Journal of Clinical Virology 66 (2015) 95–99

Contents lists available at ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

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

D. Koontz[a,*], K. Baecher[a], M. Amin[b], S. Nikolova[a], M. Gallagher[a], S. Dollard[b]

[a] Newborn Screening and Molecular Biology Branch, Centers for Disease Control and Prevention, 4770 Buford Hwy. NE, Atlanta, GA 30341, USA
[b] Measles, Mumps, Rubella, and Herpesvirus Laboratory Branch, Centers for Disease Control and Prevention, 1600 Clifton Road, Atlanta, GA 30329, USA

ARTICLE INFO

Article history:
Received 2 December 2014
Received in revised form 24 February 2015
Accepted 14 March 2015

Keywords:
qPCR
Dried blood spots
Newborn screening

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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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

* Corresponding author at: Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention, 4770 Buford Highway, MS F-24, Chamblee, GA 30341, USA. Tel.: +1 770 488 7453; fax: +1 770 488 4005.
E-mail address: duk5@cdc.gov (D. Koontz).

http://dx.doi.org/10.1016/j.jcv.2015.03.015
1386-6532/Published by Elsevier B.V.

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 7×10^2 copies/ml to a very high 1×10^6 copies/ml. Replica blood spots were prepared by dispensing 75 μl 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

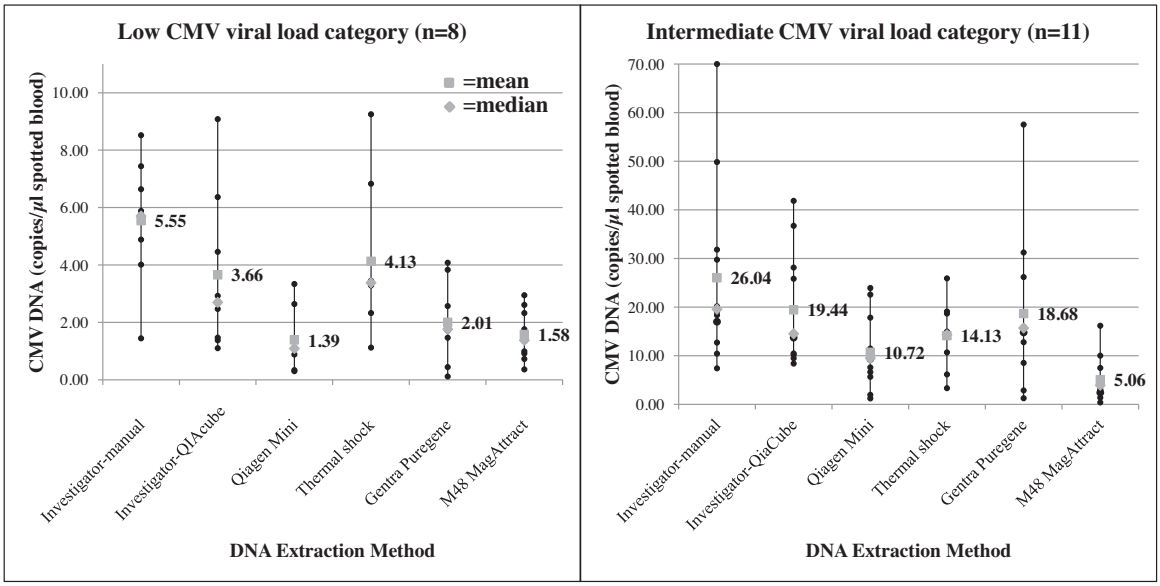

Fig. 1. Quantitative results for CMV DNA yield according to extraction method. Data points (circles) represent the average CMV DNA yield for each DBS specimen from triplicate extractions. Vertical lines show the full range of DNA yield per method. Mean (square) and median (diamond) viral DNA yields for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