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Chapter 7

Extraction of DNA from dried blood in the diagnosis of congenital CMV infection

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Summary

Viral DNA detection in dried blood spotted on filter paper, dried blood spots (DBS), is valuable in the diagnosis of viral infections, with at the moment congenital cytomegalovirus (CMV) being the most common application. CMV detection in clinical samples taken within the first 2-3 weeks after birth differentiates congenital CMV infection from the in general harmless postnatally acquired cytomegalovirus infection. DBS render the possibility to diagnose congenital CMV infection retrospectively, e.g. when late-onset hearing loss, the most frequently encountered symptom of congenital CMV infection, becomes manifest. Additionally, CMV DNA detection in DBS can be of usage in recently advocated newborn screening on congenital CMV infection. The procedure of CMV DNA detection in DBS consists of two separate steps: 1. DNA extraction from the DBS, followed by 2. CMV DNA amplification. Here, we describe two efficient methods for the extraction of DNA from DBS. Sensitivity, specificity, and applicability of the methods for high-throughput usage are discussed.

Introduction

Newborn blood taken within a few days after birth and dried on filter paper (dried blood spots, DBS), are widely used for newborn screening on metabolic diseases. Additionally, DBS have been proven valuable and are increasingly used in the diagnosis of viral infections. It is most often used in the diagnosis of congenital cytomegalovirus (CMV) infection, the most common congenital viral infection worldwide.¹ Congenital CMV infection can be retrospectively diagnosed using DBS, differentiating congenitally acquired CMV infection from postnatally acquired CMV infection, which is generally much less harmful. DBS render the possibility to diagnose congenital CMV infection when the most common symptom of congenital CMV infection, late-onset hearing loss, becomes manifest. Additionally, CMV detection in DBS can be of usage in recently advocated newborn screening on congenital CMV infection.²⁻¹¹

CMV DNA detection in DBS includes DNA extraction followed by CMV DNA amplification and is increasingly used in clinical virological laboratories worldwide. Whereas detection of CMV DNA in blood and other clinical samples is a routine diagnostic procedure, the extraction of CMV DNA from filter paper is still challenging due to the limited amount of dried blood available; one whole spot of 1 cm in diameter equals approximately 50 μ L blood, and one punch of 3 mm in diameter, frequently used for routine metabolic screening, contains as little as 3-5 μ L blood. Thus, optimal DNA extraction is crucial in the procedure for CMV DNA detection in DBS.

Currently, several non-commercial and commercial DNA extraction methods for DBS are available. A number of reports evaluating extraction methods for DBS in the diagnosis of congenital CMV infections have been published.^{3-5,12-25} Significant differences between extraction methods with respect to the analytical and clinical sensitivity are reported, ranging from 35% to 100%^{3,14-18,20,21,24-26} depending on the extraction method used and the population tested. Optimizing DNA extraction protocols, PCRs, and algorithms, e.g. by means of performing independent triplicate testing, have been shown to increase analytical sensitivity significantly.^{15,16,18} Triplicate testing (of one punch of 3 mm in diameter per tube) using the heat-shock protocol by Barbi et al¹², shown to be one of the most sensitive methods¹⁵, results in analytical sensitivities of approximately 100%, 86% and 50% for DBS with CMV DNA loads of 5-4, 4-3, and 3-2 $\log_{10}$ copies/ml, respectively.¹⁵ This indicates that limitations in sensitivity apply in the clinically relevant concentration range for congenital CMV disease (reported mean CMV DNA blood loads of 3.4²⁷, 4.0, and respectively 5.9

$\log_{10}$ copies/ ml in asymptomatic newborns with hearing loss at follow-up²⁸). In this respect, it is important to note that defined clinically important CMV DNA loads, in the absence of an international CMV DNA quantification standard, are of use only in the laboratory setting where they were obtained. For a general application, standardization of CMV DNA values obtained by different PCR protocols and different quantification standards is essential.

Specificity of CMV DNA detection using DBS has been reported to range between 99.3% and 100%.^{12,14,25} To our knowledge, transfer of CMV DNA from one DBS to another during storage has been reported once.²⁶ Transfer of CMV DNA during punching can be controlled for in the procedure (see below). However, both these potential contaminating events are not likely to be of practical significance given the above described limited analytical sensitivity.

Above mentioned advocated newborn screening for congenital CMV can only be achieved using automated, high-throughput DNA extraction methods. Currently, few methods appear suitable for 96-well format high-throughput testing.¹⁵

Here, we describe two methods for efficient extraction of DNA from DBS, used for CMV DNA detection.

Materials

DNA extraction from DBS using heat shock^{12, 24}:

1. (Automated) paper puncher
2. Positive and negative control DBS (or blanc Guthrie card/ Whatman 903 filter paper) (see *Note 1*)
3. (Eppendorf) tubes or 96-well plate
4. Minimal Essential Medium (MEM, without additives)
5. Cooler or thermal cycler (4°C)
6. Heating block or thermal cycler at 55°C and subsequently 100°C
7. (Eppendorf table) centrifuge
8. Internal control to monitor for PCR inhibition (e.g. phocine herpes virus (PhHV) DNA)

Column-based DNA extraction from DBS:

1. (Automated) paper puncher
2. Positive and negative control DBS (or blanc Guthrie card/ Whatman 903 filter paper) (see *Note 1*)

3. Microcentrifuge tube
4. QIAamp DNA Mini Kit (containing columns, collection tubes, lysis buffer (ATL, AL), proteinase K, wash and elution buffer)
5. Heating block or water bath at 85°C, 56°C, and subsequently 70°C
6. Eppendorf table centrifuge
7. Internal control to monitor for PCR inhibition (e.g. phocine herpes virus (PhHV) DNA)

Methods

DNA extraction from DBS using heat shock.^{12, 24}

1. For each test DBS (see *Notes 1 and 2*), punch one disk of 3 mm (1/8 inch) in diameter per tube or well, in triplicate. Punch one disk from a negative control DBS between each test DBS (see *Notes 1 and 3*).
2. Add 35 µLMEM, including internal control DNA (e.g. PhHV DNA, see *Note 4*) and spin the punches down (see *Note 5*).
3. Incubate at 4°C overnight (see *Note 6*).
4. Perform heat shock (e.g. in thermal cycler or heating block) according the following protocol (see *Note 7*):
 - 55°C at 60 min
 - 100°C at 7 min
 - cool rapidly to 4°C
5. Centrifugate at 3,320 x g for 15 min, or at 8,960 x g for 1-3 min (see *Note 8*).
6. Transfer the supernatant (approximately 25 µL) to an empty tube or 96-well plate and freeze at -80°C for at least 1 h (see *Note 9*).
7. Thaw; the extract is ready to use for PCR¹⁵.
8. Interpretation of PCR results of triplicates (see *Note 10*).

Column-based DNA extraction from DBS (see *Notes 11 and 12*)

1. For each test DBS (see *Notes 1 and 2*), punch one whole DBS (of approximately 1 cm in diameter, corresponding with approximately 50 µL dried blood) (see *Note 12*) in a microcentrifuge tube, in triplicates. Punch a negative control DBS between each sample (see *Note 1 and 3*).
2. Add 180 µL lysisbuffer (ATL), including internal control DNA (e.g. PhHV DNA, see *Note 4*) to each tube.
3. Incubate at 85°C for 10 min.

4. Add 20 μ L proteinase K, vortex, and incubate at 56°C for 1h.
5. Add 200 μ L lysisbuffer (AL), vortex, incubate at 70°C for 10 min.
6. Add 200 μ L ethanol 96-100%, vortex.
7. Apply the mixture (approximately 600 μ L) to column in a collection tube and centrifuge at 6,000 x g for 1 min, discard the filtrate.
8. Wash with 500 μ L washbuffer (AW1) at 6,000 x g for 1 min, discard the filtrate.
9. Wash with 500 μ L washbuffer (AW2) at 20,000 x g for 3 min, discard the filtrate.
10. Centrifuge once more at full speed for 1 min, discard the filtrate.
11. Elute the DNA with 150 μ L elution buffer (AE) after incubation for 1 min and centrifugation at 6,000 x g for 1 min.
12. The eluate is ready to use for PCR¹⁵.
13. Interpretation of PCR results of triplicates (see Note 10).

Notes

1. Positive and negative control DBS can be produced by spotting CMV DNA positive and negative (EDTA) blood on Whatman 903 filter paper (approximately 50 μ L per spot of 1 cm diameter) followed by air-drying. DBS can be stored at 4°C or at room temperature.
2. When dried on filter paper, blood spots are considered non-infectious material.
3. DNA contamination from sample to sample during punching is controlled for by testing a negative control DBS in between each test DBS.
4. PCR inhibition can be controlled for in a simultaneous reaction by adding a fixed amount of internal control (e.g. PhHV DNA) to each sample. Inhibition of internal control amplification is indicative of potential inhibition of amplification of target (CMV) DNA.

Notes specific for heat shock DNA extraction:

5. Punches must be spun down until the disks are below liquid surface level (15 min at 3,320 x g may be necessary when using a 96-well plate).
6. Incubation at 4°C overnight significantly enhances extraction efficiency.
7. During the heat shock, DNA will be extracted from the DBS.
8. Centrifugation yields sufficiently purified DNA.
9. Freezing the supernatant for at least 3 h enhances extraction efficiency (no maximum freezing time implicated).
10. Triplicate testing results in optimal sensitivity.¹⁵ Interpretation of triplicate PCR results can be performed using the flow diagram as described by Barbi et al¹³, in

which every positive result should be confirmed with at least one additional positive result, in the same run, or in case of a single positive test result (1 of the 3 replicates), by means of a confirmatory PCR procedure including DNA extraction (second run) (Figure).

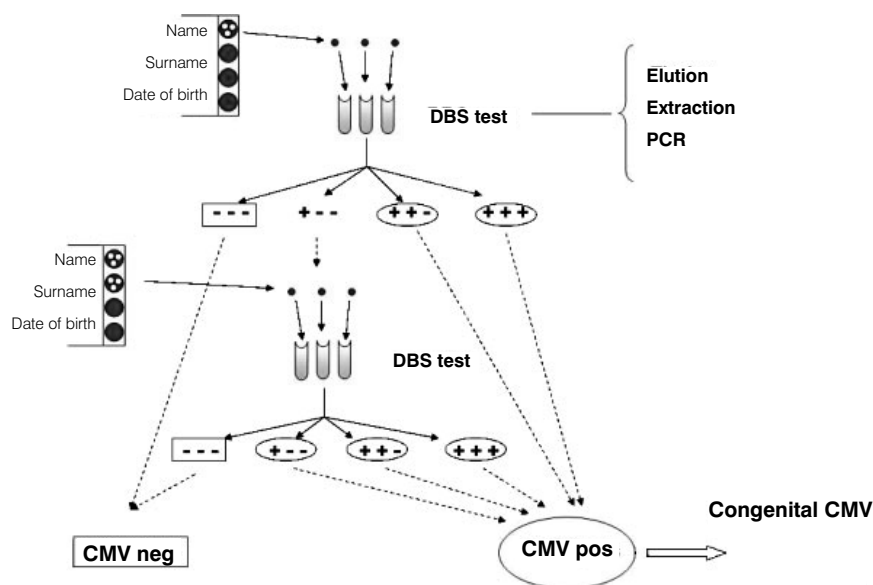


Figure Flow diagram for interpretation of triplicate testing results as proposed by Barbi et al¹³.

Notes specific for column-based DNA extraction:

11. Detailed protocol is described in the manufacturers "QIAamp DNA Mini and Blood Mini Handbook", version April 2010.

12. Using one whole DBS (diameter of 1 cm, corresponding with approximately 50 µL blood) enhances sensitivity (DNA yield) significantly, when compared to three or six punches of 3 mm in diameter as proposed in the "QIAamp DNA Mini and Blood Mini Handbook" (version April 2010).

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