Published in final edited form as:

Diagn Microbiol Infect Dis. 2012 August ; 73(4): 326–329. doi:10.1016/j.diagmicrobio.2012.04.009.

Dried Urine Spots for Detection and Quantification of Cytomegalovirus in Newborns

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Abstract

Dried urine spots (DUS) have been reported to provide a simple screening tool for congenital cytomegalovirus (CMV) infection. We developed a standardized method for CMV quantification from DUS. Two applications of 20 μ L urine remained within the rim of the filter paper disc and were used to determine the analytical performance of Towne CMV spiked into urine and applied on the discs. The measurable range spanned 3.7 to 8.0 log₁₀ copies/mL. The detection limit was 22 DNA copies/disc. Urine samples from congenitally-infected newborns and negative controls were either diluted 1:10 or applied on filter paper at the same volume. DNA copy number from DUS correlated well with copy number from 1:10 diluted urine, although there was a trend for lower levels from DUS (0.3 log₁₀ difference). Our standardized method for CMV detection and quantification may facilitate CMV studies in resource-limited areas and allow for longitudinal monitoring of viral loads in treated infants.

1. Introduction

CMV is the most common congenitally-acquired infection, causing mental retardation and deafness in infected children (Demmler, 1991). Primary infection, reactivation, or infection with a new CMV strain during pregnancy can lead to in-utero transmission of CMV. Infection during pregnancy is typically asymptomatic, and therefore is usually under-recognized. Despite the potential devastating outcomes of congenital CMV infection, screening programs are not yet available. The absence of screening for CMV during pregnancy results in approximately a 1% congenital CMV prevalence in the US amongst newborns delivered yearly (Kenneson & Cannon, 2007).

Universal newborn screening programs for metabolic and genetic disorders utilize dried blood spots (DBS), onto which a small amount of blood is applied. Research is ongoing to elucidate a simple and cost-effective method for screening of congenital CMV infection including the detection of CMV DNA from amniotic fluids of infected mothers (Lazarotto et al., 2000) and from urine, saliva or blood of their infected infants (Lanari et al., 2006; Boppana et al., 2005; Yamamoto et al., 2006; Choi et al., 2009). DBS have been reported to lack sensitivity in a large US study (Boppana et al., 2010), but a recent European

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study suggested an improved sensitivity (Leruez-Ville et al., 2011). Dried saliva spots had excellent sensitivity (Boppana et al., 2011), and reports from Japan suggest dried urine spots (DUS) could also provide a highly sensitive screening tool for congenital CMV infection by simply inserting a filter paper into the infant's diaper (Nozawa et al., 2007).. However, lack of standardization in sample collection currently limits the use of dried spots for quantitative studies. Theoretically, DUS could provide a tool not only for the detection of CMV but for longitudinal monitoring of viral loads especially in treated infants, since congenitally-infected children shed CMV for a period of months to years in both urine and saliva (Nozawa et al., 2007; Noyola et al., 2000). At this time quantification of CMV DNA is limited due to differences in the volume of urine collected on the each filter. Therefore, comparative data between patients or longitudinal data within the same patient may be difficult to interpret.

The aim of this study was to develop a standardized method for CMV quantification from DUS which could be utilized in resource-limited areas for future CMV studies. This method could be used to compare viral loads between patients or longitudinally within the same patient.

2. Materials and methods

Subjects

Forty two archived urine samples collected from congenitally-infected ($n = 27$) and non-infected neonates ($n = 15$) were tested. The Johns Hopkins IRB approved the use of samples for this study.

Filter papers

Whatman 903 neonatal cards with 5, 13 mm diameter discs were used. Each circle containing 40 μ L of urine was cut out and placed in either 1.5 ml or 2.0 ml Sarstedt tubes for manual or automated nucleic acid extraction.

DNA extraction

CMV DNA was extracted from original and 10-fold dilution of urines using the automated, BioRobot M48 instrument with the MagAttract Virus Mini M48 Kit and the Virus Mini Protocol, version 1.1, (Qiagen, Germantown, MD). 400 μ L undiluted urine or 40 μ L urine diluted in total volume of 400 μ L PBS were used. CMV DNA was extracted from urine dried on filter paper discs using either a manual (boiling) method or the automated method. For manual extraction, 50 μ L of dH₂O or PBS was added to sample tubes containing filter discs, boiled (100°C) for 30 min, followed by centrifugation at 16,000 $\times$ g for 5 min to recover the eluate. For automated extraction, 400 μ L PBS was added to the tube containing the filter paper and heated at 100 °C in a heat block for 15 min. DNA extraction was performed on the BioRobot M48 instrument with the extraction kit and protocol described above. The elution volume was 100 μ L. Manual DNA extraction using boiling and centrifugation demonstrated lower levels of CMV DNA compared to automatic extraction (data not shown), and therefore all subsequent experiments were performed using automated extraction.

Real-time PCR

The quantitative CMV real-time PCR assay is based on detection of a 151bp region from the highly conserved US17 gene (Tanaka et al., 2002). The limit of detection of the assay is 100 copies/mL (3.0 copies/reaction). The measureable range is 2.4–8.0 log₁₀ copies/mL. The PCR was performed using 50 μ L total reaction volume. This included 25 μ L of TaqMan 2 $\times$ Universal PCR Master Mix (Applied Biosystems), 1.5 μ L each of 10 μ M primers, 1 μ

L of 10 μ M FAM-labeled probe, 11 μ L of dH₂O, and 10 μ l of template. Amplification was performed on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). PCR conditions were: 50°C for 2 min, 95°C for 10 min, 45 cycles of 95°C for 15s and 60°C for 60s. Quantification standards were prepared by cloning the US17 amplicon into the pCR®2.1-TOPO® plasmid vector (Invitrogen, Carlsbad, CA). Serial 10-fold dilutions of plasmid from 7.0 to 1.0 log₁₀ copies/reaction were included with each assay and used to establish a standard curve. Assay controls included quantified CMV AD169 DNA (Advanced Biotechnologies Inc.) and quantified Towne CMV (VR 977, American Type Culture Collection, Manassas, VA) at 3.0 and 5.0 log₁₀ copies/mL. Quantitative CMV data were expressed as viral copies/ milliliter.

Analytical Performance and Correlation

(i) Measurable range was determined with serial CMV Towne dilutions (8.0 to 3.0 log₁₀ copies/mL, $n = 2$ per concentration) dried on filter paper discs. (ii) Limit of detection (LOD) was determined using serial dilution of CMV Towne, from 10,000 to 500 copies/mL. Twenty replicates at each concentration were dried onto filter paper discs and tested. The LOD was defined as the concentration at which 95% of replicates were detected. (iii) Precision of DUS was determined using two concentrations of CMV Towne (4.0 log₁₀ and 5.7 log₁₀ copies/mL). For intra-assay precision, 20 replicates of each concentration were assayed. For inter-assay precision, 20 additional replicates were tested at a different time and data were combined with the initial run of 20 replicates. (iv) Correlation between original urine and DUS necessitated the use of diluted urine to correct for the lower volume of urine in DUS (40 μ L) compared to extracted neat urine (400 μ L). Prior to determining this quantitative correlation, the effect of urine dilution on CMV quantification was assessed. CMV DNA copy number detected in 400 μ L of original urine was compared to quantity detected in a 10-fold dilution of urine in PBS that would approximate the volume of urine in a DUS (40 μ L urine and 360 μ L PBS). The CMV DNA concentration in the diluted sample was corrected mathematically to account for the dilution step. Correlation between DUS and diluted urine samples from infants was assessed.

Statistical Methods

Regression lines and their characteristics were determined using Microsoft Excel (Microsoft Office 2007; Microsoft Corp., Redmond, Wash.). The dose-response 95th percentile (with 95% confidence interval) was estimated using Probit analysis and performed with Matlab (The MathWorks, Nalick, MA).

3. Results

Urine volumes applied on DUS

The volume of urine that reproducibly remained within the border of the filter paper disc was evaluated and found to be 20 μ L applied twice, with 30 min. drying time between applications. Real-time PCR was more sensitive with two applications of 20 μ L than with a single application. Adding 40 μ L in a single application diffused outside of the disc and CMV quantification was less sensitive (data not shown). All subsequent experiments were therefore performed using two applications of 20 μ L onto each filter paper disc.

Measurable range with Towne CMV

Application of Towne virus on DUS revealed a measurable range of 3.7 to 8.0 log₁₀ copies/mL (Fig. 1). Although data points within this range demonstrated linearity, quantification was lower than expected as demonstrated by a y intercept of -0.73 .

Limit of Detection

In experiments to determine analytical sensitivity, 100% of replicates at 7500 CMV copies/mL were detected (Fig. 2). By Probit analysis, the LOD was 5403 copies/mL (95% CI 4307-7522, Fig. 2.). This was determined to be 22 CMV DNA copies/disc after accounting for extraction input/elution volumes and template volume added to the PCR reaction.

Precision

Intra- and inter-run variability of CMV quantification from DUS was greater at 4.0 log₁₀ copies/mL than at 5.7 log₁₀ copies/mL. The intra-/inter-run percent coefficient of variation was 11.9/11.6 at 4.0 log₁₀ copies/mL compared to 2.6/4.2 at 5.7 log₁₀ copies/mL (Table 1).

Correlation of DNA quantification from original and 1:10 diluted urine

11 of 14 urine samples had detectable CMV DNA in both urine diluted 1:10 and undiluted urine. There was 100% agreement between positive or negative original and diluted urine samples. However, there was a trend of higher CMV DNA levels in the diluted urines (average difference 0.3 log₁₀ copies/mL, Fig. 3).

Quantitative correlation between diluted urine and DUS

15 samples from non-infected infants had no detectable CMV DNA in diluted urine and DUS. Concentrations of CMV DNA in the 27 positive samples ranged from 4.4 to 8.8 log₁₀ copies/mL (mean = 6.5 log₁₀ copies/mL). Three samples had CMV DNA levels that were greater than the highest concentration of CMV Towne (8.0 log₁₀ copies/mL) tested for measurable range calculation. Results for these samples appear to fall close to the regression line for correlation of all samples, Data obtained by DUS and 1:10 diluted urine were linearly related (slope = 1.03) and correlated ($r = 0.97$) but not identical (Fig.4)

4. Discussion

We report on the successful use of DUS for detection and quantification of CMV DNA from urine samples. DNA copy number from DUS correlated well with copy number from 1:10 diluted urine, although there was a trend for lower level of detection. Quantification from 1:10 diluted urine was 0.3 log higher compared to original undiluted urine (Fig. 3). One possible explanation is that PCR inhibitors in urine were diluted. DUS under-quantified DNA by 0.6 log₁₀ copies/mL as compared to diluted urine (Fig. 4); therefore the difference in quantification between DUS and original urine was approximately 0.3 log₁₀ copies/mL. A possible disadvantage of DUS is that not all congenitally-infected infants would be detected since the limit of detection of the assay is 5403 copies/mL or 22 copies/disc. However, the majority of infected infants are expected to shed higher levels of CMV, and the low shedders may be at the lowest risk for any sequelae from congenital CMV infection (Lanari et al., 2006; Boppana et al., 2005). Therefore the potential advantages of a simple assay may outweigh its current limitations.

A study comparing real-time PCR from DBS with saliva rapid culture for the diagnosis of congenital CMV infection showed that DBS had a low sensitivity and therefore could not be used efficiently as a screening tool (Boppana et al., 2010). In another study that used a different methodology (single DBSs of 10 mm diameter were cut in 10–12-thin strips prior to DNA extraction) the sensitivity and specificity for detection of CMV from DBS were 95% and 98%, respectively, as compared to CMV PCR of a urine sample (Leruez-Ville et al., 2011). Several studies have compared DNA extraction methods from DBS suggesting that some methods could result in better sensitivity than others in subsequent real-time PCR assays (Soetens et al., 2008; de Vries et al., 2009). The role of DBS as a screening tool for congenital CMV infection requires additional studies. A recent study of real-time PCR from

dried saliva swabs demonstrated a high level of sensitivity (97.4%) and specificity (99.9%) compared to rapid CMV culture in screening newborns for CMV (Boppana et al., 2011). Studies from Japan have shown that dried urine spots (DUS) could provide a simple and sensitive alternative for universal CMV screening (Inoue & Koyano, 2008; Nozawa et al., 2007). In these studies filter papers were placed in infants' diapers and then 3mm discs were used for detection of CMV DNA by real-time PCR. Comparison of viral loads between CMV-infected children could not be interpreted since the volume of urine absorbed by the filter paper was not standardized. Thus, our results provide a new standardized method for quantification of CMV DNA using DUS.

Screening for presence of congenital CMV infection could be achieved by inserting a filter paper in the newborn's diaper. For this purpose, quantification may not be necessary. Our standardized assay is not intended for screening, since it requires urine collection and application of a specific volume on a DUS. Rather, our assay may provide a simple and rapid tool for CMV detection and quantification from DUS in underdeveloped or developing countries in which diagnostic capabilities for CMV are lacking. Information on CMV prevalence from resource-constrained areas is currently limited due to lack of diagnostic infrastructure (Kaye et al., 2008; Miles et al., 2008; Bates et al., 2008). The described method may allow initiation of such studies which will enhance our understanding of the epidemiology of congenital CMV.

Conclusion

DUS provide a simple method for CMV detection and quantification. Collected filter papers can be shipped and tested in well-equipped laboratories.

Acknowledgments

Supported by NIH KO8 AI074907-01A1 to RAB. We thank Robert F. Pass, University of Alabama at Birmingham, for providing some of the urine samples for this study.

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Figure 1. Measurable range of CMV DNA quantification

Dilutions of Towne virus were applied on DUS. The dotted line represents a theoretical complete agreement between expected and observed measurements

Figure 2. Limit of detection

Probability of detection corresponds to percentage of replicates detected ($n = 20$ at each concentration). Open circles, LOD data (percentage of replicates detected). Arched solid line, probit regression line. Arched dashed lines, 95% confidence intervals of the regression line. Solid vertical line, predicted concentration of 95% detection limit. Dashed vertical lines, 95% confidence intervals for 95% detection limit.

Figure 3. Correlation of CMV DNA copy number from original and 1:10 diluted urine
The dotted line represents a theoretical complete agreement between expected and observed measurements

Figure 4. Correlation of CMV DNA from original urine diluted 1:10 and DUS
The dotted line represents a theoretical complete agreement between expected and observed measurements

Table I

Precision of viral load measurements from DUS

	Intra-Run Precision¹		Inter-Run Precision²	
	4.0 log ₁₀	5.7 log ₁₀	4.0 log ₁₀	5.7 log ₁₀
Mean	3.6	5.2	3.5	5.2
SD	0.43	0.14	0.41	0.22
CV (%)³	11.9	2.6	11.6	4.2

¹Intra-run precision, 20 replicates tested at each concentration

²Inter-run precision, 10 additional replicates tested on two additional runs performed on two separate days and combined with the initial run of 20 replicates.

³CV (coefficient of variation), calculated from log transformed viral load values, is the standard deviation (SD) divided by the mean.