Supplemental Methods

Qualitative and real-time quantitative PCR

DNA was extracted from up to 200 µl aliquots of ocular fluid samples using the NucliSens nucleic acid isolation kit as recommended by the manufacturer (BioMerieux) and all samples were eluted with 50 µl of elution buffer. Real-time PCR was performed using the LightCycler (LC) 1.2 instrument (Roche Diagnostics) in a 20 µl reaction consisting of 1X LC FastStart DNA Master HybProbe mixture containing FastStart Taq polymerase, reaction buffer dNTP mix (with dUTP substituted for dTTP), and 1.0 mM $MgCl_2$ (Roche), an additional 3.0 mM $MgCl_2$, 0.5 µM of each primer, 0.2 µM of each FRET probe, 1 U uracil-DNA-glycosylase (UNG), and 10 µl of extracted DNA. The reaction mixture was preincubated for 10 min at 30° C to activate UNG and DNA was denatured and UNG inactivated at 95° C for 10 min. The template was first amplified using step-down PCR which consisted of 12 cycles of 5 sec at 95° C, 10 sec at 66° C to 55° C with a 1° C decrease in annealing temperature at each cycle, and 20 sec at 72° C. This was then followed by an additional 34 cycles of 5 sec at 95° C, 10 sec at 54° C, and 20 sec at 72° C. Separate PCR amplifications were performed for detection of CMV, HSV-1, HSV-2, and VZV (Supplemental Table 1). Prior to extraction, each sample was spiked with an internal control (pBR322 plasmid DNA) to verify successful recovery of DNA and removal of PCR inhibitors. The internal control in extracted samples was detected by amplification in a separate qualitative LC real-time PCR as described previously (Cohen et al, Human Herpesvirus 6-A, 6-B, and 7 in vitreous fluid samples. *J Med Virology* 2010; 82: 996-9).

For determination of quantitative values, a separate plasmid that contained the amplification site for each virus was generated by cloning the target region into the pCR2.1 vector (Invitrogen). After propagation and purification of each plasmid, the concentration was calculated using the absorbance at 260 nm and the plasmid molecular weight. The plasmids were linearized and then diluted in TRIS-EDTA (TE) buffer (pH 8.0) with glycogen (33.3 µg/mL), to obtain a ten-fold dilution series for the generation of the standard curves. For each positive sample, the LC software plotted the crossing threshold value on this curve to determine the number of target copies present in the amplification reaction. This value was then expressed as copies/mL based on the volume of specimen extracted and the quantity of extracted DNA added to the reaction.

Direct sequencing of CMV genome for genotypic antiviral drug resistance

Patients who developed CMV reactivation while on prophylactic valganciclovir therapy were evaluated for resistance mutations using PCR-amplified DNA. Following the isolation of CMV DNA from aqueous fluid (as described above), sequencing of the UL97 gene encoding phosphotransferase and the UL54 gene encoding DNA polymerase was performed. Two separate amplification reactions consisting of overlapping primers were used to amplify a region of approximately 650-bp (codons 426 – 638) of the UL97 gene and then approximately 2400-bp of the UL54 gene (codons 280 – 1085) were amplified using five separate amplification reactions. PCR amplification was performed on the LC 1.2 instrument and each reaction consisted of 1X LC FastStart DNA Master HybProbe mixture, 0.5 µM of the appropriate forward and reverse primers (Supplemental Table 2), 0.5 U UNG, and 10 µl of extracted DNA in a final volume of 20 µl. The reaction mixture was preincubated for 10 min at 30° C and the template amplification consisted of 45 cycles of 5 sec at 95° C, 10 sec at 65° C and 20 sec at 72° C. Following amplification, each 20-µl reaction was electrophoresed on a 1% GTG gel and the appropriately sized band for each reaction (Supplemental Table 2) was excised from the gel and the DNA purified using the QIAquick Gel Extraction kit as recommended by the manufacturer (QIAGEN). Cycle sequencing was performed using the appropriate forward and reverse primers used for amplification and all reactions were performed with the ABI Prism BigDye Terminator Cycle Sequencing kit (Applied Biosystems). Excess dye terminators were removed using the Agencourt CleanSEQ Dye-Terminator reagent (Beckman Coulter) according to guidelines recommended by the manufacturer. Fluorescence-based sequence analysis of the extension products was performed with the ABI 3100 Genetic Analyzer (Applied Biosystems) and sequences were assembled using the SeqMan Pro software (DNASTAR, Inc.). For detection of drug-conferring mutations, the determined nucleotide sequences from each gene were submitted to the University of Ulm's HCMV drug resistance mutations web-based search tool that links the sequence to a database containing all published UL97 and UL54 mutations.[3]

Supplemental Table 1. Primer and probe sequences, product size, and target DNA for each herpes virus PCR assay

Assay	Sequence	Product size (bp)	Gene target
Cytomegalovirus			
CMV.FOR primer	5'-GCCGACGGGACCACCGTGACG-3'	206	Glycoprotein B
CMV.REV primer	5'-GCTCGCTGCTCTGCGTCCAGAC-3'		
CMV.FRET.up probe	5'-GTGTTTATAATTCTGGTCGCAAAGGAC-Fluor.-3'		
CMV.FRET.dn probe	5'-Red640-GGGACCACCGTCGTCTGATGCA-Phos.-3'		
Herpes virus 1 and 2[a]		288	Glycoprotein B
HSV.FOR primer	5'-GGACGACCACGAGACCGACATG-3'		
HSV.REV primer	5'-TGCTTGAAGCGGTCGGCGGCG-3'		
HSV1.FRET.up probe	5'-GAGGTCGGTGGTGTGCCAGCCCC-Fluor.-3'		
HSV1.FRET.dn probe	5'-Red640-TCGTGCGGGTCGCGGCGTTGGC-Phos.-3'		
HSV2.FRET.up probe	5'-GTGGTGTGCCACCCCCGGCTCGT-Fluor.-3'		
HSV2.FRET.dn probe	5'-Red640-CGTGGCGACCTTCGCCGGCTTGA-Phos.-3'		
Varicella-zoster virus		267	Glycoprotein B
VZV.FOR primer	5'-CGGTGCGAACACGGGAGTATCCTCG-3'		
VZV.REV primer	5'-ATCCCTTTGCCGAGAAACCCAACGC-3'		
VZV.FRET.up probe	5'-CAGATACTAACGTCATATATTTAATCATTTC-Fluor.-3'		
VZV.FRET.dn probe	5'-Red640-TGGGCTTCTCGAAATTTATCGGGATCAAAC-Phos.-3'		

[a] Same forward and reverse primers used for both the HSV-1 and HSV-2 PCR assays.