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Short communication

Development of a real-time quantitative polymerase chain reaction assay for the detection of congenital human cytomegalovirus infection in urine samples

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ABSTRACT

A TaqMan real-time quantitative PCR (qPCR), based on amplification of HCMV UL54 gene specific sequence, was developed and compared with shell vial viral culture assay, the gold standard technique for the diagnosis of congenital HCMV infection, using urine samples collected from 110 newborns. The results indicate that this qPCR is slightly more sensitive than shell vial assay suggesting that qPCR may be considered a useful alternative for diagnosing congenital HCMV infection.

1. Methods, results and discussion

Human cytomegalovirus (HCMV) is a leading cause of congenital infection, occurring in 0.2%–2.2% of newborns [1,2], resulting in severe neurological alterations with hearing loss in some cases [2,3]. Hence, early diagnosis of congenital HCMV infection is critical for the monitoring and treatment of these patients. The diagnosis of congenital HCMV infection must be performed before the third week of life [4,5]. After this point in time, the determination of HCMV infection cannot distinguish congenital infection from postnatal infection [5]. The gold standard for diagnosing congenital HCMV infection, is represented by traditional shell vial viral culture on urine and/or saliva samples [5–7]. HCMV can often be detected in as little as 1–2 days; however, cultures that are negative for the virus must be held for 3–4 weeks to confirm the absence of HCMV because a few copies of infectious HCMV can be available in the original samples, or HCMV wild type strain may grow slowly, with a delaying a positive culture detection [5]. The qPCR assays were currently used for the determination of HCMV infection in immunocompromised hosts [8–10]. Importantly, qPCR is the reference method to determine the reactivation of HCMV infection in organ-transplanted individuals, replacing the immunofluorescence-based antigenemia [8–11]. Strikingly, the shell vial viral culture assay in urine or saliva samples, is still considered the gold standard technique for diagnosing congenital HCMV infection as the clinical use of qPCR assay to diagnose congenital HCMV infection has not yet been validated [4,12–15] although several PCR assays on urine have been conducted exhibiting an analytical sensitivity ranging from 93 to 100% [12,13,16,17]. In this study, we assessed a TaqMan qPCR for the detection of HCMV based on amplification of a conserved sequence into the UL54 HCMV gene. In addition, we evaluated the performances of this assay in comparison against the shell vial viral culture of urine from a cohort of infants with suspected of HCMV congenital infection.

In the first set of experiments, we determined the performances of our TaqMan qPCR. TaqMan qPCR was carried out in a total reaction volume of 50 μL consisting of 25 μL of TaqMan Universal Master Mix (Applied Biosystems, CA, USA), 0.5 μM of each HCMV specific primer (forward primer CMV1: 5′-ACTTTGCCGATG-TAACGTTTCTTG-3′; nucleotides 79,017–79,040, reverse primer CMV2: 5′-CGGGTCATCTACGGGGACAC-3′; nucleotides 79210-79191), 0.25 μM of HCMV specific probe (fluorogenic-FAM CMV MGB-probe, 5′-CTGGAGTTTGAAAAGGT-3′; nucleotides 79090-79065), 0.06 μM of each β-globin-specific primer (forward primer GlobF: 5′-GCCAGGGCTGGGCATAA-3′ nucleotides 2974–2990,

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reverse primer GlobR: 5′-TGTCAGAAGCAAATGTAAGCAATAG-3′; nucleotides 3008–3033), 0.25 μM of β-globin probe and (fluorogenic-VIC Glob MGB-probe: 5′-AGTCAGGGCAGAGCC-3′ nucleotides 2992–3006), 10 μL of sample represented by scalar dilutions of the pHCMVUL54 Bluescript plasmid or DNA extracted from clinical samples and 12.5 μL of double-distilled water. The sequences of selected oligonucleotides and probes are conserved and have no homologies to all sequences published in the viral and cellular gene repository by sequence comparison analysis with the NCBI Blast analysis program (http://www.ncbi.nlm.nih.gov/BLAST).

To check for adequate extraction efficiency and the absence of inhibitors, β-globin gene fragment cloned into a backbone vector (200 copies/reaction) and obtained from a commercial source (CPE internal control, diluted 1:100, Nanogen, Buttigliera, Italy) was added on samples prior to extraction procedure, as internal control. The quantitation of HCMV target was determined by the assessment of an appropriate external reference curve represented by pHCMVUL54 plasmid (from 5×10^5 to 5×10^1 copies) scalar dilutions. The pHCMVUL54 plasmid was obtained by cloning of a single copy of the UL54 gene encompassing the sequence from 2712 to 2906 nucleotides in pBluescript SK plasmid (Stratagene, La Jolla, CA, USA) using the classical cloning procedure. The purification of plasmids from bacteria was performed by Midi plasmid extraction kit (Qiagen, Hilden, Germany). The TaqMan qPCR was carried out in a Mini Opticon Real-Time PCR Detection System (Bio-Rad, Richmond, VA, USA) under the following conditions: 1 cycle at 95 °C for 5 min and 40 cycles of amplification in two steps each (94 °C for 15 s, and 58 °C for 30 s). The results were analyzed using real-time CFX Manager Software version 3.0 (Bio-Rad). All standard dilutions, controls and samples from patients were run in duplicate. The control of extraction and amplification was determined by the Ct value of internal control. To validate the sample run the internal control Ct value did not exceed cycle 38. The standard curve was considered valid when the slope was detected between −3.45 and −3.23 (95–100% efficiency). The results demonstrated that this assay encompasses at least 4 orders of magnitude with a linear relationship ($r^2 > 0.98$) and detects up to 200 copies/ml in all replicates (Table 1). This sensitivity level is similar to HCMV-specific qPCR based on amplification of alternative conserved HCMV genes including for example, the UL83 and UL55 viral genes [18–20]. Linear regression analysis with Probit indicated in 180 copies/ml the viral load detectable in at least the 95% of replicates. Probit model analysis is a type of regression analysis used in the determination of molecular assay sensitivity [21]. The reproducibility of this technique was assessed with intra- and inter-assay procedures. The intra-assay test was performed using three replicates (n = 3) of each point of scalar dilutions tested between 5×10^5 copies/ml and 5×10^2 copies/ml. The coefficient of variation (CV) is shown in Table 2. Inter-assay reproducibility was assessed with three separate experiments in triplicate with a CV ranged from 0.42% to 3.2% using the scalar dilutions between 5×10^5 copies/ml and 5×10^2 copies/ml. Assay specificity was investigated by TaqMan qPCR amplification of samples negative for HCMV DNA (n = 10). No positive amplification was detected in all replicates. Moreover, different samples positive for other *Herpesviridae* including EBV, VZV or HSV-1 DNA (n = 3 each) and negative for HCMV were targeted by TaqMan qPCR, and no cross-reactivity was detected in all samples.

We also evaluated TaqMan qPCR in comparison against traditional shell vial viral culture on urine collected within the first three weeks after birth from a cohort of infants with suspected of HCMV congenital infection. Urine samples were collected in sterile urine bags from 110 infants born at Verona Hospital from September 2013 to May 2014. The specimens were stored at 4 °C and processed within 4 h of collection. The shell vial viral culture technique was performed as previously described [22] using a monoclonal antibody to HCMV immediate early 1 (IE-1) protein. In parallel, urine samples were processed for TaqMan PCR. Urine samples (1 ml) were centrifuged at 500 × *g* and the supernatant was collected, mixed with 500 μl of DNA lysis buffer solution [5.25 M GuSCN, 50 mM TrisHCl pH 6.4, 20 mM EDTA, 1.3% (wt/vol) Triton X-100] and incubated for 10 min at room temperature. The mixture was then added to the EasyMAG vessel and 100 μL of diluted magnetic silica (50 μL silica + 50 μL ultrapure water) was subsequently added. The DNA was extracted using an EasyMAG instrument (Biomerieux) with the Generic 2.0.1 program and eluted in 65 μL of NucliSens extraction buffer 3 (Biomerieux). TaqMan qPCR was carried out with the protocol indicated above. Shell vial viral culture assay detected 29 immunofluorescence positive samples out of 110 (26%) whereas the qPCR displayed positive amplification of HCMV target in 30 out of 110 samples (27%) with a Kappa index = 0.977 (Table 3). The analysis of viral load in all positive samples showed a mean value of 15001 ± 30815 copies/ml (from 310 copies/ml to 144757 copies/ml). All 29 positive samples identified by shell vial viral culture were also identified using TaqMan qPCR. The comparison between these two technical approaches demonstrated that TaqMan qPCR is slightly more sensitive (100% considering TaqMan qPCR as a reference technique) than shell vial viral culture (95%). These results are in according to previous studies, in which shell vial viral culture was compared with HCMV DNA detection. In fact, shell vial viral culture showed a sensitivity ranging from 61.5% to 100% assuming real-time qPCR as a reference method [12,15,23–25].

Table 1
Analysis of sensitivity of TaqMan qPCR assay.

Copy number/reaction	Number of positive replicates/total replicates	%	Probit
5×10^5	10/10	100	NA[a]
5×10^4	10/10	100	NA
5×10^3	10/10	100	NA
5×10^2	10/10	100	NA
2×10^2	10/10	100	NA
1×10^2	7/10	70	5.52
5×10^1	4/10	40	4.75
0	0/10	0	NA

[a] NA: Not applicable.

Table 2
Intra-assay variation of the CMV real-time PCR assay with scalar dilutions of pHCMVUL54 plasmid.

Copy number/reaction	Intra-assay		
	Mean C_T	SD[a]	CV (%)[b]
5×10^5	25.03	0.3	1.24
5×10^4	28.83	0.14	0.5
5×10^3	32.20	0.1	0.3
5×10^2	35.38	0.13	0.37

[a] SD, standard deviations.
[b] CV, coefficient of variation.

Table 3
Comparison between TaqMan HCMV real time qPCR and shell vial viral culture in the analysis of urine samples from 110 newborns.

CMV culture	CMV PCR in urine		Total
	Positive	Negative	
Positive	29	0	29
Negative	1	80	81
Total	30	80	110

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Discordant results were observed for only a single sample, in which classical viral culture failed to detect the HCMV target. This discordant sample could be considered either a shell vial viral culture false negative or a PCR false positive; however, the clinical and laboratory follow-up of the infant determined the presence of congenital HCMV infection. The failure of shell vial viral culture to detect HCMV infection may be related to loss of HCMV viability. This is likely caused by HCMV inactivation during the sample transport at room temperature, few available copies of infectious HCMV, or slow growing HCMV wild type strain as suggested in other studies in which similar discordant results were revealed [4,12].

In conclusion, a TaqMan qPCR, based on amplification of HCMV UL54 specific sequence, was developed to measure the HCMV genome in the urine of newborns. Our results demonstrated that TaqMan qPCR and shell vial viral culture sensitivity is comparable, but TaqMan qPCR offers some advantages: including ease of management and execution, quantification of HCMV, rapid determination of positive and negative HCMV samples, possibility of semi-automation of assay and adaption of a molecular screening of congenital HCMV infection. On the contrary, the shell vial viral culture is an expensive and technically demanding method and is not suitable for large screening. On the contrary, the shell vial viral culture is an expensive and technically demanding method and is not suitable for large screening. Altogether, these results indicate that qPCR could be considered as a useful candidate to replace shell vial viral culture as the reference method, and it will be important to standardize the PCR protocol across different laboratories for large-scale screening of suspected HCMV-infected newborns.

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Author contributions

ML designed the experiments, ML, MP, and MS performed the experiments, ML, MP, MS, DG analyzed data and ML and DG wrote the manuscript.

Conflict of interests

The authors declare no conflict of interest.
All authors approve of the final article.

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