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Identification of Chromosomally Integrated Human Herpesvirus 6 by Droplet Digital PCR

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BACKGROUND: Human herpesvirus 6 (HHV-6) latently infects a majority of adults. In about 1% of the population HHV-6 exists in a chromosomally integrated form (ciHHV-6) that resides in every somatic and germ cell and can be transmitted through the germ line. Patients with ciHHV-6 have been misdiagnosed and unnecessarily treated for active HHV-6 infection, sometimes with important side effects, based on results from quantitative molecular HHV-6 tests.

METHODS: A droplet digital PCR (ddPCR) assay was developed to identify ciHHV-6 in cellular patient samples by precisely determining the ratio of HHV-6 to cellular DNA. We validated the assay on confirmed ciHHV-6 patient samples and a cell line derived from a ciHHV-6 patient, and we analyzed hematopoietic stem cell transplant patients suspected of having ciHHV-6. We additionally evaluated whether the assay could be applied to stored plasma samples from a study of clinical correlates of HHV-6.

RESULTS: The ddPCR assay accurately identified ciHHV-6 in cellular samples (buffy coat, peripheral blood mononuclear cells), giving a ratio very close to 1 HHV-6/cell [mean (SD), 1.02 (0.03)] in fluorescence in situ hybridization–confirmed sample). In stored plasma samples, the assay performance was set by design to have 100% sensitivity, which resulted in 82% specificity for ciHHV-6.

CONCLUSIONS: The possibility of ciHHV-6 is often overlooked in patients with detectable HHV-6 viral loads by quantitative PCR. Our ddPCR test provides rapid and accurate laboratory identification of ciHHV-6 from easily obtained cellular samples. In addition, the assay provides excellent sensitivity and specificity using stored plasma samples, facilitating retrospective analysis of the clinical significance of ciHHV-6.

Human herpesvirus 6 (HHV-6)[5] latently infects more than 90% of adults *(1)* and reactivates in 30%–50% of transplant recipients *(1)*. HHV-6 has 2 species, HHV-6A and HHV-6B, and both are capable of chromosomal integration *(2, 3)*. Recent studies demonstrate that about 1% of the population has HHV-6 stably integrated into the chromosome telomere regions of all somatic and germ cells, *(4–6)* and this chromosomally integrated form of the virus (ciHHV-6) can be passed from parent to child through the germline *(7)*.

The presence of ciHHV-6 in all cells of an individual complicates interpretation of HHV-6 real-time PCR testing on plasma, serum, or whole blood. Because typical plasma PCR assays for HHV-6 also detect integrated virus within any cellular DNA present in the sample, i.e., from lysed cells, patients with ciHHV-6 may be falsely diagnosed with active HHV-6 infection. Misdiagnosis is detrimental because antiviral treatment for HHV-6 involves drugs, including ganciclovir, foscarnet, and cidofovir, which are costly and carry substantial side effects.

Patients with HHV-6 viral loads of $>1\times 10^6$ copies/mL in whole blood or 1×10^4 copies/mL in plasma are currently presumed to have ciHHV-6 *(8, 9)*. However, a recent study demonstrated that qualitative or quantitative HHV-6 PCR of plasma is not sufficient to distinguish active viral replication from the chromosomally integrated form of HHV-6 *(10)*. A review of 21 case reports of confirmed ciHHV-6 patients determined that antiviral therapy was mistakenly administered to 5 asymptomatic patients presumed to have ac-

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[5] Nonstandard abbreviations: HHV-6, human herpesvirus 6; ciHHV-6, chromosomally integrated HHV-6; FISH, fluorescence in situ hybridization; ddPCR, droplet digital PCR; HCT, hematopoietic stem cell transplant; PBSC, peripheral blood stem cell; PBMC, peripheral blood mononuclear cells.
[6] Genes: *U67*, U67 protein UL95 [Human herpesvirus 6A]; *RPP30*, ribonuclease P/MRP 30kDa subunit.

tive HHV-6 infection due to high circulating HHV-6 DNA concentrations by standard real-time PCR testing *(11)*. Conversely, patients with symptomatic active HHV-6 infection can have very high circulating concentrations of HHV-6 consistent with ciHHV-6, which may confound or delay the diagnosis of active infection.

Currently, identification of ciHHV-6 requires fluorescence in situ hybridization (FISH), a lengthy procedure with limited availability, or HHV-6 PCR testing of hair follicle cells *(2)*, an atypical sample type for many molecular diagnostics laboratories. Here we utilize an emerging molecular quantification method called droplet digital PCR (ddPCR) to perform a ratio-based assay that rapidly and accurately detects ciHHV-6 from cellular samples, typically buffy coat collected from whole blood.

METHODS

DROPLET DIGITAL PCR

ddPCR uses TaqMan chemistry like real-time PCR, but it partitions the reaction into thousands of individual droplets, which are each read as positive or negative for DNA template, allowing absolute quantitation of DNA copies without the use of a standard curve *(12–14)*. Development and validation of our ciHHV-6 assay was performed in accordance with the MIQE (Minimum Information for Publication of Quantitative Digital PCR Experiments) guidelines for digital PCR *(15)*. The HHV-6 primer and probe set amplify a 150-bp region of U67 as previously described *(16)* and are as follows: 5R(A), 5′-GTTAGGATA TACCGA TGTGCGTGAT-3′; 5R(B) 5′-TACAGAT ACGGAGGCAATAGATTTG-3′; 5R probe, 5′-FAM-TCCGAAACAACTGTCTGACTGGCAAAA-BHQ1–3′. These sequences target the U67 protein UL95 [Human herpesvirus 6A] (*U67*)[6] genein a region that is conserved between HHV-6A and B. Because both HHV-6A and -B integrate, this assay does not discriminate. In our laboratory, typing is performed with a secondary PCR reaction designed to target a nonconserved region *(17)*. Ribonuclease P/MRP 30kDa subunit (*RPP30*) is a ribonuclease reference gene for cell count. The RPP30 primer and probe set amplify a 60-bp region, were provided by Bio-Rad Laboratories, and have the following sequences: RPP30 forward, 5′-GATTTGGACCTGCGAGCG-3′; RPP30 reverse, 5′GCGGCTGTCTCCACAAGT-3′; RPP30 probe, 5′-HEX-TCTGACCTGAAGGCTCTGCG CG-BHQ1–3′.

The ddPCR reaction mixture consisted of 12.5 μL of 2× ddPCR Supermix for Probes (Bio-Rad), 1.25 μL of each 20× primer–probe mix (18 μmol/L each PCR primer, 5μmol/L probe), and 10 μL of template DNA in a final volume of 25 μL. If derived from a cellular sample, the template DNA was digested with the restriction enzyme *Hind*III (New England BioLabs) before adding it to the ddPCR reaction. The digestion reaction used 5 μL template DNA, 1 μL *Hind*III, 1 μL New England BioLabs buffer 4, and 3 μL water, digested at 37 °C for 1 h and diluted 1:5 with the addition of 40 μL water. Dilute New England BioLabs buffer 4 did not inhibit these reactions, but inhibitory effects of any reaction additives on ddPCR should be determined empirically *(18)*. DNA from plasma samples was used undigested. Twenty microliters of each reaction mixture was loaded onto a disposable plastic cartridge (Bio-Rad) with 70 μL of droplet generation oil (Bio-Rad) and placed in the droplet generator (Bio-Rad). The droplets generated from each sample were transferred to a 96-well PCR plate and PCR amplification was performed on a 2720 Thermal Cycler (Applied Biosystems) with the conditions: 94 °C for 10 min, 40 cycles of 94 °C for 30 s, and 60 °C for 1 min, followed by 98 °C for 10 min and ending at 4 °C. After amplification, the plate was loaded onto the droplet reader (Bio-Rad) and the droplets from each well of the plate were automatically read at a rate of 32 wells/h. Data were analyzed with QuantaSoft analysis software (V1.3.2.0), and quantitation of target molecules was presented as copies per microliter of PCR reaction. Around 15 000 droplets (0.89 nL/droplet) were analyzed per well *(19)*. Data from any wells with <10 000 droplets analyzed were discarded. The result of HHV-6 copies/cell was obtained using the formula: HHV-6 copies/(RPP30 copies/2), because 2 copies of RPP30 are present in a diploid genome.

CELL LINES AND PATIENT SAMPLES

The Hector-2 cell line (Bioworld Consulting Laboratories) was derived from B-lymphocytes of a donor with FISH-confirmed ciHHV-6 (7) and contains 1 HHV-6 integration per cell (in chromosome 18). Hector-2 cells and whole blood samples from an institutional review board–approved ciHHV-6 registry (HHV-6 Foundation) were used to validate and calibrate the ratiometric ddPCR assay. DNA was extracted from cells previously frozen at −80 °C and the duplex ddPCR assay for HHV-6 and RPP30 performed.

Patient buffy coat, plasma, and tissue samples saved frozen at −20 to −80 °C from routine clinical HHV-6 and ciHHV-6 real-time PCR testing were evaluated for ciHHV-6 by digital PCR. Plasma and tissue samples from patients with or without real-time PCR–confirmed HHV-6 reactivation, and patients with suspected ciHHV-6 (determined a priori as increasing HHV-6 plasma DNA concentrations during the first 2 weeks after transplantation and persistent concentrations of ≥100 copies/mL in ≥80% of subsequent plasma samples) were selected from a hematopoietic

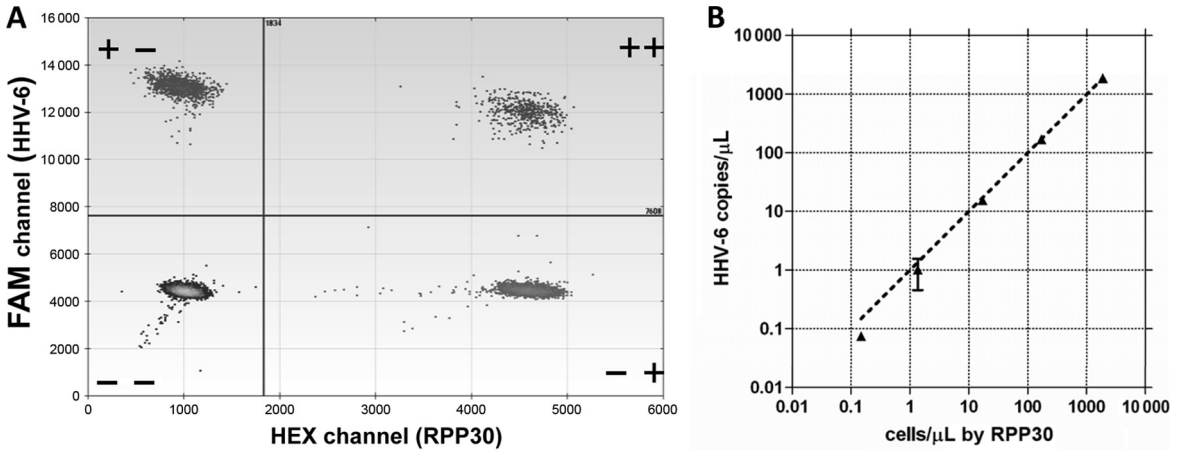

Fig. 1. (A), Droplet plot of positive and negative droplets for HHV-6/RPP30 duplex ddPCR assay using template DNA from Hector-2 ciHHV-6 positive control cells.

Upper left dots are positive for the HHV-6 FAM assay, lower right dots are positive for the RPP30 HEX assay, upper right dots are positive for both assays and lower left dots are negative for both assays. (B), Concordance of HHV-6 copy number with cell number in ciHHV-6 positive control Hector-2 cells assayed by the duplexed digital PCR assay for HHV-6 and RPP30 run in duplicate.

stem cell transplantation (HCT) study population *(20, 21)*. Study participants with HHV-6 reactivation were selected based on the following criteria: (*a*) negative sample within the first 10 days posttransplant, (*b*) 2 consecutive positive samples, (*c*) negative sample collected 7 to 10 days after the last positive result. Patients without HHV-6 reactivation had 3 consecutive negative samples collected within the same range of days posttransplant as those with reactivation, as well as a negative sample collected 7 to 10 days after the last of the 3 consecutive negative samples. Samples were run blind by a single technician.

DNA extractions of cultured cells and tissue samples were performed on a Maxwell 16 (Promega) utilizing the total viral nucleic acid extraction kit with varying volumes of cell or tissue sample extracted to 50–100 μL in water. Plasma samples were extracted on a MagnaPure LC light cycler (Roche) utilizing the DNA isolation kit I with a volume of 200 μL plasma extracted to 100 μL DNA in elution buffer. DNA was stored frozen at −20 °C until use. DNA from plasma samples was run in triplicate ddPCR reactions while DNA from cellular samples was run as a single ddPCR reaction. Use of all patient samples was approved by the University of Washington Institutional Review Board.

STATISTICAL ANALYSIS OF PLASMA RESULTS

The ratio of HHV-6 to cellular DNA in plasma samples from patients without ciHHV-6 depends on the number of lysed cells in the sample and the amount of infectious virus circulating in the blood. These could by chance have a ratio of 1 even in active infection. To distinguish active HHV-6 from ciHHV-6, we analyzed the plasma HHV-6/cell ratios of known ciHHV-6 positive and -negative samples. To aid analysis, we transformed these ratios to the absolute log scale, as ratios near 1 approach 0 on this scale, while ratios larger (or smaller) than 1 will be larger than 0. Then we set as a cutoff for differentiation the maximum observed absolute log ratio in confirmed ciHHV-6 participants and used this cutoff to define the nontransformed ratio range indicative of ciHHV-6. Specificity of this cutoff was estimated from participants with real-time PCR-confirmed HHV-6 reactivation as described above. CIs for sensitivity and specificity were computed using the normal approximation to the binomial distribution. Positive and negative predictive values were computed from sensitivity, specificity, and prevalence using Bayes' rule.

Results

ANALYTICAL PERFORMANCE OF ddPCR FOR ciHHV-6

A duplex ddPCR assay for HHV-6 and RPP30 was optimized on the QX100 Droplet Digital PCR system (Bio-Rad Laboratories). To evaluate the performance of ddPCR for the quantitation of HHV-6 vs human genome copies in cellular material, we used the Hector-2 cell line, which contains 1 HHV-6 integration per cell. The duplexed assay showed consistent, wide separation of positive and negative droplets in the FAM and HEX channels (Fig. 1A) and could detect as few as 0.1 HHV-6 copies/μL or 2 copies/reaction

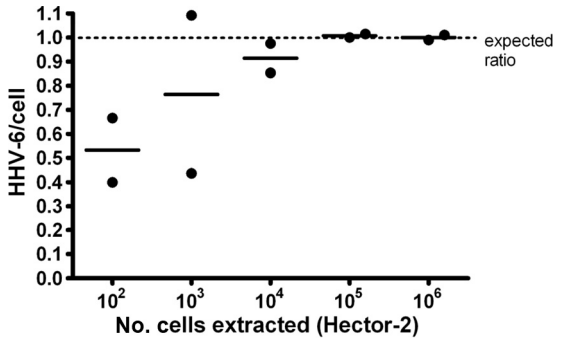

Fig. 2. Dilution series (10-fold) of Hector-2 ciHHV-6 cell line indicates that the ddPCR assay provides a precise ratio of HHV-6/cell with as few as 10^4 cells.

Bars represent the mean of 2 replicate reactions (denoted by circles).

(Fig. 1B). The false-positive background of the assay was minimal. Of 20 negative control wells, each run on a separate day, 3 had a single positive droplet for HHV-6 and none were positive for RPP30. Therefore, the cutoff for detection would conservatively be placed at 2 positive droplets/reaction, corresponding to 4 copies/reaction.

The assay provided a precise ratio of HHV-6/cell close to 1 with as few as 10^4 cells (15 cells/μL ddPCR reaction as counted by RPP30) (Fig. 2). A typical whole blood sample from a healthy individual contains $4–7 \times 10^6$ leukocytes/mL of blood, *(9)* so this cell input was well within the range applicable to buffy coat samples taken from 2–5 mL of whole blood. Moreover, the interassay precision was high. Results for 5 independent runs using Hector-2 template DNA gave a mean (SD) of 0.96 (0.03) with a CV of 3%.

ASSAY PERFORMANCE ON FISH-CONFIRMED CLINICAL SAMPLES

To confirm the performance of this assay on patient samples, we obtained samples from 2 patients with previously identified, FISH-confirmed ciHHV-6. FISH results and patient background were provided for both of these patients in previous publications [patient 1 is sibling 3 *(7)* and patient B *(22)*, and patient 2 is the father of sibling 3 *(7)*]. Only 2 buffy coat samples were available given the low number of confirmed samples available worldwide. Buffy coat samples from the 2 patients resulted in ratios near 1 HHV-6/cell by the ddPCR assay (patient 1, 1.01, and patient 2, 1.05). The striking precision of this assay on buffy coat samples underscores its potential utility as a rapid diagnostic tool for identification of ciHHV-6 from blood samples.

ciHHV-6 STATUS OF HCT STUDY PATIENTS

We analyzed samples from 4 HCT patients who were candidates for a study of HHV-6 reactivation but were excluded from that study for suspected ciHHV-6 *(20, 21)*. Three of the 4 patients (patients 30, 199, and 329) had white blood cell samples that gave a ratio of HHV-6/cell very close to 1 (Table 1). In the cases of patients 199 and 329, donor white blood cell samples were available to confirm that ciHHV-6 was transferred from

Table 1. Suspected ciHHV-6–positive patient cell and plasma samples from HCT study.

Patient	Sample type[a]	Days posttransplant	HHV-6/cell
Patients with ciHHV-6 donor			
30	PBSC	22	0.83
30	Plasma	81	0.97
199	Pretransplant PBMC	NA	0.01
199-donor	PBMC	NA	0.99
199	PBMC	95	0.99
199	Plasma	92	0.57
329-donor	PBSC	NA	1.04
329	PBMC	84	0.98
329	Plasma	1334	1.32
ciHHV-6 patient with non–ciHHV-6 donor			
98	PBMC	145	0.002
98	Plasma	838	0.18

[a] Samples are posttransplant unless otherwise noted.

donor to recipient through stem cell transplantation (Table 1). Patient 30 (Table 1) was particularly interesting, with a peripheral blood stem cell (PBSC) HHV-6/cell ratio of 0.83 on a PBSC sample taken 22 days posttransplant. This patient received a nonmyeloablative transplant and had bone marrow chimerism of 99.8% donor 6 days later, at 28 days posttransplant. Thus, the ratio of HHV-6/cell in the tested PBSC sample most likely corresponds to the ratio of donor and recipient stem cells present at day 22. The fourth patient (patient 98) was suspected of being ciHHV-6–positive and receiving a transplant from a ciHHV-6–negative donor. Consistent with this, posttransplant peripheral blood mononuclear cells (PBMC) showed a very low HHV-6/cell ratio (Table 1). Although pretransplant PBMC were not available, we were able to obtain 2 posttransplant formalin-fixed, paraffin-embedded skin tissue samples. Since skin is not derived from the donor hematopoietic cells, these samples should reflect the recipient's pretransplant ciHHV-6 status. Although the cell numbers available from these samples were not adequate to calculate an accurate HHV-6/cell ratio, the samples were positive for HHV-6, supporting the hypothesis of recipient ciHHV-6.

It is worth noting that these 4 patients were singled out as possible carriers of ciHHV-6 on the basis of HHV-6 plasma viral load levels that were being monitored regularly over time in the context of a research study. However, in a typical clinical setting, HHV-6 viral load levels are not routinely interrogated over time, and without a high index of suspicion and a rapid molecular assay available, ciHHV-6 status could easily go unrecognized.

IDENTIFICATION OF ciHHV-6 IN RESIDUAL CLINICAL CELLULAR SAMPLES

Further analyses utilizing the ddPCR assay were performed on cellular samples left over from routine clinical HHV-6 testing from 8 patients. Although 5 of the patients were clearly negative for ciHHV-6, 3 patients had samples that indicated possible ciHHV-6 by ddPCR testing (Table 2). Patient A had a buffy coat sample with an HHV-6/cell ratio that indicated 2 chromosomal integrations, a rare but documented condition *(23)*. Patient B had HHV-6 viremia with HHV-6–associated hepatitis, and a liver biopsy sample was tested for ciHHV-6 by ddPCR. The analysis demonstrated 1.2 HHV-6/cell, but the liver biopsy was a poor sample with only 1 to 2 cells/μL, prompting testing of additional samples from multiple sites (see Table 2). These samples all had ratios close to 0, which ruled out ciHHV-6 and suggested that the ratio near 1 in the liver tissue was likely due to an active HHV-6 infection. An in-depth report of this patient's presentation and clinical course has been accepted for publication *(24)*. Patient C had a buffy coat sample with a ratio precisely indicating a single chromosomal integration.

Table 2. Residual clinical cellular samples tested for ciHHV-6.

Patient	Sample	Sample type	HHV-6/cell
A	1	Buffy coat	2.07
B	1	Pretransplant PBMC	0
B	2	Donor PBMC[a]	0
B	3	Buffy coat[a]	0.03
B	4	Bone marrow[a]	0.03
B	5	Liver biopsy	1.2
C	1	Buffy coat	0.99

[a] Donor-derived sample.

SUITABILITY OF PLASMA SAMPLES FOR ddPCR ciHHV-6 ANALYSIS

Identification of ciHHV-6 status from plasma samples is of interest to researchers working from sample registries, many of which save only plasma samples. Until now the field has assumed that ciHHV-6 status cannot be determined from plasma because the integrated form of HHV-6 resides in cells. However, cellular DNA is known to be released into plasma due to cell lysis during sample processing and handling *(6)*. We therefore asked whether the ddPCR assay might be applicable to plasma samples.

To evaluate the performance of the assay in plasma, we determined the HHV-6/cell ratios in plasma samples from the 9 available ciHHV-6 patients (6 from the ciHHV-6 registry, 3 from the HCT study). The 2 FISH-confirmed ciHHV-6 patients who contributed buffy coat samples described earlier also contributed plasma samples. Both of these plasma samples resulted in ratios close to 1 (0.72, 0.95), but as predicted, results were not as precise as with the cellular samples. It is worth noting that the real-time PCR results in plasma from the FISH-confirmed ciHHV-6 patients were in the 1000–2000 copies/mL range. Such concentrations can be observed in active HHV-6 infection without ciHHV-6. Thus, if a physician were presented with a plasma viral load at this level without prior knowledge of the patient's ciHHV-6 status, the patient could be administered unnecessary treatment for active HHV-6 infection. Plasma samples from the 3 ciHHV-6 positive HCT patients also resulted in ratios close to 1 HHV-6/cell (Table 1, plasma samples; Fig. 3). Based on these data, absolute $\log_{10}$ ratios from 0 to 0.25, corresponding to 0.56–1.78 HHV-6/cell, were identified as the range in which ciHHV-6 would be suspected from tests

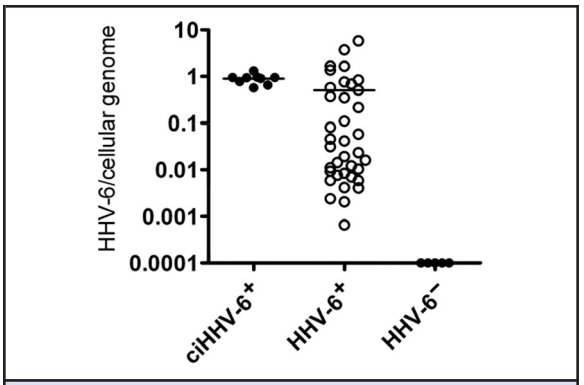

Fig. 3. HHV-6/cell ddPCR results for plasma samples from hematopoietic stem cell transplant study patients with ciHHV-6 (ciHHV-6$^+$), reactivated HHV-6 infection (HHV-6$^+$), or no HHV-6 reactivation (HHV-6$^-$).

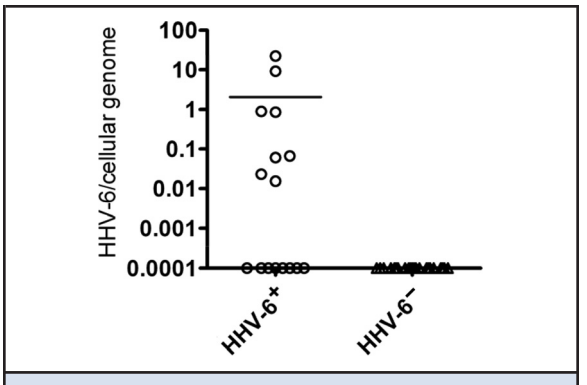

Fig. 4. HHV-6/cell ddPCR results for plasma samples from residual clinical samples that were leftover from routine qPCR testing for HHV-6 viral load and were positive by qPCR (HHV-6$^+$).

Negative control samples (HHV-6$^-$) are leftover samples from routine qPCR testing for human cytomegalovirus.

on plasma samples (Fig. 3), chosen to achieve 100% sensitivity among these persons.

To investigate the specificity of the assay on plasma, we used control patients with active HHV-6 infection and without ciHHV-6 (Fig. 3). The control patients were selected from the population of patients in an HCT study *(20, 21)* who reactivated HHV-6 but were negative for ciHHV-6 as determined by subsequent negative results on longitudinal real-time PCR monitoring, or who were presumed ciHHV-6 negative because they never had positive HHV-6 PCR results on longitudinal testing. From the cohort of 38 available reactivation patients, 7 had ratios outside our cutoff of 0.56–1.78. Additionally, 5 samples from 5 nonreactivators were tested and found to be negative. On the basis of this, we calculated a specificity of the plasma assay for ciHHV-6 of 82% (12%).

We further investigated the use of the ddPCR assay on plasma by testing 16 residual plasma samples that previously tested positive for HHV-6 by routine clinical real-time PCR testing but whose ciHHV6 status was unconfirmed. Fourteen of these patients were ciHHV-6 negative according to our cutoff range of 0.56–1.78 (Fig. 4). On the basis of the reported population frequency of ciHHV-6 [1% (4–6)], we would expect none of the 16 patients to be positive for ciHHV-6. By using this assumption, the specificity of the assay in plasma would be 88%, which falls within the range of our previous results.

Discussion

We have developed a rapid, precise assay for identifying patients with a chromosomally integrated and heritable form of HHV-6. The assay uses ddPCR technology (Fig. 1A) to identify integrations from cellular samples by precisely assaying the ratio between HHV-6 and cellular genomic DNA (Fig. 1B). A similar method for detecting ciHHV-6 by real-time PCR was described by Ward and colleagues, which relied on quantification of HHV-6 in tandem with quantification of β-globin to determine cell number (or genome equivalent copy number) *(2)*. However, ratiometric assays utilizing real-time PCR are problematic because of inherent assay imprecision *(14)*, which results in a broad distribution of ratios. As demonstrated here, the extreme precision of ddPCR provides highly precise HHV-6/cell ratios and thus avoids this problem.

Our assay has been validated on samples confirmed for chromosomal integration by FISH, the current diagnostic gold standard for ciHHV-6 (Fig. 2). Notably, in these FISH-confirmed cases, plasma HHV-6 viral loads by real-time PCR were lower than the levels that have been suggested for suspicion of ciHHV-6 in acellular fluids ($>3.5 \log_{10}$) *(9)*, calling attention to the fact that an HHV-6 real-time PCR result alone cannot identify ciHHV-6. The need for a more robust, rapid clinical assay for ciHHV-6 is met by utilizing ddPCR, which provides a precise ratio of HHV-6/cellular DNA. For clinical use, this ddPCR assay should be performed on patient buffy coat samples obtained from whole blood to identify patients with ciHHV-6.

The clinical significance of ciHHV-6 is an area of active research. Several recent reports have suggested links between ciHHV-6 and long-term sequelae; *(22, 25–29)*; however, no larger studies have systematically examined how often clinical disease occurs in persons with ciHHV-6. In addition to preventing misdiagnosis of active HHV-6 infection, our ddPCR assay should also help identify individuals with ciHHV-6 to

aid in determining how this condition may contribute to disease states. The assay could also prove useful in the transplant setting, as data from the HCT study population presented here highlight the need for screening patients and potential donors for ciHHV-6.

Our ddPCR assay is a powerful tool for rapid distinction of active infection from ciHHV-6 in cellular samples, but caution must be taken with certain samples. Samples with low genomic DNA content, such as the liver biopsy sample described in Table 2, may yield imprecise ratios that make distinguishing ciHHV-6 from active infection difficult. In cases such as these, it is advisable to obtain additional tissue or cellular samples to determine the likelihood of ciHHV-6. Additionally, in rare cases a patient may have multiple HHV-6 genomic integrations, such as in patient A (Table 2). Although only 1 case has been reported *(23)*, with a high-quality cellular sample a ratio of 2 HHV6/cell it is likely indicative of ciHHV-6.

Beyond demonstrating its clear clinical utility when using cellular samples, we have also shown that our ddPCR assay is suitable for plasma samples when cellular samples are not available, such as for large retrospective studies, for which plasma is often the only sample stored long term. Based on our estimates of sensitivity (100%), specificity [82% (12%)], and the prevalence in the overall population (1%), the negative predictive value of the plasma test (using an absolute $\log_{10}$ ratio <0.25) is estimated at 100%, and thus the test is ideal for ruling out ciHHV-6. In contrast, the positive predictive value, the proportion of positive results that are true positives, would be only 5%–8% in the general population (although it would be substantially higher in populations selected for a higher pretest probability of ciHHV-6); thus, the plasma test would be best suited for screening samples for additional follow-up. We should note that the number of cellular genomes in an acellular specimen such as plasma varies according to length of time between sample collection and fractionation, so the ratio cutoffs described here may not be directly transferable to other laboratories. Finally, for clinical testing, we would reemphasize that the optimal specimen type is buffy coat cells from whole blood, which permit the definitive identification of ciHHV-6. This assay is the first clinically available viral diagnostic test that uses ddPCR and paves the path for additional genetic diagnostic assays based on digital PCR technology.

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