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## Evaluation of DNA extraction methods for the detection of *Cytomegalovirus* in dried blood spots

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### Abstract

**Background**—Dried blood spots (DBS) are collected universally from newborns and may be valuable for the diagnosis of congenital *Cytomegalovirus* (CMV) infection. The reported analytical sensitivity for DBS testing compared to urine or saliva varies greatly across CMV studies. The purpose of this study was to directly compare the performance of various DNA extraction methods for identification of CMV in DBS including those used most often in CMV studies.

**Study design**—Whatman® Grade 903 filter paper cards were spotted with blood samples from 25 organ transplant recipients who had confirmed CMV viremia. Six DNA extraction methods were compared for relative yield of viral and cellular DNA: 2 manual solution-based methods (Gentra Puregene, thermal shock), 2 manual silica column-based methods (QIAamp DNA Mini, QIAamp DNA Investigator), and 2 automated methods (M48 MagAttract Mini, QIAcube Investigator). DBS extractions were performed in triplicate followed by real-time quantitative PCR (qPCR).

**Results**—For extraction of both viral and cellular DNA, two methods (QIAamp DNA Investigator and thermal shock) consistently gave the highest yields, and two methods (M48 MagAttract Mini and QIAamp DNA Mini) consistently gave the lowest yields. There was an average 3-fold difference in DNA yield between the highest and lowest yield methods.

**Conclusion**—The choice of DNA extraction method is a major factor in the ability to detect low levels of CMV in DBS and can largely account for the wide range of DBS sensitivities reported in studies to date.

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#### Competing interests

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention or the Agency for Toxic Substances and Disease Registry. Mention of any company or product is for identification only and does not imply endorsement.

#### Ethical approval

Not required.

## Keywords

qPCR; Dried blood spots; Newborn screening

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## 1. Background

Human *Cytomegalovirus* (CMV) is a leading cause of congenital infections worldwide. The frequency of congenital CMV infection varies in different populations but on average is approximately 0.7% of live births, with 15–20% of infected children developing permanent disability including hearing loss, vision loss, and cognitive impairment [1–3]. The most common of these disabilities is hearing loss for which congenital CMV infection is a major cause in young children second only to genetic mutations [4].

US newborns are currently screened within the first week of life for a wide range of birth defects through the collection of blood on filter paper in the form of dried blood spots (DBS). DBS have been shown to provide >95% sensitivity compared to urine or saliva for the retrospective diagnosis of congenital *Cytomegalovirus* infection in children born with CMV-associated symptoms or born to mothers who had primary CMV infection during pregnancy [5,6]. In contrast, in studies where CMV screening was performed on unselected newborn populations the reported sensitivity of DBS relative to urine or saliva has varied widely from 28 to 80% [7–9]. Because of numerous differences between studies, it was difficult to ascertain the reason for the wide range in results. To establish that lab methods are an important variable in DBS testing sensitivity, de Vries and others compared available DNA extraction methods for DBS and showed large differences in performance among the methods [10].

## 2. Objectives

The aim of our study was to extend previous method comparisons and include the two DNA extraction methods most frequently used in CMV studies (QIAamp DNA Mini and thermal shock) [6,8,11–14] and the automated method used by the largest CMV newborn screening study to date (M48 MagAttract Mini) [9]. The goal was to contribute additional important information relevant to the ongoing debate over the potential utility of DBS for CMV testing in newborns.

## 3. Study design

### 3.1. Blood samples and dried blood spots

De-identified CMV DNA positive EDTA whole blood from 25 organ transplant recipients was kindly provided by The Cleveland Clinic Foundation, Dept. of Clinical Pathology, Cleveland, OH. Blood specimens had CMV viral loads ranging from a low of  $7 \times 10^2$  copies/ml to a very high  $1 \times 10^6$  copies/ml. Replica blood spots were prepared by dispensing 75  $\mu$ l of blood onto the circles of Whatman® 903 Specimen Collection Paper. After drying the spots overnight, punches were prepared manually for DNA extraction methods with negative control punches between each sample. Remaining DBS material was stored at –20

°C with desiccant. CMV DNA-negative EDTA whole blood from healthy volunteers was spotted and used as negative controls.

### 3.2. Extraction of DNA from DBS

DNA was extracted from DBS using the following six extraction methods: (1) QIAamp DNA Investigator kit, (2) QIAamp DNA Investigator kit with QIAcube automation, (3) QIAamp DNA Mini kit, (4) MagAttract DNA Mini kit with BioRobot M48 automation, (5) thermal shock, and (6) Gentra Puregene. Sample input for all methods was 3 punches of 3.2 mm in size with the exception of the thermal shock method which used one 6 mm punch. Input volume of whole blood was calculated based on the area of the blood spots extracted. Samples were extracted in triplicate for each method. With the exception of thermal shock, all extraction methods were kit-based (Qiagen, Valencia, CA) and DNA extracted following the manufacture's protocols for isolation of total DNA from DBS. Carrier RNA was added to Buffer AL as recommended for small amounts of DNA. DNA extracted using thermal shock followed the method developed by Shibata and modified by Barbi [14,15]. Briefly, one 6-mm punch was soaked in 60 µl minimum essential medium (MEM) at room temperature for 2 h with shaking (300 rpm) followed by incubation at 56 °C for 1 h, and incubation at 100 °C for 7 min. Samples were placed on ice for at least 2 min, spun in a centrifuge at 14,000 rpm for 5 min, and stored at -80 °C overnight. Prior to PCR testing, samples were thawed and transferred to a DNA IQ Spin Basket (Promega) inserted into an elution tube, centrifuged at 14,000 rpm for 3 min and the liquid flow through used directly for qPCR.

### 3.3. Real-time PCR

Viral DNA was amplified using primers and probes that target the conserved envelope glycoprotein B as described [9] with addition of TaqMan Universal PCR master mix and an exogenous internal positive control (Applied Biosystems, Foster City, CA). PCR testing was performed in triplicate for all samples. AD169 (Advanced Biotechnologies) was used as quantitation standard. PCR cycling on the ABI 7900HT (Applied Biosystems) was as follows: 95 °C, 10 min.; 95 °C, 15 s., 60 °C, 1 min for 45 cycles; 4 °C hold. Genomic DNA was quantified using the same reaction conditions. The following primers and probe that target the cellular RNaseP gene were used: forward primer: 5'-AGATTTGGACCTGCGAGCG-3'; reverse primer: 5'-GAGCGGCTGCTCCACAAGT; probe: FAM-5'-TTCTGACCTGAAGGCTCTGCGCG-3'.

### 3.4. Data analysis

For quantitative results, negative samples were included as zero when calculating mean viral loads. CMV quantitation results were used to classify specimens into three viral load categories: low (<10 copies/µl spotted blood), intermediate (~10–100 copies/µl spotted blood), and high (>100 copies/µl spotted blood). For qualitative results, DBS samples were counted positive when two or more of the triplicate PCR reactions tested positive.

## 4. Results

### 4.1. Quantitative results

Fig. 1 shows the DNA yields for six extraction methods used on DBS made with low CMV viral load blood (left panel,  $n = 8$ ) and intermediate CMV viral load blood (right panel,  $n = 11$ ). For low viral load specimens, manual extraction with Investigator and thermal shock produced the highest DNA yields; Qiagen Mini and M48 MagAttract gave the lowest yields. There was a 3-fold difference in mean DNA yield between the two highest and two lowest yield extraction methods ( $p < 0.0001$ , student's  $t$ -test). For intermediate viral load specimens, manual Investigator and QIAcube Investigator produced the highest CMV DNA yields; Qiagen Mini and M48 MagAttract gave the lowest CMV DNA yields representing a 3-fold difference between the mean DNA yields for the two highest and two lowest yield extraction methods ( $p < 0.0006$ , student's  $t$ -test). For high viral load specimens, differences between methods were not significant.

Mean CMV DNA yields expressed as  $\log_{10}$  for the low or intermediate viral load categories are displayed together in Fig. 2. For the low viral load category samples, the manual Investigator and thermal shock methods gave yields of CMV DNA significantly greater than those obtained from the Qiagen Mini ( $p = 0.05$ , student's  $t$ -test) or the M48 MagAttract ( $p = 0.05$ , Student's  $t$ -test). For the intermediate viral load category samples, all methods gave yields of CMV DNA significantly greater than those obtained from the M48 MagAttract ( $p = 0.05$ , student's  $t$ -test). Moreover, the manual Investigator and QIAcube Investigator methods gave yields of CMV DNA significantly greater than those obtained from the Qiagen Mini kit ( $p = 0.05$ , student's  $t$ -test).

For extraction of genomic DNA, relative performance of the various extraction methods was similar to that seen for viral DNA. Fig. 3 shows the results of qPCR for RNaseP performed on the same DNA extracts as that used for CMV qPCR. Investigator and thermal shock produced the highest DNA yields; Qiagen Mini and M48 MagAttract gave the lowest yields. There was a 3-fold difference in DNA yield between the two highest and the two lowest yield extraction methods.

### 4.2. Qualitative results

In addition to measuring DNA yields among extraction methods, we compared CMV positive and negative results for each method. Table 1 lists the percent of samples in each viral load category that was identified as positive for each extraction method. For specimens with low viral load, CMV DNA detection ranged from 100% to 46% of samples. The top two methods for detection of CMV in DBS were thermal shock (100%) and manual Investigator (88%). The lowest detection rates were seen with M48 MagAttract and Qiagen Mini (58% and 46%, respectively), with these differences being significant ( $p < 0.01$ , student's  $t$ -test).

For DBS specimens in the intermediate viral load category, CMV detection rates were higher with 3 methods showing 100% CMV detection and the lowest method showing 85% detection (Table 1), with this difference being significant ( $p < 0.05$ , student's  $t$ -test). When testing DBS with high CMV loads, all extraction methods identified 100% of DBS

specimens as positive. All CMV DNA negative control samples tested negative, and there was no PCR inhibition seen with any of the extraction methods.

## 5. Discussion

Our study found large and consistent differences in the relative performance of six DNA extraction methods for DBS measuring yields for both genomic and viral DNA at multiple concentrations. The highest yield methods were Investigator manual and thermal shock; the lowest yield methods were Qiagen mini and M48 MagAttract. Two of the above mentioned methods, thermal shock [14–18] and Qiagen mini [6,9,11–13], have been used by several CMV studies and showed variable sensitivities for identifying CMV infection in children. But the studies varied widely, mainly regarding the sample size and the extent to which it was enriched for infants with symptomatic CMV infection. The largest CMV newborn screening study to date was the CMV and Hearing Multicenter Screening (CHIMES) study that used the M48 MagAttract method for DBS testing. DBS sensitivity observed in CHIMES was the lowest reported to date at 28–34% compared to saliva and it was concluded that DBS would not be suitable for newborn screening [9]. However, our study showed that M48 MagAttract had the lowest DNA yield of the 6 methods evaluated which was likely a contributing factor to the poor performance of DBS testing in CHIMES [9].

A major strength of the present study is the comprehensive expertise at CDC for DBS diagnostic testing. The Newborn Screening and Molecular Biology Branch at CDC manufacturers and validates quality assurance materials for all biomarkers associated with the core conditions on the Recommended Uniform Screening Panel. A second strength of the study was the use of blood from patients with CMV viremia, as opposed to the often used CMV uninfected blood spiked with laboratory strain AD169. The results of our study were consistent with those from the DBS method comparison by de Vries [10], which examined seven different extraction methods (four of which are in common with our study) and also used blood from patients with CMV viremia. Concordant with our study, the Investigator manual and thermal shock methods showed superior performance for detection of CMV. A third DBS methods comparison by Gohring [19] included four methods and showed the Qiagen Mini kit performed much better than thermal shock (referred to as heat). However the Gohring study used AD169-spiked blood instead of naturally infected blood, and their thermal shock (heat) method did not include the important pre-incubation of DBS at either 4 °C overnight [10] or at room temperature for 2 h.

Methods that are under consideration for newborn screening need to be sensitive, adaptable to automation, and cost-effective. Extraction methods used in our study that provided the best results for CMV detection from DBS (Investigator manual and thermal shock) are low throughput and thus in their current form are not suitable for newborn screening. However with sufficient market demand, these methods could potentially be developed for higher throughput and lower cost. The thermal shock protocol has the advantage of very low cost over the other methods tested but does have the disadvantage of an overnight freezing step. This will be addressed in future work.

For CMV newborn screening, it is clear DBS would offer lower analytical sensitivity than saliva or urine. However, only 15–20% of children with congenital CMV infection develop permanent disabilities [2,3] and they are largely the children born with higher viral loads [20–22]. Thus, DBS-based detection may offer adequate clinical sensitivity. Moreover, amplification methodologies continue to improve and provide increased sensitivity. Atkinson recently reported enhanced detection of CMV from DBS using a single tube nested PCR [23]. In conclusion, our study demonstrates that DBS warrant further consideration for identification of newborns at risk for disability from congenital CMV infection.

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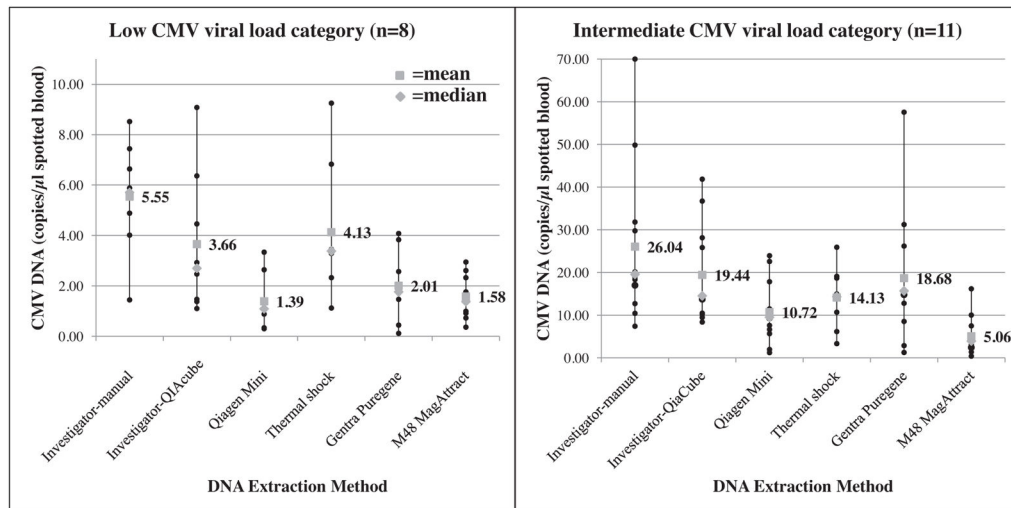
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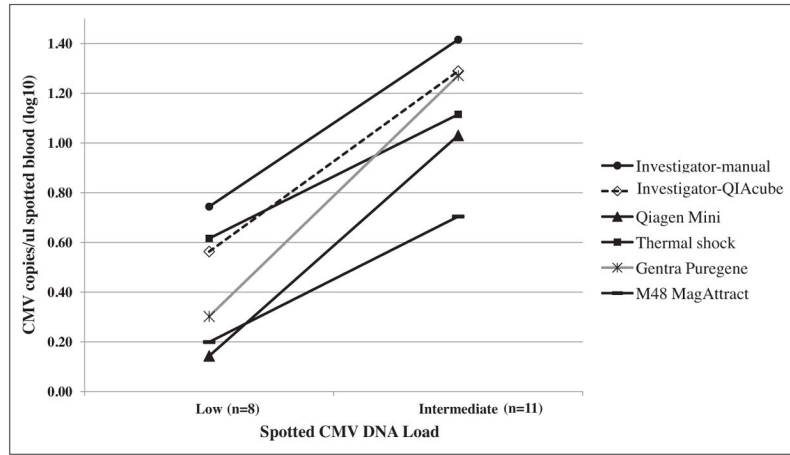


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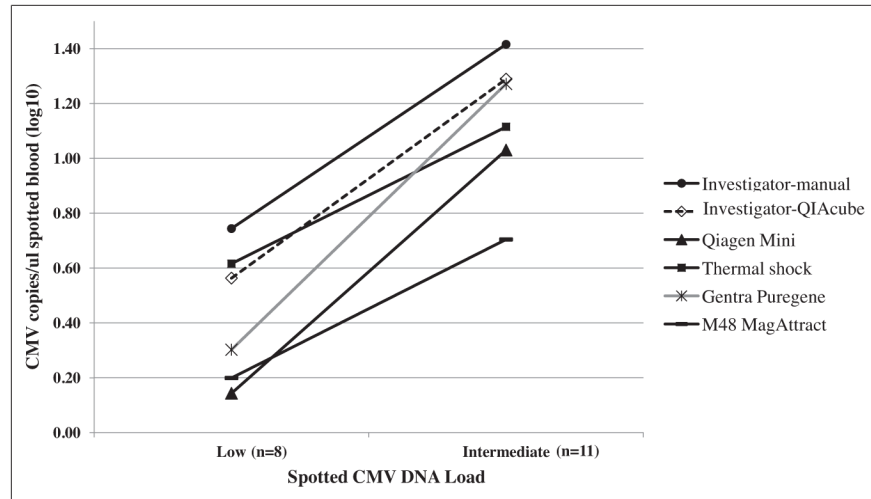


**Fig. 1.** Quantitative results for CMV DNA yield according to extraction method. Data points (circles) represent the average CMV DNA yield for each DBS specimen from triplicate extractions. Vertical lines show the full range of DNA yield per method. Mean (square) and median (diamond) viral DNA yields for the complete sample set are shown for each extraction method, with numeric values for means shown.





**Fig. 2.** Overall quantitative results for CMV DNA yield from six extraction methods. Average DNA yields for all specimens combined is shown for each extraction method, for low and intermediate viral load categories.



**Fig. 3.** Quantitative results for extraction of total genomic DNA: the mean genomic DNA yield measured by qPCR targeting housekeeping gene RNase P. Replica extractions quantified in duplicate were performed for all 25 blood samples.

**Table 1**

Qualitative assessment. Percentage of CMV-positive DBS in which CMV was detected following various extractions methods.

| <b>Method</b>          | <b>Low viral load category (<i>n</i> = 8) (%)</b> | <b>Intermediate viral load category (<i>n</i> = 11) (%)</b> |
|------------------------|---|---|
| Investigator – manual  | 88  | 97  |
| Investigator – QIAcube | 79  | 100   |
| Qiagen mini            | 46  | 91  |
| Thermal shock          | 100   | 100   |
| Gentra puregene        | 67  | 100   |
| M48 MagAttract         | 58  | 85  |

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