positive results presented greater variability relative to the group with negative results. It is also clear that the median gestational age at diagnosis for the group with positive results was greater (13 weeks) than that shown by the group with negative results (12 weeks), although no statistically significant difference was noted between the two groups with the Mann-Whitney test ($p = 0.197$).

Another fact to be observed on figure 1 is that the group with negative results showed an isolated maximal point, i.e., the 24-week gestational age at diagnosis is distant from the second highest gestational age at diagnosis (18 weeks) for this group. In the groups with positive urine culture results, two cases were seen with gestational ages at diagnosis (24 and 26 weeks) distant from the other cases.

Gestational age at the time of amniocentesis for amniotic fluid for PCR varied from 15 to 30 weeks. Distribution of this variable according to the group can be noted on figure 2, where it is clear that the group with positive results presented greater variability when compared to the group with negative results. Additionally, median gestational age at the time of amniocentesis for the group with positive results was higher (18 weeks) than for the group with negative results (16 weeks), and this difference proved statistically significant upon use of the Mann-Whitney test ($p = 0.016$).

Of the pregnant women evaluated, approximately 12% (95% CI - [6%; 23%]) presented a positive PCR result. The study proposal was to evaluate sensitivity, specificity, and positive and negative predictive values in order to assess use of PCR in comparison with the gold standard, which is neonate urine testing. Sensitivity (proportion of positive results correctly identified by PCR) was
30.8% (95% CI [16.5%-50.0%]), specificity (proportion of negatives correctly identified by PCR) was 100.0% (95% CI [91.0%-100.0%]) (table 1). In other words, the test proposed showed greater specificity than sensitivity. The positive predictive value, i.e., proportion of patients with positive PCR results who truly had positive neonate urine tests, was 100.0% (95% CI [67.6%-100.0%]). On the other hand, the negative predictive value (proportion of patients with negative PCR results who were correctly identified) was 68.4% (95% CI [55.5%-79.0%]).

Table 1. Distribution of patients by results of newborn urine and PCR

<table>
<thead>
<tr>
<th>PCR</th>
<th>Newborn urine</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>total</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>8</td>
<td>-</td>
<td>8 (12.3%)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>18</td>
<td>39</td>
<td>57 (87.7%)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>26 (40.0%)</td>
<td>39 (60.0%)</td>
<td>65 (100.0%)</td>
<td></td>
</tr>
</tbody>
</table>

A ROC curve was then constructed to determine the diagnostic accuracy of the test. The area under the curve is used to quantify the accuracy. According to figure 3, this area is 98% with a 95% CI given by [0.951; 1.000]. An area under the curve equal to 1.0 indicates the perfect separation of the evaluated test between the two groups. As to the value obtained for this area, it can be interpreted as follows: 98.0% of the time, a randomly selected subject with a positive PCR result has a gestational age at the time of amniocentesis greater than that of a randomly selected subject with negative PCR results. Additionally, we can affirm that there is evidence that gestational age at the time of amniocentesis distinguishes the patients between the two groups, since a 95% confidence interval does not contain the value 0.5 (a value in which there is no evidence of PCR differences between the two groups).

Given the evidence that gestational age at the time of amniocentesis is able to differentiate the patients from both groups (positive PCR and negative PCR), it is necessary to find a cut-off point with the greatest chance of detecting a positive result.

According to what can be noted on table 2, no statistically significant difference was seen in the proportion of positive results between the gestational age under 16 weeks at time of amniocentesis and the gestational age greater than or equal to 16 weeks at the time of amniocentesis (p = 0.185). As of the gestational age of 17 weeks at the time of amniocentesis, a statistically significant difference is observed in the proportion of positive results between the gestational age under 17 weeks at the time of amniocentesis and the gestational age greater than or equal to 17 weeks at the time of amniocentesis (p = 0.002). It is evident that as the gestational age at puncture increases, the difference in proportion of positive results also increases, and for the gestational age of 20 weeks, this difference exceeds 50%.

Table 2. Distribution of patients by gestational age at puncture and PCR result

<table>
<thead>
<tr>
<th>GA puncture (weeks)</th>
<th>PCR Positive</th>
<th>Negative</th>
<th>p value+</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;16</td>
<td>-</td>
<td>14 (100.0%)</td>
<td>0.185</td>
</tr>
<tr>
<td>≥16</td>
<td>8 (15.7%)</td>
<td>43 (84.3%)</td>
<td>0.002</td>
</tr>
<tr>
<td>&lt;17</td>
<td>-</td>
<td>34 (100.0%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>≥17</td>
<td>8 (25.8%)</td>
<td>23 (74.2%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>&lt;18</td>
<td>-</td>
<td>41 (100.0%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>≥18</td>
<td>8 (33.3%)</td>
<td>16 (66.7%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>&lt;19</td>
<td>-</td>
<td>48 (100.0%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>≥19</td>
<td>8 (47.1%)</td>
<td>9 (52.9%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>&lt;20</td>
<td>-</td>
<td>50 (100.0%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>≥20</td>
<td>8 (53.3%)</td>
<td>7 (46.7%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>&lt;21</td>
<td>1 (1.9%)</td>
<td>53 (98.1%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>≥21</td>
<td>7 (63.6%)</td>
<td>4 (36.4%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>&lt;22</td>
<td>1 (1.8%)</td>
<td>55 (98.2%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>≥22</td>
<td>7 (77.8%)</td>
<td>2 (22.2%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>&lt;23</td>
<td>2 (3.5%)</td>
<td>56 (96.5%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>≥23</td>
<td>6 (85.7%)</td>
<td>1 (14.3%)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Table 3. Distribution of patients by gestational age at puncture

Based on these data, sensitivity, specificity, PPV, and NPV of the PCR test relative to the gold standard (newborn urine testing) for the gestational age at the time of amniocentesis as of the 20th week were calculated, as is displayed on table 3.

Associating the urine culture results with the gestational age at the time of the PCR we obtained the distribution shown on table 4, with statistical significance.

According to table 4, above, one can observe that of the eight cases presenting both positive tests, the median gestational age at the time of amniocentesis was 24 weeks, a result higher than that seen among the results with negative PCR and positive urine testing (16 weeks) or negative PCR and negative urine testing (16 weeks). By means of the Kruskal-Wallis test, a statistically significant difference for the gestational age at amniocentesis was observed as per the results presented by both tests (p
We also point out that avidity testing is not a part of our routine procedures, because when the test result suggests a past infection, it limits the date to 12 weeks, which is exactly the median gestational age at diagnosis in our study.

According to what was proposed by Revello e Gerna(22), the avidity test should be performed, along with IgM and IgG tests, up to the 10th week of gestation in order to distinguish late versus recently acquired infection. Even so, it is not possible to differentiate a late infection from a recurrence(23) or from a reinfection by another strain(24), although some authors recommend the use of the avidity test since IgM can be positive for up to 300 days after the primary infection, leading to many false positives in the current ultra-sensitive serologic tests.

There is no doubt in medical literature(22,25) that currently the best tool for diagnosis of fetal involvement by the disease in question is PCR determination in amniotic fluid in order to identify viral DNA.

Our data show a global sensitivity for amniotic fluid PCR in the diagnosis of fetal CMV equal to 30.8% (95% CI [16.5%-50.0%]), and a specificity of 100.0% (95% CI [91.0%-100.0%]). The positive predictive value was 100.0% (95% CI [67.6%-100.0%]), and the negative predictive value was 68.4% (95% CI [55.5%-79.0%]).

Initially, these results were not very encouraging, but we noted that as the gestational age at amniocentesis increased, the number of positive urine culture tests decreased. We observed that 87.5% of the fetuses at risk for congenital infection with CMV detected by PCR testing were positive urine tests (p = 0.964). In other words, the gestational age at amniocentesis was greater among those who had both positive urine and positive PCR results (p = 0.001). No statistically significant difference in the gestational age at amniocentesis was noted among those who predicted negative PCR results and positive or negative urine tests (p = 0.964). In other words, the gestational age at amniocentesis was greater among those who had both positive urine and positive PCR results.

The results of PCR testing strongly influenced obstetric practice. It is important to recognize that the higher the sensitivity of the test, the less the observed number of diagnosis. For example, 87.5% of the fetuses at risk for congenital infection with CMV detected by PCR testing were positive urine tests. Similarly, 87.5% of the fetuses at risk for congenital infection with CMV detected by PCR testing were positive urine tests.

### Table 3. Sensitivity, specificity, predictive values for different gestational ages at puncture for CMV detection

<table>
<thead>
<tr>
<th>GA puncture (weeks)</th>
<th>PCR</th>
<th>NB urine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pos</td>
<td>Neg</td>
</tr>
<tr>
<td>≥20s</td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>72.7</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>[43.4;90.3]</td>
<td>[67.6;100.0]</td>
</tr>
<tr>
<td>≥21s</td>
<td>7</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>87.5</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>[43.8;100.0]</td>
<td>[64.6;100.0]</td>
</tr>
<tr>
<td>≥22s</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>[64.6;100.0]</td>
<td>[9.5;90.5]</td>
</tr>
<tr>
<td>≥23s</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>[61.0;100.0]</td>
<td>[20.7;100.0]</td>
</tr>
</tbody>
</table>

### Table 4. Descriptive measures of gestational age at puncture in terms of PCR result related to result of viral culture in newborn urine

<table>
<thead>
<tr>
<th>PCR</th>
<th>URINE</th>
<th>n</th>
<th>Mean</th>
<th>Standard deviation</th>
<th>Median</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>Positive</td>
<td>8</td>
<td>24.3</td>
<td>3.0</td>
<td>24.0</td>
<td>20.0</td>
<td>30.0</td>
</tr>
<tr>
<td>Positive</td>
<td>Negative</td>
<td>18</td>
<td>17.0</td>
<td>2.0</td>
<td>16.0</td>
<td>15.0</td>
<td>22.0</td>
</tr>
<tr>
<td>Negative</td>
<td>Positive</td>
<td>39</td>
<td>16.8</td>
<td>2.0</td>
<td>16.0</td>
<td>15.0</td>
<td>24.0</td>
</tr>
</tbody>
</table>

### DISCUSSION

It is interesting to observe that the median gestational age at the time of diagnosis of CMV was situated between 12 and 13 weeks. This shows a peculiar characteristic of serologic tracking in our population. Since serologic testing is not routinely done before conception, we adopt the obstetric practice of diagnosing a primary infection only when positive IgM is detected by ELISA testing in the mother’s serum during the first trimester.

This explains the small number of diagnoses we have made(21). In a prospective serologic assessment of 7140 women, 44 cases of congenital infection were identified: eight by reactivations, 22 women who presented seroconversion, 20 who initially were IgM positive. In other words, if we considered merely positive IgM detection, we could probably make diagnoses in only 31% of the fetuses at risk for congenital infection with this disease. The authors advocate the use of serologic tracking using ELISE during preconception, during the first trimester, and at the end of the gestation(21).

This characteristic of our protocol for serologic detection of CMV during pregnancy, associated with the resistance on the part of obstetricians for performing the tracking, may explain the small number of patients in this study, considering that it covered an eight-year interval, despite being a prevalent disease. If we consider 0.80% of primary disease in our population at UNIFESP(6), and the fact that this number only represents 31% of the fetuses at risk for CMV infections(25), we can suppose that the subjects in our study represent a sub product of 35,080 patients accompanied during this period.

We also point out that avidity testing is not a part of our routine procedures, because when the test result suggests a past infection, it limits the date to 12 weeks, which is exactly the median gestational age at diagnosis in our study.

According to what was proposed by Revello e Gerna(22), the avidity test should be performed, along with IgM and IgG tests, up to the 10th week of gestation in order to distinguish late versus recently acquired infection. Even so, it is not possible to differentiate a late infection from a recurrence(23) or from a reinfection by another strain(24), although some authors recommend the use of the avidity test since IgM can be positive for up to 300 days after the primary infection, leading to many false positives in the current ultra-sensitive serologic tests.

There is no doubt in medical literature(22,25) that currently the best tool for diagnosis of fetal involvement by the disease in question is PCR determination in amniotic fluid in order to identify viral DNA.

Our study sought to assess the efficacy of this instrument when used quantitatively. We carried out a retrospective evaluation of cases between 1997 and 2004.

Our data show a global sensitivity for amniotic fluid PCR in the diagnosis of fetal CMV equal to 30.8% (95% CI [16.5%-50.0%]), and a specificity of 100.0% (95% CI [91.0%-100.0%]). The positive predictive value was 100.0% (95% CI [67.6%-100.0%]), and the negative predictive value was 68.4% (95% CI [55.5%-79.0%]).

Initially, these results were not very encouraging, but we noted that as the gestational age at amniocentesis increased, the number of positive urine culture tests decreased. We observed that 87.5% of the fetuses at risk for congenital infection with CMV detected by PCR testing were positive urine tests.
increased. The group with negative urine cultures had a median gestational age of 16 weeks, and the group with positive urine cultures had a median gestational age of 18 weeks, with $p = 0.016$ as per the Mann-Whitney test. This was because the patients referred to us with higher gestational ages already had ultrasound abnormalities, and the diagnosis of cytomegalovirus was made retrospectively in maternal serum, contrary to the other cases referred to us because of abnormal maternal serum tests.

In evaluating PCR as a diagnostic instrument, we perceive that positivity increased as the gestational age at amniocentesis increased. For each additional week in gestational age at amniocentesis, the number of positive results also increases, with $p < 0.001$ according to Fisher’s Exact Test, which is also confirmed by the ROC curve, with an area of 98%.

A possible bias of the observation that the gestational age at amniocentesis influences the results would be the fact that more fetuses where infected when the maternal serum diagnosis was made at a later time, as shown above, and this would lead to a false conclusion that the precision of the PCR test improved over the weeks.

This misconception was cleared up when we analyzed PCR together with the neonate urine cultures, where the median obtained for the group with positive urine cultures and PCR results was 24 weeks and for those with negative PCR results and positive or negative urine cultures it was 16 weeks, proving a statistically significant difference by the Krustal-Wallis test.

CONCLUSION

We have demonstrated that when we advance in gestational age, the test under study is optimized; as of the 21st week of gestation, we obtained a sensitivity of 87.5% (95% CI [52.9%-97.8%]), specificity of 100.0% (95% CI [43.8%-100.0%]), positive predictive value of 100.0% (95% CI [52.9%-97.8%]), specificity of 100.0% (95% CI [64.6%-100.0%]), and negative predictive value of 75.0% (95% CI [30.1%-95.4%]). As of the 23rd week of gestation, results were 100% for all these criteria.

REFERENCES