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

Albert H. Park, MD; David Mann, MD; Marc E. Error, MD; Matthew Miller, BA; Matthew A. Firpo, PhD; Yong Wang, PhD; Stephen C. Alder, PhD; Mark R. Schleiss, MD

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.
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.8

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).9 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 1 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).

ENZYME-LINKED 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.10 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 double-walled 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).

Figure 1. Experimental process showing the animal outcomes for each step in the experimental design. ABR indicates auditory brainstem response; CMV, cytomegalovirus; ELISA, enzyme-linked immunosorbent assay; and PCR, polymerase chain reaction.

Figure 2. Body mass of cytomegalovirus (CMV)–infected and CMV-uninfected pups. The mean body mass was determined at birth and at various postnatal time points for pups from sham-inoculated dams (CMV-negative dams) and from pups from CMV-inoculated dams (CMV-positive dams). Data represent the mean (SE).
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 in the tissues. The primers were synthesized by the DNA/Science) in a volume of 20 μL, using real-time quantitative PCR (LightCycler; Roche Applied Biosystems) containing 110 ng antibody (TaqStart; Clon-tech Laboratories). The CMV culture at a concentration of 1 × 10⁵ 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.

**REAL-TIME PCR STUDIES OF BLOOD, URINE, AND SALIVA**

Total DNA was extracted from specimens obtained from experimental and control animals using a mini kit (QIAamp DNA; Qiagen, Inc) according to the manufacturer’s recommended protocol. The DNA concentrations were determined using a spectrophotometer (Nanodrop; Nanodrop Technologies).

The GP83-specific primer pair, upstream primer UL83F6 (5’-CGACGACGATGCAGAAAAC-3’) and downstream primer UL83B11 (5’-TCCCTGGTCTCAACGAGGGTC-3’), were used in this study to detect the presence of gpCMV DNA in the tissues. The primers were synthesized by the DNA/Peptide Shared Resource facility of the Huntsman Cancer Institute, University of Utah. Under optimized conditions, this primer pair amplifies a 225-base pair region, corresponding to Asp402 through Ser473 of the GP83 protein, and generates a single band of expected size on agarose gel. The PCR was performed using real-time quantitative PCR (LightCycler; Roche Applied Science) in a volume of 20 μL, containing the following: 2 μL DNA, 1.5mM magnesium chloride, 70mM Tris-hydrochloride (pH 8.3), 5 μg bovine serum albumin, 0.2mM each deoxyribonucleotide triphosphate, 0.5μM each primer, 1:30 000 dilution of nucleic gel acid stain (SYBR Green I; Molecular Probes, Life Technologies), and 0.4 U Taq DNA polymerase (Roche Applied Science) containing 110 ng antibody (TaqStart; Clon-tech Laboratories). The CMV culture at a concentration of 1 × 10⁵ 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.

**RESULTS**

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 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 for both ears. The peak-to-peak response amplitude begins to rise. Hearing loss was defined as at least a 15-dB elevation 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.

**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 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).

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**STATISTICAL 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.
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.

### 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>Blood</th>
<th>Urine</th>
<th>Saliva</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>CMV</td>
<td></td>
<td>CMV</td>
</tr>
<tr>
<td></td>
<td>Inoculated</td>
<td>Not Inoculated</td>
<td>Inoculated</td>
</tr>
<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>Value (95% CI)</th>
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</thead>
<tbody>
<tr>
<td>Sensitivity, %</td>
<td>29.0 (14.2-48.0)</td>
</tr>
<tr>
<td>Specificity, %</td>
<td>100.0 (63.1-100.0)</td>
</tr>
<tr>
<td>Positive likelihood ratio</td>
<td>5.3 (0.3-83.3)</td>
</tr>
<tr>
<td>Negative likelihood ratio</td>
<td>0.7 (0.6-1.0)</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>100.0 (66.4-100.0)</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>26.7 (12.3-45.9)</td>
</tr>
</tbody>
</table>

* 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.
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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Correspondence: Albert H. Park, MD, Division of Otolaryngology—Head and Neck Surgery, Department of Surgery, University of Utah School of Medicine, 50 N Medical Dr, Room 3C 120, Salt Lake City, UT 84132 (albert.park@hsc.utah.edu).

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.

REFERENCES