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Evaluation of rapid and sensitive DNA extraction methods for detection of cytomegalovirus in dried blood spots

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ABSTRACT

Background: Dried blood spots (DBS), collected universally from newborns in the U.S., could be used as a matrix for the detection of cytomegalovirus (CMV) infection in infants. However, sensitivity to detect CMV in DBS as compared to saliva and urine is variable across studies largely due to the DNA extraction method. Thermal shock, a widely used DNA extraction method, is highly sensitive for the detection of CMV in DBS, however, the processing time required is not practical for high-throughput testing.

Objective: To determine if rapid and cost-effective DNA extraction methods amenable to newborn screening (NBS) could achieve the same sensitivity as the thermal shock method.

Study design: DBS were prepared from CMV positive blood samples from 20 organ transplant recipients. Three DNA extraction methods were compared for relative yield and sensitivity of detection of CMV DNA: thermal shock, KOH Tris buffer, and DNA Extract All. CMV DNA was detected by real-time quantitative polymerase chain reaction (qPCR).

Results: The KOH Tris and DNA Extract All methods gave higher yields and sensitivity of CMV detection in DBS than thermal shock, which were significantly greater when viral loads were ≤ 10,000 copies/ml blood. Both methods gave faster turnaround times than thermal shock and would be better suited for NBS.

Conclusions: The choice of DNA extraction method greatly influences the ability to detect low levels of CMV DNA in DBS. Moreover, development of highly sensitive yet rapid methods for CMV detection could help facilitate future newborn screening of CMV in DBS.

1. Introduction

Cytomegalovirus (CMV) is a leading cause of sensory neural hearing loss (SNHL) in newborns worldwide (Grosse et al., 2008). Other common sequelae include cognitive and motor deficits, seizures, microcephaly, and vision loss (Dreher et al., 2014). The prevalence of CMV infection varies considerably among different study populations but is reported to be on average 0.7% of live births, with 10–15% symptomatic at birth (Dollard et al., 2007; Kenneson and Cannon, 2007). However, the true burden of CMV infection may change with the identification of CMV-infected infants through universal NBS (Dollard et al., 2007).

Dried blood spots (DBS) collected in US newborns as well as many nations worldwide are currently tested for a variety of treatable, life threatening conditions. Potential benefits to adding CMV to the NBS panel would be early detection and intervention to prevent the onset or progression of SNHL and identification of infants at risk for late-onset SNHL and other CMV-associated disabilities. Interventions might include antiviral drugs, cochlear implants, or non-pharmaceutical therapies (Cannon et al., 2014). The gold standard for diagnosis of congenital CMV infection is PCR of DNA from saliva or urine (Yamamoto et al., 2001; Bopanna et al., 2011); however, these samples are not collected for NBS. Studies to determine the appropriateness of using DBS for CMV NBS have had varied results. Prospective and retrospective studies testing for CMV in DBS have shown a sensitivity close to that of urine or saliva in children born with CMV-associated symptoms or born to mothers who had primary CMV infection during pregnancy (Barbi et al., 2000; Leruez-Ville et al., 2011). Other studies have shown a significant association between increasing CMV blood viral loads in children born with CMV and risk for developing sequelae (Bradford et al., 2005; Walter et al., 2008). In contrast, studies screening for CMV in unselected newborn populations reported that the sensitivity of DBS relative to urine or saliva varied widely from 28 to 100% (Yamamoto et al., 2001; Barbi et al., 2000; Johansson et al., 1997; Boppana and Ross, 2010). Moreover, sensitivity of CMV detection in DBS is strongly correlated with the DNA extraction method used (de

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Vries et al., 2009; Koontz et al., 2015; Vauloup-Fellous et al., 2007; Gohring et al., 2010). In addition to the widely used thermal shock method (Yamamoto et al., 2001; de Vries et al., 2009; Koontz et al., 2015; Gohring et al., 2010; Shibata et al., 1994; Barbi et al., 1996; Kharrazi et al., 2010), other extraction methods such as silica-based columns (Leruez-Ville et al., 2011; Walter et al., 2008; de Vries et al., 2009; Koontz et al., 2015; Vauloup-Fellous et al., 2007; Gohring et al., 2010), magnetic bead technologies (Boppana and Ross, 2010; de Vries et al., 2009; Koontz et al., 2015; Gohring et al., 2010), and phenol-chloroform (Leruez-Ville et al., 2011; Johansson et al., 1997; Vauloup-Fellous et al., 2007; Gohring et al., 2010) have been used. We have previously shown that thermal shock performs well compared to column and bead-based extraction methods (Koontz et al., 2015). This study presents rapid and inexpensive alternatives to thermal shock that are amenable to screening without sacrificing sensitivity in the detection of CMV in DBS.

2. Materials and methods

2.1. DNA extraction from blood samples

Subsequent to institutional review board approval, CMV positive blood from 20 adult organ transplant recipients of unspecified age was collected, de-identified, and sent to CDC by the Cleveland Clinic. Residual de-identified CMV seronegative umbilical cord blood was obtained from the Carolinas Cord Blood Bank at Duke University and used as CMV negative controls. Prior to spotting, DNA was extracted from 100 µl of the CMV positive blood samples using the QIAamp DNA mini kit (Qiagen, Valencia, CA). Replica blood spots of each sample were prepared by dispensing 75 µl of blood onto Whatman® 903 Filter Paper. After drying overnight, 3.2 mm punches were prepared manually for DNA extraction, and the remaining DBS material was stored in sealed bags at -20^{0}C with desiccant. DNA was extracted using thermal shock (Shibata et al., 1994; Barbi et al., 1996), KOH Tris buffer (Baker et al., 2016), and DNA Extract All (Applied Biosystems, Foster City, CA). The DNA Extract All method was modified by reducing the lysis and stabilization solutions to 25 µl each for a 50 µl final volume, consistent with the other extraction methods. The DNA elution volume for all methods was 50 µl. See Table 1 for method comparisons.

2.2. Real-time quantitative PCR and analysis

CMV DNA was amplified by real-time qPCR as previously described (Koontz et al., 2015) with the following modifications: Perfecta qPCR ToughMix, Low Rox (Quanta Biosciences, Beverly, MA) master mix was used, and the assay was run on the ViiA7 (Applied Biosystems). Volume of CMV DNA input was 5 µl, and all testing was performed in duplicate. To minimize inter-plate variation, all three extraction methods for each DNA sample were run on the same plate, and the threshold for all runs was adjusted to a calibrator sample located in the same well in all plates. A standard curve was generated using a 10-fold serial dilution of CMV NIST (National Institute of Standards and Technology, Gaithersburg, MA) standard DNA ranging from 16,000 to 0.625 copies/µl. CMV negative DBS were pre-tested for CMV and included in every run. CMV quantitation results from whole blood were used to classify specimens into low (≤10,000 copies/ml blood) and high (> 10,000 copies/ml blood) CMV viral load categories based on generally accepted viral load thresholds for defining CMV infection in organ transplant recipients (Haijing et al., 2003; Razonable and Hayden, 2013; Eshraghi and Hekmat, 2015). CMV viral loads in DBS were expressed as copies/µl spotted blood, and a two-tailed *t*-test was used to determine significant differences between methods. Volume of whole blood contained in each 3.2 mm punch was assumed to be approximately 3 µl based on an average DBS diameter of 15 mm (Hall et al., 2015).

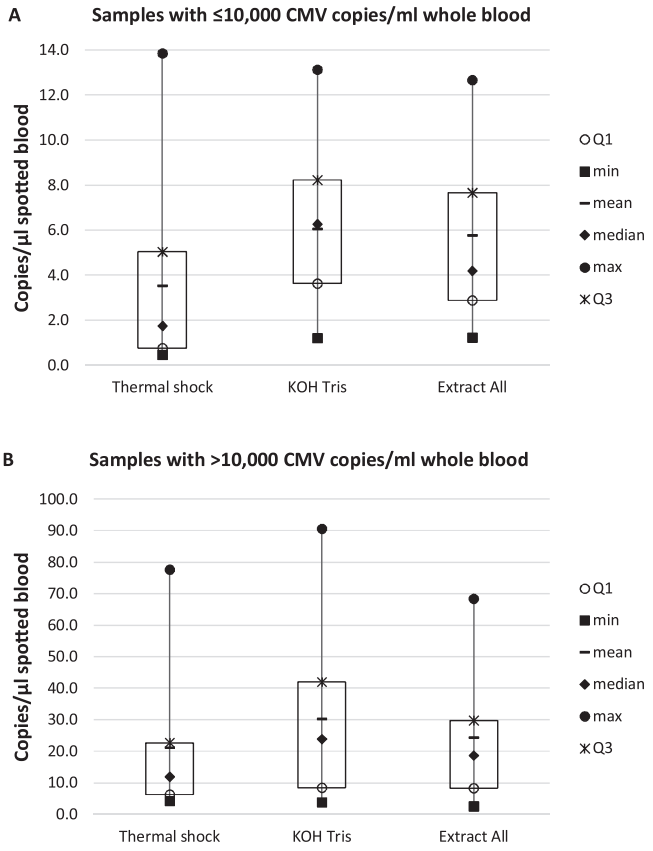

Fig. 1. Quantitative Results for CMV DNA yield according to extraction method. A) DBS specimens in the ≤10,000 CMV copies/ml category were prepared from blood samples ranging from 1,000 to 9,900 CMV copies/ml. B) DBS specimens in the > 10,000 CMV copies/ml category were prepared from blood samples ranging from 11,000 to 191,000 CMV copies/ml. The boxes represent the interquartile range with horizontal bars for mean values and diamonds for median values. The minimum and maximum values are represented by squares and circles, respectively.

3. Results

3.1. Quantitative results

The quantitative results for each DBS extraction method is presented in Fig. 1 separated into low viral load (≤10,000 copies/ml blood-

Table 1
Methods Evaluated for Extraction of CMV DNA from DBS. Comparison of steps and approximate time and cost required per method.

DNA Extraction method	Extraction format	Processing steps	Approximate time required	Estimated cost/sample
Thermal shock	2 ml tube	2 hrs @ room temperature, 1 hr @ 55 °C, 7 min @ 100 °C, 2 min @ 4 °C on ice, 1 min spin @ 14,000 rpm, 2 hr freeze	6hr	$0.20
KOH Tris*	96-well plate	10 min @ room temperature, 40 min @ 99 °C, 30 min spin @ 3700 rpm	90min	$0.80
DNA Extract All	96-well plate	3min in lysis solution @ 95 °C, 2 min @ room temperature, add stabilization solution, spin briefly	10min	$1.20

* KOH Tris buffer is now available commercially as Extracta™ DBS (QuantaBio, Gaithersburg, MD).

Table 2
Qualitative Assessment. Overall percentage of DBS punches that tested positive for CMV DNA for each extraction method. Triplicate extractions of 10 samples in each category were tested in duplicate for CMV representing a total of 60 tests.

DNA Extraction Method	Low Viral Load Category (n = 10)	High Viral Load Category (n = 10)
Thermal Shock	60% (36/60)	90% (54/60)
KOH Tris buffer	80% (48/60)	98% (59/60)
Extract All	83% (50/60)	98% (59/60)

Fig. 1A) and high viral load (> 10,000 copies/ml blood-Fig. 1B) categories. For low viral load specimens, CMV DNA yield using thermal shock was significantly lower compared to the KOH Tris and Extract All methods ($p < 0.05$, paired *t*-test). For high viral load specimens, thermal shock gave the lowest CMV DNA yield, and the KOH Tris method yielded significantly higher quantities of CMV DNA than the other two methods ($p < 0.05$, paired *t*-test). For all methods, CMV DNA negative controls showed no amplification, and an exogenous internal positive control consistently gave strong amplification, which indicated no signs of PCR inhibition.

3.2. Qualitative results

The number of positive CMV results obtained from six qPCR runs (triplicate extractions run in duplicate) per sample was determined for each extraction method. Table 2 lists the overall percentage of samples in each viral load category that were identified as positive for each extraction method. For specimens with low viral load, the KOH Tris and Extract All methods showed significantly increased sensitivity of detection than thermal shock ($p < 0.05$, paired *t*-test). CMV detection rates were not significantly different in the high viral load category between the three methods.

4. Discussion

This is the first feasibility study of low cost and rapid DNA extraction methods for the detection of CMV in DBS that would be amenable to the NBS workflow. The KOH Tris and Extract All methods performed notably better than thermal shock in both yield of CMV DNA and sensitivity of detection in DBS. Historically, the thermal shock method has been performed using a 6 mm or greater DBS punch or multiple 3 mm punches and has shown to have superior yield of CMV DNA than other methods published in the literature (Yamamoto et al., 2001; Barbi et al., 2000; de Vries et al., 2009; Koontz et al., 2015; Shibata et al., 1994; Barbi et al., 1996; Kharrazi et al., 2010). There is a consistent increase of DNA concentration with increased addition of DBS material to sample extractions (Gohring et al., 2010; Saavedra-Matiz et al., 2013). However, in keeping with standard NBS laboratory practice, our study focused on the use of only one 3.2 mm punch per sample extraction. NBS labs have limited amounts of DBS material on which to perform mandated testing, therefore, the DBS material requirement of any additional test needs to be carefully considered. Additionally, methods need to be sensitive, automatable, within the NBS required turnaround time, and cost-effective. We found that the KOH Tris and Extract All methods fit these criteria. The thermal shock method requires significantly more time and, based on results of this study and our previous study using a 6 mm punch (Koontz et al., 2015), resulted in a significant loss in CMV detection sensitivity and reproducibility using a 3.2 mm punch. However, DNA extraction from a 3.2 mm punch using the KOH Tris and Extract All methods showed no significant decrease in CMV detection compared to thermal shock extraction using a 6 mm punch (data not shown).

For CMV NBS, it has been shown that DBS would offer lower analytical sensitivity than saliva or urine (Yamamoto et al., 2001; Johansson et al., 1997; Boppana and Ross, 2010). While the collection of saliva is not difficult, the infrastructure to collect and process these specimens in NBS laboratories does not currently exist (Grosse et al., 2009). Of CMV infected children, only 15–20% develop permanent disabilities (Dollard et al., 2007; Kenneson and Cannon, 2007), which are largely children born with higher viral loads (Bradford et al., 2005; Boppana et al., 2005). This may make the use of DBS more feasible since sensitivity of detection is greatest with higher viral loads. However, studies to discern a prognostic viral load cutoff are lacking, yet essential to understanding the validity of screening for CMV in the newborn period. A prospective study currently being conducted by the Minnesota Department of Health, University of Minnesota, and Centers for Disease Control is testing paired saliva and DBS specimens from unselected infants to assess the utility of DBS for the identification of CMV-related sequelae (personal communication S. Dollard).

This study demonstrates that rapid and cost-effective DNA extraction methods can sensitively detect CMV DNA from a 3.2 mm DBS punch relative to proven methods. Both the DNA extraction method and the qPCR detection method are compatible with ongoing molecular tests in NBS laboratories. If adequate clinical sensitivity that satisfies a pre-determined prognostic threshold is achieved in DBS, the case for universal screening of newborns for CMV in the U.S. would be strengthened.

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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.

CRediT authorship contribution statement

D. Koontz: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Visualization. **S. Dollard:** Methodology, Validation, Formal analysis, Writing - review & editing, Visualization. **S. Cordovado:** Writing - review & editing, Resources, Visualization.

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