Evaluation of Cytomegalovirus (CMV) DNA Quantification in Dried Blood Spots: Retrospective Study of CMV Congenital Infection

Christelle Vauloup-Fellous,1 Aurélie Ducroux,2 Vincent Couloigner,3 Sandrine Marlin,4 Olivier Picone,5 Julie Galimand,2 Natalie Loundon,4 Françoise Denoyelle,4 Liliane Grangeot-Keros,1 and Marianne Leruez-Ville2*

Service de Microbiologie Immunologie Biologique, Assistance Publique-Hôpitaux de Paris, Hôpital Antoine Bécère, Université Paris Sud 11, 157 Avenue de la Porte de Trivaux, 92141 Clamart, France;2 Laboratoire de Virologie, Hôpital Necker-Enfants-Malades, Assistance Publique-Hôpitaux de Paris, Université Paris Descartes, EA 36-20, 149 rue de Sèvres, 75015 Paris, France3; Service d’ORL Pédiatrique CHU Necker-Enfants-Malades, Assistance Publique-Hôpitaux de Paris, Université Paris Descartes, 149 rue de Sèvres, 75015 Paris, France4; Service d’ORL Hôpital Trousseau, Assistance Publique-Hôpitaux de Paris, 26 Avenue du Dr. Arnold Netter, 75571 Paris, Cedex 12, France5; and Service de Gynécologie-Obstétrique, Assistance Publique-Hôpitaux de Paris, Hôpital Antoine Bécère, Université Paris Sud 11, 157 Avenue de la Porte de Trivaux, 92141 Clamart, France5

Received 20 August 2007/Returned for modification 24 August 2007/Accepted 13 September 2007

We compared two protocols for extracting DNA from dried blood spots for cytomegalovirus (CMV) DNA detection and quantification by real-time PCR. Both extraction methods were reliable for the retrospective diagnosis of CMV congenital infection. Quantification of CMV DNA was valuable after normalization of viral loads with albumin gene PCR amplification results.

Human cytomegalovirus (CMV) is the main organism responsible for congenital infection and permanent deafness in young children in developing countries (13). Policies for screening during pregnancy and at birth have not been implemented in European countries or in the United States, essentially because there is no well-established treatment for pregnant women or for newborns with CMV infection (7). Retrospective diagnosis of congenital infection has been achieved by PCR detection of the CMV DNA in dried blood spots (DBS) stored on perinatal Guthrie cards (2, 5, 6, 8, 9, 14, 16, 17). Only one protocol (heat DNA extraction, followed by nested PCR) has been extensively evaluated in a clinical setting, with excellent sensitivity and specificity compared to that of viral isolation in the urine (1, 2). However, lower sensitivities (63 and 71%) were reported using the same protocol (5, 16). Alternative methods based on either phenol-chloroform or silica extraction protocols have been proposed, but their sensitivities (81% and 100%, respectively) were studied only with small numbers of patients (9, 14).

In the current study, we compared two DNA extraction protocols (phenol-chloroform versus silica-based technology) followed by quantitative in-house real-time CMV DNA-specific PCR amplification (12). Indeed, knowing the CMV DNA load in DBS could provide unique insights regarding the pathogenesis and outcome of CMV congenital infection, especially the relationship between viral load and the risk of hearing loss (4, 10).

DNA extraction using a whole DBS cut into thin strips with single-use scissors was performed using two protocols. In protocol one, the strips were submerged twice in 1.5 ml of washing solution (10 mM NaCl, 10 mM EDTA) for 30 min at room temperature. Then, 150 μl of lysis buffer (0.32% NaOH) was added onto the strips, and the lysate was recovered after centrifugation (at 10,000 × g for 2 min) and supplemented with 30 μl of neutralization solution (1 M Tris, pH 7.5) before DNA extraction with a QIAamp DNA blood mini-kit (QIAGEN, Courtaboeuf, France). Protocol two was performed as described previously (15). Briefly, the strips were submerged in 400 μl of extraction buffer and incubated at 56°C for 1 h. The supernatant was recovered after centrifugation and purified by phenol-chloroform extraction, followed by ethanol precipitation.

CMV DNA-specific PCR amplification and human albumin PCR amplification were carried out with in-house real-time PCR assays in duplicate (11, 12). The normalized value of the CMV DNA load was expressed as the number of CMV genome copies per 107 cells.

The 45% and 95% sensitivity values of the assays were calculated with a nonlinear regression sigmoidal model (Graph Pad). Nonparametric Spearman correlation coefficients were used to assess the association between continuous variables. Median copy numbers obtained from DBS from different groups of patients were compared by the Mann-Whitney U test. A P value of <0.05 was accepted as statistically significant.

For sensitivity assessment, a prequantified CMV DNA-positive whole-blood sample obtained from an infected patient who had undergone transplantation was diluted in CMV-negative whole blood at concentrations of 104, 105, 5 × 105, 106, 5 × 106, 107, and 10 genome copies/ml. Fifty μl (10 spots for each of the three highest concentrations and 20 spots for each of the other four dilutions) were applied to Guthrie cards (903 specimen collection paper; Whatman, Maidstone, England). With extraction protocol one, the CMV DNA-specific PCR was positive for 100% (15/15) of the spots loaded with the
three highest concentrations of genome copies/ml; positive for 80% (8/10), 50% (5/10), and 10% (1/10) of the spots loaded with, respectively, $10^3$, $5 \times 10^2$, and $10^2$ genome copies/ml; and positive for 0% (0/10) of the spots loaded with 10 genome copies/ml. The 45% sensitivity of the assay was 421 (2.6 log_{10}) genome copies/ml, and the 95% sensitivity was 4,000 (3.6 log_{10}) genome copies/ml. With extraction protocol two, CMV DNA-specific PCR was positive for 100% (15/15) of the spots loaded with, respectively, 10^3, 5 \times 10^2, and 10^2 genome copies/ml, and the 45% sensitivity of the assay was 189 (2.28 log_{10}) genome copies/ml, and the 95% sensitivity was 4,000 (3.6 log_{10}) genome copies/ml.

Guthrie cards of 76 children were analyzed. Fourteen were from neonates who had CMV congenital infection (proven by PCR or found to be culture positive in a urine sample obtained at birth), of which 8 were asymptomatic at birth and 6 were symptomatic; 20 were from noninfected neonates (proven by PCR or who were culture negative according to a urine sample obtained at birth); and 42 were from children (age 1 to 72 months) diagnosed with hearing loss ($n = 35$) or with other symptoms compatible with CMV congenital infection ($n = 7$). All Guthrie cards collected on the third day of life were retrieved from the Regional Screening Laboratories after obtaining parents’ consent. Similar results were obtained with both protocols (Table 1): albumin gene amplification was positive for all extracts, and CMV DNA was detected in 100% (14/14) of the DBS from CMV-infected neonates (patients A1 to A14), in 0% (0/20) of the DBS from noninfected neonates, in 34% (12/35) of the DBS from children with hearing loss (patients B1 to B12), and in 28% (2/7) of the DBS from children with other symptoms (patients C1 and C2). With protocol one, the PCR was positive for only one duplicate for two DBS: in case A7, DNA extraction efficiency was poor (low albumin loading), and in case A14, the CMV viral load was very low (16 copies/ml). With protocol two, CMV DNA was detected in 100% (14/14) of the DBS from CMV-infected neonates (patients A1 to A14), in 0% (0/20) of the DBS from noninfected neonates, in 34% (12/35) of the DBS from children with hearing loss (patients B1 to B12), and in 28% (2/7) of the DBS from children with other symptoms (patients C1 and C2). With protocol one, the PCR was positive for only one duplicate for two DBS: in case A7, DNA extraction efficiency was poor (low albumin load), and in case A14, the CMV viral load was very low (16 copies/ml).

Our results show that the detection threshold was slightly lower with the phenol-chloroform extraction than with the silica-based extraction (protocol one). However, clinical sensitivity values of the two extraction protocols were similar, and protocol one appeared to be more convenient for routine testing (less time-consuming and no exposure risk of phenol toxicity). CMV DNA quantification performances were therefore analyzed following the extraction method with protocol one, as follows: 50 µl of 14 prequantified CMV DNA-specific PCR positive whole-blood samples (ranging from 1,000 to 4,000,000 [3.0 to 6.6 log_{10}] genome copies/ml) from 14 infected patients who had undergone transplantation were submitted for direct extraction with a QIAamp DNA blood mini-kit and were also spotted in triplicate on Guthrie cards. Viral loads obtained from DBS were underestimated compared to those obtained from fresh whole-blood samples, with a median difference of 0.73 log_{10} genome copies/ml (range, 0.19 to 1.18 log_{10}). Following normalization of viral loads with albumin PCR results,
the median difference dropped to 0.27 log_{10} genome copies/ml (range, 0.03 to 0.7 log_{10}), with high correlation (r = 0.94; P < 0.006, Spearman correlation).

The median normalized CMV DNA viral load was significantly higher for the six symptomatic neonates (patients A9 to A14; 58,260 [4.76 log_{10}] genome copies/10^9 cells; range, 16 to 720,000) than for the seven asymptomatic ones (patients A1 to A8; 140 [2.14 log_{10}] genome copies/10^9 cells; range, 57 to 460; Mann-Whitney U test, P = 0.045). The median normalized CMV DNA viral load of the 12 children with hearing loss (patients B1 to B12) was significantly higher (2,100 [3.32 log_{10}] genome copies/10^9 cells; range, 165 to 120,000) than the median value for the 7 asymptomatic neonates (Mann-Whitney U test, P = 0.002) (Fig. 1).

The threshold values of the CMV DNA-specific PCR in DBS reported here, following any of our two DNA extraction protocols, was in the same range as those (e.g., 2,000 or 4,000 genome copies/ml) reported by two other groups (3, 14). These protocols, was in the same range as those (e.g., 2,000 or 4,000 genome copies/ml) reported by two other groups (3, 14). These protocols, was in the same range as those (e.g., 2,000 or 4,000 genome copies/ml) reported by two other groups (3, 14). These

REFERENCES


