Comparison of Serologic Assays and PCR for Diagnosis of Human Herpesvirus 8 Infection

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A variety of assays for the diagnosis human herpesvirus 8 (HHV-8) infection have been reported. We compared several such assays with a panel of 88 specimens from human immunodeficiency virus (HIV)-infected patients with Kaposi’s sarcoma (KS) (current-KS patients; n = 30), HIV-infected patients who later developed KS (later-KS patients; n = 13), HIV-infected patients without KS (no-KS patients; n = 25), and healthy blood donors (n = 20). PCR assays were also performed with purified peripheral blood mononuclear cells (PBMCs) to confirm positive serologic test results. The order of sensitivity of the serologic assays (most to least) in detecting HHV-8 infection in current-KS patients was the mouse monoclonal antibody-enhanced immunofluorescence assay (MIFA) for lytic antigen (97%), the orfK8.1 peptide enzyme immunoassay (EIA) (87%), the orf65 peptide EIA (87%), MIFA for latent antigen (83%), the Advanced Biotechnologies, Inc., EIA (80%), and the orf65 immunoblot assay (80%). Combination of the results of the two peptide EIAs (combined peptide EIAs) increased the sensitivity to 93%. For detection of infection in later-KS patients, the MIFA for lytic antigen (100%), the orfK8.1 peptide EIA (85%), and combined peptide EIAs (92%) were the most sensitive. Smaller percentages of no-KS patients were found to be positive (16 to 56%). Most positive specimens from the current-KS and later-KS groups were positive by multiple assays, while positive specimens from the no-KS group tended to be positive only by a single assay. PCR with PBMCs for portions of the HHV-8 orf65 and gB genes were positive for less than half of current-KS and later-KS patients and even fewer of the no-KS patients. The concordance between serologic assays was high. We propose screening by the combined peptide EIAs. For specimens that test weakly positive, we recommend that MIFA for lytic antigen be done. A positive result with a titer of $\geq 1:40$ would be called HHV-8 positive. A negative or low titer would be called HHV-8 negative. If a population has a high percentage of persons who test positive by the combined peptide EIAs, then a MIFA could be performed with the negative specimens to determine if any positive specimens are being missed. Alternatively, if a population has a low percentage that test positive, then a MIFA could be performed with a subset of the negative specimens for the same reason. As described above, only a titer of $\geq 1:40$ would be considered HHV-8 positive.

Following the discovery in 1994 of human herpesvirus 8 (HHV-8) and its association with Kaposi’s sarcoma (KS) (7, 11, 14, 30, 31), body-cavity-based lymphoma (BCBL) or primary effusion lymphoma (PEL) (6), and multicentric Castleman’s disease (41), a variety of laboratory assays have been developed for the detection of HHV-8 infection. These include (i) serologic assays for HHV-8 antibodies such as immunofluorescence antibody assays (IFAs) against both the lytic and latent antigens of the virus (12, 18, 20, 21, 24, 28, 39, 40; J. J. Goedert, D. H. Kedes, and D. Ganem, Letter, Lancet 349: 1368, 1997), (ii) immunoblot assays with a variety of viral proteins (12, 28), and (iii) enzyme immunoassays (EIAs) with either whole viral lysates (8), synthetic peptides (9, 33), or recombinant peptide-carrier protein conjugates (1, 40). PCR-based assays have also been used to detect viral DNA in a variety of tissues and body fluids (6, 7, 38, 41) including peripheral blood mononuclear cells (PBMCs) (16, 26, 45), lymph node biopsy specimens (3), plasma (21, 26), serum (2, 26, 45), sputum (45), saliva (4, 5, 21), nasal secretions (4), prostate biopsy specimens (10, 29), and semen (13, 15, 26, 44) (results of tests with the last two types of specimens are controversial [10, 21, 35, 43; J. A. Ambroziak, D. J. Blackbourn, B. G. Herndier, R. G. Glogau, J. H. Gullert, A. R. McDonald, E. T. Lennette, and J. A. Levy, Letter, Science 268: 582–583, 1995]). Viral culture is currently used primarily to investigate virus-host interactions and is not practical for diagnostic purposes (32). To evaluate assays for use for the diagnosis of HHV-8 infection, we conducted a comparative study of several different types of assays and developed an algorithm that would be useful for clinical testing.

MATERIALS AND METHODS

Study populations. Serum and PBMCs from four groups of individuals who participated in Centers for Disease Control and Prevention studies conducted between 1983 and 1994 were tested. The first group comprised 30 men who had reported that they had had sex with men (MSM) and who had clinical KS at the time of specimen collection; all but 1 of these men were human immunodeficiency virus (HIV) seropositive (current-KS patients). PBMCs were available from all but one patient. The second group comprised 13 HIV-positive MSM who did not have clinical KS at the time of specimen collection but who later developed KS (within 5 years) (later-KS patients). The third group comprised 25 HIV-seropositive MSM who did not have clinical KS and who did not develop KS in the 5 years after specimen collection (no-KS patients). The fourth group comprised 20 HIV-negative healthy blood donors, 10 men and 10 women (controls). All patients were residents in the Atlanta, Ga., metropolitan area.

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MIFA. Antigen and slides were prepared for the monoclonal antibody-enhanced immunofluorescence assay (MIFA), by the procedure of Lennette et al. (24), with minor modifications. Briefly, BCBL-1 cells (AIDS Research and Reference Reagents Program, National Institutes of Health, Bethesda, Md.) were passaged 1:10 in medium with or without 20 ng of tetradecanoyl phorbol ester acetate (TPA; Gibco BRL, Grand Island, N.Y.) per ml and were cultured for 6 days. Cells were distributed on slides (50 µL/well), air dried, fixed in cold acetone for 5 min, and then stored at −20°C. Slides were incubated with human serum (1:10 dilution in phosphate-buffered saline) at 37°C for 30 min, washed, and incubated with mouse anti-human immunoglobulin G (IgG) hybridoma supernatant (HP6508; American Type Culture Collection) at 37°C for 30 min. The cells were then incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Cappel, Durham, N.C.) at a 1:100 dilution in phosphate-buffered saline at 37°C for 30 min, washed, and air dried. Coverslips were mounted with buffered glycerol (BBI Microbiology Systems, Cockeysville, Md.), and the slides were stored in the dark.

Positive staining by MIFA for lytic antigen (MIFA lytic) was typically a bright uniform fluorescence present in 1 to 10% of the cell population. Positive staining by MIFA for latent nuclear antigen (MIFA latent) was of lower intensity and punctate and was usually present in 50 to 90% of the cells.

orf65 IB assay. The immunoblot (IB) assay was performed by the method of Yamamoto et al. (46) but was modified as follows: the HHV-8 orf65 C-terminal region (264 bp; orf65 gene coordinates, nucleic acids 256 to 519) was expressed as an epitope-tagged fusion protein in E. coli and was then partially purified by preparative electrophoresis. Partially purified protein was separated in 15% polyacrylamide gels and was then transferred to nitrocellulose sheets.

The sheets were treated with serum samples diluted 1:10 by using an IB manifold (Bio-Rad Laboratories, Hercules, Calif). Gel strips were incubated at room temperature for 3 h and then at 4°C overnight. The blot was washed and then incubated with anti-IgG–alkaline phosphatase conjugate (Bio-Rad Laboratories) (1:10,000) at room temperature for 2 h. The blots were developed with alkaline phosphatase substrate for 20 min, washed, and then air dried. The antibody reactivity of each sample was determined by comparison to the reactivities of positive controls (antibody to an enterokinase recognition site on the fusion protein [Invitrogen, San Diego, Calif.] and a KS-positive patient serum specimen). The band of interest corresponds to a protein with a molecular mass of approximately 28.6 kDa.

ABI EIA. The HHV-8 IgG antibody enzyme-linked immunosorbent assay (ELISA; a gift of Dharam Ablashi, Advanced Biotechnologies, Inc. [ABI], Columbia, Md.) was performed as directed in the instructions for use (ELISA; a gift of Dharam Ablashi, Advanced Biotechnologies, Inc. [ABI], Columbia, Md.). The band of interest corresponds to a protein with a molecular mass of approximately 28.6 kDa.

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Peptide EIA. Peptides were synthesized according to the manufacturer’s protocol on an automatic synthesizer (model 432A; Applied Biosystems, Foster City, Calif.), partially purified by reverse-phase high-performance liquid chromatography (Bio-Rad Laboratories), lyophilized, and stored desiccated at room temperature until use. The orf65 peptide was derived from the C-terminal portion (amino acids 140 to 170) of the gene product (33). Its sequence is ASLTTLASS TTETAAAPAV DARKPSSGSKK K. The orfKs1 peptide was derived from the N-terminal portion (amino acids 32 to 62) of the gene product (36). Its sequence is RSHLFGQWEG WSGQVYQYDL GRMNCSYENMT.

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Positive control sera for peptide EIA were followed (34). The mean corrected optical density at 450 nm for the 20 control specimens plus 5 standard deviations was arbitrarily chosen as the assay cutoff for each peptide. The results of the two individual peptide EIAs were combined into a new “assay” for each individual sample. Samples of either of the two peptide EIAs was positive, the new assay (combined peptide EIAs) was considered positive.

The titers in samples positive by MIFA lytic, orfKs1 peptide EIA, and orf65 peptide EIA were also determined: the MIFA lytic at 3 twofold dilutions beginning with 1:10 and the peptide EIAs at 1:100, 1:400, 1:1,600, and 1:3,200.

PCR. DNA was purified from PBMC specimens with the QIAamp blood kit (QIAGEN, Chatsworth, Calif.) by following the manufacturer’s directions, eluted with double-distilled water, and then quantified by fluorometry. The concentration of the purified DNA was then adjusted to 100 µg/ml, and 0.5 µg of DNA was used for each PCR.

We developed two primer sets (gB-N and gB-C) that target the gB gene (orf8) and that had detection limits of 5 to 10 copies of HHV-8 genomic DNA (estimated by serial dilutions of a BCBL-1 lysate). The gB-N (358-bp amplimer) primer sequences are 5′-GCCACCCGGGAGGCTGTT-3′ and 5′-TGGGTG ATGCGCGACTGTC-3′, respectively. For gB-C (534-bp amplifier), the sequences are 5′-ATGGCGGACTCTGTC-3′ and 5′-AATTACCCGGACCTCAGTCTTCC-3′. The outer primer set amplifies a 572-bp segment from the end of orf64 to the middle of orf65, while the inner primer set amplifies a 467-bp orf65 segment.

The orf64-orf65 PCR mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 200 µM each deoxynucleoside triphosphate, 1.25 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer Corp., Foster City, Calif.), and 30 pmol of each primer. Cycling parameters for the outer primer set were 95°C for 10 min for 1 cycle, 95°C for 45 s, and 72°C for 30 s for 40 cycles and 72°C for 7 min. The amplification primer of primary PCR amplification was used for nested PCR. Cycling parameters for the inner primer set were 95°C for 10 min for 1 cycle, followed by 95°C for 45 s and 70°C for 45 s for 40 cycles and 72°C for 7 min. The PCR products were separated by electrophoresis in a 1% agarose gel. They were stained with ethidium bromide and read under UV light.

PCR amplification for each primer set was performed by two different operators with two sets of independently coded specimens. In addition, each PCR operator had his or her own operational controls, which include three to four duplicate positive and negative controls. All positive control and negative control reactions were run with the same sample reaction mixture with water as a template for every 10 PCRs as negative controls. After breaking the code, if there was any trace of contamination, e.g., any negative control was identified as being positive, or the sensitivity of the experiment was low, the results were recalculated and the experiment was repeated. Samples for a specific primer set were called positive for purposes of test comparisons if the results of both operators were in agreement. If there was discordance between the operators, the results were called indeterminate.

RESULTS

Results of single serologic assays. HHV-8 serology results by assay for each study population are given in Table 1. The assays are presented in general order of positivity for the populations studied. HHV-8 antibodies were detected in 80 to 97% of the current-KS group, 54 to 100% of the later-KS group, 16 to 56% of the no-KS group, and 0 to 10% of the control group. Antibody titers were much lower in specimens from controls, who were positive only by the MIFA lytic and MIFA latent assays, than in the vast majority of specimens from the other groups (data not shown).

Table 2 compares the patterns of reactivity of the subjects by study group according to the different assays. Among the individuals in the current-KS group, two-thirds were positive by all the assays, but only two persons were positive by two or fewer assays. Although one current-KS patient was negative by all the assays, the clinical diagnosis of KS for this patient was based on a reported medical history and could not be documented (the institution where the diagnosis was made had since closed). Thus, it is possible that this KS diagnosis was erroneous. Among the individuals in the later-KS group, more than half were positive by five or more assays and all were positive by at least two assays. Among persons in the no-KS group, approximately one-third were positive by three or more assays, about half were positive by only one or two assays, and 16% were negative by all assays. Among the controls, most...
(75%) were negative by all assays, 25% were positive by only one assay, and none were positive by more than one assay.

**Concordance between assays.** The concordance between pairs of assays is shown in Table 3. The MIFA lytic and the combined peptide EIAs had the greatest concordance. Although the concordance between the combined peptide EIAs and the individual peptide EIAs was higher, this was expected because results from the individual assays were combined to give the combined peptide EIA results. The concordance rates between the MIFA lytic and combined peptide EIAs were highest when testing the current-KS and later-KS groups (97 and 92%, respectively) but were lower when testing the controls and no-KS groups (85 and 80%, respectively).

With regard to assay specificity, all specimens positive by only one of the two EIAs were positive by at least one other assay, suggesting that they are each specific. Of the specimens positive by the MIFA lytic and negative by the combined peptide EIAs, only 20% were positive by the other assays. At this time it is unclear whether the MIFA is more sensitive or less specific than the EIAs. Positive results by the MIFA lytic and the ABI EIA were all confirmed by other assays, suggesting that they are both specific. Among specimens positive by the orf65 IB assay, 13% (6 of 45) were not positive by any other assay, indicating a lower specificity for this assay.

We also performed limited tests for determination of titers in serum by the MIFA lytic and the two peptide EIAs. Relative antibody titers by the MIFA lytic tended to be higher for the current-KS and later-KS groups than for the no-KS group (1:10, 1:20) for the controls. Titers by the peptide EIAs tended to be higher for the current-KS and later-KS groups than for the no-KS group, but these differences were not statistically significant (data not shown).

**PCR results.** Fewer subjects were positive for HHV-8 by PCR (Table 4) than by the serologic assays. No control subject was PCR positive. About 40 to 50% of the current-KS group and about 30% of the later-KS group were positive by either PCR method. Very few (5 to 10%) of the subjects in the no-KS group were PCR positive.

**DISCUSSION**

**Sensitivity for detection of KS.** The order of sensitivity (from greatest to least) of the serologic assays for the detection of HHV-8 antibodies in KS patients was as follows: MIFA lytic, combined peptide EIAs, orfK8.1 peptide EIA, orf65 peptide EIA, MIFA latent, ABI EIA, and, last, orf65 IB.

**IFAs.** Most individuals with KS were positive by the MIFA lytic and multiple other assays. Positivity for lytic antigen alone was observed for 16% of the no-KS group and 10% of the controls. Whether these are true positives remains to be determined.

IFA for lytic antigens has generally used two basic methods: either MIFA as the modified method of Lennette et al. (24) or a standard IFA. In this study, we used the former, which uses a mouse anti-human IgG monoclonal antibody and TPA-induced BCBL-1 cells; the latter uses either butyrate or TPA-induced BC-1 or BCBL-1 cells. Previous results reported for

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**TABLE 1. HHV-8 serology results by assay and study population**

<table>
<thead>
<tr>
<th>Study population</th>
<th>No. of subjects</th>
<th>Mean age (yr [range])</th>
<th>MIFA lytic</th>
<th>orfK8.1 peptide EIA</th>
<th>orf65 peptide EIA</th>
<th>MIFA latent</th>
<th>ABI EIA</th>
<th>orf65 IB assay</th>
<th>Combined peptide EIAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current KS</td>
<td>30</td>
<td>37 (24–49)</td>
<td>97</td>
<td>87</td>
<td>87</td>
<td>83</td>
<td>80 (3)</td>
<td>80</td>
<td>93</td>
</tr>
<tr>
<td>Later KS</td>
<td>13</td>
<td>38 (29–50)</td>
<td>100</td>
<td>85</td>
<td>54</td>
<td>77</td>
<td>62 (15)</td>
<td>54</td>
<td>92</td>
</tr>
<tr>
<td>No KS</td>
<td>25</td>
<td>38 (27–55)</td>
<td>64</td>
<td>28</td>
<td>32</td>
<td>20</td>
<td>16 (4)</td>
<td>52</td>
<td>36</td>
</tr>
<tr>
<td>Controls</td>
<td>20</td>
<td>10</td>
<td>10</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Indeterminate values as per manufacturer’s definition are given in parentheses.

**TABLE 2. HHV-8 serology results, cumulative by study population**

<table>
<thead>
<tr>
<th>Assay result</th>
<th>No. of assays positive</th>
<th>No. of subjects with assay result</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIFA lytic</td>
<td>orfK8.1 peptide EIA</td>
<td>orf65 peptide EIA</td>
</tr>
<tr>
<td>Current KS</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td>Later KS</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>No KS</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Controls</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
<td>13</td>
</tr>
</tbody>
</table>

*Total number positive.

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patients with either African KS or HIV-positive individuals with KS are similar to our own, ranging from 96 to 100% by MIFA and from 65 to 100% by the standard assay (21, 28, 40). Among HIV-positive MSM without KS, assay results have ranged from 90 to 100% positive by MIFA and from 13 to 55% positive by standard IFA. Twenty percent of blood donors or healthy adults were HHV-8 positive by the MIFA, but none were positive by the standard IFA. In a comparison of HHV-8 serologic assays, Rabkin et al. (37) found that the MIFA lytic (93% sensitivity) and the IFA for lytic antigen (91% sensitivity) were best at detecting KS. The other assays—which included a whole-virus ELISA, a recombinant orf65 protein ELISA, an IFA for latent antigen (run at a 1:150 dilution), and the MIFA latent—were less sensitive (67 to 72%). The least sensitive was a recombinant orf26 (minor capsid antigen) protein ELISA.

Compared to the results found by Lennette et al. (24), the rates of HHV-8 infection in our study were comparable for KS patients but lower for HIV-positive MSM without KS and healthy controls. These differences may be due to differences in the prevalence of HHV-8 infection in the populations from which these specimens were drawn or differences in the ways in which the assays were run. Studies that have used other assays have failed to detect as high a rate of infection as Lennette et al. (24) found in HIV-positive men (90 to 100%) and healthy blood donors (20%). They also found a high rate of HHV-8 seropositivity in adult women (15% of 54). These findings have raised questions about the specificity of this assay since studies that have used other assays have found much lower rates in similar populations. Zhu et al. (47) reported data obtained by IFAs for both latent and lytic antigens. Among 104 HIV-positive MSM with KS, 84 and 99% were positive by these assays, respectively. Among 77 HIV-positive MSM without KS, these rates were 56 and 78%, respectively, while among 84 HIV-negative men (either blood donors or from the general population [46]), the rates were 8 and 12%, respectively.

In the HIV-positive KS group, only two of the individuals positive by the IFA for lytic antigen were not confirmed to be positive by the IB assay for a variety of HHV-8 recombinant proteins. We found some individuals (n = 8) who were positive by this assay but on no other assays, including PCR. Whether all of these persons are truly HHV-8 infected is unknown. In our study, however, the MIFA remains the most sensitive for individuals with KS, those who later developed KS, and HIV-positive individuals without KS.

**Peptide and other EIA.s.** The next most sensitive assays in our study were the peptide EIAs. Both were comparable in detecting HHV-8 infection among the current-KS and no-KS groups, while the orfK8.1 peptide assay was more sensitive in detecting infection in the later-KS group. All specimens positive by these assays were also positive by the MIFA lytic. Among specimens with discordant results between the peptide EIAs and the MIFA lytic, all were MIFA positive but were negative by one of the peptide EIAs. Of these, approximately half of those whose results were discordant with those of the orfK8.1 peptide EIA or the orf65 peptide EIA were positive by at least one other assay. This supports the contention that many specimens with discordant results represent true positives. Similarly, all specimens with discordant results between the two EIAs were positive by at least one other assay, suggesting that they are probably true positives. Use of the combined results of the individual peptide EIAs (combined peptide EIAs) increased the overall sensitivity over that obtained with the individual peptide EIAs and was the second most sensitive method for the detection of HHV-8 infection among current-KS and later-KS patients. Among specimens negative by the combined peptide EIAs and positive by the MIFA lytic, only 20% were confirmed to be positive by the other assays. This may be due to either the greater sensitivity or the lower specificity of the MIFA lytic.

Other data obtained by EIAs with peptides or recombinant proteins of HHV-8 are limited. Studies of assays with five recombinant antigens, orf65 (23, 39), orf26 (1), orfK8.1 (23), orf52 (23), and orfK12 (23), and an assay with an orf26 peptide coupled to bovine serum albumin (9) have been reported. The recombinant orf26 assay was the least sensitive and produced results the least concordant with those of other assays in a comparative study of HHV-8 serologic assays (37). In the original report, this assay was positive for only about one-third of HIV-positive persons with KS (1). The orf52 and orfK12 assays were also relatively insensitive in detecting infection in this group, although the latter assay was positive for 66% of HIV-positive MSM from the United States. The three assays detected HHV-8 in 60 to 82% of HIV-positive KS patients (with the exception of Lang et al. (23), who found that only 37% of HIV-positive KS patients were positive for HHV-8 by the orf65 assay). 94 to 100% of classic-KS patients, and 83% of

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### TABLE 3. Concordance between serologic assays

<table>
<thead>
<tr>
<th>Assay</th>
<th>MIFA lytic</th>
<th>MIFA latent</th>
<th>orfK8.1 peptide EIA</th>
<th>orf65 peptide EIA</th>
<th>orf65 IB assay</th>
<th>Combined peptide EIAs</th>
<th>ABI EIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIFA lytic</td>
<td>100</td>
<td>73 (0.485)</td>
<td>84 (0.682)</td>
<td>81 (0.622)</td>
<td>69 (0.404)</td>
<td>89 (0.764)</td>
<td>73 (0.508)</td>
</tr>
<tr>
<td>MIFA latent</td>
<td>100</td>
<td>86 (0.727)</td>
<td>88 (0.728)</td>
<td>77 (0.521)</td>
<td>69 (0.387)</td>
<td>81 (0.616)</td>
<td>82 (0.633)</td>
</tr>
<tr>
<td>orfK8.1 peptide EIA</td>
<td>100</td>
<td>85 (0.705)</td>
<td>97 (0.778)</td>
<td>95 (0.709)</td>
<td>94 (0.909)</td>
<td>90 (0.818)</td>
<td></td>
</tr>
<tr>
<td>orf65 peptide EIA</td>
<td>100</td>
<td>84 (0.728)</td>
<td>91 (0.820)</td>
<td>86 (0.678)</td>
<td>83 (0.635)</td>
<td>72 (0.434)</td>
<td></td>
</tr>
<tr>
<td>orf65 IB assay</td>
<td>100</td>
<td>83 (0.635)</td>
<td>72 (0.434)</td>
<td>85 (0.710)</td>
<td></td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Combined peptide EIAs</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABI EIA</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

*Strength of agreement: <0.00, poor; 0 to 0.20, slight; 0.21 to 0.40, fair; 0.41 to 0.60, moderate; 0.61 to 0.80, substantial; 0.81 to 0.99, almost perfect; 1.00, perfect.

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### TABLE 4. HHV-8 PCR results

<table>
<thead>
<tr>
<th>Study population</th>
<th>No. of subjects</th>
<th>% Positive</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>orf65&lt;sup&gt;a&lt;/sup&gt;</td>
<td>gB-N</td>
</tr>
<tr>
<td></td>
<td>1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2</td>
</tr>
<tr>
<td>Current KS</td>
<td>29</td>
<td>41 (7)&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Later KS</td>
<td>13</td>
<td>31</td>
</tr>
<tr>
<td>No KS</td>
<td>25</td>
<td>4</td>
</tr>
<tr>
<td>Controls</td>
<td>20</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Nested PCR.
<sup>b</sup> Operator.
<sup>f</sup> Indeterminate values (only faint bands present or discordant results on repetition), in percent, are given in parentheses.
later-KS patients. They were also HHV-8 positive for 27 to 34% of HIV-positive MSM without KS and 2 to 20% of blood donors. Lang et al. (23) found a higher rate of HHV-8 positivity (74%) in HIV-positive MSM from the United States. The sensitivities of our EIAs exceed those of the other EIAs reported for the current-KS group and are comparable to the sensitivities of the other assays for the no-KS group. No persons in the control group were positive by our assays, in contrast to the 20% infection rate found by the orf65 IB assay (9), although the numbers of healthy individuals tested in this study was small.

The ABI EIA uses a purified virion lysate (37). The rate of HHV-8 seropositivity by this assay was 73 to 92% for HIV-positive KS patients, 93 to 100% for classic-KS patients, and 84% for later-KS patients. Among the HIV-positive patients without KS, 27 to 50% were HHV-8 positive. None of the blood donors tested positive by this assay. Our results by this assay for the current-KS group were within the range of results from previously published reports. Among those in the later-KS group, we found fewer positive individuals than Chatlynne et al. (8) did for a similarly composed pre-KS group (8). It was of interest that all of the ABI EIA-positive and -negative persons in the control group were positive by our assays, implying that although it is specific, the ABI EIA is less sensitive than the other EIAs for the no-KS group. All those who were orfK8.1 peptide EIA positive but ABI EIA negative were also positive by other assays, implying that although it is specific, the ABI EIA missed some true positives.

**IB assays.** The orf65 IB assay was the least sensitive assay that we examined. Although the results of this assay had 86% concordance with those of the orf65 EIA, 75% of specimens that were positive by the orf65 IB assay but not by the orf65 EIA were negative by all other assays. This raises questions about the specificity of the orf65 IB assay as it is currently constituted.

Data on other HHV-8 serologic assays in the IB assay format are limited. One study used the p226-234 protein (orf73 protein [19]) derived from a BC-1 cell (12), and another used the orf65 protein (25). These assays detected HHV-8 antibodies in 80 to 89% of HIV-positive KS patients, 18 to 20% of HIV-positive patients without KS, and 0 to 11% of blood donors. The orf65 IB assay also detected infection in 93 and 95% of posttransplant and classic KS patients, respectively. Recently, Zhu et al. (47) reported serologic data obtained by assays performed with additional recombinant lytic antigens orf65, orfK8.1A, and orfK8.1B and latent antigen orf73 in an IB assay format. Among HIV-positive MSM with KS, the proportions found to be HHV-8 positive were 95, 93, 90, and 89%, respectively. Among HIV-positive MSM without KS, these proportions were 73, 69, 60, and 60%, respectively, while those for HIV-negative men were 13, 11, 8, and 10%, respectively. Lang et al. (23) have also reported on the use of recombinant orfK8.1, orf65, orfK12, and orf52 in the IB assay format. Among seven HIV-positive KS patients, five, five, two, and two were positive by these assays, respectively. Katano et al. (17) tested a recombinant orf59 IB assay but found only 32% of specimens from HIV-positive KS patients to be HHV-8 positive. Our results obtained by the orf65 IB assay are comparable to those of Lin et al. (25) and Lang et al. (23), although we found a larger percentage of our no-KS group to be HHV-8 positive than Lin et al. (25) did. Our results were lower than those of Zhu et al. (47). The latter’s results obtained with recombinant orf56 and orfK8.1A in an IB assay format are comparable to our results with the immunogenic peptides of these proteins in an EIA format.

**PCR.** PCR is insensitive in detecting HHV-8 infection in PBMCs. Using nested PCR for orf65, we found less than half of the current-KS group and about 30% of the later-KS group to be HHV-8 positive. One-step PCRs for the detection of HHV-8 in PBMCs have often used the primers for the 233-bp fragment of orf26 originally described by Chang et al. (7). Summary data from a series of studies that used both one-step and nested PCR showed an overall HHV-8 positivity rate of 53% for HIV-positive KS patients (range, 33 to 91%), 10% for HIV-positive patients without KS (range, 3 to 38%), and 0.2% for healthy adult or blood donor controls (range, 0 to 9%, with higher rates detected in Italy) (42). PCR testing was done in an attempt to provide confirmatory data for HHV-8 seropositivity. Our results for the orf65 gene fragment for the current-KS and no-KS groups are comparable to those reported previously for the orf26 gene fragment. Despite the current low detection rate of PCR, it may still be useful for confirming the results for specimens with indeterminate serologic assay results.

**Summary and conclusions.** On the basis of our evaluation of serologic assays for the detection of HHV-8 infection in individuals with KS, no single assay is completely sensitive and specific. The single-peptide assays may lack sensitivity since they detect antibodies against a single protein fragment. Both of the peptide assays used in the present study are based on lytic cycle antigens which may be present intermittently in vivo. Combination of the results of several peptide assays (combined peptide EIAs), as we have shown here, can increase sensitivity. Also, we have found that the use of a combination of several peptides in a single assay may actually increase sensitivity (L. L. Lam, T. J. Spira, and C. P. Pau, unpublished data). The ABI EIA and the orf65 IB assay also had lower sensitivities than the other assays. The ABI EIA is slightly less sensitive than the orfK8.1 peptide EIA, but it is commercially available. The MIFA latent appears to be specific, but it is not as sensitive as some of the other assays. The MIFA lytic is highly sensitive but may not be completely specific. Because of its high sensitivity, it could be used as a screening assay, which would then require confirmation by a second assay. IFAs, by nature, require extensive quality control both for the activation of the lytic antigens in the BCBL cell line (to ensure that an adequate percentage of cells are expressing the antigens) and for the interpretation of the slides. These assays also require more time and effort, and interpretation of results is subject to observer variability. EIAs, while they still require quality control in the preparation of the slides, these assays also require more time and effort, and interpretation of results is subject to observer variability. EIAs, while they still require quality control in the preparation of the slides, produce more objective results in their readout for samples with positive results. Determination of the cutoffs between positive and negative results requires careful consideration in the choice of both the actual cutoffs and the negative controls to be used in setting them.

We currently propose screening for HHV-8 by peptide EIAs. Specimens that test weakly positive by both assays (for example, above the cutoff but with an optical density of <0.6) or weakly positive by one and negative by the other may not be true positives. For these “indeterminate” specimens, we recommend that testing by MIFA lytic be done. A sample with an indeterminate EIA result and with a titer by MIFA of $\geq 1:40$ would be considered HHV-8 positive. A sample with a negative MIFA result or a low titer would be called HHV-8 negative. If a population has a high percentage of persons positive by the peptide EIA, then a MIFA could be performed with the samples that tested negative to determine if any positive specimens are being missed by the peptide EIA. Alternatively, if a population has a low percentage of samples that test positive by the peptide EIA, then for the same reason a MIFA could be performed with a randomly selected subset of the samples that test negative. As described above, a sample with a MIFA titer of $\geq 1:40$ would be called HHV-8 positive and a sample with a
negative or low titer would be called HHV-8 negative. By application of this algorithm to samples from our current-KS and later-KS groups, 93% of samples would be positive by the combined peptide ELAs and an additional 5% (two of the three remaining samples) would be positive by the MIFA lytic. This proposed testing algorithm is consistent with some of the recommendations of Zhu et al. (47), who maintain that it is critical to use both IFA and IB assay to determine HHV-8 positivity, especially with HIV-negative asymptomatic individuals. Sera reactive by at least two tests would be considered positive. They also recommended performing the IFA with a range of serum dilutions of from 1:10 to 1:160 rather than with a single dilution and establishing the cutoff for positivity at ≥1:40. Our strategy differs primarily in not requiring IFA confirmation of results for sera positive by the peptide ELA. We also establish a category of indeterminate results.

Accurate diagnostic assays for HHV-8 infection are necessary to move from seroprevalence studies to studies of the pathogenesis of infection and prognostic indicators for the development of KS, BCBL, and multicentric Castleman’s disease. HHV-8 diagnostic assays show lower overall concordance when they are used to screen sera from individuals without KS compared to the concordance achieved when they are used to screen sera from those with KS. The assays do, however, identify similar trends in seroprevalence in various epidemiologic and ethnic groups, suggesting that they all detect HHV-8 antibodies, but it is not yet apparent which of the assays is most accurate. While there are clear differences in assay sensitivities, as seen when various assay formats and viral antigens are applied to KS patient sera, the question of assay specificity is more controversial and harder to address. The specificity of the highly sensitive lytic IFAs has been called into question because these assays detect higher percentages of positive blood donors without corroboration of the results by other assays, but it cannot be determined at this time whether these assays are more cross-reactive or simply more sensitive than the other assays. Specificity is a challenging issue to resolve because there are no known clinical correlates of primary, chronic, or latent HHV-8 infection other than KS, PEL, and multicentric Castleman’s disease. Assays based on individual HHV-8 proteins and peptides appear to sacrifice some sensitivity, but they indicate that patients can sometimes react to one viral epitope and not another, suggesting that different antibody profiles can develop during the course of HHV-8 infection (23). Newer diagnostic assays will be developed on the basis of combinations of specific viral epitopes. These assays will likely enhance assay sensitivity while increasing specificity, and this may enhance our understanding of the implications of this infection.

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