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Consistent viral DNA quantification after prolonged storage at ambient temperature

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Abstract

Long-term storage of biological specimens at low temperatures is costly and impractical in resource limited settings, where the disease burden of chronic viral infections is highest, and the need for research greatest. We examined the necessity of cold storage by comparing the quantity of HHV-8 DNA recovered from swab samples before and after 9–11 months of storage at temperatures of –20 °C, 4 °C and 37 °C. Quantitative levels of HHV-8 DNA remained consistent for laboratory or mucosal swab samples regardless of storage temperature. Freezer storage is determined to be not necessary for mucosal samples destined for HHV-8 DNA quantification.

Keywords

real-time PCR; HHV-8; room temperature; storage; freezing

1. Introduction

The development of sensitive and specific polymerase chain reaction (PCR) assays for herpesvirus DNA has enabled detailed studies of systemic and mucosal reactivation of these pathogens in the human host.^{1–4} Herpesvirus PCR assays are used widely both for clinical care (e.g., plasma CMV monitoring after transplant) and for research purposes. As high throughput methods are developed and optimized, large numbers of samples can be processed relatively rapidly. We, and others, have shown that the quantitative amount of DNA remains the same after prolonged storage of either original samples at –20 °C or extracted DNA at 4 °C.^{5–7} However, a persistent cost of this approach has been the routine

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freezing or refrigeration of samples prior to any processing, as well as after DNA extraction. Freezer storage requires additional physical space over shelf storage, reliable electricity, temperature monitoring and warning devices, and personnel to maintain. For international studies, shipping of samples that must be maintained at -20°C is especially costly and burdensome. Because many viruses such as HHV-8 have high prevalence in resource-limited settings, cost-effective means of storage and shipping are essential for international collaborations studying the biology of such infections.

Our previous studies have relied on collection of mucosal samples by participants at home, and intermittent delivery to the clinic.⁸ In the initial studies comparing viral culture to HSV PCR, we showed that warm temperatures during transport adversely affect the ability to recover virus in tissue culture.⁹ The extent to which transport and storage temperatures affect the quantification of DNA from other herpesviruses is not known. The current study aims to demonstrate whether mucosal samples can be stored at local ambient temperature without adversely impacting HHV-8 DNA quantification, thereby reducing storage costs and facilitating viral mucosal shedding studies in resource-limited settings.

2. Materials and methods

Real-time PCR

DNA was extracted from 200 μl buffer of each vial using QIAamp 96 blood kit and eluted into 100 μl of Qiagen AE buffer (QIAGEN); and 10 μl of extracted DNA was then used for each PCR reaction. HHV-8 real-time PCR, which detects ORF73 gene, was applied to quantify the amount of HHV-8 DNA.^{10,11}

Cell culture Samples

Culture cell line V115P was used to create 3 identical test sets for testing 3 different storage temperatures, 4°C , -20°C and 37°C . The line consists of Vero cells infected with the recombinant HHV-8 virus rKSHV.219, which contains a 4.8 kb insert cassette of RFP/GFP/PURO; latently infected cells are puromycin resistant and green; when virus enters the lytic cycle, cells express RFP and turn red¹². After centrifugation, the cell pellet of V115P was resuspended and serial dilutions (neat, 10, 10^2 , 10^4 , 10^6 and 10^7) were made in 1x PBS. The supernatant of V115P cell culture was also serially diluted with 1xPBS (10^2 , 10^4 , 10^6 and 10^7). To mimic mucosal swab samples, 100 μl of each dilution point was spiked onto sterile swabs, air dried for 30 minutes, and the swabs were then put into the vial containing 1ml of 1x digestion buffer (100 mM KCl, 10 mM Tris [pH 8.0], 25 mM EDTA, 0.5% IGAPAL). To control the possibility of swab influence of the DNA recovery, 100 μl of the samples from the same dilution series were spiked into another vial with digestion buffer directly. Fifteen replicates were made for each dilution point, both with and without swabs, for a total of 285 samples. These replicates received no further processing (e.g. DNA was not extracted) and were divided equally into 3 boxes. The first box of 95 samples was placed in an incubator at 37°C , representing a tropical ambient temperature. The second box of 95 was stored at 4°C and the third box at -20°C . Quantity of HHV-8 was measured prior to storage and then again after 9 months had passed.

Clinical samples

We used 210 swabs collected from 38 people for a natural history study, previously found by PCR to be above 500 copies/ml and subsequently stored at -20°C . Most (91%) were oral swabs, with the remainder rectal (6%), urethral (2%), and penile (<1%). Samples were selected to allow approximately equal numbers in the following quantity ranges: 2.5–2.9, 3.0–3.9, 4.0–4.9, 5.0–5.9, and 6.0–6.9 $\log_{10}$ copies/ml. Each quantity range was randomly divided into three temperature storage groups: either 37°C , 4°C or -20°C . Samples were first quantified again using PCR since they had been stored for various periods of time, then stored at the designated temperature for 11 months. After storage they were subsequently re-quantified and compared to the quantification immediately prior to the 11-month storage.

Laboratory operation

Good Laboratory Practices (GLP) include requiring that reagents used pass required quality control (QC) tests before use; calibration of PCR curves with standards ensures that quantification is comparable between runs.

3. Results

In the cell culture samples, 14% percent of results were discordant between runs, with 7% positive only prior to storage and 7% positive only following storage (Table 1). Nearly all of the discordant samples were in the lower tertile of copy number when positive; with the discordant samples equally distributed between storage methods. The correlation coefficients for HHV-8 copy number comparing samples prior to storage and 9 months later were 0.91 for 37°C , 0.88 for 4°C and 0.90 for -20°C (Figure 1). Using linear regression to relate yield following storage to pre-storage quantity, we found that the difference in HHV-8 quantities after storage was estimated to be 10–19% below the original (slopes of 0.90 at 37°C , 0.81 at 4°C , and 0.81 at -20°C ; $p = 0.14$ for the difference in slopes between 37°C and -20°C and $p = 0.96$ for the difference in slopes between 4°C and -20°C). Among the positive samples, the great majority of the post-storage quantities were within 1 $\log_{10}$ copy/ml of the initial result (79% at 37°C , 91% at 4°C , and 70% at -20°C). Most samples were concordant positive (49%) or concordant negative (37%) at both runs. We further separated the samples based on whether swabs were used or not, and again assessed correlation in quantity between initial and post-storage samples; the correlations at each storage temperature changed by no more than 3% versus when all samples were combined for analysis.

Of the 210 clinical samples, 79% (157/198) were still positive immediately prior to storage while 12 samples had insufficient volume for quantification (Table 1). Detection of HHV-8 DNA was largely consistent in the 198 before versus after storage, with 71% of paired test results concordant positive and 17% concordant negative. The remaining 12% had discordant results following storage, with 16 samples (7 at 37°C , 3 at 4°C and 6 at -20°C) positive only before storage, and 8 samples (3 at 4°C and 5 at -20°C) positive for HHV-8 only after storage. As above, the samples positive at only one time point were all of modest quantity. In comparing HHV-8 quantity, the correlation coefficient between pre- and post-storage quantities was 0.88 at 37°C , 0.83 at 4°C and 0.84 at -20°C (Figure 2). The

$\log_{10}$ viral quantity after storage was estimated at 12 to 32% lower than the original (slopes from linear regression analysis were 0.88 at 37 °C, 0.68 at 4 °C and 0.80 at -20 °C; $p = .35$ for the difference in the slopes, 37 °C versus -20 °C). Among the positive samples, 47% of the stored specimens were within 1 $\log_{10}$ copies/ml of the initial result when stored at 37 °C, and 95% had similar agreement when stored at -20 °C.

4. Discussion

Our results show that ambient storage of non-blood samples containing herpesvirus DNA retains the quantity of viral copies over extended periods with minimal degradation. The consistency in correlation and slope demonstrates the lack of advantage of lower temperature storage. If prolonged ambient storage impacted the copy number detected, the slopes of the regression lines between pre- and post-storage samples would differ by storage temperature. While the rates of agreement within 1 log in pre- versus post-storage quantitation appeared to be lower in clinical samples stored at 37 °C versus -20 °C, indicating perhaps greater variability in quantity at the higher temperature, the consistency in slope reassures us that there is no discernably larger decline in quantity over storage with the lower temperature. That is, though variability in quantity may increase, the consistent slopes indicates that on average, higher temperature storage is no more degrading to the initial quantity than cold storage.

While some initially positive samples were negative after storage, an equal number of initially negative samples were positive following storage. This phenomenon of discrepancy commonly occurs even in replicate aliquots taken simultaneously from the same low concentration sample; it is the consequence of using small volume aliquots, some of which may not contain any DNA. Imperfect sensitivity at low concentration has been well described for this assay and others^{13,14}.

Our study is limited by the range of temperatures considered, between -20 °C and 37 °C. Temperatures higher than 37 °C (99 °F) may be common in some tropical research settings and samples stored under such conditions may accordingly experience greater degrees of degradation than what we observed. While our conclusions do not apply directly to settings outside the range of tested temperatures, our long duration of storage (9–11 months) without noticeable degradation might indicate that storage at even higher temperatures is potentially feasible.

Previous work on optimizing storage techniques for similar samples is fairly limited. Our lab had reported in 2002 that there was no loss of quality in PCR assay results for HSV DNA after 16 months of storage, but temperatures were kept at -20 °C for specimens (mucosal swabs) and 4 °C for extracted HSV DNA.⁵ Interestingly, while we found no degradation in quantity by freezing HSV DNA over 16 months, Speicher et al.¹⁵ found substantial degradation at -80 °C over 14 months while sampling HHV-8 DNA in Kenya. One possible explanation of the DNA stability in our study is our buffer which contains high concentrations of EDTA and salt. EDTA inhibits DNase activity, and salt stabilizes the DNA. Hasan et al reported no loss of sample quantity for both herpesvirus DNA (EBV and CMV) and *N. meningitidis* after 30 days at room temperature, but only if stored as purified

DNA. In contrast, they found that CMV in serum stored at room temperature had a half-life of less than one day when quantified by PCR.¹⁶ A study investigating the stability of viral DNA in CSF samples found no decline in HSV quantity using PCR after one month regardless of storage temperature.⁷ However, another study found that storing dried blood spots at 37 °C for 1 year significantly diminished the yield of HIV-1 DNA testing.¹⁷ Our experiments focused on HHV-8, reflecting our interest in oncogenic viruses in sub-Saharan Africa, and the reality that studies on HHV-8 are impractical in more developed parts of the world due to the low prevalence of HHV-8 infection. However, these results are likely to also be applicable to other herpesviruses, which have similar genomes and virion structure.

5. Conclusions

Based on these data, mucosal samples from our sub-Saharan African sites are now stored locally at ambient temperature, shipped to us without cryopreservation, and then stored at ambient temperature here in Seattle, WA. This change in practice has resulted in considerable time and cost savings, enabling more comprehensive studies of the biology of HHV-8. Further studies are needed before these storage practices can be extended to samples from other herpesviruses.

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Highlights

- Herpesvirus musocal samples and extracted DNA are commonly frozen to maintain integrity.
- ~500 samples stored at 37 °C correlated strongly with pre-storage quantities ($\rho > 0.88$).
- Findings were consistent using both laboratory dilution samples and clinical samples.

Figure 1.

Correlation in viral copy number measured by HHV-8 DNA PCR prior to and after 9 months storage of HHV-8 DNA cell culture dilutions, at 37 °C, 4 °C and -20 °C. Values below the limit of detection of 2.2 log₁₀ are true negatives, jittered for visibility.

Figure 2. Correlation in viral copy number measured by HHV-8 DNA PCR prior to and after 11 months storage at 37 °C, 4 °C and -20 °C. Values below the limit of detection 2.2 log₁₀ are true negatives, jittered for visibility.

Table 1

Detection of HHV8 DNA by PCR, before and after storage.

		N(%)					
Cell cultures (n=285)		Post storage (9 months)					
		37°C		4°C		-20 °C	
		Positive	Negative	Positive	Negative	Positive	Negative
Prior to storage	Positive (n=160)	47 (49%)	8 (8%)	46 (48%)	4 (4%)	47 (49%)	8 (8%)
	Negative (n=125)	4 (4%)	36 (38%)	10 (11%)	35 (37%)	6 (6%)	34 (36%)

Clinical samples (n=198)		Post storage (11 months)					
		37°C		4°C		-20 °C	
		Positive	Negative	Positive	Negative	Positive	Negative
Prior to storage	Positive (n=157)	49 (74%)	7 (11%)	51 (76%)	3 (4%)	41 (63%)	6 (9%)
	Negative (n=41)	0 (0%)	10 (15%)	3 (4%)	10 (15%)	5 (8%)	13 (20%)