

SELECTION OF REFERENCE GROUPS AND CUT-POINTS IN THE DEVELOPMENT OF HHV-8 SEROLOGIC ASSAYS

J.N. Martin, B. Forghani, and D.H. Osmond. University of California, San Francisco and the California Department of Health Services.

The lack of agreement among HHV-8 serologic assays has occurred, in part, because certain assays target different antibodies for which inherent sensitivity and specificity for HHV-8 infection differ. In other instances, however, lack of agreement may be the result of varying approaches to the selection of reference groups and assay cut-points used during assay development. Not only might this lead to interassay disagreement, but it also leaves in question the validity of sensitivity and specificity claims for any one assay. When assembling reference groups, valid estimates of sensitivity and specificity depend upon challenging an assay with a wide spectrum of individuals who are HHV-8-infected ("true positive" reference group) and persons who are not ("true negative" group). A wide spectrum is hardest in practice to achieve in the "true positive" group, where including solely KS patients can result in sensitivity estimates that are unrealistically inflated. One approach to extend the spectrum of "true positives" to include HHV-8-infected persons without KS is to select well-characterized persons without KS who have risk factors for HHV-8 (e.g., homosexual men) and who test positive on established assays with known high specificity (e.g., LANA IFA). Other approaches include using diluted serum from KS patients or serum collected prior to the KS diagnosis. The lack of a gold standard for non-infection makes assembling a "true negative" group problematic, but a high probability that subjects are uninfected can be achieved by selecting persons known to be both at low risk for KS and lacking HHV-8 risk factors (e.g. virginal women and young children). Finally, both reference groups must be sufficiently large to allow for statistically precise estimates of sensitivity and specificity. Once reference groups are assembled, assuming that no single cut-point can completely distinguish "true positives" from "true negatives", an assay is best characterized by a family of cut-points that can be represented in a receiver-operating characteristics (ROC) curve. In this case, no single correct cut-point exists; which cut-point to use depends upon the desired use of the assay.

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SEROLOGIC ASSAYS FOR DETECTION OF HHV-8 ANTIBODIES

Dollard, SM, C-P Pau, TJ Spira, and PE Pellett

Centers for Disease Control and Prevention, Atlanta, GA 30333

We have refined and standardized three HHV-8 serologic assays. They include a monoclonal antibody-enhanced lytic IFA (mIFA) similar to that described by Lennette et al. (Lancet 348:858-861, 1996) and ELAs based on peptides derived from ORFs 65 and K8.1. These assays were evaluated on panels of well-characterized specimens and applied in epidemiologic studies involving several thousand specimens, including the collaborative REDS study (described in a separate abstract). We have identified strengths and weaknesses of each of the assays and have developed approaches to improve their reproducibility. To summarize, reproducibility in the mIFA is dependent on lot-to-lot variation in the induced BCBL cells and the antihuman monoclonal antibody, and interpretation of slides that can be more subjective than other assay formats. Nonetheless, the mIFA remains among the most sensitive assays for HHV-8 and therefore it continues to be useful. A small number of sera from KS patients are positive only by the mIFA at a 1:20 dilution but sera from individuals in low risk groups are sometimes reactive at this dilution in the absence of corroboration from other assays. To increase specificity, we use a 1:40 dilution as the cutoff. The peptide ELAs require careful evaluation of each lot of plates based on an acceptable coefficient of variation and signal to noise ratio. The ELA cutoff is set at 5 SD above the mean of 20 negative control sera taken from a panel of 100 sera that approximate the general population and follow a normal distribution. The cutoff established in this manner nearly coincides with the inflection point in population-derived curves of rank-ordered OD values, supporting the it as being reasonable for discriminating uninfected and infected populations. Our current testing algorithm for population studies involves screening with the two peptide assays. Positive in either assay with an OD ≥ 0.6 is reported as positive. Sera weakly reactive in either or both ELAs (OD between the cutoff and 0.6) are confirmed by mIFA at a 1:40 dilution; mIFA positive is reported as positive, mIFA negative is reported as negative.

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SEROLOGICAL RESPONSES TO HHV-8 (KSHV).

Bala Chandran. Univ. Kansas Medical Center, Kansas City, KS.

HHV-8 serological responses were measured by a) immunofluorescence assays (IFA) using acetone fixed uninduced and phorbol ester induced HHV-8 positive BCBL-1 cells and negative BJAB cells and b) by Western blot assays (WB) using recombinant HHV-8 lytic (ORF 65 and K8.1A) and latent (ORF 73) replicative cycle proteins. Before fixation, BCBL-1 cells were monitored for the production of ORFs 73, 59 and K8.1A with appropriate antibodies. For IFA, sera were diluted from 1:10 to 1:160, mixed with a constant quantity of anti-ORF59 (lytic protein) monoclonal antibodies, tested with anti-human FITC (green) and anti-mouse rhodamine (red) antibodies and counter stained with Evans blue. Sera showing specific reactivity at a dilution of 1:40 and above were considered positive and tested by further dilutions. For WB, affinity purified ORFs 73, 65, K8.1A and GST proteins were run in SDS-PAGE, visualized and the major bands were cut, electroeluted, concentrated and titrated. About 300 ng of each antigen was used per lane and sera were tested at a dilution of 1:100. High levels of antibodies against HHV-8 latent and lytic antigens were detected in the HIV + KS+ sera after KS diagnosis (titer range 1:320->1:10,240). About 49 to 80 months prior to the development of KS in these individuals, similar levels of LANA and lytic antibodies (GMT=108) were detected. Between 31 and 48 months before KS diagnosis, the LANA antibody levels increased significantly (GMT= 363). Between 7 to 15 months before the development of KS, there was a substantial increase in the lytic antibody titers (GMT=416). Two types of antibody responses were detected in the sera from HIV+ KS negative homosexual men. In majority of the sera with and without detectable HHV-8 DNA in the PBMC, significantly low levels of HHV-8 antibodies were detected. In contrast, in a subgroup of sera from HIV+KS negative homosexual men, higher levels of antibodies against HHV-8 lytic and latent antigens were detected. This suggests reactivation and lytic HHV-8 replication under the conditions of HIV-induced reduced immuno-surveillance preceding the development of clinical AIDS-KS.

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PERFORMANCE OF HHV-8 SEROLOGICAL ASSAYS FOR TESTING SERA FROM DIVERSE POPULATIONS

Eric A. Engels², Michael Sinclair³, Joseph Gastwirth², P. Bradley Goebel¹, Andrea Stossel¹, Robert Biggar², James Goedert², Denise Whitby¹. 1. SAIC Frederick NCI-FCRDC, 2. Div. of Cancer Epidemiology and Genetics, NCI, 3. Mathematica Inc., NJ.

We recently characterized sensitivity and specificity of serological assays for HHV-8 infection, using sera from KS patients and U.S. subjects with hemophilia and their spouses. (Engels et al. *JAIDS*, In press) In that study, we used ELISAs to recombinant ORF.K8.1, ORF.65 and the LANA IFA. However, many epidemiologic studies will use such assays to assess HHV-8 infection rates in more diverse populations, and the performance characteristics of serological tests in these settings is unknown. To address this issue, we have extended our studies to include sera from diverse populations, namely, elderly Mediterranean subjects, subjects from the highly KS endemic region of Congo (formerly Zaire) and two ethnically distinct populations of Botswana. Using a novel analytic approach, the sensitivity and specificity of our current assays were assessed; some variation in test performance across populations was noted. Prevalence of HHV-8 infection was found to be high in the African populations and lower in the Mediterranean subjects.

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