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Development of a novel single tube nested PCR for enhanced detection of cytomegalovirus DNA from dried blood spots

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ABSTRACT

Newborn screening for congenital cytomegalovirus (CCMV) using dried blood spots (DBS) has been proposed because many developed countries have DBS screening programmes in place for other diseases. The aim of this study was to develop a rapid, single tube nested polymerase chain reaction (PCR) method for enhanced detection of CMV from DBS compared to existing (single target) real time PCRs. The new method was compared with existing real time PCRs for sensitivity and specificity. Overall sensitivity of the single target PCR assays in both asymptomatic and symptomatic infants with laboratory confirmed congenital CMV was 69% (CMV PCR or culture positive before day 21 of life). In contrast, the single tube nested assay had an increased sensitivity of 81% with100% specificity. Overall the assay detected CMV from a DBS equivalent to an original blood sample which contained 500 IU/ml. In conclusion this single tube nested methodology allows simultaneous amplification and detection of CMV DNA in 1.5 h removing the associated contamination risk of a two step nested PCR. Owing to its increased sensitivity, it has the potential to be used as a screening assay and ultimately allow early identification and intervention for children with congenital CMV.

1. Introduction

Congenital cytomegalovirus (CCMV) infection occurs in 0.7% of births causing long-term sensorineural hearing loss (SNHL) and neurological impairment in a significant proportion of infected infants (Dollard et al., 2007). Approximately 12.7% of those born with symptoms and 13.5% who are asymptomatic at birth, face a significant risk of developing late sequelae commonly SNHL (Dollard et al., 2007). Such is the burden of disease caused by CMV that the Institute of Medicine ranked the development of a CMV vaccine as the highest priority (Stratton et al., 1999). Recently, encouraging results have been published from CMV vaccine trials (Griffiths et al., 2011; Pass et al., 2009) but decades will be required to control CMV infection in the community even if a vaccine is used for universal immunisation (Griffiths, 2012). Therefore there is still an urgent need to identify infected children early for interventions to protect speech and language development, such as hearing aids, cochlear implants or speech therapy. In addition, a randomised controlled trial of ganciclovir, given for six weeks to babies born with CNS symptoms of CCMV, reported significant protection against progressive hearing impairment (Kimberlin et al., 2003).

The diagnosis of CCMV is challenging. Perinatal CMV infection is acquired commonly at birth or through breast feeding. Although this does not cause CNS damage, the timing of sampling is pivotal because a diagnosis of CCMV cannot be made with certainty in children unless samples are available within 21 days of birth. Early accurate diagnosis of CCMV is essential to allow prompt recognition of sequelae and provide the opportunity for treatment. Dried blood spots (DBS) are taken routinely after birth in many countries for biochemical and genetic analysis and are stored for prolonged periods of time. Numerous proof-of-concept studies conducted over the last decade have shown that CMV can be detected in DBS (Barbi et al., 2000; Shibata et al., 1994). Owing to the timing of sample acquisition, DBS have been shown to be useful in retrospective diagnosis of child presenting with compatible symptoms later in infancy or childhood (Barbi et al., 2006; Vauloup-Fellous et al., 2007; Walter et al., 2008). These studies have used a variety of patient populations and methods for both extraction of nucleic acid and CMV detection from the DBS matrix, resulting in a wide range of reported sensitivities (28–100%) mainly attributable to methodology (Boppana et al., 2010; de Vries et al., 2009; Vauloup-Fellous et al., 2007). The sensitivity reported by the largest reported study was about 30% (Boppana et al., 2010).

Currently, no country screens for CCMV; the use of DBS for newborn screening has been proposed. DBS samples are by definition small-volume collections often with only 10–80 μl of whole blood available for analysis, so highly sensitive detection methods such

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as nested PCR are needed. Published methods with high sensitivity use manual DNA extraction methods and nested PCR with gel based detection which are not suitable for high throughput due to the inherent potential for cross contamination and the labour intensive nucleic acid extraction step.

This study describes the development and validation of a novel single tube nested PCR for enhanced detection of CMV from DBS which has the potential for use as a high throughput screening assay to facilitate the prompt identification and intervention in children with CCMV.

2. Materials and methods

2.1. Samples

DBS samples were tested from 4 sample sets.

1. Artificial CMV negative and positive DBS prepared from the World Health Organisation 1st International standard for CMV (who/35/10.2138 report). Serial dilutions from 500,000 IU/ml to 100 IU/ml were prepared in a whole blood matrix and 50 μl absorbed onto standard Whatman 903™ DBS cards obtained from the Royal Free Hospital neonatal unit. The prepared cards were dried for a minimum of 48 h prior to testing.
2. 20 DBS samples from newborns with CCMV infection were obtained from an earlier published study. The cases were Children with diagnosed or suspected congenital CMV born in the UK between 2001 and 2002 were reported by paediatricians via the BPSU notification system (BPSU 17th Annual report, 2002–2003). Cases were confirmed on the basis of PCR or virus isolation from urine, blood, saliva or tissue taken at biopsy within 3 weeks of birth. The cards had been stored in standard UK storage conditions and all were collected within 21 days of life.
3. The 2011 CMV DBS panel (ref CMV/DBS11) obtained from Quality Control for Molecular Diagnostics (QCMD, Glasgow UK). The panel comprised 10 DBS. Each spot was prepared from a whole blood matrix with varying concentrations of CMV (AD169 strain). The stated CMV concentration ranged from 625 to 20,000 copies/ml. One DBS was equivalent to 50 μl of whole blood.
4. DBS samples received as part of an ethically approved study (Benefits of Extended Screening Testing (BEST Study) from 6 children who failed their newborn hearing screen and had CCMV (cases were confirmed on the basis of PCR or from urine or saliva); samples were taken within 3 weeks of birth (manuscript in preparation).

The specificity of the assay was determined by analysing 200 blood samples from donor/recipient CMV negative solid organ transplant patients. These samples were received for routine post transplant CMV screening and were CMV negative in our diagnostic RT PCR assay (Atabani et al., 2012).

2.2. DNA extraction from DBS

A 6.5 mm diameter (33 mm^2) circle of DBS was used for extraction. This size was chosen because this is a realistic amount of sample that remains after newborn screening.

To prevent contamination the scissors used to cut the DBS were cleaned with 0.1 M hydrochloric acid between cards.

DNA was extracted from DBS using the QIAsymphony automated extraction system with the QIAsymphony DNA Mini Kit (Qiagen, Hilden, Germany) following manual pre-treatment: DBS was added to 400 μl Buffer ATL and 20 μl proteinase K, and incubated at 56 °C for 30 min. The resulting supernatant was transferred into a 2 ml tube, without disturbing the digested DBS, and loaded onto the QIAsymphony SP. Extraction was carried out using the VirusBlood200_V5_DSP protocol with an elution volume of 60 μl. Each DBS extract was analysed in triplicate.

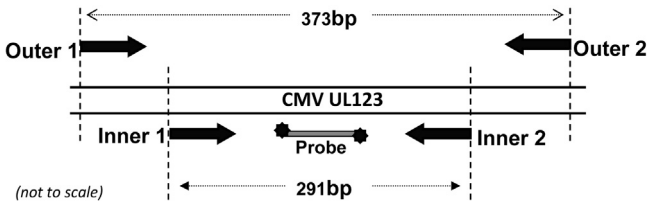

Fig. 1. Schematic diagram of single tube nested PCR (not to scale). A single tube nested PCR was developed to amplify a target in exon 4 of the major immediate early region of CMV (UL123). The thermodynamic profile was adapted so that initial 15 cycles of PCR yield a 373 bp product from the outer primers (Outer 1 and 2). A second PCR cycle incorporated the inner primers (inner 1 and 2) to yield a 291 bp product. A internal double quenched probe allowed simultaneous amplification and detection of the amplicon in real time.

2.3. DNA extraction from whole blood

DNA was extracted using the QIAsymphony automated extraction system with the QIAsymphony DNA Mini Kit (Qiagen, Hilden, Germany). Extraction was carried out using the VirusBlood200_V5_DSP protocol with an elution volume of 60 μl. Each blood sample was analysed in triplicate.

2.4. Single tube nested PCR strategy

A single tube nested PCR that amplified a target in exon 4 of the major immediate early region of CMV (UL123) was developed.

Two sets of primers were modified from a previously published method (Taylor-Wiedeman et al., 1991). External primers (outer 1 and 2) were extended to give an annealing temperature at least 10 °C higher than the internal primers (inner 1 and 2). First round PCR amplification was performed at 68 °C, which allowed only the external primers to bind and amplify target sequence. The extension temperature was reduced to 55 °C so that the target amplified with both the external and internal primers. A FAM/ZEN double quenched probe was degraded during the extension and the resulting fluorescent monitored in real time. Double quenching was used to reduce background and improve reporter signal. Primers and probe were purchased from Integrated DNA Technologies (Leuven, Belgium). A schematic diagram of the nested PCR (Fig. 1) and the primer and probe sequences for the single tube nested PCR are summarised in Table 1.

DNA amplification was performed in 30 μl total reaction volume. Each reaction contained 10 μl of DNA extract 15 μl QuantiFAST mastermix (Qiagen, Hilden Germany), 1 μM of each primer and 0.2 μM of probe. Real time PCR was carried out in a TaqMan 7500 system (Applied Biosystems, Foster City, CA, USA).

PCR conditions were: template denaturation and activation of Taq polymerase for 10 min at 95 °C was first followed by 15 cycles of 95 °C for 15 s and 68 °C for 45 s. This was followed by a second cycling step of 95 °C and 55 °C for 30 cycles. Data acquisition occurred during the second cycling step (55 °C extension).

2.5. CMV gB and UL69 PCR

The single round real time CMV gB and UL69 PCRs were used as previously described (Atkinson et al., 2009) for comparison with standard diagnostic methodologies.

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