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Coinfection with Human Herpesvirus 6 Variants A and B in Lung Tissue

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Human herpesvirus 6 (HHV-6) variant B is frequently identified in peripheral blood, but identification of HHV-6 variant A is relatively rare. We devised a PCR-based method for sensitive, simultaneous detection of both HHV-6 variants. The method was applied to 34 lung tissue specimens that were previously shown to contain HHV-6 DNA. A total of 22 lung tissue samples showed coinfections with HHV-6 variants A and B, 2 had only HHV-6 variant A DNA, and 10 had only HHV-6 variant B DNA. The prevalences of coinfections in lung tissues from healthy controls (54% coinfected) and in those from bone marrow transplant patients with pneumonia (67% coinfected) were similar. These data indicate that coinfections of HHV-6 variants A and B commonly occur in lung tissues of healthy and diseased individuals.

Young children with primary human herpesvirus 6 (HHV-6) infections typically have HHV-6 variant B isolated from their peripheral blood cells (18). In contrast, HHV-6 variant A is only infrequently isolated from children with primary infections and occasionally from immunosuppressed patients (20). The prevalence of HHV-6 variant A in peripheral blood appears to be lower than that of HHV-6 variant B infection, although the natural history of HHV-6 variant A remains largely unexplored.

Variant A and B sequences diverge by about 4% (2, 11, 22), enabling the variants to be distinguished on the basis of restriction fragment length polymorphisms. Differential reactivities with monoclonal antibodies (2, 8, 10), in vitro growth characteristics (17), and PCR (2, 4, 21, 31) have also been used to identify HHV-6 variants.

We and others have previously shown that HHV-6 DNA is commonly present in lung tissues from both healthy and diseased individuals (12). Amplification of HHV-6 DNAs from lung tissue specimens from 15 bone marrow transplant patients with pneumonia and 15 victims of sudden death showed that 29 of 30 contained HHV-6 DNA; the one HHV-6 DNA-negative lung specimen was from an HHV-6-seronegative infant. This report describes the use of a newly developed PCR-based identification method to evaluate which HHV-6 variants are present in HHV-6 DNA-containing lung tissues.

(Portions of this work have been presented previously [15a]).

MATERIALS AND METHODS

Subjects. With variant-specific PCR, lung tissue specimens from 34 subjects, including 15 bone marrow transplant patients with pneumonia, 13 organ donors who were victims of sudden death, 5 surgical patients undergoing partial lung resections, and 1 autopsy patient, were studied (Table 1). Details for the 15 bone marrow transplant patients and 12 of the victims of sudden death (subjects C1 to C12) were previously reported (12). One organ donor, subject C13, died from a myocardial infarction. The five surgical resections involved cases of pulmonary carcinoid tumor (subjects S1 and S3), pulmonary aspergillosis (S2), chronic obstructive pulmonary disease (S5), and lung adenocarcinoma (S6). The autopsied patient (S4) died at home of tricuspid atresia. In the five surgical cases and one autopsy case, grossly normal areas of lung tissue were sampled for PCR. Research use of all human specimens in this study was approved by our Institutional Review Board.

Variant identification. Variant-specific identification was accomplished by using a dimorphic region of the HHV-6 large tegument protein (LTP) gene in HHV-6 strain SIE previously characterized by Aubin et al. (2, 3). The 249-bp SIE-1 and SIE-2 (SIE-1/2) product contains seven polymorphisms that consistently differentiated HHV-6 variant A from HHV-6 variant B (Fig. 1). DNA samples from 10 previously cultivated and characterized HHV-6 strains (U-1102 [25], GS [29], SIE [1], Z-29 [6], MA and TA [24], HTLHV [5], SF [26], and H622 and H623 [11]) were used to test the specificity and sensitivity of the variant-common and variant-specific primers and probes.

The results for a subset of specimens were verified by repeating the procedures with freshly extracted DNA from separate tissues that had never been exposed to the PCR laboratory. Stringent precautions to eliminate contamination included use of separate laboratories and equipment for pre- and postamplification procedures, with restricted entry and use of surgical caps, shoe covers, fresh gowns, face masks, and gloves. Amplifications of pure HHV-6A DNA by variant B-specific methods never revealed variant B contamination and vice versa.

Variant identification method 1: variant-common amplification of LTP gene with variant-specific probes. Variant-common amplification was done with SIE-1/2 PCR primers (2, 3), which amplify a 249-bp region of the LTP (Fig. 1 and Table 2). We redesigned the primer sets previously reported by Aubin et al. (3) to reduce self-annealing and false priming. In our hands, the redesigned SIE-1/2 primer set reported here was substantially more amplification efficient than previous primer sets for this genomic region (data not shown). Detection of HHV-6 in lung tissues was done as previously reported, using different HHV-6 primers (12, 13, 15). Frozen tissue sections were digested with proteinase K, extracted with phenol and chloroform, and precipitated with glycogen and ethanol. Amplification reactions were initiated by using hot start. The final concentrations and amounts in the amplification reaction mixtures were 12.5 mM Tris (pH 8.3), 62.5 mM KCl, 2.5 mM $MgCl_2$, 2 U of *Taq* polymerase (Perkin-Elmer), 10% glycerol, 5×10^{13} copies of each primer (0.83 μM), and 200 μM (each) deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP). The temperature settings were 99°C for 3 min, 40 cycles of 55°C for 1 min, 72°C for 1 min, and 99°C for 1 min, and finally 72°C for 10 min.

Amplification products were detected by liquid hybridization as previously described (16). The formamide concentrations were optimized for each probe: variant-common detection used the LTP-Com probe (Table 2) with 32% formamide, HHV-6 variant A detection used the LTP-A2 probe with 20% formamide, and HHV-6 variant B detection used the LTP-B2 probe with 27% formamide. Hybridization of the 249-bp SIE-1/2 product with the other variant-specific probes, LTP-A3 and LTP-B3 (Fig. 1 and Table 2), produced results equivalent to those of hybridization with LTP-A2 and LTP-B2. The hybrids were detected by electrophoresis in a nondenaturing acrylamide gel that was dried and autoradiographed (14).

One negative control was included for each specimen during the first round of PCR, when the presence of HHV-6 DNA was first documented (12). Subsequent amplifications for variant identification, as reported here, included at least one negative control (PCR reagents without added DNA) per run. Positive controls included HHV-6 strains U-1102 (19) and Z-29 (6).

Variant identification method 2: variant-specific PCR of LTP gene. Variant-specific amplification was done with two primer sets: HHV-6 variant A DNAs

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TABLE 1. Results of variant identification in lung tissues by methods 1 and 3[a]

Virus strain or subject[b]	Result with the indicated probe and DNA digestion					HHV-6 variant[c]	HHV-6 DNA level[d]
	LTP-Com, none	LTP-A2		LTP-B2			
		None	*Taq*I	None	*Taq*I		
U1102	+	+	+	−	−	A	
Z-29	+	−	−	+	−	B	
1	+	+	+	−	−	A	1,000,000
2	+	−	−	+	−	B	320,000
3	+	+	+	+	−	A, B	280,000
4	+	+	+	+	−	A, B	63,000
5	+	+	+	+	−	A, B	28,000
6	+	−	−	+	−	B	20,000
7	+	+	+	+	−	A, B	930
8	+	+	+	+	−	A, B	740
9	+	+	+	+	−	A, B	630
10	+	+	+	+	−	A, B	320
11	+	+	+	+	−	A, B	140
12	+	−	−	+	−	B	31
13	+	+	+	+	−	A, B	15
14	+	−	−	+	−	B	13
15	+	+	+	+	−	A, B	3
C1	+	−	−	+	−	B	1,000
C2	+	−	−	+	−	B	1,000
C3	+	+	+	+	−	A, B	500
C4	+	−	−	+	−	B	500
C5	+	+	+	+	−	A, B	200
C6	+	+	+	+	−	A, B	100
C7	+	+	+	+	−	A, B	100
C8	+	+	+	+	−	A, B	100
C9	+	+	+	+	−	A, B	100
C10	+	+	+	+	−	A, B	100
C11	+	−	−	+	−	B	100
C12	+	−	−	+	−	B	20
C13	+	−	−	+	−	B	5,000
S1	+	+	+	−	−	A	20
S2	+	+	+	+	−	A, B	10
S3	+	+	+	+	−	A, B	1,000
S4	+	+	+	+	−	A, B	500
S5	+	+	+	+	−	A, B	50
S6	+	+	+	+	−	A, B	500

[a] Method 1, variant-common PCR with variant-specific probes (no DNA digestion); method 3, variant-common PCR with *Taq*I digestion.

[b] Subjects 1 to 15, bone marrow transplant patients with pneumonia; C1 to C13, accidental death victims; and S1 to S6, surgical patients.

[c] Consensus variant identifications.

[d] Expressed as HHV-6 genome equivalents per million cell genome equivalents, as previously reported (12).

were amplified with primers LTP-A1 and LTP-A2, resulting in a 146-bp product that was detected by hybridization with the LTP-A3 probe, and HHV-6 variant B DNAs were amplified with primers LTP-B1 and LTP-B2, resulting in a 147-bp product detected with the LTP-B3 probe (Table 2). Variant-specific amplification methods were similar to those described above except that HHV-6 variant A cycling temperatures were 47, 62, and 99°C and LTP-A3 hybridizations used 20% formamide, and HHV-6 variant B cycling temperatures were 51, 68, and 99°C and LTP-B3 hybridizations used 24% formamide.

Variant identification method 3: variant-specific digestion with *Taq*I. Two *Taq*I endonuclease restriction sites in the 249-bp SIE-1/2 product were identified from previously published sequence data (2) (Fig. 1). One site near the SIE-1 primer is conserved between HHV-6 variants. Therefore, that site is cut by *Taq*I regardless of whether the PCR products are derived from variant A or variant B. The other *Taq*I site falls within the LTP-B2 probe sequence, and only HHV-6 variant B DNAs are susceptible to *Taq*I cutting at that site (Fig. 1).

The SIE-1/2 PCR products from each specimen were separately hybridized six times: hybridizations with the LTP-Com, LTP-A2, and LTP-B2 probes were performed on *Taq*I-digested and undigested PCR products (Fig. 2).

Variant identification method 4: variant-common amplification of NGH with variant-specific probes. Variant-common amplification of the glycoprotein H gene (NGH) was essentially similar to the procedure described above for variant-common amplification of the LTP gene and variant identification by variant-specific probes. Sequence data comprising the amino-terminal end of the NGH from multiple strains (22) was used to generate conserved primers (Table 2) that amplify both HHV-6 variants, producing a 417-bp product. The variant A-specific probe, NGH-AP, and the variant B-specific probe, NGH-BP, specifically identified PCR products from laboratory strains of A and B variants, respectively, whereas the variant-common probe, NGH-Com, identified PCR products from both variants. Amplification conditions were the same as for variant-common PCR with the LTP primers except that cycling was performed at 97, 50, and 72°C. Liquid hybridizations with the variant-specific NGH probes NGH-AP, NGH-BP, and NGH-Com were carried out in 20, 17, and 24% formamide, respectively, using the hybridization temperatures described above.

RESULTS

Characterization of PCR for variant identification. All of the specimens in this study were shown to contain HHV-6 DNA by quantitative PCR with the 5R HHV-6 primer set, as previously described (Table 1) (12, 15). Quantitative determinations of HHV-6 DNA for subjects 1 through 15 and C1 through C12 were previously published (12). Previously unpublished quantitation of HHV-6 DNA for subjects C13 and S1 through S6 was done by the same method.

All 10 of the previously characterized HHV-6 isolates were successfully detected with the SIE-1/2 (variant-common) primer set and the SIE-Com probe. SIE-1/2 PCR products from three variant A isolates (U1102, SIE, and GS) hybridized with the variant A-specific probes (LTP-A1 and LTP-A3), and SIE-1/2 PCR products from seven variant B isolates (Z-29, MA, TA, HTLHV, SF, H622, and H623) hybridized with the HHV-6 variant B-specific probes (LTP-B2 and LTP-B3) (prototype variants shown in Fig. 2). Similar characterization of the NGH-1 and NGH-2 primers and NGH variant-specific probes also showed specificity with the characterized strains. However, the NGH amplifications were generally less consistent among various strains than amplifications with the LTP primer sets. This is apparently related to the variable primer binding sites in the NGH region: two nucleotide variations in the primer binding sites were observed among 10 HHV-6 isolates that were previously characterized (22), and better amplification efficiency was observed with the LTP primers. Dilutions of purified HHV-6 DNA indicated sensitivities of at least 10 genomes with the LTP primer sets and 100 genomes with the NGH primers. None of the HHV-6 primer sets amplified other herpesvirus DNA (HSV-1, HSV-2, Epstein-Barr virus, cytomegalovirus, or varicella-zoster virus) or human genomic DNA. The HHV-6 variant A- and variant B-specific probes did not cross-hybridize with HHV-6 variant B and variant A PCR products, respectively. Mixing experiments using purified HHV-6 variant A and B DNA showed that coamplification of the variants produced specific bands for both variants when as little as 1% of the viral DNA was from one variant. When 100 copies of HHV-6 variant A DNA were mixed with a 100-fold excess (10,000 copies) of HHV-6 variant B DNA, the HHV-6 variant A products were clearly present after amplification, and the reverse was also true.

The variant-specific hybridization results were confirmed with two additional methods. First, the variant-common products were digested with *Taq*I, cutting HHV-6 variant A products once and cutting HHV-6 variant B products twice (Fig. 1 and 2). *Taq*I digestion of HHV-6 variant B products eliminated hybridization with the HHV-6 variant B-specific probe, but such digestion of HHV-6 variant A products did not affect hybridization with the HHV-6 variant A-specific probe (Fig. 2 and Table 1). Second, variant-specific amplification was accomplished with two other primer sets, LTP-A1 with LTP-A2

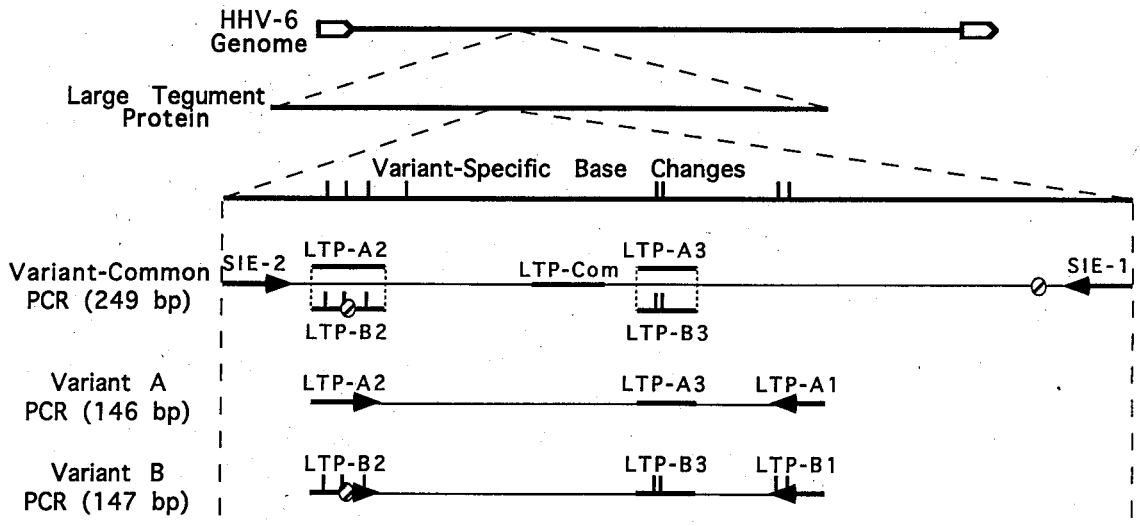

FIG. 1. Diagram of primers and probes. Arrow, primer; short horizontal thick lines, probes; hatched circles, *Taq*I sites.

and LTP-B1 with LTP-B2. Weak cross-reactivity was observed when more than 10,000 HHV-6 variant A genomes were primed with the HHV-6 variant B primer set and probed with the variant-common primer and vice versa, as detected by the variant-common probe. However, when variant-specific probes (LTP-A3 and LTP-B3) were used to identify the variant-specific PCR products, completely specific variant detection was achieved.

The NGH primer set produced variant-common products from another part of the viral genome, and variants were identified by using variant-specific probes. The specificity of this approach was also tested with the HHV-6 strains mentioned above; the NGH primer set identified all tested strains successfully.

Variant-specific analyses of lung tissues. The 34 lung tissue samples from the bone marrow transplant patients and controls were evaluated separately by three variant identification methods described above: variant-common PCR with variant-specific probes, variant-common PCR with *Taq*I digestion, and variant-specific PCR with variant-specific probes (Table 3). Detailed results of the variant-common PCR and *Taq*I digestion are presented in Table 1. The results of variant identification were identical for all three methods except for patient 1.

Fourteen of 15 lung specimens from the bone marrow transplant patients were further tested by variant-common amplification of the amino terminus of the NGH with variant-specific probes of the PCR products. Complete concurrence among all four methods was demonstrated for 7 of 14 specimens, including five variant A and B coinfections and two variant B-only infections. In 5 of 14 specimens (patients 7, 10, 11, 13, and 15), the NGH method failed to detect variant A when the other methods indicated coinfection. For patients 1 and 2, the NGH primer set detected coinfection with variants A and B, although the variant B signals were weak in both cases. All three LTP-based methods detected only variant B in lung tissues from patient 2, while the NGH method detected both variants. This discrepancy could be due to a contamination event, although the coinfection was verified by performing the NGH method on two different lung tissue samples from this patient. Alternatively, the coinfection detected by the NGH method could be due to sequence variation between strains in the NGH region that was not present in the LTP region. We feel that the three methods using the LTP gene described above are the best measure of HHV-6 variants because the NGH region had sequence variability in the primer binding sites and less efficient amplification, as described above.

To summarize the variant identification results for all control and pneumonic lung specimens, 2 of the 34 (6%) contained only HHV-6 variant A DNA, 10 (29%) contained only variant B DNA, and 22 (65%) had both variant A and variant B genotypes. Patients with only variant B had a median HHV-6 DNA level of 750 HHV-6 genome equivalents per million cell equivalents (range, 13 to 320,000), and those with HHV-6 variant A and B coinfections had a median HHV-6 DNA level of 260 (range, 3 to 280,000). The highest level of HHV-6 DNA was found in patient 1, whose lung tissue had only variant A. The prevalence of HHV-6 variant A and B coinfections was not substantially different for samples from the bone marrow transplant patients with pneumonia (10 of 15 [67%]) and the healthy lung tissue specimens (7 of 13 [54%]) and surgically resected tissue specimens (5 of 6 [83%]).

DISCUSSION

Our data indicate that coinfection of human lung tissue with both HHV-6 variants is common. Sixty-five percent of the 34 lung tissue specimens examined in this study contained viral

TABLE 2. Primer and probe sequences for variant-specific HHV-6 detection

Oligonucleotide	Sequence (5′→3′)
SIE-1	GAT CCG ACG CCT ACA AAC AC
SIE-2	TAC CGC ATC CTT GAC ATA TTA C
LTP-Com	TAG GAA AAG ATT TGA GAT CGT GAA A
LTP-A1	GGC TGA TTA GGA TTA ATA
LTP-A2	ACA GAA GTT GAC CTC ATT
LTP-A3P	TCT AAT TTC AAC TGG ACC
LTP-B1	GGG CTG ATT AGG ATT CAT G
LTP-B2	ACG GAA GTC GAC CTG ATT
LTP-B3P	TCT CAA TTC AAC TGG ACC
NGH-1	TCC TCA AGT GGT GAA ACT GTC CAA T
NGH-2	ATC GGA GCC GAG TAA GCA TTT AGG G
NGH-AP	TCG AGC CAT TGT AGA AAT
NGH-BP	GAG AGC CAT TGT AAA AAT
NGH-Com	ACG ACA CTC GGA TAT ATC

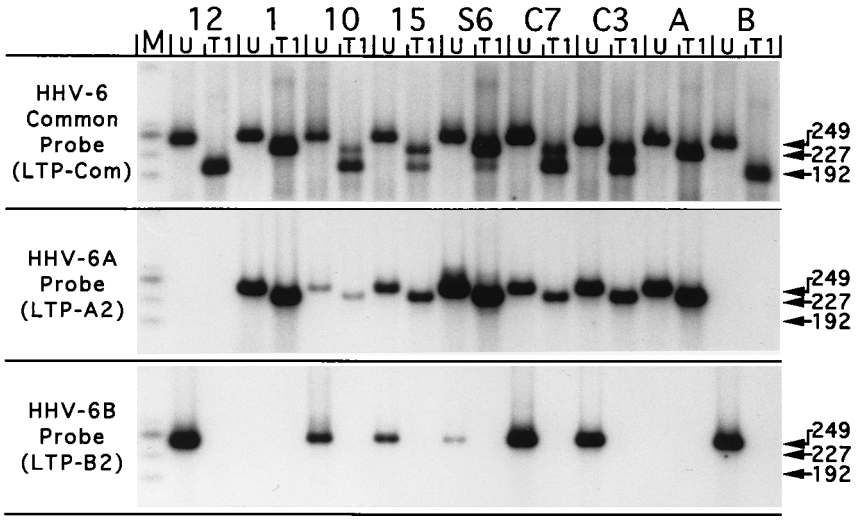

FIG. 2. Autoradiogram of hybridized PCR products from selected amplification reactions. Lanes contain undigested (U) or *Taq*I-digested (T1) products or ^{32}P-end-labeled ϕX173 *Hae*III-cut DNA size markers (M). Each pair of undigested and digested PCR products is designated as in Table 1. A and B, prototype HHV-6 variants A (U1102) and B (Z-29), respectively. Products from each amplification reaction were hybridized with a variant-common probe (LTP-Com), variant A-specific probe (LTP-A2), or variant-B specific probe (LTP-B2). The full-length SIE-1/2 variant-common product appears at 249 bp (all three panels), the *Taq*I-digested variant A product appears at 227 bp (top and middle panels), and the *Taq*I-digested variant B product appears at 192 bp (top panel). *Taq*I digestion abrogates hybridization of the variant B probe with variant B PCR products, as shown in the bottom panel and predicted by the diagram in Fig. 1.

DNAs from both HHV-6 variants. Overall, 71% of lung tissues contained HHV-6 variant A DNA (including single and double infections). Lung tissues from healthy and diseased individuals had similar prevalences of HHV-6 variant A and B coinfections. Also, the HHV-6 DNA levels in lung tissues of patients with single versus double infections were similar.

While these data demonstrate the presence of both HHV-6 variants in lung tissues, the cell types harboring these viruses were not identified in this study. Immunocytochemistry of postmortem specimens from bone marrow transplant patients with pneumonitis showed HHV-6-reactive antigens in pulmonary lymphocytes (9), macrophages, and pneumocytes (28). One previous study used a variant-specific monoclonal antibody to detect the presence of two variants in lung tissue from an infant with pneumonitis (23). The potential for more than one strain to infect the same patient is reminiscent of similar findings with cytomegalovirus (27).

TABLE 3. Summary of variant identification results for lung tissues from bone marrow transplant patients

Patient	Variant identification by method[a]:			Consensus
	1	2	3	
1	A	AB	A	A
2	B	B	B	B
3	AB	AB	AB	AB
4	AB	AB	AB	AB
5	AB	AB	AB	AB
6	B	B	B	B
7	AB	AB	AB	AB
8	AB	AB	AB	AB
9	AB	AB	AB	AB
10	AB	AB	AB	AB
11	AB	AB	AB	AB
12	B	B	B	B
13	AB	AB	AB	AB
14	B	B	B	B
15	AB	AB	AB	AB

[a] Variant identification methods: 1, variant-common amplification of LTP gene with variant-specific probes; 2, variant-specific amplification of LTP gene with variant-specific probes; 3, variant-common amplification of LTP gene with variant-specific *Taq*I digestion. AB, variants A and B.

We can say little about other potential reservoirs of coinfection by variants A and B in these subjects. Unfortunately, matched peripheral blood mononuclear cell specimens were not available from any of the 34 subjects whose lungs were studied. In another study, we were able to detect only variant B in peripheral blood mononuclear cells of bone marrow transplant patients. Further studies defining the location of variant A in immunocompromised patients are needed. These findings are consistent with previous studies that also identified HHV-6 variant B as the predominant variant in peripheral blood mononuclear cells from bone marrow transplant patients (21, 30) and from infants undergoing primary infection (18). However, the variant A genome was more common in Kaposi's sarcoma tissues (7).

Our variant-specific PCR methods provide a high degree of specificity and sensitivity; three methods were used to obtain results for all specimens, and four methods were used for 14 of the 15 bone marrow transplant specimens. First, variant-common amplification products were separately probed with HHV-6 variant A-specific and variant B-specific oligonucleotides. This provides positive identification of both variants, in contrast to some previously published single restriction digest methods that rely on the absence of digestion to identify one of the variants (17). Second, the *Taq*I restriction method validated the identity of the PCR products and confirmed the presence of coinfections. Third, every specimen was independently amplified with variant-specific PCR primers, also confirming the results of the other two tests. Fourth, amplification of a different gene, the NGH, was also performed. Variant detection with the NGH primer set failed to detect some coinfections identified by the other three methods, probably because of sequence variation in the NGH and inefficient amplification by the NGH primers. Thus, several polymorphisms were assessed before the variant identification was assigned. Therefore, our PCR-based variant identification method appears to provide reliable documentation of coinfections that

may be missed by other methods. Having now documented agreement among the three LTP-based methods reported here, we feel that the use of just the SIE-1/2 primers with variant-specific probes is sufficient for future variant identification.

In conclusion, HHV-6 variants A and B coinfected lung tissues from patients with pneumonia, patients undergoing partial lung resection, and otherwise healthy victims of accidental death.

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