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

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Abstract (195 words)

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 hours 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.

Keywords: Congenital Cytomegalovirus, Dried Blood Spot, Nested PCR, Newborn Screening

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 10-80μl of whole blood available for analysis, so highly sensitive detection methods such 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 Material 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 500,000IU/ml - 100 IU/ml were prepared in a whole blood matrix and 50μl 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 hours 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 2001-2002 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 625-20,000 copies/ml. One DBS was equivalent to 50µl 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 6.5mm diameter (33mm^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 0.1M 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 minutes. 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.

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, 2) were extended to give an annealing temperature at least 10°C higher than the internal primers (inner 1,2). 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 (figure 1) and the primer and probe sequences for the single tube nested PCR are summarised in Table 1.

DNA amplification was performed in 30µl total reaction volume. Each reaction contained 10µl of DNA extract 15µl QuantiFAST mastermix (Qiagen, Hilden Germany), 1µM of each primer and 0.2µM 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 minutes at 95°C was first followed by 15 cycles of 95°C for 15 seconds and 68°C for 45 seconds. 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).

2.6 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.

3 Results

3.1 Detection limit of single tube nested PCR

The sensitivity of the single tube nested PCR assay was determined using triplicate testing of DBS spotted with serial dilutions of the WHO CMV international standard. The lowest positive dilution (1/3 triplicate positive) correlated with an original blood sample which contained 500 IU/ml CMV.

The QCMD samples showed a similar detection limit. The lowest CMV concentration DBS sample (stated as 625 copies/ml) giving was positive in the nested assay (2/3 positive triplicates).

3.2 Specificity of single tube nested PCR

None of the 200 CMV negative blood samples showed amplification when tested in triplicate in the single tube nested PCR assay.

3.3 Performance and evaluation of single tube Nested PCR with DBS sample

Results obtained with the single tube nested PCR assay were compared with those generated using the CMV gB assay. All samples were tested in parallel using the equivalent input.

In the QCMD panel all 9 CMV positive samples were identified correctly (100%) in the single tube nested PCR assay. In comparison only 6/9 (67%) positive samples were identified with the CMV gB assay. The one true CMV negative DBS showed no amplification in both assays. The DBS with discordant results corresponded to the 3 samples with the lowest viral loads 625, 1250 and 2500 copies/ml (viral loads stated in the QCMD final report). To confirm that enhanced detection was not due to target region or primer design, the QCMD panel was also tested using a real time PCR CMV UL69 assay. The same 6/9 samples tested positive in the assay for the UL69 region as the gB assay.

DBS samples obtained from 20 children with laboratory confirmed CCMV gave positive results in 18/20 (90%) samples with the single tube nested assay. 16/20 samples tested positive in the CMV gB real time assay (80%), again showing enhanced detection with the single tube nested PCR (figure 2). The two negative samples in the single tube assay were also negative in the gB assay. In the DBS received from infants identified through the BEST study (failed NHSP) 3/6 cards tested positive by the single tube assay (50%) compared with 2/6 with the single round PCR (33%). Reviewing laboratory results in the three negative DBS from children identified through the BEST study, two children had blood samples taken for CMV PCR in the neonatal period both of which were undetectable (<200 copies/ml).

Overall sensitivity of the single tube nested PCR assay in identifying neonates with confirmed CCMV was 21/26 (81%; 95% CI, 60.6%-93.4%). In contrast the single step gB real time PCR had a sensitivity of 18/26 (69%;95% CI, 48.2%-85.6%) giving an increased detection rate of 12% in children with laboratory diagnosed CCMV infection.

After analysis of the clinical data from the 20 confirmed CCMV children the two negative samples had asymptomatic presentation with a normal clinical outcome (no problems reported, apparently normal development) at follow up (20.8 and 20.5 months after birth; (table 2). On further investigation, the additional positive DBS in the CCMV failed NHSP samples had a sample of whole blood tested in the neonatal

period (prior to the DBS being taken) with a viral load of 7,700 copies/ml. The child presented with unilateral SNHL and subependymal cysts on cranial imaging and received 6 weeks' treatment with valganciclovir.

Overall the outcome was known in 25/26 CCMV children. The mean follow up period was 19.9 months (SD 4.6 months). The single tube nested PCR detected CMV DNA in 20/25 samples compared to the gB assay with 17/25 testing positive. On further investigation of the 3 samples positive which tested only with the nested PCR, only one DBS was from a symptomatic infant with mild SNHL at follow up. The two other DBS samples were from symptomatic children with bi –lateral hearing loss at follow-up (table 2).

4 Discussion

This study demonstrates enhanced detection of CMV DNA from DBS using a novel single tube nested protocol when compared with a single round PCR. PCR based testing for viral targets often report high sensitivities but use a much larger sample volume. For example whilst an HIV RNA viral load assay may detect a sensitivity of 50 copies/ml an original volume of sample of 1ml would be required. The hypothesis was that that the low sensitivity reported from DBS is due to small sample volume (typically between 10 and 80ul) and that a detection method with high sensitivity would be required in order to detect these very small quantities of CMV.

Proof of concept studies already exist reporting detection of CMV from DBS. However the methods reporting the highest sensitivities for CMV DNA detection from DBS are not suitable for high throughput screening because of labour intensive methodologies and/or detection. This study is the first to report a sensitive method which has the potential to be used as a high throughput screening tool.

Newborn screening for CMV has been suggested using saliva or urine samples due to their reported high sensitivities (Boppana et al. 2011). However DBS have the advantage that newborn screening programmes using DBS (Guthrie cards) are already in place in many countries. Thus DBS samples are available on every child born without any additional sample requirements.

Recently a study by Boppana et al reported DBS to have low sensitivity when compared to saliva rapid culture. (Boppana et al, 2010) It has been shown that DNA extraction and CMV detection methods can have significant effect on the reported assay sensitivity. (de Vries, et al 2009);(Soetens et al. 2008) highlighting the importance of assay methodology. This study shows that existing DBS methodology can be improved; however a study to address the methods clinical sensitivity compared to urine or saliva in the context of CCMV newborn screening is warranted.

In the current study, the overall sensitivity was 81%. Interestingly the 5 CMV negative samples from confirmed CCMV cases were asymptomatic infants with normal outcome at follow up. Studies using PCR quantitation have shown that viral load in urine and blood at birth is higher in symptomatic babies compared to asymptomatic babies, but more importantly that level of viraemia correlates with

future sensorineural hearing loss (Boppana et al. 2005;Bradford et al. 2005). In solid organ transplant patients quantitative studies have likewise shown that risk of disease is associated with high viral load (Cope et al. 1997). A sigmoid relationship has been proposed recently for the severity of SNHL versus CMV viral load at birth (Walter et al, 2008). However this ‘threshold effect’ is novel in the context of newborn screening and denotes that a test without 100% analytical sensitivity that nevertheless detected all children at risk of developing disease would be deemed acceptable for the purposes of screening. Our data seem to support this as the DBS from the asymptomatic children with normal outcome are CMV PCR negative in contrast to the symptomatic children with severe outcome who had CMV detected in all DBS screened. This possibility should now be evaluated in much larger numbers of cases and controls to determine whether an assay with less than 100% sensitivity for infection could nevertheless identify all cases destined to develop disease.

Traditionally newborn screening for genetic and biochemical disorders was based on mass spectrometry. The first DNA based newborn screen for severe combined immunodeficiency (SCID) was included in the US screening core panel in 2010. The assay is based on the detection and quantitation of T cell receptor excision circles (TREC) from DBS by real time PCR (Kwan et al. 2013). Currently the UK is running a pilot study for SCID screening and, if implemented, the TREC assay will be the first DNA based screen to be added to the UK screening panel. This will provide the

expertise and instrumentation required for DNA extraction and real time PCR. Once these are in place it will provide the ideal opportunity for the addition of new molecular based tests into the newborn screening repertoire including those for CMV.

This data show that detection of CMV from DBS is possible and that inclusion of CMV screening in the newborn programme is feasible. Overall this will allow a greater number of infected infants to be identified early and provide the opportunity for intervention.

5 Conclusions

A novel real time single tube nested PCR was developed for improved detection of CCMV from DBS. This rapid single tube nested PCR allows detection of CMV in 1.5hrs and removes the associated contamination risk of a two step nested PCR and/or gel based detection method. A large scale evaluation is warranted.

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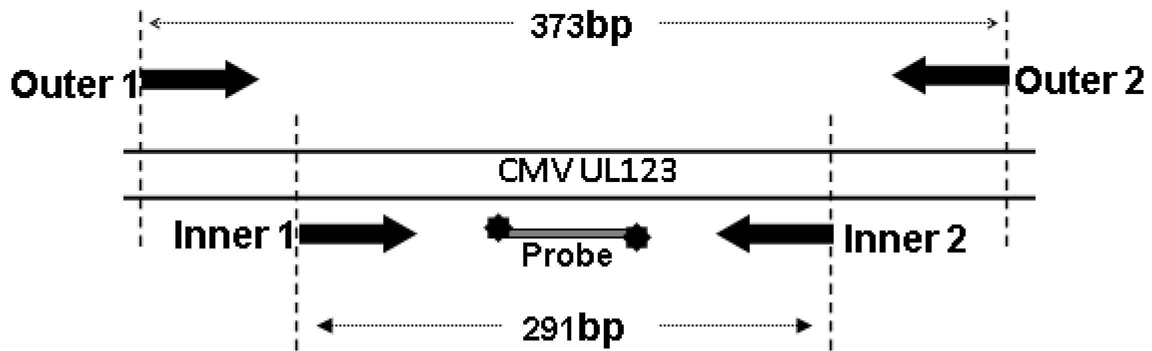

Figure 1: Schematic diagram of single tube nested PCR (not to scale)

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 373bp product from the outer primers (Outer1,2). A second PCR cycle incorporated the inner primers (inner 1,2) to yield a 291bp product. A internal double quenched probe allowed simultaneous amplification and detection of the amplicon in real time.

Table 1 Primers and probes used for single tube nested PCR

Primer /probe Name	*(Sequence 5'-3')*	*Tm*	*Nucleotide position[a]*
Outer 1	GGTCACTAGTGACGCTTGTATGATGACCATGTACGG	74	172906-172941
Outer 2	GATAGTCGCGGGTACAGGGGACTCTG	71	172568-172594
Inner 1	AGTGAGTTCTGTCGGGTGCT	58	172870-172889
Inner 2	GTGACACCAGAGAATCAGAGGA	58	172598-172619
Probe	6 FAM-AGG AGA CTA /ZEN/GTG TGA TGCTGG CCA A-IB®FQ	66	172828-172852

FAM, 6-carboxyfluorescein; ZEN, internal quencher; IB®FQ Iowa Black dark quencher

[a] *Indicates nucleotide positive in CMV Merlin; GenBank: AY446894.2*

Figure 2- Sensitivity of single round and nested PCR for detecting CMV from DBS in each sample set

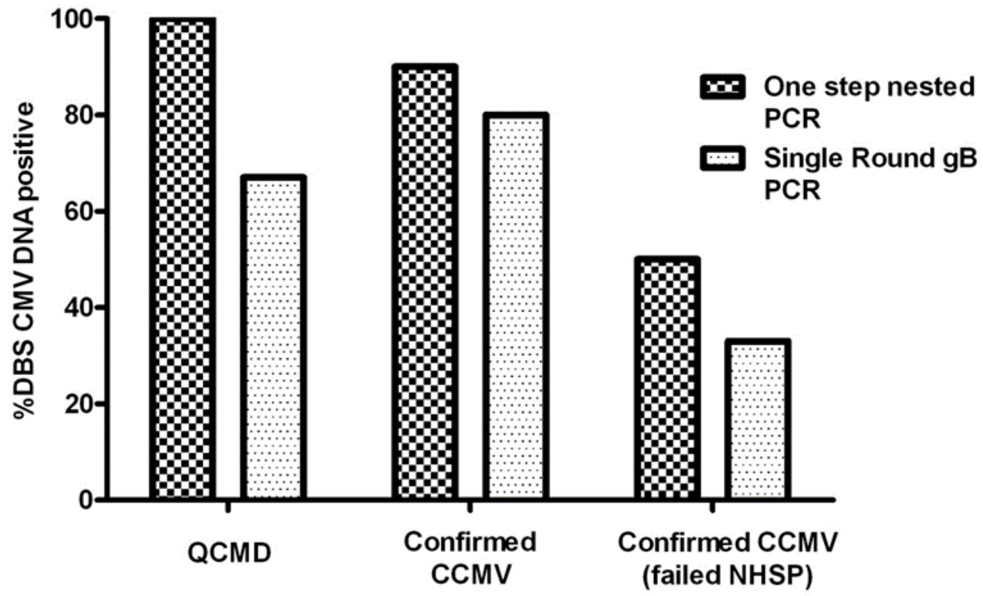

Table 2- Correlation between neonatal presentation of CCMV, clinical outcome and the proportion of DBS testing positive.

Neonatal Presentation	Outcome*	Number of DBS testing positive with Single round PCR	Number of DBS testing positive with nested single tube PCR
Asymptomatic	Normal	3/7	3/7
Symptomatic	Normal	2/2	2/2
Symptomatic	Mild	**5/6**	**6/6**
Symptomatic	Moderate	**5/7**	**7/7**
Symptomatic	Severe	3/3	3/3

***Outcome: Normal**- No reported problems **Mild**: Unilateral hearing loss, mild cerebral palsy, mild language delay, **Moderate:** Bi-lateral profound deafness, deafness and other problem **Severe**: Multiple serious problems e.g. Severe global delay