Comparative Analysis of Detection Methods for Congenital Cytomegalovirus Infection in a Guinea Pig Model

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Objective: To assess the validity of the guinea pig as a model for congenital cytomegalovirus (CMV) infection by comparing the effectiveness of detecting the virus by real-time polymerase chain reaction (PCR) in blood, urine, and saliva.

Design: Case-control study.

Setting: Academic research.

Subjects: Eleven pregnant Hartley guinea pigs.

Main Outcome Measures: Blood, urine, and saliva samples were collected from guinea pig pups delivered from pregnant dams inoculated with guinea pig CMV. These samples were then evaluated for the presence of guinea pig CMV by real-time PCR assuming 100% transmission.

Results: Thirty-one pups delivered from 9 inoculated pregnant dams and 8 uninfected control pups underwent testing for guinea pig CMV and for auditory brainstem response hearing loss. Repeated-measures analysis of variance demonstrated no statistically significantly lower weight for the infected pups compared with the noninfected control pups. Six infected pups demonstrated auditory brainstem response hearing loss. The sensitivity and specificity of the real-time PCR assay on saliva samples were 74.2% and 100.0%, respectively. The sensitivity of the real-time PCR on blood and urine samples was significantly lower than that on saliva samples.

Conclusions: Real-time PCR assays of blood, urine, and saliva revealed that saliva samples show high sensitivity and specificity for detecting congenital CMV infection in guinea pigs. This finding is consistent with recent screening studies in human newborns. The guinea pig may be a good animal model in which to compare different diagnostic assays for congenital CMV infection.

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Cytomegalovirus (CMV), a member of the Betaherpesvirinae subfamily, is the most common infectious cause of congenital sensorineural hearing loss. Approximately 40,000 infants are born in the United States each year with congenital CMV infection. Current estimates suggest that up to 40% of all nonsyndromic sensorineural hearing loss in infants is caused by congenital CMV infection. The cost associated with congenital CMV infection has been estimated to be $4 billion a year.

The diagnosis of congenital CMV infection requires laboratory testing of samples from neonates within the first 3 weeks of life because the finding of CMV beyond this period may be due to natal acquisition of infection, most commonly from breastfeeding. Moreover, postnatal acquisition of CMV infection is not associated with sensorineural hearing loss. Barbi et al proposed testing of neonatal dried blood spot samples in children with suspected congenital CMV. Enthusiasm for dried blood spot assays as a diagnostic tool has been tempered because of the findings by Boppana et al that a 2-primer dried blood spot polymerase chain reaction (PCR) assay identified CMV infection in only 34.4% of infected neonates.

Unfortunately, the resources required to evaluate potential diagnostic assays are substantial and burdensome. A subsequent study by Boppana et al comparing liquid and dried saliva specimens involved 8 institutions and 34,989 infants. The earlier study by Boppana and colleagues comparing dried blood spot assays with saliva cultures had included 20,448 infants from 7 medical centers. As other diagnostic assays become available, it will become important to develop less costly measures to assess efficacy.
In the quest for a better diagnostic option, we chose to use a small animal model to evaluate various diagnostic methods to reliably identify neonatal CMV infection. Elevated auditory brainstem response (ABR) thresholds and evidence of the virus in guinea pig pup temporal bones transplacentally infected with CMV were previously demonstrated. The goal of this study was to compare the diagnostic accuracy of real-time PCR in blood, urine, and saliva in a guinea pig model.

**METHODS**

**PREPARATION OF VIRUS AND CELLS**

Guinea pig CMV (gpCMV) (strain 22122; American Type Culture Collection) was used for these studies. Viral stocks were propagated on guinea pig fibroblast lung cells as previously described.

**PREGNANCY, BREEDING, AND VIRAL CHALLENGE**

Hartley guinea pigs (Elm Hill Laboratories) were used for these experiments; pregnant dams were purchased. Preinoculation serum samples were obtained to ensure no previous exposure to gpCMV by testing for CMV antibody using enzyme-linked immunosorbent assay (ELISA). Only seronegative dams were used. Animals infected with gpCMV were housed separately from gpCMV-uninfected controls to avoid cross-contamination. The University of Utah Institutional Animal Care and Use Committee approved and monitored this study.

At day 39 (second trimester), pregnant dams were divided into 2 groups. Group 1 dams were inoculated 3 times with 1 × 10⁶ plaque-forming units (pfu) of gpCMV administered subcutaneously. The 3 doses were given during the course of one week. Group 2 dams, the observational control group, underwent an identical course of subcutaneous injections of normal saline. Following delivery, newborn guinea pigs were housed with their mothers until they were weaned from their mother’s milk.

**SAMPLE COLLECTION**

Blood, urine, and saliva samples were collected from the sedated pups at age 6 weeks. All pups had been weaned from their mother’s milk by age 3 weeks to reduce the possibility of a false-positive result from CMV being detected in the breast milk. Animals were placed in an induction chamber with 4% isoflurane and supplemental oxygen. On sedation, enuresis occurred, and the urine was collected. At the time of sedation, saliva samples were obtained by placing a 5-mm disk of surgical absorbable gelatin sponge (Gelfoam; Pfizer, Inc) in the pup’s mouth. The saturated disk was retrieved after 1 minute. Approximately 200 μL of blood was collected via toenail clipping and was mixed with buffered citrate solution (1:10).

**ENZYMELINKED IMMUNOSORBENT ASSAY**

ELISA was performed on blood samples from each pregnant dam to confirm seronegativity before inoculation and seropositivity after inoculation. ELISA antibody to gpCMV was determined by the modification of a method used to detect guinea pig antibody to herpes simplex virus. The gpCMV antigen was prepared from gpCMV-infected and gpCMV-uninfected strain 2 primary fetal guinea pig cell monolayers, and peroxidase-conjugated rabbit anti–guinea pig immunoglobulin was used for detection. The ELISA titer was defined as the reciprocal of the highest dilution that produced an absorbance exceeding 0.10 and twice the absorbance against control fetal guinea pig antigen.

**HEARING ASSESSMENT**

The ABRs for the pups were obtained at 4 weeks following delivery. The ear canal and tympanic membrane were first visualized via an operating microscope and were cleaned of wax when necessary. The guinea pig pups were placed in a doublewalled acoustically isolated chamber (Industrial Acoustics Company) and were anesthetized with 4% isoflurane. An electrostatic speaker (ES-1; Tucker-Davis Technologies) with a 1.5-cm-long polyethylene tube was placed against the ear canal to make a closed acoustic system. Recording electrodes were placed at the vertex and mastoid, with a remote ground under the skin in the rump area. Signals were amplified with a preamplifier (RA4; Tucker-Davis Technologies) and were digitized. The ABRs (1000 trials) were filtered from 100 to 3000 Hz and averaged with processor (RA16BA; Tucker-Davis Technologies) controlled by software (BioSigRP; Tucker-Davis Technologies).
primer UL83B11 (5'-Asp402 through Ser473 of the GP83 protein, and generates a single primer pair amplifies a 225–base pair region, corresponding to Peptide Shared Resource facility of the Huntsman Cancer Institute. These primers were used in this study to detect the presence of gpCMV DNA using real-time quantitative PCR (LightCycler; Roche Applied Science). The CMV culture at a concentration of 1 x 10^6 pfu/mL was used as positive control, and a sample with no DNA added was used as negative control. Four replicates were measured for each sample. The following cycling program was used: 95°C for 2 minutes, followed by 45 four-step cycles, each comprising denaturation at 93°C for 0 seconds, annealing at 64°C for 10 seconds, extension at 72°C for 10 seconds, and detection at 88°C for 2 seconds. Measurement of the PCR amplicon concentration was performed at the last step of each cycle. After amplification, a melting curve was acquired in the range of 55 to 95°C with a ramp speed of 0.1°C per second to verify amplification of a single moiety of expected melting temperature. In addition, appropriate amplicon size was verified by electrophoresis in a 2% agarose gel containing 0.5 µg/mL ethidium bromide.

**STORAGE ANALYSIS**

Data were entered into a software program (Excel; Microsoft) and were imported elsewhere for analyses (IBM SPSS Statistics version 19.0; SPSS Inc; and STATA/IC 11.2; StataCorp LP). Control and infected pup weights were compared using a repeated-measures analysis of variance. Sensitivity, specificity, positive likelihood ratio, negative likelihood ratio, positive predictive value, and negative predictive value, as well as 95% CIs, were calculated for blood, urine, and saliva samples assuming 100% transplacental inoculation of all pups.

**RESULTS**

**OFFSPRING OUTCOMES**

Eleven pregnant Hartley guinea pigs were procured for this study. All 11 animals were seronegative for CMV on initial ELISA. Nine pregnant dams were inoculated with gpCMV during the second trimester. Two pregnant dams were injected with normal saline carrier and were used as controls. Eight female guinea pigs tested were gpCMV seropositive at 4 weeks after inoculation. One female guinea pig was not tested because it had died at 3 weeks after inoculation; all 4 pups born to that guinea pig were seropositive. Both control animals remained seronegative at 4 weeks after delivery (Figure 1). Thirty-two pups were delivered from 9 inoculated pregnant dams. Six pups were stillborn. One pup died of anesthetic complications during ABR testing. Eight pups were delivered from 2 pregnant noninoculated controls; no pups from these dams were stillborn. Weights of the pups were obtained for both groups from birth to age 42 days. The mass differences between pups from inoculated dams vs pups from control dams did not reach the level of significance by repeated-measures analysis of variance (P = .07), indicating that CMV infection was not deleterious to pup growth (Figure 2).

**AUDITORY BRAINSTEM RESPONSES**

Representative normal and abnormal ABR thresholds are shown in Figure 3. Six of 31 pups born to dams inoculated with gpCMV demonstrated a hearing loss. One of 8 pups in the control group also had elevated hearing thresholds in any tested frequencies. Asymmetry was defined as at least a 10-dB difference between the left and right ear thresholds in any tested frequencies.
thresholds. The markedly elevated hearing thresholds in 6 pups with hearing loss are shown in Figure 4.

**COMPARISON OF gpCMV PCR FOR BLOOD, URINE, AND SALIVA**

Blood, urine, and saliva were compared in the inoculated and control animals by real-time PCR assays. The sensitivity and specificity of the real-time PCR assay on saliva samples for detecting congenital gpCMV infection were 74.2% and 100.0%, respectively. For the real-time PCR assay, the blood and urine samples showed lower sensitivity, 29.0% and 100.0%, respectively (Table).

This study demonstrates that real-time PCR analysis of saliva has a higher sensitivity than real-time PCR analysis of blood or urine for correctly identifying gpCMV infection in newborn guinea pig pups. The low sensitivity for gpCMV detection in blood compared with saliva is consistent with the clinical trial by Boppana et al. Among 9026 infants screened using a 2-primer dried blood spot PCR, 11 of 32 (34%) screened positive for CMV. A single-primer real-time PCR assay yielded an even lower sensitivity of 28%. In another trial by Boppana et al, real-time PCR samples of wet and dry saliva were compared with rapid saliva cultures. The sensitivity for liquid saliva PCR assay was 100%; the sensitivity for dry saliva PCR assay was 95%.

One would have expected a comparable sensitivity with urine PCR and saliva PCR. However, this result was not demonstrated in our study. Koyano et al collected urine samples onto filter cards from more than 21 000 newborns, that were analyzed by PCR for CMV. Congenital CMV infection was identified in 0.31% of the newborns. Unfortunately, no urine culture or other sample was used for comparison to calculate the sensitivity or specificity.

The sensitivity and specificity calculations were obtained assuming that the inoculated dams transmitted gpCMV to all their offspring. As has been used in clinical trials, a urine or saliva culture may have been a more appropriate standard for comparison. A lower transmission rate would have resulted in a higher sensitivity for all the samples tested but would not have affected our findings that the sensitivity for saliva is highest among the samples tested.

![Figure 4. Auditory brainstem response (ABR) thresholds in guinea pig pups by group.](image)

**Table. Comparison of Detection by Real-time Polymerase Chain Reaction (PCR) Assays in Blood, Urine, and Saliva Samples for Congenital Cytomegalovirus (CMV) Infection in Guinea Pigs**

<table>
<thead>
<tr>
<th>Variable</th>
<th>CMV Inoculated</th>
<th>CMV Not Inoculated</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR positive, No.</td>
<td>9</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>PCR negative, No.</td>
<td>22</td>
<td>8</td>
<td>30</td>
</tr>
<tr>
<td>Total, No.</td>
<td>31</td>
<td>8</td>
<td>39</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Blood</th>
<th>Urine</th>
<th>Saliva</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity, %</td>
<td>29.0</td>
<td>29.0</td>
<td>74.2</td>
</tr>
<tr>
<td>(95% CI)</td>
<td>(14.2-48.0)</td>
<td>(14.2-48.0)</td>
<td>(55.4-88.1)</td>
</tr>
<tr>
<td>Specificity, %</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>(95% CI)</td>
<td>(63.1-100.0)</td>
<td>(63.1-100.0)</td>
<td>(63.1-100.0)</td>
</tr>
<tr>
<td>Positive likelihood ratio&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.3 (0.3-83.3)</td>
<td>5.3 (0.3-83.3)</td>
<td>13.2 (0.9-197.0)</td>
</tr>
<tr>
<td>Negative likelihood ratio&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.7 (0.6-1.0)</td>
<td>0.7 (0.6-1.0)</td>
<td>0.3 (0.2-0.5)</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>100.0 (66.4-100.0)</td>
<td>100.0 (66.4-100.0)</td>
<td>100.0 (85.2-100.0)</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>26.7 (12.3-45.9)</td>
<td>26.7 (12.3-45.9)</td>
<td>50.0 (24.7-75.3)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Likelihood ratios are estimated using the substitution formula; 0.5 is added to all cell frequencies before the calculation. Accuracy measures are given as percentages.

**COMMENT**

This study demonstrates that real-time PCR analysis of saliva has a higher sensitivity than real-time PCR analysis of blood or urine for correctly identifying gpCMV infection in newborn guinea pig pups. The low sensitivity for gpCMV detection in blood compared with saliva is consistent with the clinical trial by Boppana et al. Among 9026 infants screened using a 2-primer dried blood spot PCR, 11 of 32 (34%) screened positive for CMV. A single-primer real-time PCR assay yielded an even lower sensitivity of 28%. In another trial by Boppana et al, real-time PCR samples of wet and dry saliva were compared with rapid saliva cultures. The sensitivity for liquid saliva PCR assay was 100%; the sensitivity for dry saliva PCR assay was 95%.

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The sensitivity and specificity calculations were obtained assuming that the inoculated dams transmitted gpCMV to all their offspring. As has been used in clinical trials, a urine or saliva culture may have been a more appropriate standard for comparison. A lower transmission rate would have resulted in a higher sensitivity for all the samples tested but would not have affected our findings that the sensitivity for saliva is highest among the samples tested.
Although auditory testing was not the focus of this study, we performed click and tone burst testing of all the inoculated and control pups. We demonstrated a higher incidence of hearing loss in the inoculated animals and elevated thresholds compared with the control pups. These findings are similar to the results of an earlier study from our group that demonstrated analogous characteristics of hearing loss seen in children with congenital CMV-induced sensorineural hearing loss.

The similar birth weight and subsequent weight gains for the 2 groups are compatible with earlier observations of an asymptomatic phenotype with this model. Prior research demonstrated no signs of an inflammatory response in the brain, salivary gland, lung, liver, or kidney using histologic studies. Most congenitally infected infants who go on to develop sensorineural hearing loss also appear normal and healthy at birth and clinically have no evidence of end-organ disease. Despite the subclinical evidence of infection, a 10% to 15% risk for the development of sensorineural hearing loss exists in humans nonetheless.

In conclusion, a reliable method to screen newborns for congenital CMV is needed to identify infants at risk of developing hearing loss. Because clinical trials are costly and difficult to complete, the guinea pig model for CMV infection is an attractive alternative to evaluate potential new diagnostic or screening tests. The results of this study recapitulate prior clinical trials for saliva and dry blood spot assays and validate this model for future studies.

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Author Contributions: Drs Park, Mann, Wang, and Schleiss had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Park, Mann, Error, Firpo, Alder, and Schleiss. Acquisition of data: Park, Mann, Error, Miller, Firpo, and Wang. Analysis and interpretation of data: Park, Mann, Error, Miller, Firpo, and Alder. Drafting of the manuscript: Park, Mann, Firpo, and Alder. Critical revision of the manuscript for important intellectual content: Park, Mann, Error, Miller, Firpo, Wang, Alder, and Schleiss. Statistical analysis: Miller, Firpo, Wang, Alder, and Schleiss. Obtained funding: Park. Administrative, technical, and material support: Park, Mann, Error, Firpo, and Schleiss. Study supervision: Park, Mann, and Firpo.

Conflict of Interest Disclosures: None reported.

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REFERENCES