Congenital cytomegalovirus infection : disease burden and screening tools : towards newborn screening

Vries, J.J.C. de

Chapter 6

Evaluation of DNA extraction methods for dried blood spots in the diagnosis of congenital cytomegalovirus infection

Jutte JC de Vries, Eric CJ Claas, Aloys CM Kroes, Ann CTM Vossen

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Department of Medical Microbiology, Leiden University Medical Center, Leiden, The Netherlands

Abstract

Background

Dried blood spots (DBS) may be valuable in the diagnosis of congenital cytomegalovirus (CMV) infection. However, the 2007 European Quality Control for Molecular Diagnostics (QCMD) proficiency testing programme showed that CMV DNA detection in DBS was lacking sensitivity in a considerable number of participating laboratories.

Objective

To compare DNA extraction methods for DBS for detecting CMV. Sensitivity and applicability of the methods for high-throughput usage were assessed.

Study design

Guthrie cards were spotted with CMV DNA-positive whole blood (n = 15). DNA was extracted from the DBS using different extraction methods, followed by CMV amplification by means of real-time PCR.

Results

Significant differences between the extraction methods with respect to the sensitivity were found. Optimal sensitivity was achieved when samples were tested in triplicate, demonstrating that the methods in general operated around their detection limits. Triplicate testing using the protocol by Barbi et al. [Barbi M, et al. Cytomegalovirus DNA detection in Guthrie cards: a powerful tool for diagnosing congenital infection. *J Clin Virol* 2000;17:159–65], representing the most sensitive methods, resulted in sensitivities of 100%, 86%, and 50% for DBS with CMV DNA loads of 5–4, 4–3, and 3–2 $\log_{10}$ copies/ml, respectively. This indicates that sensitivity limitations apply in the clinically relevant concentration range. Few methods appeared suitable for 96-well format high-throughput testing.

Discussion

When considering universal neonatal screening for congenital CMV infection, an assay which is both sensitive and applicable for high-throughput testing is required. The protocol by Barbi et al. and the BioRobot Universal System appear appropriate candidates currently available for 96-well format application in neonatal screening using DBS.

Introduction

Cytomegalovirus (CMV) infection is the most common cause of congenital infection worldwide with an overall birth prevalence of 6–7 per 1000 births.[1,2] About 12% of the live-born infants with congenital CMV infection are symptomatic at birth.[1,2] Of the children asymptomatic at birth, an additional 11–13.5% will develop permanent sequelae in the following years.[1–3] The most frequently encountered symptom of congenital CMV infection is sensorineural hearing loss (SNHL). Congenital CMV infection is responsible for 15–20% of SNHL in infants and children.[4,5]

Neonatal blood collected on filter paper within the first week of life (dried blood spots, DBS) has been proven useful for (retrospectively) diagnosing congenital cytomegalovirus (CMV) infection. The sensitivity of CMV DNA detection in DBS reported in literature is 71–100%, depending on the method used and the population tested.[6–11] However, the 2007 Quality Control for Molecular Diagnostics (QCMD) proficiency testing programme in which 33 European and South African laboratories participated, showed that CMV DNA detection in DBS was lacking sensitivity in a considerable number of participants. Only 50% of the laboratories were able to detect CMV DNA in a DBS sample with a load of 9.4×10^3 (4.0 $\log_{10}$) copies/ml whole blood.[12]

Currently, several non-commercial and commercial DNA extraction methods for DBS are available. A number of reports evaluating DNA extraction methods for DBS have been published. However, comparison of these data is complicated by inter-study differences, such as the origin of the samples, and the input and output volumes.[6–11]

The aim of our study was to test a panel of DNA extraction methods for DBS currently available. CMV-positive whole blood samples from transplant patients were spotted and DNA was extracted using the various methods, with identical input and output volumes, followed by CMV DNA amplification by real-time PCR. Sensitivity and applicability of the methods for high-throughput usage were determined.

Methods

Dried blood spots (DBS)

DBS samples were prepared by spotting CMV-positive EDTA-anticoagulated whole blood from transplant recipients with a broad range of CMV DNA loads (range 2–5 $\log_{10}$ copies/ml whole blood, $n = 15$) on Whatman 903 filter paper (kindly provided by Bert Elvers, RIVM, The Netherlands). The samples were air-dried, stored at room

temperature and tested within 3 months after spotting. In addition, CMV DNA-negative EDTA-anticoagulated whole blood from CMV-seronegative healty volunteers was spotted and used as negative controls. Furthermore, DBS from the QCMD CMV DBS 2007 panel (manufactured by Sandro Binda and Maria Barbi, Dept. of Public Health-Microbiology-Virology, University of Milan, Italy) were used to further analyse the protocol previously published by Barbi et al.,[6] representing the most sensitive methods.

Extraction of DNA from whole blood

CMV loads of the EDTA-anticoagulated whole blood from transplant recipients were determined prior to spotting using 200 µl for DNA extraction with the MagNa Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics, Almere, The Netherlands). DNA extraction was followed by CMV amplification (see Section 2.12).

Extraction of DNA from DBS

DNA was extracted from DBS using the following extraction methods: the protocol described by Barbi et al.,[6] the QIAamp DNA Investigator Kit (QIAGEN), the BioRobot Universal System (QIAGEN), the MagNA Pure LC (Roche Diagnostics), the NucliSens easyMAG (bioMeriéux), the QIAsymphony (QIAGEN), and Dynabeads Silane (Invitrogen). Sample input per tube/well was 3 punches, each measuring 3.2 mm in diameter, corresponding with approximately 9 µl dried blood per tube/well for all extraction methods tested. DBS were punched using an automated plate punch type 1296-071 (Perkin Elmer-Wallac, Zaventem, Belgium). For all extraction methods, samples were tested in triplicate with a negative control punch between each sample. Output volume was 100 µl for all extraction methods tested. DNA extraction was followed by CMV amplification (see Section 2.12).

Since the above-mentioned fixed input and output volumes meant a significant deviation from the original protocol by Barbi et al. (dictating 1 punch input and 35 µl output volume), the original protocol by Barbi et al. (unmodified) was tested as well.

Extraction of DNA from DBS using the protocol by Barbi et al. (unmodified)

DNA was extracted from DBS using the protocol described by Barbi et al.[6] (details obtained by personal communication). One punch of 3.2 mm per tube (in triplicate) was incubated at 4 °C overnight in 35 µl Minimum Essential Medium (+Earle's, +25 mM HEPES, −l-glutamine, Gibco/Life Technologies, Breda, The Netherlands) without additives in 96-well cluster tube strips. An aliquot of phocine herpes virus (PhHV) was

added as nucleic acid isolation and PCR inhibition control, as described previously.[13] Incubation was followed by heating at 55 °C for 60 min, and 100 °C for 7 min in a thermal cycler. After rapid cooling at 4 °C, the sample was centrifuged at 3220 × g for 15 min. The supernatant was transferred to a 96-well plate, frozen at −80 °C for at least 1 h, and thawed. This protocol resulted in an output solution which was approximately 20% more concentrated than when using the modified protocol by Barbi et al. described below.

Extraction of DNA from DBS using the protocol by Barbi et al. (modified)
Essentially the same procedure was followed for the modified protocol by Barbi et al., except that 3 punches of 3.2 mm per tube (in triplicate) were incubated in 125 µl Minimum Essential Medium, obtaining an output volume of 100 µl.

Extraction of DNA from DBS using the QIAamp DNA Investigator Kit
DNA was extracted using the QIAamp DNA Investigator Kit (column-based manual extraction) following the protocol "Isolation of total DNA from FTA and Guthrie cards" with a modification in the elution buffer according to the manufacturer's recommendations. Briefly, 280 µl buffer ATL and 20 µl proteinase K were added to the punches in screw-capped tubes, followed by vortexing, and incubation at 56 °C while shaking at 900 rpm for 1 h. After addition of 300 µl buffer AL (with 1 µg carrier RNA and internal PhHV control), the mix was pulse-vortexed and incubated at 70 °C while shaking at 900 rpm for 10 min. Additionally, 150 µl ethanol (96–100%) was added, the sample was pulse-vortexed, and the mix was transferred to the QIAamp MinElute column and centrifuged. The column was washed with 500 µl buffer AW1, 700 µl buffer AW2, and 700 µl ethanol (96–100%) subsequently, followed by drying of the column membrane at room temperature for 10 min, and eluting of DNA with 100 µl buffer AE (provided with the QIAamp DNA Mini Kit).

Extraction of DNA from DBS using the BioRobot Universal System
DNA extraction using the BioRobot Universal System (columnbased automated extraction) was performed using the QIAamp Investigator BioRobot Kit with manual pretreatment according to the manufacturer's recommendations. Tests were performed by QIAGEN application specialists in application laboratory Hilden, Germany. Briefly, 280 µl buffer ATL (with 2.75 µg carrier RNA and internal PhHV control) and 20 µl proteinase K were added manually to the punches in a QIAGEN 96-well S-Block. Samples were incubated at 56 °C overnight while shaking at 900 rpm in

a heatable shaker (Eppendorf Thermomixer Comfort with Thermoblock for Microtiter and Deepwell Plates with lid). After pretreatment, the supernatant was transferred manually to an empty S-Block and loaded on the BioRobot Universal System running the protocol "QIAamp DNA BloodCard UNIV" with an input volume of 300 and 100 µl elution volume.

Extraction of DNA from DBS using the MagNA Pure LC

DNA extraction using the MagNA Pure LC (magnetic particlebased automated extraction) was performed with manual pretreatment according to manufacturer's recommendations (Ref. 14, with minor modifications). The MagNA Pure LC DNA Isolation Kit III (Bacteria, Fungi) (Roche Diagnostics, Almere, The Netherlands) was used. Briefly, pretreatment was performed by adding a premix of 230 µl Bacteria Lysis/Binding Buffer, 20 µl proteinase K, and internal PhHV control to the punches in screw-capped tubes, vortexing and incubating at room temperature overnight. The following day, the mixture was incubated at 95 °C for 10 min, centrifuged briefly, and cooled at 4 °C. Supernatant was transferred manually to the MagNA Pure LC running the protocol "DNA Isolation Kit III" with an input volume of 200 µl and an elution volume of 100 µl.

Extraction of DNA from DBS using the QIAsymphony

DNA extraction using the QIAsymphony (magnetic particlebased automated extraction) was performed using the QIAsymphony DNA Mini Kit with manual pretreatment according to manufacturer's recommendations since this method was originally not designed for application of DBS (however currently in development). Pretreatment was performed following the QIAsymphony protocol "Pretreatment of Tissues" with minor modifications. Briefly, 180 µl buffer ATL (with added internal PhHV control) and 20 µl proteinase K were added to the punches in screw-capped tubes, followed by incubation at 56 °C with shaking at 900 rpm overnight. Supernatant was loaded manually on the QIAsymphony (magnetic particles based) running the protocol "Purification of DNA from tissues, cultured cells and bacterial cultures/DNA Tissue Low Content" with an input volume of 200 and 100 µl elution volume.

Extraction of DNA from DBS using the easyMAG

DNA extraction using the easyMAG (magnetic particle-based automated extraction) was performed using the NucliSENS easy-MAG Extraction Kit with manual pretreatment according to manufacturer's recommendations (Ref. 9, with minor modifications).

Briefly, punches were transferred into 2 ml NucliSens easyMAG lysis buffer in 10 ml lidded glass tubes, and incubated by gently rocking on a roller in horizontal position at room temperature for 30 min. After spinning down potential filter fibers at 1500 × g for 15 s, supernatant was loaded on the easyMag manually, running the off board extraction protocol (Generic, version 2.0.1). Internal PhHV control was added to extraction buffer 3, the elution volume was 100 µl.

Extraction of DNA from DBS using Dynabeads Silane

Dynabeads Silane extraction (magnetic particle-based manual extraction) was performed using the Dynabeads Silane viral NA kit with pretreatment according to manufacturer's suggestions since this method was originally not designed for application of DBS (however currently in development). Briefly, 200 µl phosphate buffered saline was added to the punches in screw-capped tubes and incubated at 85 °C for 10 min, followed by incubation with 20 µl proteinase K (20 mg/ml, Invitrogen/ Life Technologies, Breda, The Netherlands) at 55 °C for 10 min. Additionally, the mixture was incubated with 300 µl viral NA lysis buffer (including internal PhHV control) on a rotating wheel at room temperature for 10 min. The supernatant was transferred to an empty tube and suspended in 150 µl isopropanol and 50 µl Dynabeads suspension (silica-like magnetic beads) and incubated on a rotating wheel at room temperature for 10 min. Using the magnet, supernatant was removed and the Dynabeads were washed twice with 850 µl Washing Buffer 1 and 450 µl Washing Buffer 2. After drying the bead-pellet at room temperature for 10 min, the pellet was resuspended in 100 µl viral NA elution buffer and incubated at 70 °C for 3 min. Using the magnet, beads were separated from the supernatant, which was harvested.

Quantitative real-time PCR

CMV DNA amplification was performed by means of an internally controlled quantitative real-time PCR as described previously[13] with minor modifications. Briefly, 10 µl of DNA extract was added to 40 µl PCR pre-mixture obtaining final concentrations of 0.5 µM forward CMV primer, 0.5 µM reverse CMV primer, 0.2 µM CMV TaqMan probe, 0.3 µM forward PhHV primer, 0.3 µM reverse PhHV primer, 0.05 µM PhHV TaqMan probe, 3 mM $MgCl_2$, and 25 µl HotStar Master mix (QIAGEN, Hilden, Germany). The PCR running 50 cycli was carried out in an iQ5 Multi-colour Real-Time PCR Detection System (BioRad, Veenendaal, The Netherlands), amplifying a 126-bp fragment from the CMV immediate-early antigen region. Quantification was performed using a dilution series of titrated CMV (Advanced Biotechnologies Inc., Columbia, MD, USA) as an external standard.

Qualitative and quantitative data analysis

Qualitative data of DBS (n = 15), tested in triplicate, were analysed as follows. DBS were counted positive when ≥1 of the triplicates tested positive.[7] Additionally, to compare single and triplicate testing, ordinal means of the triplicates were calculated and considered the result of single testing (thereby enhancing the distinctive character compared with true single testing). Statistical analysis of ordinal data was performed using the Wilcoxon signed ranks test (two-tailed). The sign test (two-tailed) was used for comparison of single and triplicate testing.

In the quantitative data analysis, undetected samples were assigned the minimum detected load and mean loads were calculated per spotted CMV load categories. CMV DNA loads detected in 3 punches of each 3.2 mm, corresponding with in total approximately 9 µl dried blood, were converted to CMV DNA loads per ml spotted whole blood.

High-throughput applicability

Throughput characteristics determined were the maximum number of tubes/wells per run and the applicability of an automated system.

Results

Qualitative results

Qualitative results of the extraction methods tested are shown in Fig. 1(A) and (B). Fig. 1(A) shows the number of detected CMV-positive DBS (%) per method, comparing single testing (left) with triplicate testing (the DBS was counted positive when ≥1 of the triplicates was positive,[7] right). Single testing of DBS resulted in CMV DNA detection ranging from 32% (4.8/15) using the extraction method Dynabeads Silane, to 73% (11.0/15) using the protocol by Barbi et al. (unmodified). The highest number of samples were detected using the protocol by Barbi et al. (unmodified), the QIAamp DNA Investigator Kit (71%, 10.7/15), the BioRobot Universal System (67%, 10.0/15), the modified protocol by Barbi et al. (67%, 10.0/15), and MagNA Pure LC (62%, 9.3/15), respectively. The protocol by Barbi et al. (unmodified) was significantly more sensitive than extraction using the QIAsymphony (54%, 8.2/15, P = 0.031, Wilcoxon signed ranks test), the easyMAG (53%, 8.0/15, P = 0.031) and Dynabeads Silane (P = 0.003). Extraction using Dynabeads Silane was significantly less sensitive than all other extraction methods tested (P ≤ 0.039). For all methods, sensitivity was enhanced when testing was performed in triplicate compared with single testing (P = 0.008, sign test).

Fig. 1(B) shows the number of detected CMV-positive DBS (%) per spotted CMV DNA load category resulting from single testing. DBS with low CMV DNA loads (2–3 $\log_{10}$ copies/ml whole blood, n = 2) were not detected by four out of eight methods in any of the triplicates. When testing DBS with moderate CMV loads (3–4 $\log_{10}$ copies/ml whole blood, n = 7), the number of detected samples varied from 17% (1.2/7) using the extraction method Dynabeads Silane, to 67% (4.7/7) using the protocol by Barbi et al. (unmodified). DBS with high CMV DNA loads (4–5 $\log_{10}$ copies/ml whole blood, n = 6) tested positive in all triplicates using the protocol by Barbi et al. (unmodified and modified), and the QIAamp DNA Investigator Kit.

Sensitivity of CMV DNA detection per CMV load category was increased when samples were tested in triplicate (not shown in graph). When tested in triplicate, all DBS with CMV DNA loads of 3–4 $\log_{10}$ copies/ml (100%, 7/7) were detected using the BioRobot Universal System. All DBS with CMV DNA loads of 4–5 $\log_{10}$ copies/ml (100%, 6/6) were detected by all methods tested, except for Dynabeads Silane. Triplicate testing using the protocol by Barbi et al. (unmodified) resulted in sensitivities of 50% (1/2), 86% (6/7), and 100% (6/6) for spotted CMV DNA loads of 2–3, 3–4, and 4–5 $\log_{10}$ copies/ml, respectively.

All 120 CMV DNA-negative control samples (15 per extraction method) tested negative. No PCR inhibition was found using any of the extraction methods.

Quantitative results

Quantitative results of the DNA extraction methods tested are shown in Fig. 1(C). Depicted are the detected mean CMV DNA loads of triplicates per spotted CMV DNA load categories. Detected CMV DNA loads in DBS with spotted CMV DNA loads of 2–3, and 3–4 $\log_{10}$ copies/ml were lower than the spotted load category in six out of eight and five out of eight methods tested, respectively. CMV DNA loads detected in DBS with high spotted CMV DNA loads (4–5 $\log_{10}$ copies/ml) were within the ranges of the spotted load category in seven out of eight methods tested.

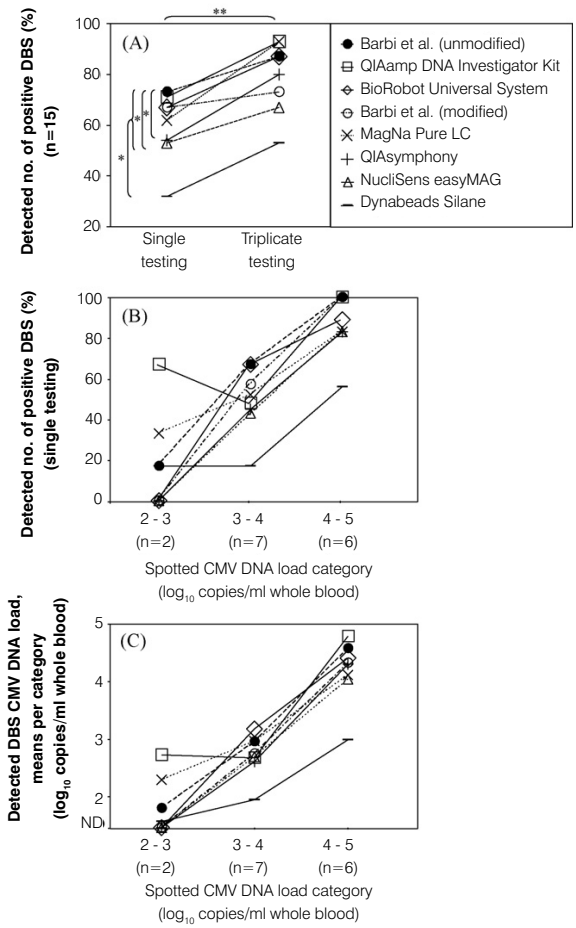

Figure 1 (A) Qualitative results of CMV DNA detection from DBS of the extraction methods tested, demonstrating the effect of triplicate testing on sensitivity. Left: single testing, right: triplicate testing (DBS was counted positive when ≥1 of the triplicates was positive). $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.
(B) Qualitative results of CMV detection from DBS of the extraction methods, per spotted CMV DNA load category, after single testing.
(C) Quantitative results of CMV DNA detection from DBS of the extraction methods tested, per spotted CMV DNA load category. Depicted are detected mean CMV DNA loads of triplicates per category. DBS, dried blood spot; CMV, cytomegalovirus.

QCMD panel

The QCMD CMV DBS 2007 panel (manufactured by Sandro Binda and Maria Barbi, Dept. of Public Health-Microbiology-Virology, University of Milan, Italy) was used to test the extraction method by Barbi et al. (unmodified, tested and analysed in triplicate), representing the most sensitive methods. Results are shown in Table 1. DBS with spotted CMV DNA loads from 3.9×10^6 (6.6 $\log_{10}$)to9.4×10^3 (4.0 $\log_{10}$) copies/ml were detected in all triplicates. One out of two DBS with spotted CMV DNA loads of 7.3×10^2 (2.9 $\log_{10}$) copies/ml was detected (in 1/3 triplicates). Only 50% and 4% of the QCMD CMV DBS 2007 participants detected CMV DNA in DBS with spotted loads of 9.4×10^3 (4.0 $\log_{10}$) and 7.3×10^2 (2.9 $\log_{10}$) copies/ml, respectively.[12]

Table 1 Qualitative and quantitative results of CMV detection in the QCMD CMV DBS 2007 panel using the DNA extraction protocol by Barbi et al. (unmodified), and the qualitative results of all QCMD participants. Quoted with permission of QCMD. QCMD, Quality Control for Molecular Diagnostics; CMV, cytomegalovirus; DBS, dried blood spot.

QCMD CMV DBS 2007 panel[a]	Results using Barbi et al. (unmodified)		Results of all QCMD participants
Spotted viral load (copies/ml whole blood)	Qualitative results	Detected viral load (copies/ml whole blood)	% correct qualitative results
3.9×10^6	Positive (3/3 triplicates)	2.2×10^6	100
9.6×10^5	Positive (3/3)	3.0×10^5	96
8.8×10^4	Positive (3/3)	4.4×10^4	93
9.4×10^3	Positive (3/3)	5.0×10^3	52
9.4×10^3	Positive (3/3)	4.2×10^3	48
7.3×10^2	Positive (1/3)	1.1×10^2	7
7.3×10^2	Negative	–	0
Negative	Negative	–	96
Negative	Negative	–	96

[a] Panel manufactured by Sandro Binda and Maria Barbi, the Dept. of Public Health-Microbiology-Virology, University of Milan, Italy.

High-throughput applicability

Throughput characteristics of the DNA extraction methods tested are shown in Table 2. Methods applicable for 96-well format (32 samples/run when testing in triplicate) were the protocol by Barbi et al., the BioRobot Universal System, and the QIAsymphony. All automated systems tested required a manual pretreatment step (no primary tube input format for DBS was available).

Table 2 Throughput characteristics of the DNA extraction methods tested.

	No. of tubes/ wells per run	Manual/ automated	Input type	Throughput
Dynabeads Silane[a] (Invitrogen)	1–16	Manual	Tube	Low
QIAamp DNA Investigator Kit[b] (QIAGEN)	1–24	Manual	Tube	Low
NucliSens easyMAG[a] (bioMérieux)	1–24	Automated[c]	Tube	Medium
MagNA Pure LC[a] (Roche Diagnostics)	1–32	Automated[c]	Tube	Medium
Barbi et al. (un)modified	1–96	Manual	Tube or 96-well plate	Medium
QIAsymphony[a] (QIAGEN)	1–96	Automated[c]	Tube or 96-well plate	High
BioRobot Universal System[b] (QIAGEN)	8–96	Automated[c]	96-Well plate	High

[a] Magnetic particle-based extraction.
[b] Column-based extraction.
[c] Manual pretreatment step, no primary tube input format for DBS available.

Discussion

The data presented here show that sensitivity of CMV DNA detection in DBS varies widely depending on the DNA extraction method used. The most sensitive methods were the protocol described by Barbi et al. (unmodified and modified), the QIAamp DNA Investigator Kit, the BioRobot Universal System, and the MagNA Pure LC. Interestingly, the unmodified protocol by Barbi et al. using only 1 punch was not less sensitive than the modified protocol by Barbi et al. using 3 punches, probably resulting from a DNA concentration effect: the unmodified protocol by Barbi et al. resulted in an output solution which was approximately 20% more concentrated than the modified protocol. For all extraction methods, optimal sensitivity was achieved when samples were tested in triplicate. Triplicate testing using the protocol by Barbi et al. resulted in sensitivities of 100%, 86%, and 50% for DBS with CMV DNA loads of 5–4, 4–3, and 3–2 $\log_{10}$ copies/ml, respectively. DBS with low spotted CMV loads had lower detected loads reflecting the presence of not detected samples. The protocol by Barbi et al., the QIAsymphony, and the BioRobot Universal System were suitable for 96-well format testing, which would be a requirement for application in newborn screening laboratories. It must be stressed that in the automated systems tested, pretreatment had to be performed manually (lacking primary tube input for DBS), thereby significantly increasing hands-on time. Considering cost-efficacy, the protocol by Barbi et al. has the advantage of the lower costs (<0.30€ per sample, triplicate testing) compared to the other methods tested (7–15€ per sample, triplicate testing).

Several reports have been published comparing a limited amount of DNA extraction methods for DBS.[6–11] However, comparison of these data is complicated by inter-study differences. Potential variables influencing the sensitivity are the origin of the DBS sample (e.g. spiked virus versus clinical samples from symptomatic or asymptomatic patients with congenital CMV infection), the amount of dried blood volume used, the elution volume, and the amplification method. The QIAamp DNA Blood Mini Kit (QIAGEN) has been reported to have a 95% sensitivity at a spotted CMV DNA load of 3.6 $\log_{10}$ copies/ml in an experiment with diluted blood from a transplant recipient, using a whole DBS (50 µl dried blood).[10] A modified QIAamp DNA Micro Kit (QIAGEN) protocol has been described to have a sensitivity of 100% when testing DBS from seven neonates with congenital CMV (of whom three known to be symptomatic), using a whole DBS.[8] Soetens et al. reported 73% sensitivity of extraction by the NucliSens easyMAG when testing DBS from 53 asymptomatic and 2 symptomatic congenital infected neonates, using a whole DBS.[9] Considering DNA extraction by means of heat shock, Yamamoto et al. reported a 71.4% sensitivity of heat shock in combination with a nested PCR when testing DBS from seven congenitally infected children (of whom five symptomatic), using 3 $\times$ 6 mm punches.[11] The highest detection rate using heat shock was reported by Barbi et al., whose method had a 100% sensitivity when testing DBS from 72 congenital infected babies (of whom 26 symptomatic), using one 3 mm punch tested in triplicate followed by nested PCR.[6] In our study, the influence of potential differences was excluded by using identical clinical samples (samples from transplant recipients, containing both extra-and intracellular CMV DNA), identical input and output volumes, and an identical amplification assay for all extraction methods tested.

The sample size in our study was small, but partially amended by calculating ordinal means of triplicates, thereby enhancing the differences. However, the power of the study did not yet allow to detect potential other statistically significant differences between the extraction methods.

A number of studies have been published on the viral load levels in whole blood of neonates with congenitally infected CMV. Halwachs-Baumann et al. reported a median viral load of 2.3×10^3 (3.4 $\log_{10}$) copies/ml cord vein blood in 18 neonates with congenital CMV. No significant difference was found in virus load between children that were symptomatic (n = 7) or asymptomatic (n = 11) at birth.[15] In contrast, Boppana et al. reported a mean peripheral blood CMV DNA load of 4.0×10^5 (5.6 $\log_{10}$)copies/ml in congenitally infected symptomatic newborns (n = 18), which was significantly higher than the mean load of asymptomatic newborns:

8.2×10^4 (4.9 $\log_{10}$) copies/ml (n = 58). Among asymptomatic newborns, those with hearing loss at follow-up had a significantly higher mean CMV DNA load (8.7×10^5, 5.9 $\log_{10}$ copies/ml, n =4) than those with normal hearing (1.1×10^4, 4.0 $\log_{10}$ copies/ml, n = 54).[16] The results of Boppana et al. corresponded with data from Lanari et al. and Revello et al., both reporting a significantly higher mean CMV DNA load in symptomatic newborns (3.2 $\log_{10}$ copies/10^5 PMNLs and 3000 copies/10^5 PBL, respectively) than in asymptomatic newborns (2.8 $\log_{10}$ copies/10^5 PMNLs and 30 copies/10^5 PBL, respectively).[17,18] In our study, the 86% sensitivity of CMV DNA detection in DBS using the extraction protocol by Barbi et al. was 3–4 $\log_{10}$ copies/ml. This sensitivity combined with the median viral load of 3.4 $\log_{10}$ copies/ml mentioned by Halwachs-Baumann et al. would implicate that a significant amount of cases with congenital CMV would not be detected even using one of the most sensitive methods available. In contrast, when considering the mean viral loads of 4.0 and 5.9 $\log_{10}$ copies/ml in asymptomatic newborns with respectively normal hearing and hearing loss at follow-up mentioned by Boppana et al., the clinical significance of loads below the detection limit are debatable.

The usage of dried urine specimens on filter paper (placed in diapers) has been suggested by Nozawa et al. as urine generally contains higher CMV loads than blood.[19] Though not evaluated in our study, it is likely that the above described extraction methods will be applicable to dried urine specimens on filter paper as well. When considering universal neonatal screening for congenital CMV infection, a cost-efficient assay which is both sensitive and applicable for 96-well format testing, using only a very small amount of dried blood, is required. In our hands, the protocol by Barbi et al. and the BioRobot Universal System appear appropriate candidates currently available for application in neonatal screening. Further studies are needed to optimize test characteristics (e.g. primary tube input) and to assess the clinical relevance of the detection limit in the intended population of asymptomatic newborns at risk for developing hearing loss later in life.

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