



## Diagnosis of HHV8 infection

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**B**efore 1994, the idea that an infectious agent played a role in Kaposi's sarcoma, was nothing more than a strong suspicion. In that year, human herpesvirus 8 (HHV8) was identified in KS tissue. HHV8, also known as Kaposi's sarcoma-associated Herpesvirus (KSHV), belongs to the *Gammaherpesvirinae* subfamily of genus *Rhadinovirus* and is the first member of the genus shown to infect humans.

The discovery of the role of HHV8 in KS is a good example of how molecular and epidemiological approaches can work together to identify previously unknown infectious agents. Subsequent research has shown that HHV8 is associated with all variants of KS (AIDS KS, classic KS, endemic KS and iatrogenic KS) as well as with two rare lymphoproliferative diseases: primary effusion lymphoma (PEL) and the multicentric Castleman's disease (MCD). In recent years, molecular and seroepidemiological studies have investigated the prevalence of HHV8 infection, its modes of transmission and its oncogenic potential. Yet despite these efforts, there are still many aspects of the epidemiology and pathogenesis of HHV8 which need to be clarified. And there is still no gold standard for laboratory detection of infection.

HHV8 is an enveloped DNA virus with an icosahedral capsid composed of four structural proteins. Three (encoded by ORF 25, 26 and 62) show significant homologies to the capsid protein of other Herpesviruses; the small protein encoded by ORF 65 is peculiar to HHV8 and has been proposed as a target for antibody detection.

The viral genome consists of approximately 160 Kb of linear double stranded DNA, in which researchers have identified nearly 90 open reading frames (ORF). In addition to conserved gamma-herpesvirus genes, the genome contains genes which are unique to HHV8; at least 12 genes of obvious host derivation encode cellular homologs. These viral genes are of special importance in HHV8 pathogenesis. Their products stimulate cell division, inhibit

apoptosis and modulate immune function; four have independent growth transforming properties. One gene of particular interest is K1, a transforming gene. Different strains of K1 show up to 30% divergence in the amino acid sequences of their gene products. Six distinct subtypes (A, B, C, D, E and N) have been identified, each with its own distinctive geographical distribution. Subtypes A and C predominate in Europe, subtype B in Africa; the other subtypes are rare and are mainly confined to small areas of Oceania and South America. It is unclear whether different strains have differing levels of virulence or infectivity. In any one host there is only one strain of K1. This makes the gene suitable for molecular epidemiology. In its latent and lytic phases, HHV8 infects several different types of cell, including normal B lymphocytes, monocytes, endothelial cells, and oral epithelia. Low levels of latent HHV8 can be maintained as nuclear episomal structures, in circulating B lymphocytes and monocytes, which may serve as reservoirs for the virus. In a small percentage (5-10%) of infected cells, HHV8 can replicate lytically, transmitting the virus to other cell types. It is in this phase that the virus can be detected in peripheral blood samples. Like other Herpesviruses, HHV8 tightly regulates gene expression: in latently infected cells only a handful of latent genes are expressed, some at a low level. These genes are required for persistence of the viral genome. Most tumor cells, in KS and PEL, are latently infected. The genes expressed in these cells (LANA-1, v-cyclin, vFLIP) are involved in overcoming G1 arrest and in tumor growth.

Lytic genes activate viral replication and release new virus particles through cellular lysis; in general, productive infection is not consistent with the transforming activity of the virus. A small subset of tumor cells express genes for lytic HHV8 infection (v-bcl2 and IL-6). These genes may contribute to overcome cell cycle arrest. It is possible they play an important role in the initiation and progression of HHV8 associated

tumors. The laboratory diagnosis of HHV8 infection is based on serological tests and molecular assays for nucleic acid detection.

Many important results on the prevalence and epidemiology of HHV8 have used serological assays to detect antibodies for the virus. Much early work was based on Immunofluorescence assay (IFA) using HHV8-infected cell lines established from PEL. These cell lines are latently infected with HHV8; the lytic replication cycle can be induced by incubating cultures with tetradecanoyl phorbol-acetate (TPA). Subsequent research developed a range of different IFAs allowing the detection both of latent and of lytic viral antigens. In general, lytic antibodies appear before latent antibodies, assays for latent antigens are usually less sensitive than lytic antigen-based tests.

In our own research, we have successfully used IFA with BC-3, EBV-negative B-cell line latently infected with HHV8. By testing human sera with uninduced and TPA-induced BC-3 cells we were able to detect latent and lytic antibodies both in KS patients and non-KS patients. In these assays specific staining patterns are associated respectively with latent and lytic antigens. Speckled nuclear fluorescence, indicating reactivity against latent antigens, was seen both in latently infected and in TPA-induced cells. In most cases, cytoplasmic fluorescence was observed only after TPA-induction to activate the lytic cycle and release viral particles. In our study, we found antibodies to latent antigens in virtually all KS patients, though at lower titers than antibodies to lytic antigens. In non-KS patients, we observed a much lower rate of infection. In these patients the nuclear fluorescence typical of latently infected cells was observed less frequently, probably because these patients have lower serum titers. The sensitivity of the test was high, yielding 100% positive results in patients with classic or iatrogenic KS and approximately 95% of positive results in patients with AIDS-related KS. The specificity of the HHV8-positive reactions was measured using supplementary tests. Sera positive for HHV8 failed to react when tested with HHV8/EBV negative cells (Ramos) and with Hep2 cells, routinely used for detection of antinuclear autoantibodies; HHV8 IFA titers were unchanged after absorption with TPA-induced EBV-producer cells (P3HR1); there was no evidence of cross-reaction with EBV-infected cells.

Yet despite these positive results, and similar studies by other workers in the field, researchers have yet to attain a full understanding of the real specificity and sensitivity of lytic and latent antibody detection. To date, the most frequently used serological tests include panels of antigens of both types. The last few years have seen the development of immunoenzymatic assays and Western-blotting using purified or recombi-

nant antigens. However, in the absence of a method for reliably culturing the virus, it has not been possible to establish the accuracy of these methods. The lack of "reference" specimens representing true HHV8-infected and non-infected individuals prevents reliable comparisons between assays or between the results obtained by different laboratories.

An alternative, an highly effective tool for HHV8 diagnosis is the detection of nucleic acid by molecular techniques. The first applications of qualitative DNA amplification reactions (PCR) for the diagnosis of HHV8 used a nucleotide sequence in ORF 26 (VP3), first identified by Chang *et al.* Primer sets based on this sequence were widely used to investigate the natural history of HHV8 infection. Subsequent studies have broadened the range of available tests.

Molecular techniques have very high sensitivity. This, however, is also their biggest drawback. Only with very stringent experimental control it is possible to avoid false positives. These problems can be overcome by applying international recommendations. According to these recommendations control measures must always include:

1. separate processing of the specimens and reaction mixtures
2. screening for the presence of amplification inhibitors
3. negative controls for the extraction and amplification steps
4. confirmation of all positive results using alternative primer sets targeting another genomic region

In our experience, qualitative PCR under appropriate experimental conditions is a useful tool for detecting viral DNA in KS lesions, peripheral blood mononuclear cells (PBMC), urine, saliva and oral mucosal cells from KS patients. Lower rates of HHV8 DNA detection were found in patients without KS. This supported the view that HHV8 is specifically associated with KS and that the presence of the virus precedes development of KS.

Our studies showed a relatively high prevalence of HHV8 in the Italian population, compared to other populations in Europe. This picture is compatible with findings by other authors who have reported high prevalence in sub-Saharan Africa, intermediate prevalence in Mediterranean areas and very low HHV8 prevalence in northern Europe and United States. Similar patterns of prevalence have been reported for KS, which is particularly widespread in certain regions of Italy. Molecular amplification have been used to investigate the spread of the virus through the general population. Yet, despite the steady accumulation of data, the complete picture remains somewhat unclear. What seems likely is modes of transmission differ between areas with high and with low prevalence of the virus and between people with different risk factors.

Quantitative molecular assays make it possible to collect important information on active replication sites and on viral load. In particular, real-time PCR can be successfully used to monitor viral reactivation, development or progression of disease and antiviral therapy. This approach is less time-consuming than standard PCR and is highly reproducible. It also limits the amplification product handling, reducing the risk of contamination. As a result it is well-suited to the needs of large-scale investigations. By using real-time PCR, we were able to measure HHV8 copy number in peripheral blood from KS patients. The findings showed a correlation between patients' clinical stage and their viral load. They thus support suggestions from previous studies that quantitative PCR could be very useful in the evaluation of at risk patients, or in measuring the effectiveness of treatment. The common drawback of immunological and molecular methods is the lack of reference specimens for inter-laboratory testing. We nonetheless suggest that molecular methods at least can play an important role in the evaluation of at-risk patients and development or progression of disease.

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