Comparison of DNA Extraction Methods from Small Samples of Newborn Screening Cards Suitable for Retrospective Perinatal Viral Research

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Reliable detection of viral DNA in stored newborn screening cards (NSC) would give important insight into possible silent infection during pregnancy and around birth. We sought a DNA extraction method with sufficient sensitivity to detect low copy numbers of viral DNA from small punch samples of NSC. Blank NSC were spotted with seronegative EDTA-blood and seropositive EBV EDTA-blood. DNA was extracted with commercial and noncommercial DNA extraction methods and quantified on a spectrofluorometer using a PicoGreen dsDNA quantification kit. Serial dilutions of purified viral DNA controls determined the sensitivity of the amplification protocol, and seropositive EBV EDTA-blood amplified by nested PCR (nPCR) validated the DNA extraction methods. There were considerable differences between the commercial and noncommercial DNA extraction methods ($P=0.014$; $P=0.016$). Commercial kits compared favorably, but the QIamp DNA micro kit with an added forensic filter step was marginally more sensitive. The mean DNA yield from this method was 3 ng/μl. The limit of detection was 10 viral genome copies in a 50-μl reaction. EBV nPCR detection in neat and 1:10 diluted DNA extracts could be replicated reliably. We conclude that the QIamp Micro DNA extraction method with the added forensic spin-filter step was suitable for retrospective DNA viral assays from NSC.

KEY WORDS: nPCR, dried blood spots, cytomegalovirus

INTRODUCTION

Cytomegalovirus (CMV) is the most common congenital infection, which occurs in 0.6–2% of all newborns,[1,2] is asymptomatic in up to 90% of babies, and can affect the neonate's developing neurological system.[3] Several studies have reported that prenatal infection of CMV in the early stages of pregnancy may be associated with poorer neurological outcomes.[4–6] Other herpes viruses, such as herpes simplex virus-1 (HSV-1),[7] HSV-2,[7] varicella zoster virus (VZV),[8,9] Epstein-Barr virus (EBV),[10–12] human herpes virus-6 (HHV-6),[13–15] HHV-7,[16] and HHV-8,[17] have also been shown to be detrimental to the unborn child. Unless samples have been taken for viral detection within the first 21 days of life, it is not possible to determine if later neurological sequelae may be a result of infection during the perinatal period.[6,18] Neonatal blood is collected routinely onto newborn screening cards (NSC) in the first 3–5 days of life to test for a variety of metabolic and endocrine disorders.[1] NSC have been identified as an important resource to retrospectively diagnose possible congenital infections in infants who have later presented with unexplained neurological sequalae.[6,18,19] In many cases, they are the only available resource for detection for neurotropic viruses in the perinatal period.[7,19] It is possible that viral infection during pregnancy may trigger an abnormal fetal inflammatory response in genetically vulnerable pregnancies, or prior viral infection sensitizes the fetus to subsequent bacterial infections.[15,20–22]

Use of NSC for the detection of viral nucleic acids has been well documented.[7,18,23] Barbi et al.[24] have demonstrated 100% sensitivity compared with "Gold-standard" methods of virus isolation from urine and/or saliva. Recently, use of NSC have been utilized for diagnosis of congenital viral infections in an Australian laboratory where sensitivities of 100% were achieved for HSV-1 and HSV-2 compared with direct fluorescent antibody and serology.[25] Nested PCR (nPCR) assays developed by

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McIver et al.[25] for herpes viruses reported 100% specificity. A major consideration in using dried blood spots (DBS) from NSC is obtaining the DNA quantity required for viral screening. In most cases, only a small sample of the NSC is available for testing.

The aim of this study was to find a suitable DNA extraction method sensitive enough to detect low copy numbers of viral DNA from small punch samples of NSC that could be applied to retrospective viral research.

MATERIALS AND METHODS

Determination of Limits of Detection

Using a simple replication experiment, 50 μl seronegative EDTA-blood and seropositve EBV EDTA-blood were pipetted in duplicate onto blank NSC (Whatman-903, Schleicher & Schuell, Keene, NH, USA) and allowed to dry at room temperature for 48 h. A stainless-steel puncher (McGill, Marengo, IL, USA) was used to collect three, 3.2-mm punches from the 50-μl aliquots for each of the DNA extraction methods examined. Thirty blank punches on clean filter paper were made between each card to minimize carryover contamination.[7] It was determined that the average diameter of a bloodspot from 50 μl blood was 12 ± 2 mm.

Calculation of Detectable Copy Numbers Based on Extraction Volume and Expected Viral Load

The majority of published methods uses between three and six, 3-mm-diameter punches or one, 10-mm-diameter punch for viral nucleic acid detection.[7,18,24,26] The following calculations are based on three, 3.2-mm punches, as it was desirable to use as little of the valuable archived material as possible. The area of a 12-mm-diameter bloodspot is 113.097 mm^2, and the area of a 3.2-mm-diameter punch from that bloodspot is 8.04 mm^2. Theoretically, 14 × 3.2 mm punches could be taken from one bloodspot, with each punch containing 3.55 μl blood. The total volume of blood extractable from three, 3.2-mm punches is 10.66 μl. According to the literature, viral load during congenital CMV infection rarely exceeds 10^6 copies/ml[18] and in asymptomatic infants with normal hearing, averages 1.1×10^4–1.5×10^4 copies/ml.[27] Literature detailing typical viral loads in congenital herpes virus infections other than CMV is scarce and does not permit estimation of the expected viral load.

Assuming infected infants have a viral load of 1.1×10^4–1.5×10^4 copies/ml (11–15 copies/μl), 10.66 μl blood (3×3.2 mm punches)/extraction would contain 117.26–159.9 viral copies. The final extraction volume would be 60 μl; therefore, each microliter would contain 1.95–2.67 copies. Extracted DNA (5 μl) for PCR use should theoretically contain 9.75–13.35 copies. Therefore, if the sensitivity of our PCR assay is able to detect nine viral copies, then in principal, the assay would be able to detect CMV in DBS.

DNA Extraction Methods

In this study, we compared commercial and noncommercial DNA extraction protocols; QIAamp DNA micro kit (Qiagen, Stanford, CA, USA), QIAamp DNA mini kit (Qiagen), MEM heat-extraction protocol,[24] and finally, a Chelex-lysis protocol.[28] For this experiment, we used DBS from a symptomatic EBV-infected patient in the absence of a sufficient amount of CMV-infected blood for research.

DNA was extracted using the QIamp DNA micro kit (first edition, August 2003) and QIamp DNA mini kit (second edition, November 2007) from the protocol, "DNA Purification from Dried Blood Spots", with slight modifications. Three, 3.2-mm-diameter punches were used, and the final eluting volume was 60 μl. A third extraction method was trialed with the QIAamp DNA micro kit with the modifications described above, as well as a further step in which additional lysate was collected with a forensic spin filter[19] (Axygen, Union City, CA, USA). Three, 3.2-mm-diameter blood spots were placed in 180 μl buffer ATL and 20 μl Proteinase K (Roche, Mannheim, Germany) and vortexed for 10 s. The samples were then placed in a thermomixer and incubated at 56°C with shaking at 900 rpm for 1 h. The DBS were retrieved with a pipette tip and placed into a forensic spin filter and centrifuged at 6000 *g* for 5 min at room temperature. Approximately 20 μl extra lysate was recovered and transferred back to the original lysate and mixed with 200 μl buffer AL and 1 μl carrier RNA, which was dissolved in buffer AE and then added to buffer AL prior to mixing with original lysate. The rest of the protocol followed the manufacturer's instructions, until the final step, where the DNA was eluted in 60 μl AE buffer.

A fourth MEM heat-extraction protocol, as described by Barbi et al.,[24] was implemented with no modifications. Finally, a Chelex-lysis DNA extraction protocol was used.[28] Three, 3.2-mm blood spots were washed in 1 mL PBS, 0.1% Tween 20, for 10 min with shaking and transferred to a new, 1.5-mL Eppendorf tube containing 60 μl nuclease-free water. Chelex-lysis solution [10 μl; 50% w/v Chelex-100 (Bio-Rad, Hercules, CA, USA), 2% w/v SDS, 0.1 M Tris, 5 mM EDTA] and 1 mg Proteinase K (Roche) were added and incubated at 60°C for 30 min and then 95°C for a further 30 min. The Chelex was pelleted by centrifugation at 20,000 *g* for 1 min and remained in the tube for storage at –20°C. Prior to using the supernatant for each PCR reaction, the Chelex was repelleted.[28]

Statistical Analysis

The mean DNA concentration with 95% confidence interval was calculated for all kits tested. Mean differences were calculated in comparison with the QIamp micro/forensic kit and compared pair-wise using ANOVA (no adjustment made for multiple comparisons). Calculations were performed in PASW Statistics (SPSS Inc., Chicago, IL) 17.0.2, and differences were considered significant at $P \leq 0.05$.

Quantification and Quality of DNA

DNA from each extraction protocol was quantified on a spectrofluorometer (Gemini XS SPECTRAmax, Molecular Devices, Sunnyvale, CA, USA) using the PicoGreen dsDNA quantification kit, as per the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

Controls and PCR

Purified HSV-1, HSV-2, VZV, EBV, and CMV DNA (Vircell, Santa Fe, Spain) were used for controls by preparing serial dilutions of 1×10^4–1.5×10^4 copies/5 μl TE (10,000–15,000 copies/5 μl TE, where TE=10 mM Tris-Cl, pH 7.5, 1 mM EDTA), 1000–1500 copies/5 μl TE, 100–150 copies/5 μl TE, 10–15 copies/5 μl TE, and 1–1.5 copies/5 μl TE. To ascertain the viral detection quantitatively, DNA extracts from seronegative EDTA-blood spots were spiked with 1 μl/5 μl TE of control DNA and amplified by nPCR in a Mastercycler® ep (Eppendorf, Hamburg, Germany) using primers published previously for HSV-1,[25] HSV-2,[25] VZV,[25] EBV,[25] and CMV.[23] With the exception of CMV, where the related, published amplification protocol was followed,[23] the remaining DNA controls were assayed in singleplex reactions, as opposed to the published multiplex reaction, with minor modifications.[25] The final 50-μl PCR mixture (first and second rounds) consisted of 1× GoTaq Green buffer, 2 mM $MgCl_2$ (Promega, Madison, WI, USA), 0.2 mM dNTPs (Bioline, London, UK), 0.20 μM previously published HSV-1,[25] HSV-2,[25] VZV,[25] and EBV[25] primers (outer and inner sense; Sigma-Aldrich, St. Louis, MO, USA), 1.5 U Taq Hot Start polymerase (Promega), and 5 μl neat and 1:10 diluted template (round one) or 1 μl round one product (round two). First-round cycling parameters included initial denaturing at 94°C for 2 min, followed by 30 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 1 min, with a final extension of 72°C for 7 min. Second-round cycling parameters included denaturing at 94°C for 2 min, 30 cycles of 94°C for 30 s, 60°C for 45 s, and 72°C for 45 s with a final extension of 72°C for 7 min.[25] Amplification products, after the second nPCR round, were visualized in a 2% agarose TBE gel containing 1× GelRed (Biotium, Hayward, CA, USA) and 500 ng/μl pUC19/*Hpa*II DNA (Geneworks, Adelaide, Australia) on a Bio-Rad Gel-Doc system. Each DNA extraction method from the seropositve EBV EDTA-blood was subject to the same nPCR conditions, as described for the controls, and replicated four times.

RESULTS

The yield distributions were comparable between the commercial DNA extraction methods ranging from 2.3 to 3 ng/μl but significantly greater than the noncommercial methods, 0.4 ng/μl (Table 1). The difference reached significance for MEM heat-extraction and Chelex-lysis methods ($P=0.014$, and $P=0.016$, respectively; Table 2).

Each purified DNA control was detectable by nPCR at 10–15 copies/5 μl TE, depending on their starting concentration. Occasionally, we were able to detect a result from a lower dilution of 1–1.5 copies/5 μl TE. This was not always repeatable, therefore, not reliable enough to be used as a control. We considered the sensitivity of the PCR assay to be 10–15 copies/5 μl TE of purified DNA for each reaction, equating to 11,200–16,800 copies/mL whole blood.

Results from the seropositive EBV nPCR were comparable between each of the commercial DNA extraction methods (Table 1). At a neat DNA concentration, the Chelex-lysis method did not yield consistent results, but the same samples successfully amplified at a 1:10 dilution. We were unable to generate a PCR result for the MEM heat-extraction method at either concentration (Table 1).

TABLE 1

Quantification and nPCR Results from DNA Extraction Methods

Method	PicoGreen ng/μl	EBV PCR neat	EBV PCR 1:10
QIamp micro kit/forensic filter	3 (1–4.8)	4/4	4/4
QIamp micro kit	2.4 (0.2–7.08)	4/4	3/4
QIamp mini kit	2.3 (0.49–4.48)	4/4	3/4
MEM heat-extraction	0.4 (0.17–0.53)	0/4	0/4
Chelex-lysis	0.4 (0.04–0.69)	2/4	4/4

TABLE 2

Comparison of Five DNA Extraction Methods

Comparison kit (A)	Kits compared (B)	Mean difference (A–B)	Sig.	95% Confidence interval for difference
QIamp Micro/forensic	QIamp micro	.634	.528	−1.424–2.691
	QIamp mini	.609	.544	−1.449–2.667
	MEM	2.658[a]	.014	.600–4.716
	Chelex	2.606[a]	.016	.548–4.664

[a]The mean difference between the comparator kit is significant at the .05 level.
Shaded gray represents commercial kits.

DISCUSSION

There have been several studies examining DNA extraction methods suitable for NSC with varying results as a result of differences in DBS volume, final elution volume, amplification protocols, and spiked or seropositive clinical samples from asymptomatic or symptomatic donors.[7,19,24,29–31] We found that each of the commercial DNA extraction methods trialed was more reliable compared with the noncommercial DNA extraction methods with regard to DNA quantity and nPCR results. The commercial DNA extraction methods have the added advantage of incorporating a column-based process to remove residual contaminants, ensuring the integrity of the DNA extract. As a result of the low viral load in asymptomatic, congenitally infected neonates and the small amount of NSC available to us, we chose to adopt the QIamp DNA micro extraction method with the added forensic filter step. By adding the forensic spin-filter step, we increased the final DNA yield, an added advantage when working with a small amount of starting material. Overall, the performance of this method gave more positive results and maximized on DNA quantity. Our nPCR results were also more sensitive between the commercial and noncommercial DNA extraction methods. Moreover, we found that each of the commercial methods successfully amplified EBV DNA, but this did not hold true for the noncommercial methods. We were unable to replicate the results of Barbi et al.,[24] who reported the highest detection rate for the MEM heat-extraction method. A study by Göhring et al.[29] also found this method inefficient in their laboratory and suggested that hemoglobin contaminants may have been an inhibitory factor. The Chelex DNA extraction method was more consistent when the template was used at a 1:10 dilution. This correlates with inhibitory agents being diluted in the DNA extract. The only available virus-positive clinical sample was EBV-seropositive EDTA-blood from a symptomatic donor of unknown viral load, which would be expected to be higher in symptomatic compared with asymptomatic cases. Approximately 90% of congenital CMV cases are asymptomatic. We estimated that 5 μl extracted DNA would, in principle, need to contain nine viral copies, and we saw 10 copies; therefore, we would not recommend diluting the final DNA extract for PCR viral detection. False-negative results may arise in samples from asymptomatic infants, from whom viral load falls below our detectable limit from failure of the DNA extraction or from PCR inhibition.

The purpose of seeking a sensitive DNA extraction method was to identify a research protocol for retrospective diagnosis of neurotropic viruses that may be associated with unexplained neurological sequelae not diagnosed until after the perinatal period. More recently, we have implemented the QIamp micro DNA extraction method with the added forensic filter step to assay DNA of nine herpes viruses in our NSC cohort. This method of DNA extraction meets our criteria. The Qiamp micro kit, with the added forensic filter step, gave better use of a finite resource.

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