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Multicenter Comparison of PCR Assays for Detection of Human Herpesvirus 8 DNA in Semen

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Reported prevalences of human herpesvirus 8 (HHV-8) (Kaposi's sarcoma-associated herpesvirus) in semen have ranged widely. This is possibly due to differences in assay sensitivity, geographic or population-based differences in the true presence of the virus in semen, and PCR contamination. This study assessed interlaboratory sensitivity and reproducibility in the analysis of blinded experimental panels, each consisting of 48 specimens and being composed of semen specimens from different healthy artificial-insemination donors ($n = 30$) and human immunodeficiency virus (HIV)-infected patients ($n = 7$) plus positive ($n = 4$) and negative ($n = 7$) controls. The experimental panels analyzed in each laboratory were identical except for being independently coded. Of 10 experiments done in five laboratories, 5 experiments from three laboratories had evidence of PCR contamination; all instances of contamination were in the context of nested PCR procedures. In the experiments with no false-positive results, HHV-8 DNA was detected in three (8%) of the 37 semen specimens (two from artificial-insemination donors and one from an HIV-positive patient) but in only 3 (1.6%) of the 184 PCRs in which these specimens were analyzed. This suggests that HHV-8 DNA is present in semen at concentrations that can be too low to allow its consistent detection. This study emphasizes the importance of performing blinded, multi-institution experiments to provide a coherent basis for comparing results and to motivate standardization of methods.

Human herpesvirus 8 (HHV-8) (or Kaposi's sarcoma [KS]-associated herpesvirus) is detected in essentially all KS lesions (6), in which it is present in the spindle cells that are a major pathologic hallmark of the disease (4, 13, 23). An etiologic association between the virus and the disease is suggested by the temporal association between the onset of KS and the development of both detectable HHV-8 DNA in the blood and reactivity in HHV-8 serologic assays (9, 20). In addition, HHV-8 encodes genes that can modulate cellular growth properties and possibly play a direct role in KS pathogenesis (22).

Because the epidemiology of AIDS-associated KS suggests that the disease is caused by a sexually transmitted agent other than human immunodeficiency virus (HIV) type 1 (2), semen has been studied as a possible route of HHV-8 transmission. In addition to the possibility of interrupting transmission if the virus is transmitted via semen, it is important to ascertain whether this potentially oncogenic virus is present in semen donated for use in artificial insemination. The prevalences of HHV-8 DNA in prostate and semen as measured by PCR have ranged from zero to over 90% (1, 7, 8, 10–12, 15–19, 23, 24). Possible explanations for this discrepancy include HIV status, geographic or population-based differences in HHV-8 prevalence, laboratory-to-laboratory differences in assay sensitivity, and PCR contamination. These possibilities are not mutually exclusive. This study was designed to compare the sensitivity

and reproducibility of PCR detection of HHV-8 DNA in semen among five laboratories.

MATERIALS AND METHODS

Experimental panels. Experimental panels each consisted of 48 specimens allocated as follows: 7 semen specimens from different HIV-positive men who have sex with men; 30 semen specimens obtained from different healthy artificial-insemination donors; 4 positive control BCBL-1 cell specimens containing approximately 1,000, 100, 10, and 1 HHV-8 genomic copies per 10 μ l; 3 aliquots of Molt-3 cells (a T-cell line that is presumed to be HHV-8 free and that was propagated in a room not used for HHV-8 work by someone who did not work with HHV-8); and 4 aliquots of sterile distilled water. Several experimental panels that were identical in composition but independently coded were prepared.

Semen specimens were prepared as 3:1 dilutions of semen with a semen stabilization buffer (100 mM NaCl, 5.365 mM KCl, 2.721 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.492 mM MgCl_2 , 12.85 mM sodium lactate, 0.321 mM NaH_2PO_4 , 30.949 mM NaHCO_3 , 20.0 mM HEPES, 50.0 mM sucrose, 133.2 mM glycine, 5.51 mM glucose, 28% [vol/vol] glycerol, 1 to 2% human serum albumin) (obtained as a gift from an artificial-insemination bank) that is used to stabilize semen for the purpose of artificial insemination. Semen was divided into aliquots by volume, not by cell density. Semen manipulations were done in a biological safety cabinet located in a laboratory that had not been used for any HHV-8-related work and were performed by a person who had not previously done HHV-8 laboratory work. Experimental panels were shipped to collaborating laboratories on dry ice.

PCR methods. Each laboratory followed its standard procedures for HHV-8 PCR; thus, procedures varied from laboratory to laboratory.

(i) **Laboratory A.** Specimens were separated into cell pellets and supernatants by centrifugation at $800 \times g$ for 5 min. One hundred microliters of lysis buffer (10 mM Tris-HCl [pH 8.5], 1% lauryl ether, 0.2 mg of proteinase K per ml) and 1/10 volume of $10\times$ lysis buffer were added to the cell pellets and supernatants, respectively. The mixtures were incubated at 55°C for 3 h, followed by 98°C for 10 min. Ten-microliter aliquots were used in first-round PCRs. Primer sets were from both open reading frame (ORF) 25 (experiment A1) and ORF 26 (experiment A2) (6, 15, 20). Amplifications were performed in a total reaction volume of 0.1 ml as described previously (15). The supernatant fractions were all negative for HHV-8 with both primer sets in un-nested and nested PCRs. Several precautions were taken to prevent false-positive results: (i) rooms for pre- and post-PCR procedures were physically separated; (ii) reagents were prepared in

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TABLE 1. Detection of HHV-8 DNA in semen and controls

Expt	Nested PCR performed	No. of HHV-8 PCR-positive specimens among:				Result for positive controls with the following no. of HHV-8 copies/10 µl of template (n = 1 each):			
		Semen from:		Negative controls		1,000	100	10	1
		HIV-positive MSM ^a (n = 7)	Artificial-insemination donors (n = 30)	Molt-3 cells (n = 3)	Water (n = 4)				
A1	Yes	0	1	1	0	+	+	+	—
A2	Yes	0	0	0	0	+	+	+	—
B1	No	1	5	0	0	+	+	—	—
B2	Yes	2	13	1	2	+	+	—	+
C1	No	0	0	0	0	+	+	+	—
C2	No	0	0	0	0	+	+	+	—
D1	Yes	0	4	0	2	+	+	+	+
D2	Yes	0	1	0	0	+	+	—	—
D3	Yes	0	3	0	2	+	+	—	+
E	Yes	1 ^b	1	0	0	+	+	—	—

^a MSM, men who have sex with men.^b n = 6 due to one insufficient specimen.

large batches and stored in small aliquots; (iii) equipment such as the microcentrifuge, water baths, pipettes, tube racks, and other small equipment was designated for PCR work only; (iv) gloves were changed frequently; and (v) aerosol-barrier pipette tips, PCR tubes, and autoclaved, diethylpyrocarbonate-treated water were sterilized by UV irradiation prior to PCR. In addition, serial dilutions of positive controls (BCBL-1 cell DNA) with known HHV-8 genome copy numbers were included to monitor the sensitivity and semiquantitation of the PCR products. Negative controls were interspersed after every five experimental samples.

(ii) **Laboratory B.** Specimens were mixed with 600 µl of NET-SDS buffer (100 mM NaCl, 10 mM Tris [pH 8], 1 mM EDTA, 0.1% sodium dodecyl sulfate [SDS]), followed by addition of 8 µl of 10-mg/ml proteinase K and then incubation at 50°C for 1.5 h. The specimens were then extracted twice with phenol and once with chloroform, followed by two ethanol precipitations; DNA was suspended in 50 µl of 10 mM Tris HCl–0.1 mM EDTA, pH 8.0. All samples (2-µl aliquots) were first assayed by PCR for the actin gene to confirm the presence of DNA, and 5 µl was assayed with the HHV-8 ORF 26 primer set (KS-1,2) described by Chang et al. (6), using KS-2 plus an internal primer (5'TGCAGC AGCTGTTGGTGTACCAT) in nested reactions. Amplification specificity was confirmed by Southern blot hybridization with a digoxigenin-labeled, cloned 120-bp internal amplicon generated by using primers 5'CTGCAGCAGCTGTT GGTGTACCAC and 5'CCGTGTTGTCTACGTCCA.

(iii) **Laboratory C.** Specimens were thawed at room temperature, and 50 µl of each specimen was denatured in guanidinium lysis buffer, phenol-chloroform extracted, precipitated with isopropanol, washed once with 75% ethanol, and then resuspended in 50 µl of 10 mM Tris (pH 8.0); 10 µl of DNA was used for each PCR. Two primer pairs, KS-1,2 and KS-A,B (experiments C1 and C3, respectively), were used to detect HHV-8 DNA. KS-1,2 amplifies the ORF 26 region as described by Chang et al. (6), and KS-A,B amplifies a segment of ORF 25 (14). PCR and liquid hybridization amplicon detection conditions were as previously described (8, 14). For semiquantitation, log₁₀ dilutions (10 to 10,000 copies) of plasmid DNA containing KS-1,2 target DNA were simultaneously amplified as part of each PCR run. The intensity of the specific specimen band was visually compared to this standard curve. To ensure that negative PCR results were not due to inhibition of PCR, each KS-1,2 PCR mixture was spiked with 50 copies of an internal positive control DNA, KS-fly2. KS-fly2 DNA is identical to the KS-1,2 target DNA sequence except that the hybridization probe sequence is replaced by a 21-bp *Drosophila melanogaster* DNA sequence (5'TG TCCTACCAACTACAATCCG). All negative PCR results from this laboratory had positive KS-fly2 controls. Negative controls were built in by matching every three specimens with an aliquot of uninfected HSB-2 cells processed in parallel from DNA extraction through autoradiography. In addition, each PCR run included at least one control containing all PCR reagents without any added DNA.

(iv) **Laboratory D.** Cells were pelleted by centrifugation at 2,000 × g. DNA was extracted from cell pellets by digestion with proteinase K (100 µg/ml) in the presence of 0.2% SDS and 40 mM 1,4-dithiothreitol, followed by phenol-chloroform extraction and then ethanol precipitation. Samples were suspended in 50 µl of 10 mM Tris HCl–0.1 mM EDTA (pH 8.0) and then spectrophotometrically quantified. About 500 ng of DNA was used in each PCR; when the amount of DNA recovered was insufficient for quantification, 10 µl of the suspension was used. Ten negative control samples, consisting of salmon sperm DNA in Tris-EDTA buffer, that were processed in parallel were always negative. Twelve nested-PCR-negative controls lacking DNA template were included in each PCR experiment and were always negative. Both kinds of negative control specimens were inserted between sets of five or six experimental specimens. Experiments

D1 and D3 were done with one primer set that amplifies a segment of ORF 26 (18), and experiment D2 was done with another that amplifies a segment of ORF 26 (5). The nested PCR products from experiment D1 were hybridized with a previously described internal probe (18); all specimens positive by ethidium bromide staining were also hybridization positive. The sensitivity of the nested PCR was between 1 and 10 molecules for experiments D1 and D3. Experiment D2 was of approximately 10-fold-lower sensitivity, and extra bands appeared during the nested PCR. These extra bands were not normally observed with these primers and were traced to a specific lot of *Taq* DNA polymerase. Several precautions were taken to avoid PCR contamination; extraction of DNA and PCRs were done in separate rooms with dedicated equipment that was extensively washed after each experiment.

(v) **Laboratory E.** Specimen processing, PCR primers that amplify a segment of ORF 26, and amplification conditions were described previously (11) (experiment E). DNA was eluted in 20 µl of water, and 200 ng or 4 µl was used in each PCR. The negative result for the 10-copy BCBL-1 specimen may have been due to an error either in aliquoting the cells or during DNA preparation, because the specimen was also negative for *env*-3, a diploid endogenous virus. PCR for *env*-2 was positive in unnested reactions for all but one semen specimen.

RESULTS

Blinded, otherwise identical panels consisting of semen specimens and both positive and negative controls were analyzed by solution-based PCR in five laboratories, including laboratories that have described results representing both ends of the prevalence spectrum in previous work. Both nested and nonnested PCR assays were used. In some laboratories, the materials were assayed more than once, with the same or different primers. For analysis and discussion, each attempt was treated as a separate experiment.

Controls. The results from the blinded control materials are summarized in Table 1. In all experiments, the positive controls were identified as positive down to the level of 100 copies per 10 µl of template. False-positive results for the negative controls occurred in experiments A1, B2, D1, and D3. The only positive results obtained from the positive control with 1 copy per 10 µl occurred in experiments with false-positive results for the negative controls (experiments B2, D1, and D3), raising the possibility that the apparent high sensitivity was due to PCR contamination. Positive results for negative controls occurred only in experiments that employed nested PCR.

Semen. As indicated by false positives for the negative controls, experiments A1, B2, D1, and D3 were affected by PCR contamination. Experiment B1 must be considered to be possibly contaminated because (i) it identified the highest percentage of semen specimens as being positive in spite of being sensitive only down to the level of 100 copies per 10 µl and (ii) three of the seven negative controls were positive in the sub-

sequent nested PCR assay (experiment B2). Thus, of the 10 solution PCR experiments, at least half (A1, B1, B2, D1, and D3) had results consistent with at least a low level of PCR contamination. In the experiments with no evidence for contamination (A2, C1, C2, D2, and E), three (8%) of the semen specimens were positive; none of the three was positive in more than one experiment. Overall, the 37 semen specimens were positive in 3 (1.6%) of 184 PCRs done in experiments with no evidence for contamination. Of note is that two of the three positive semen specimens were obtained from healthy donors.

Experiment C1 included a semiquantitative component. The observed values of 500, 20, and 10 copies for what turned out to be the positive controls corresponded well with their calculated values of 1,000, 100, and 10, respectively.

In addition to the solution PCR experiments described above, the blinded panel was also analyzed by *in situ* PCR, with many specimens testing positive. However, the positive and negative control cells were destroyed by the freeze-thaw cycle and could not be analyzed, precluding meaningful interpretation of the experiment. It will be important to study the sensitivity and specificity of *in situ* PCR in an experiment designed for that purpose.

DISCUSSION

It is important to understand the prevalence and routes of transmission of HHV-8. If the virus primarily infects people who have or are likely to develop KS (high infection-to-disease ratio), strategies for interrupting its transmission would differ from those for a situation in which the virus is widespread in the population and the associated disease is rare. Hypotheses regarding HHV-8 mechanisms of persistence and pathogenesis differ markedly between the extremes of high (low prevalence) and low (high prevalence) infection-to-disease ratios.

This work was intended to answer one of the questions that have emerged because of the widely varying values that have been reported for the prevalence of HHV-8 DNA in semen, specifically, whether similar results would be obtained in different laboratories from the same experimental materials. The answer is that in experiments with no evidence for contamination, absolute sensitivities were similar and agreement on semen specimens was high, with only sporadic semen specimens being identified as positive. Several other observations can be made based on these results.

(i) PCR contamination appears to have occurred in three of the five laboratories, as evidenced by positive reactions in presumed negative controls. Contamination was evident only in experiments that included a nested PCR step. While it is impossible to state with absolute certainty that the controls were truly negative, these materials were prepared before aliquoting of the semen specimens and the preparation was done in a cell culture room that had not been used for any HHV-8 work. Although the positive controls were shipped in the same container as the other specimens, contamination during shipping seems unlikely. If such contamination had occurred, many more samples beyond the negative controls would have been contaminated. PCR contamination may have occurred during specimen extraction and manipulation because of equipment contamination that may have been associated with the high DNA load in some of the positive controls included with the test panel. For laboratory D, no contamination of extraction control specimens consisting of buffer and salmon sperm DNA was detected in other work. In those studies, positive control specimens, KS lesions, and any other samples except semen from HIV-negative donors were processed in dedicated rooms

with dedicated equipment (17, 18). Nonetheless, because KS lesions possibly harbor HHV-8 at copy numbers higher than those included in the positive controls, and because it is difficult to preidentify all high-copy-number specimens, laboratory methods need to be sufficiently rigorous for this level of DNA load not to be a source of contamination.

Although it is impossible to extrapolate unequivocally from these results to previously published results, it is worth noting that two of the laboratories that identified negative controls as positive previously reported high prevalences of HHV-8 in semen; those that reported all negative controls as negative have reported low HHV-8 DNA prevalence in semen. Geographic or population-based differences in HHV-8 presence in semen remain possible explanations for the previously published observations from Italy (17). In further studies, both DNA samples and aliquots of unprocessed semen from Italy were found to be HHV-8 DNA positive in a blinded analysis performed in the United Kingdom (19).

(ii) Several independent observations suggest that a previously unrecognized variable in analyses of semen specimens from AIDS patients is oligospermia, or reduced sperm production, which is sometimes observed in HIV-infected men (21). Consistent with this hypothesis, one laboratory found that no sperm were visible by light microscopy in two of the seven specimens from HIV-positive men, and two laboratories noted that one of these specimens was clear, rather than having the customary milky appearance of semen; very small cell pellets were visible after centrifugation. Subsequent studies should control for volume, semen cell count, and sperm concentration.

(iii) DNA extracted from approximately 7.5 μ l of neat semen was analyzed in each solution PCR. Thus, DNA extracted from an equivalent of about 37.5 μ l of neat semen from lysates that represented a total neat semen volume of 262.5 μ l from each specimen was analyzed in the five runs for which there was no evidence for contamination. The results can be interpreted to indicate that HHV-8 DNA is occasionally present in the semen of both healthy donors and HIV-infected men (7 and 14% of ejaculates, respectively) but at concentrations that preclude its frequent or reliable detection by PCR (1.3 and 6% of the corresponding reactions were positive, respectively). However, given that no specimen was positive in more than one assay and that the positive assays were not confirmed with a different primer set or by sequence analysis, we cannot exclude the possibility that the positive results in the "clean" experiments resulted from low-level contamination.

(iv) An additional variable that should be considered in subsequent studies of semen specimens obtained from artificial-insemination banks is the possibility that the human serum albumin used in the semen stabilization buffer is contaminated (perhaps intermittently and at a low concentration) with HHV-8 DNA present in the pooled serum from which albumin is prepared. Although this may seem unlikely, the frequent detection of HHV-8 DNA in the blood of KS and AIDS patients, plus the recent description of a blood donor from whose blood HHV-8 could be repeatedly cultured (3), lends credence to this hypothesis and suggests that albumin used to prepare semen stabilization buffers should be tested for the presence of HHV-8 DNA.

This study offers several lessons. It is clear that PCR contamination of semen samples can and does occur. This contamination appears to be related to the low copy numbers of HHV-8 DNA in semen and the use of nested PCR methods for their amplification. Experimental designs need to include sufficient negative controls to allow discrimination of true-positive results from intermittent false-positive results. We recom-

mend the following: (i) confirmation of positive PCR results by amplification of at least a second genetic locus and by performing nucleotide sequence heterogeneity analyses to demonstrate that experimental amplimers are derived from independent sources, (ii) not processing materials with known or presumed high viral DNA loads together with specimens with low viral DNA loads, and (iii) assessment of incorporation of biochemical methods for amplimer inactivation, such as uracil incorporation or digestion, into PCR procedures. *In situ* PCR methods need to be tested in blinded comparisons similar to those done in this study. This study emphasizes the importance of performing blinded, multi-institution experiments to provide a coherent basis for comparing results and to motivate standardization of methods.

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