Real-Time PCR Quantification of Human Cytomegalovirus DNA in Amniotic Fluid Samples from Mothers with Primary Infection

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Received 6 April 2001/Returned for modification 7 July 2001/Accepted 19 January 2002

A real-time PCR assay was developed to quantify human cytomegalovirus (HCMV) DNA in amniotic fluid (AF) samples collected from 30 pregnant women with primary HCMV infection as detected either from HCMV-immunoglobulin G (IgG) seroconversion or by the presence of HCMV-specific IgG and IgM associated with a low IgG avidity. Clinical information available for each case included ultrasonographic examination and fetal or newborn outcome. HCMV infection of fetuses or newborns was confirmed for the 30 studied cases. AF samples were subdivided into three groups. In group A (n = 13), fetuses presented major ultrasound abnormalities, and pregnancy was terminated. In group B (n = 13), fetuses had normal ultrasound findings, the pregnancy went to term, and the newborns were asymptomatic at birth. In group C (n = 4), fetuses had no or minor ultrasonographic signs, and pregnancy was terminated. The HCMV DNA load values in AF samples were significantly higher in group A (median, 2.8 × 10^6 genome equivalents [GE/ml]) than in group B (median, 8 × 10^5 GE/ml) (P = 0.014). Our findings suggest that HCMV load level in AF samples correlates with fetal clinical outcome but might also be dependent on other factors, such as the gestational age at the time of AF sampling and the time elapsed since maternal infection.

Human cytomegalovirus (HCMV) is the most common cause of viral intrauterine infection in developed countries, affecting 0.5 to 2% of all live births (1, 32, 40). Although the possibility of severe symptomatic fetal infection following recurrent maternal infection has been reported (8), fetal damage is mostly related to primary maternal infection (16, 38, 39). In this case, the transmission of the virus to the fetus may occur in 20 to 50% of pregnancies (18, 27, 38). It has been reported that HCMV transmission rates increase with gestational age and that a major risk of transmission is observed for seroconversion occurring late in pregnancy (5).

Congenitally infected infants are asymptomatic at birth in about 90% of cases, and for symptomatic infants, infection ranges from mild to severe disseminated life-threatening disease resulting in up to 20% perinatal mortality (9, 16, 22, 28, 31, 37). Up to 90% of the surviving symptomatic newborns will exhibit psychomotor and perceptual sequelae such as sensorineural hearing loss, mental retardation, cerebellar atrophy, seizures, and visual defects (2). Additionally, 10% of the infants who are asymptomatic at birth will later develop complications, mainly neurodevelopmental defects and deafness (7, 15, 40).

The diagnosis of maternal primary HCMV infection is based mostly on serological testing, including HCMV immunoglobulin G (IgG) seroconversion and the presence of specific HCMV IgG and IgM (35). Moreover, the direct detection of viral components, including pp65 antigenemia and DNAemia, can be helpful for acute infection diagnosis. Prenatal diagnosis of HCMV congenital infection relies on virus isolation from amniotic fluid (AF) and/or viral DNA detection by PCR in AF samples (4, 12, 24, 25, 36, 42). In a previous study, we reported that the combination of culture and PCR on AF samples allows a reliable prenatal diagnosis, with 72% sensitivity and 97.6% specificity (18). However, HCMV detection in AF samples does not allow us to discriminate among infected fetuses those who will develop a symptomatic disease from those who will remain asymptomatic. Several factors, such as the gestational age at the time of maternal HCMV infection, the level of viral replication in both mother and fetus, possible differences in viral virulence, and the immune response, might influence the outcome of fetal infection (3, 10, 19).

Regarding HCMV infection during pregnancy, the challenge is not only to detect fetal infection, but also to determine whether the infection will have any clinical consequences, as 90% of infected newborns are asymptomatic. Concerning the need to distinguish HCMV infection from HCMV disease, quantification of HCMV DNA in plasma or leukocytes has been proposed to be more specifically associated with the disease in immunocompromised patients (6, 44). The prognosis value of the quantification of HCMV DNA in AF samples has recently been evaluated, but the association between viral load and clinical outcome remains controversial (20, 34). Real-time PCR provides an accurate means of quantifying viral DNA, with the major advantage of avoiding post-PCR handling that can be the source of DNA carryover, and several studies have reported the utility of this technique for the quantification of HCMV DNA in blood or urine (17, 26, 29, 41, 43). However, to our knowledge, real-time PCR had never been used for the quantification of HCMV DNA in AF samples.

In the present study, a real-time PCR assay was developed to...
quantify HCMV DNA in AF samples. This assay was used on AF samples collected from 30 women with primary HCMV infection as detected either from HCMV-IgG seroconversion or by the presence of HCMV-specific IgG and IgM associated with a low IgG avidity. These AF samples had previously been found positive by viral culture isolation and qualitative PCR, and congenital infection was confirmed in each case. AF samples were selected because comprehensive clinical and laboratory information was available. Real-time PCR quantification was performed in order to investigate whether the amount of HCMV DNA in AF samples could be related to the clinical condition and outcome of infected fetuses.

MATERIALS AND METHODS

Patients and samples. Patients were selected from a previously reported cohort (18) of 19,456 pregnant women serologically screened for HCMV infection and attending different health care centers in France: Paris (Antoine Beclere Hospital and Saint Vincent de Paul Hospital), Caen (University Hospital), and Limoges (Dupuytren Hospital). In this cohort, 152 pregnant women had HCMV primary infection according to the following criteria: detection of HCMV-IgG seroconversion or detection of HCMV-specific IgG and IgM associated with a low IgG avidity (<30%). Ninety-five AF samples were collected from 95 of these 152 women for diagnosis of fetal HCMV infection by virus detection using both culture and PCR as previously described (18).

In the present study, 30 HCMV culture- and PCR-positive AF samples from 30 women with primary HCMV infection were selected for quantification of HCMV DNA. These 30 cases were selected because all the mothers had undergone monthly ultrasonographic examination to detect possible abnormalities of the congenitally infected fetuses and because clinical information on fetal or neonatal outcome was available. HCMV infection in neonates was confirmed by virus isolation from urine during the first week after birth. HCMV infection in abortus fetuses was confirmed in fetal tissues by virus isolation and histologic examination. Ten HCMV-negative AF samples collected from pregnant women with no history of HCMV infection to investigate intrauterine growth retardation were used as a control group for HCMV DNA quantification. None of the control group infants were HCMV infected at birth. This study was conducted according to the ethical rules of the University Hospital of Caen, France.

Real-time PCR quantification of HCMV DNA in AF samples. AF samples were kept frozen at −80°C prior to HCMV DNA quantification. DNA was extracted from 200 μl of AF sample using the QIAamp DNA blood mini kit (Qiagen S.A., Courtaboeuf, France), according to the manufacturer’s instructions. DNA was eluted in 100 μl of distilled water and kept at −20°C before handling.

Real-time PCR was performed according to a technique described previously (17). The LightCycler (Roche Molecular Diagnostic, Meylan, France) with a sequence determination system based on an exonuclease assay using a TaqMan probe. The exonuclease assay using TaqMan type probes can be used with the LightCycler technology with results equivalent with no history of HCMV infection to investigate intrauterine growth retardation were used as a control group for HCMV DNA quantification. None of the control group infants were HCMV infected at birth. This study was conducted according to the ethical rules of the University Hospital of Caen, France.

As a control for cross-contamination, a sample consisting of distilled water was included in each DNA extraction procedure and PCR run.

Specificity, sensitivity, and reproducibility of the real-time PCR assay. To confirm the specificity of the primers and probe, DNA extracted from other human herpesviruses (herpes simplex virus types 1 and 2, varicella-zoster virus, and human herpesvirus 6) was tested in the real-time PCR assay. All these samples were negative after 45 cycles of amplification, and no cross-reactivity was observed (data not shown).

Plasmid pKS-pp65K7 was used to evaluate the sensitivity of the assay and to construct the standard curve for HCMV DNA quantification. The plasmid dilution corresponding to an input of 2 × 106 to 2 GE per assay was tested in duplicate in the real-time PCR assay. The Ct values were plotted against the number of GE input in the reaction (2 to 20,000 GE). The correlation coefficient was 0.999, and the slope was −3.37. The amplification efficiency, calculated as [10(-1/slope)−1] × 100, was 98%.

FIG. 1. Standard curve for HCMV DNA quantification. Plasmid pKS-pp65K7, containing one copy of the UL 83 target sequence, was used to construct the standard curve for HCMV DNA quantification. Tenfold serial dilutions of plasmid corresponding to an input of 2 × 103 to 2 GE per assay were tested in duplicate in the real-time PCR assay. The Ct values were plotted against the number of GE input in the reaction (2 to 20,000 GE). The correlation coefficient was 0.999, and the slope was −3.37. The amplification efficiency, calculated as [10(-1/slope)−1] × 100, was 98%.

Plasmid pKS-pp65K7 was used to evaluate the sensitivity of the assay and to construct the standard curve for HCMV DNA quantification. The plasmid dilution corresponding to an input of 2 GE per reaction was repeatedly detected with 100% sensitivity. Thus, according to the dilution factors during the DNA extraction procedure, the sensitivity of the assay was approximately 500 GE of the number of genome equivalents per milliliter.

As a control for cross-contamination, a sample consisting of distilled water was included in each DNA extraction procedure and PCR run.

Specificity, sensitivity, and reproducibility of the real-time PCR assay. To confirm the specificity of the primers and probe, DNA extracted from other human herpesviruses (herpes simplex virus types 1 and 2, varicella-zoster virus, and human herpesvirus 6) was tested in the real-time PCR assay. All these samples were negative after 45 cycles of amplification, and no cross-reactivity was observed (data not shown).

Plasmid pKS-pp65K7 was used to evaluate the sensitivity of the assay and to construct the standard curve for HCMV DNA quantification. The plasmid dilution corresponding to an input of 2 × 106 to 2 GE per assay was tested in duplicate and used to construct the standard curve by plotting the GE values against the measured Ct values (Fig. 1). The linear correlation between the Ct and the logarithm of GE values was repeatedly greater than 0.995.

The intra-assay reproducibility was evaluated using eight replicates of plasmid dilutions corresponding to an input of 2 × 106, 2 × 105, and 2 GE per reaction that were tested in the same experiment. The coefficient of variation of the Ct obtained for each dilution was, respectively, 1.7, 0.7, 0.7, and 2.4%.

To estimate the interassay variability of the real-time PCR assay, DNA extracted from the supernatant of an MRC5 cell culture infected with the HCMV AD169 strain was quantified in five independent PCR runs. The mean GE value ± the standard deviation was 1,886 ± 186 GE, and the coefficient of variation was 9.9%.

Statistical analysis. The nonparametric Kruskal-Wallis test or Mann-Whitney U test was used to compare HCMV DNA load values in AF samples according to the clinical outcome of the infected fetuses. The correlation between the
In all cases in group A, pregnancy was terminated at the par-
lomegaly, hydrocephaly, and intrauterine growth retardation.
bowel, intracranial microcalci-
fetuses. Group A included 13 fetuses with at least two of the
clinical status of the newborns or examination of the aborted

diagnosis and during gestation, outcome of pregnancy, and

ication in AF samples are reported in Table 1.
DNA quanti-
infection and AF sampling, fetal clinical outcome, and HCMV

time of AF sampling, time elapsed between maternal primary
considered signi-
cific. Group B in-
cluded 13 fetuses with normal ultrasound
ndings. All the

cation in AF samples.

RESULTS

HCMV DNA quantification in AF samples. In the HCMV-
negative control group, the 10 AF samples were negative by
real-time PCR after 45 cycles of amplification. For women with
HCMV primary infection, the 30 AF samples tested were all
confirmed to be HCMV positive with the quantitative assay,
with C_{T} values of less than 45 cycles. The results concerning
time of AF sampling, time elapsed between maternal primary
infection and AF sampling, fetal clinical outcome, and HCMV
data are reported in Table 1.

Fetuses were classified into three groups (A, B, and C),
according to ultrasonographic findings at the time of prenatal
diagnosis and during gestation, outcome of pregnancy, and
clinical status of the newborns or examination of the aborted
fetuses. Group A included 13 fetuses with at least two of the
following ultrasound abnormalities: ascites, hyderekogenic
bowel, intracranial microcalcifications, microcephaly, ventricu-
losegaly, hydrocephaly, and intrauterine growth retardation.
In all cases in group A, pregnancy was terminated at the par-
ents’ request. Examination of fetuses showed clinically appar-
ent infection with multiple organ system involvement, and
HCMV infection was virologically confirmed. Group B in-
cluded 13 fetuses with normal ultrasound findings. All the
pregnancies in group B went to term, and the newborns were
HCMV infected but asymptomatic at birth. Group C included
four fetuses with no (n = 2) or minor (n = 2; isolated growth
retardation) ultrasonographic signs. Pregnancies in group C
were terminated at the parents’ request. Examination of the
fetuses showed no overt malformation or abnormality, but
HCMV infection was virologically confirmed.

Results of HCMV DNA quantification in AF samples from
groups A, B, and C are shown in Fig. 2. The median GE values
were 2.8 \times 10^5 \text{ GE/ml} (range, 1.2 \times 10^5 to 2.7 \times 10^6 \text{ GE/ml}),
8 \times 10^3 \text{ GE/ml} (range, 3.8 \times 10^2 to 1 \times 10^6 \text{ GE/ml}), and 2.5
\times 10^5 \text{ GE/ml} (range, 9 \times 10^3 to 5.3 \times 10^5 \text{ GE/ml}), in groups
A, B, and C, respectively. Overall analysis of the data indicated
that the HCMV DNA load values were not evenly distributed
among the three groups (Kruskal-Wallis test; \( P = 0.028 \)). The
HCMV DNA load values were significantly higher in group A
than in group B (Mann-Whitney U test; \( P = 0.014 \)), whereas
the GE values in group C were not significantly different from
those of groups A and B.

We further investigated whether the difference in HCMV

<table>
<thead>
<tr>
<th>Group and patient no.</th>
<th>AF collection date (wk of gestation)*</th>
<th>HCMV DNA load in AF (GE/ml)</th>
<th>Ultrasound abnormalities</th>
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<tbody>
<tr>
<td>A (n = 13)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td>23</td>
<td>1.2 \times 10^3</td>
<td>Hyperechogenic bowel/microcephaly</td>
</tr>
<tr>
<td>A2</td>
<td>14 (8)</td>
<td>2.2 \times 10^3</td>
<td>Microcephaly/intracranial microcalci</td>
</tr>
<tr>
<td>A3</td>
<td>19</td>
<td>3 \times 10^4</td>
<td>Growth retardation/hydrocephaly/intracranial microcalci</td>
</tr>
<tr>
<td>A4</td>
<td>20</td>
<td>8 \times 10^4</td>
<td>Ventriculomegaly/microcephaloi intracranial microcalci</td>
</tr>
<tr>
<td>A5</td>
<td>28</td>
<td>1 \times 10^4</td>
<td>Hyperechogenic bowel/intracranial microcalci/growth retardation</td>
</tr>
<tr>
<td>A6</td>
<td>20 (12)</td>
<td>1.3 \times 10^5</td>
<td>Ventriculomegaly/growth retardation/intracranial microcali</td>
</tr>
<tr>
<td>A7</td>
<td>29</td>
<td>2.8 \times 10^4</td>
<td>Hyperechogenic bowel/growth retardation/ascites</td>
</tr>
<tr>
<td>A8</td>
<td>25</td>
<td>3.1 \times 10^5</td>
<td>Growth retardation/hydrocephal/ventriculomegaly</td>
</tr>
<tr>
<td>A9</td>
<td>27</td>
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<td>Hyperechogenic bowl/microcephaly/intracranial microcalci</td>
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<td>7.25 \times 10^5</td>
<td>Hyperechogenic boil/intracranial microcalci/growth retardation</td>
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<td>Growth retardation/intracranial microcalci/ventriculomegaly</td>
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<td>32 (18)</td>
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<td>Growth retardation/hyperechogenic bowl</td>
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<td>A13</td>
<td>38 (30)</td>
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<td>Microcephali/intracranial microcalci</td>
</tr>
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<td>B (n = 13)</td>
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<td></td>
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<td>18 (3)</td>
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</tr>
<tr>
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<td>32</td>
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<td>21</td>
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</tr>
<tr>
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<td>17 (7)</td>
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<td>None</td>
</tr>
<tr>
<td>C3</td>
<td>20</td>
<td>3.4 \times 10^4</td>
<td>Growth retardation</td>
</tr>
<tr>
<td>C4</td>
<td>22</td>
<td>5.3 \times 10^4</td>
<td>Growth retardation</td>
</tr>
</tbody>
</table>

* Time elapsed (weeks) between maternal primary infection and AF sampling is indicated in parentheses when available.

HCMV DNA load values and the weeks of gestation at the time of AF sampling
(or the period of time between maternal infection and AF sampling) was exam-
ined by the nonparametric Spearman rank test. \( P \) values of less than 0.05 were
considered significant.
DNA load observed between groups A and B could be related to the gestational age at the time of AF sampling. AF samples were collected at an average time of 26 weeks of gestation (median, 27 weeks; range, 14 to 38 weeks) for group A, and 25 weeks of gestation (median, 25 weeks; range, 18 to 32 weeks) for group B. Strikingly, as shown in Fig. 3, a significant correlation between HCMV DNA load and gestational age at the time of sampling was found for group A (Spearman rank test; \( P = 0.003 \)) but not for group B. The time interval between maternal primary infection and AF sampling might also affect HCMV DNA load level. Unfortunately, maternal infection could be precisely dated in only four cases in group A, five in group B, and two in group C (Table 1). The overall analysis of these 11 cases indicated that the amount of HCMV DNA detected in AF samples correlated with the time interval between maternal primary infection and AF sample collection (Spearman rank test; correlation coefficient \( r = 0.810; P = 0.01 \)).

**DISCUSSION**

HCMV DNA detection in AF samples by qualitative PCR has been proposed as a useful tool for the prenatal diagnosis of HCMV congenital infection (4, 14, 18, 33, 36). However, this technique does not allow us to tell which infected fetuses will be symptomatic and which will remain asymptomatic. HCMV DNA quantification in AF samples has been proposed as a means to possibly evaluate the risk that a fetus will develop infection or disease (12, 18, 23). In this study, a real-time PCR assay was developed to quantify HCMV DNA in AF samples.

This assay had a large dynamic range and good reproducibility and sensitivity, as reported for other HCMV DNA quantitative assays using real-time PCR (17, 26, 29, 41, 43). This assay was used to quantify HCMV DNA in 30 AF samples from mothers with primary HCMV infection.

To our knowledge, HCMV DNA quantification in AF samples of congenitally infected fetuses has only been reported in two other studies (20, 34). Revello et al. reported that the DNA levels in AF samples from 21 fetuses were not statistically different between symptomatic and asymptomatic fetuses, although the median DNA level was higher in symptomatic fetuses (34). More recently, Guerra et al. (20) reported that an HCMV DNA load of \( >10^5 \) GE/ml in AF samples was predictive of symptomatic infection. Our results tend to confirm that HCMV DNA levels are significantly higher in symptomatic fetuses than in asymptomatic ones.
Interpreting HCMV DNA load in AF samples should consider its predictive value for both fetal infection and clinical outcome of infected fetuses. Regarding fetal infection, Guerra et al. (20) reported that the presence of >10^5 GE/ml in AF samples predicted mother-child infection with 100% probability. These authors also reported that among 21 PCR-positive fetuses with DNA levels in AF samples of <10^5 GE/ml, 17 were found to be uninfected at birth, while 4 were confirmed to be infected. In our study, HCMV infection was confirmed for the two fetuses with a viral load of <10^5 GE/ml. The fact that only two fetuses had a low viral load did not allow us to determine whether low DNA levels in AF samples may influence fetal infection. However, our findings that fetuses with a low viral load were confirmed to be infected are in agreement with those of Revello et al. (34), who found that all PCR-positive fetuses, including those with low amounts of viral DNA, were infected at birth, suggesting that congenital HCMV infection is not likely to be cleared in utero, even when the viral load is low.

Regarding the use of HCMV DNA quantification in AF samples as a prognosis marker of HCMV disease in fetuses, one should remain cautious in interpreting the correlation between HCMV load and clinical state. In particular, we did not attempt to propose a threshold value predictive of symptomatic infection because in some cases, low viral loads were associated with severe ultrasound abnormalities and conversely, high viral loads were found in asymptomatic fetuses (Table 1). Moreover, irrespective of the fetal outcome, HCMV DNA load levels in AF samples might be related to other factors, such as the gestational age at the time of AF sampling or the period of time elapsed between maternal primary infection and AF sampling, two factors that have been reported to be important for antenatal diagnosis efficiency (4, 11, 13). For instance, prenatal diagnosis performed before 22 weeks of pregnancy might lead to false-negative results (11, 13, 18), since fetal diuresis is efficient only after 21 weeks.

Our data indicated that the HCMV DNA load correlated with the gestational age at the time of AF sampling for group A (symptomatic fetuses) but not for group B, suggesting that viral replication could tend to increase more rapidly in symptomatic fetuses than in asymptomatic ones during the course of intrauterine HCMV infection. In this regard, the predictive value of HCMV DNA quantification in AF samples could rely on the increase in the viral load during pregnancy rather than on a threshold value. However, it will be hard to specify the kinetics of the viral load, considering the difficulty in justifying sequential AF sampling.

The time of primary infection in mothers is often difficult to determine, since most infections are asymptomatic and sequential serological testing is not always performed during pregnancy. In our study, primary infection was actually dated in only 11 of the 30 cases. Irrespective of the fetal outcome, the HCMV DNA load correlated with the time elapsed since maternal infection. Among these 11 cases, women from group A were infected earlier in the course of pregnancy and AF samples were collected later compared to group B. This should lead to a cautious interpretation of the difference in HCMV DNA levels observed between symptomatic (group A) and asymptomatic (group B) fetuses, mainly because it was not possible to assess that the time intervals between primary maternal infection and AF sample collection were similar in both groups. In contrast, Revello et al. (34) found no correlation between HCMV DNA load and time elapsed between maternal infection and prenatal diagnosis. However, the DNA quantification methods used in the two studies were different, and the populations studied were not equivalent regarding the time interval elapsed between maternal infection and AF sample testing. Considering the small number of cases, the contradiction is difficult to interpret and deserves further investigation.

In summary, real-time PCR can be efficiently used to quantify HCMV DNA in AF samples, and the use of standardized techniques, including internal controls, should improve the inter- and intra-assay reproducibility. According to our findings, HCMV load level in AF samples might be indicative of the fetal clinical outcome, but might also be dependent on other factors, such as the gestational age at the time of AF sampling and the period of time elapsed since maternal infection. To what extent these factors could influence HCMV DNA load for symptomatic and asymptomatic fetuses remains to be determined. Additional studies are needed before we can propose HCMV DNA quantification in AF samples as a prognosis marker for the clinical handling of pregnant women with primary HCMV infection.

ACKNOWLEDGMENTS

We thank the obstetricians, pediatricians, and pathologists who are members of the French group “CMV and Pregnancy.”

REFERENCES


