

Real-Time PCR Diagnostics Failure Caused by Nucleotide Variability within Exon 4 of the Human Cytomegalovirus Major Immediate-Early Gene[∇]

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Here we report how variability in the human cytomegalovirus genome sequence may seriously affect the outcome of its molecular diagnosis. A real-time quantitative PCR assay targeting the major immediate-early gene failed to detect the viral load in some, but not all, clinical samples from hematoonological patients. By amplification and sequencing the DNA across the regions targeted by this assay we found a number of nucleotide substitutions which accounted for decreased primer/probe binding. This decreased the sensitivity of the assay up to 1,000-fold.

Human cytomegalovirus (CMV) persists in the host organism for a lifetime and becomes frequently reactivated in immunocompromised patients, such as recipients of bone marrow or solid organ transplants (1, 15, 20). Establishment of a reliable real-time quantitative PCR assay (Q-PCR) for detection of CMV is crucial for successful management of CMV infection. Despite the importance of precise diagnosis of CMV, a generally accepted methodology has not yet been established. Optimal detection of CMV DNA in all clinical samples can be achieved only if primer sequences capable of detecting all the CMV strains are used. A number of different genes are used as a target for quantitative detection of CMV, including the major immediate-early (MIE) gene (5, 10, 12), the phosphoprotein 65 (pp65) gene (6, 7, 18), and the glycoprotein B (gB) gene (9, 11). Some groups use duplex assays to detect two different genes simultaneously (2, 8).

The coding sequence for the MIE gene consists of three exons and has long been assumed to be highly conserved due to the important function that MIE plays in the CMV life cycle and from sequence comparisons of the MIE genes from laboratory strains AD169 and Towne. Later studies described significant sequence variability in some parts of this gene, mainly in its exon 4 (3, 4, 16, 21). Zwegberg Wirtgert et al. (21) found the MIE exon 4 sequence to be more variable than the CMV DNA polymerase gene or the glycoprotein B gene. Despite these findings, the MIE gene is still a popular target sequence for routine CMV diagnostics.

In our laboratory, we simultaneously used the Q-PCR assay published by Tanaka et al. (19) that detects a part of exon 4 of the MIE gene (primers RQ1 and RQ2 and RQ probe) and an in-house qualitative PCR assay that detects a region between exon 2 and exon 4 of the same gene (primers CMV 2-4F and

CMV 2-4R). Table 1 has further details on the assays and primer/probe sequences. We compared the sensitivities of these two methods by amplifying 10-fold dilutions of 1×10^7 copies of CMV DNA, which had been isolated from the CMV strain AD169 (each dilution was tested in triplicate). The Q-PCR has proven to be about 10 times more sensitive than the qualitative method. The Q-PCR detection limit was 10 copies of CMV DNA apart from 100 copies detected by qualitative PCR.

Between January 2004 and January 2005 we assayed 1,854 samples from 363 patients including immunocompromised patients with hematological disorders, patients with bone marrow transplants, and/or recipients of peripheral blood stem cells. DNA isolated from 4×10^6 peripheral blood leukocytes (using the QIAamp blood kit [QIAGEN, Germany]) was used as a template. The Q-PCR detected virus reactivation in 64 patients. In contrast, Q-PCR repeatedly failed to detect any viral load in 18 patients who were previously shown as CMV positive using the less-sensitive qualitative method. We hypothesized that such a discrepancy could be explained by primer/probe mismatch due to sequence variability in the MIE exon 4.

Therefore, we performed a sequence analysis of the Q-PCR target region (nucleotide positions 2719 to 2919, GenBank accession no. M21295) in samples from all 18 patients with false-negative results. We designed two pairs of primers spanning this region and used nested PCR to obtain sufficient amounts of PCR product for sequencing (Table 1 shows primer sequences and locations). Both strands were sequenced using the inner primers and the BigDye Terminator v1.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an ABI PRISM 310 Analyzer. As a control group we sequenced 10 samples in which viral copies were detected by Q-PCR. We used Clustal W (freeware, www.ebi.ac.uk/clustalw/) to align the obtained sequences with the available sequences of laboratory strains AD169, Towne, and Toledo (results are compiled in Fig. 1).

We found a number of nucleotide substitutions in both primers and probe sequence in all 18 false-negative samples. In

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TABLE 1. Primers, probes, and PCR specifications used for amplification of various parts of the MIE gene^a

Primer/probe designation	Sequence, 5'→3'	Genomic coordinate ^b	Cycling conditions
RQ1	GACTAGTGTGATGCTGGCCAAG	2719	95°C/10 min → 50 times (95°C/15 s → 60°C/1 min)
RQ2	GCTACAATAGCCTCTTCCTCATCTG	2919	
RQ probe	AGCCTGAGGTTATCAGTGTAATGAAGCGCC	2758	
CMV 2-4F	GACCCTGATAATCCTGACGA	1506	95°C/10 min → 35 times (95°C/30 s → 53°C/30 s → 72°C/30 s) → 72°C/7 min
CMV 2-4R	ATGTGCTCCTTGATTCTATG	2073	
exon4celyF	GAAATTCCTGCGCCTTTA	2101	95°C/3 min → 35 times (95°C/1 min → 53°C/1 min → 72°C/2 min) → 72°C/7 min
exon4celyR	AGCACCATCCTCCTCTTCCT	3169	
CMV4F	AGTGAGTTCTGTCGGGTGCT	2678	95°C/3 min → 25 times (95°C/30 s → 53°C/30 s → 72°C/1 min) → 72°C/7 min
CMV4R	CCCTCCTCCTCTTCCTCATC	3075	

^a RQ1, RQ2, and RQ probe, primers and probe for Q-PCR; CMV 2-4F and CMV 2-4R, primers for qualitative PCR; exon4celyF, exon4celyR, CMV4F, and CMV4R, primers for nested PCR and consecutive sequencing.

^b MIE gene sequence (GenBank accession no. M21295).

seven samples, there were substitutions in the forward primer sequence, and all samples had substitutions in the probe and reverse primer regions. When the sequences were compared to the sequences of different laboratory strains, they were found to be more similar to strains Towne and Toledo than to AD169, which served as the template for designing the assay (19). Sequences of all 10 samples of the control group were homologous to the laboratory strain AD169. No association between the sequence of MIE exon 4 and sex, age, clinical diagnosis, and the progression of CMV infection was observed.

We also investigated the effects of primer/probe template mismatches on PCR efficiency. We cloned the PCR products (amplified with primers CMV4F and CMV4R) from AD169 (no mismatch), sample 13 (four mismatches), and sample 3 (six mismatches) into plasmids and used them as templates for real-time PCR (each sample was tested in triplicate). Comparisons of average cycle threshold values for 10-fold dilutions (10⁶ to 10² copies/tube) revealed major differences between plasmid DNA with mismatches and that without mismatches. On average, plasmid derived from sample 13 (four mismatches) was amplified 6.4 cycles later than the mismatch-free

plasmid. This represents sensitivity almost 100 times lower (assuming the average 3.32-cycle difference between 10-fold dilutions of standard DNA). Plasmid derived from sample 3 (six mismatches) was amplified about 12.3 cycles later than mismatch-free plasmid. This represents sensitivity over 1,000 times lower.

Genetic variability of CMV has been reported several times (13, 14), and this study documents how seriously this variability can influence PCR results. Considering that CMV is the most frequent cause of life-threatening infections in immunocompromised patients, its diagnosis must be fast and precise. Our data show that the assay published by Tanaka et al. (19) would not detect CMV in 21.9% of positive samples (18/82) from our patients. These samples would have been misjudged as CMV negative without simultaneous use of two PCR assays, and patients could therefore have been at risk of progression of a fatal CMV disease.

Based on our experience and previously published data we strongly recommend not using exon 4 of the MIE gene for routine PCR diagnostics. Schaade et al. (17) suggests that novel real-time PCR assays should be rigorously tested on

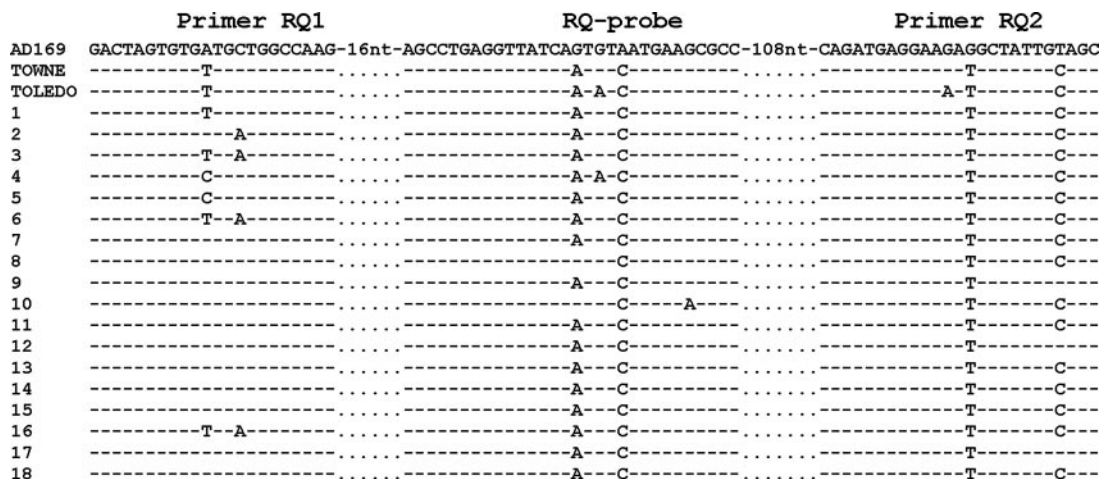


FIG. 1. Sequence comparison of selected areas of the MIE gene exon 4 of the laboratory strains AD169, Towne, and Toledo with those of clinical isolates. Dashes indicate homology to AD169.

large panels of viral isolates. However, the existence of rare or newly arising variants cannot be excluded.

Nucleotide sequence accession number. The GenBank accession number for the MIE gene sequence is M21295.

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