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Expert Working Group on HHV-6 and 7 Laboratory Diagnosis and Testing

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Expert Working Group on HHV-6 and 7 Laboratory Diagnosis and Testing

March 7-8, 2000

Canadian Science Centre for Human and Animal Health
Winnipeg, Canada

Bureau of Microbiology*
Bureau of Infectious Diseases**
Reference Laboratory Capacity Program for Infectious Disease Surveillance
Laboratory Centre for Disease Control***
Health Canada

Health Canada re-alignment:
* Laboratory for Human and Animal Health
** Centre for Infectious Disease Prevention and Control
***Population and Public Health Branch

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Introduction

Although human herpesviruses have a very long history, it is only in the last 14 years that human herpesviruses-6 and 7 (HHV-6 and 7) have been identified and the importance of these pathogens in human disease has been realized. At the present time a number of different laboratory diagnostic techniques are available to detect HHV-6 and 7. Over the past few years in Canada, there has been a steady increase in the number of HHV-6 and 7 diagnostic tests requested by clinicians. Unfortunately, clear-cut guidelines as to the best method(s) to use for HHV-6 and 7 testing in particular patient populations are not available. The currently available HHV-6 and 7 diagnostic methods have not been thoroughly assessed in the clinical context. This is urgently required for meaningful diagnostic testing for HHV-6 and 7.

With funding from the Division of Bloodborne Pathogens and the Reference Laboratory Capacity Program for Infectious Disease Surveillance (Bureau of Infectious Diseases, Laboratory Centre for Disease Control), the Viral Exanthemata Laboratory (Bureau of Microbiology) hosted a 2-day meeting of an Expert Working Group on HHV-6/7 Laboratory Diagnosis and Testing, from March 7-8, 2000, in Winnipeg (see Appendix 1 for participant list). The goal of the meeting was to bring together clinical and laboratory researchers in HHV-6 and 7 from across Canada as well as internationally recognized experts to discuss and make recommendations to improve the current status of HHV-6 and 7 diagnostic testing. Improving diagnostic testing methods for HHV-6 and 7 will not only enable reliable diagnosis of these viruses in the clinical laboratory, but it will in turn facilitate a better understanding of the link between these herpesviruses and human disease.

This collection of articles is a summary of the Expert Working Group meeting and includes the group's recommendations on current HHV-6 and 7 testing as well as steps needed to improve the standard of this testing.

Recommendations

1. Currently, the most appropriate clinical scenarios in which HHV-6 laboratory diagnosis is indicated appear to be

   (a) primary infection in children < 3 years of age presenting with febrile illness with or without rash.

   (b) primary infection or viral reactivation in immunocompromised individuals such as AIDS patients or transplantation patients.

   (c) mononucleosis-like syndrome in patients without heterophile antibodies or antibodies specific to Epstein-Barr virus (EBV).

2. Laboratory diagnosis of an active or recent HHV-6 infection may be achieved by one of the following:

   (a) demonstration of an HHV-6-specific antibody seroconversion or significant change in antibody titre between acute and convalescent paired sera.

   (b) demonstration of low avidity HHV-6-specific antibody in serum (associated with primary infection).

   (c) positive polymerase chain reaction (PCR) from serum or plasma.

   (d) positive reverse transcription PCR (RT-PCR) from peripheral blood mononuclear cells (PBMCs) targeting a gene activated in the lytic cycle.

   (e) a combination of test results, which may indicate a primary infection, such as

      (i) IgG negative and serum/plasma/whole blood PCR positive.

      (ii) IgM positive or serum/plasma PCR positive in children < 3 years of age.

3. Further evaluation in the clinical context (specificity, sensitivity, predictive values) needs to be done to improve confidence in and reliability of HHV-6 laboratory testing:

   (a) comparison of PCR and serologic methods in various patient populations (including infants and immunocompromised individuals).

   (b) comparison between different serologic methods (immunofluorescence assay [IFA], enzyme-linked immunosorbent assay [ELISA], purified recombinant antigens).

   (c) comparison between different PCR protocols that target different genomic regions and are performed on different types of specimens (urine, saliva, serum, plasma, PBMC). Additionally, quantitative PCR and RT-PCR require further exploration.

   (d) differentiation of HHV-6 variants in the clinical virology laboratory, for which PCR methods are very useful. This will lead to a better understanding of the roles of each agent (HHV-6A and HHV-6B) in human disease.

4. HHV-7 laboratory diagnosis:

   (a) Currently, HHV-7 has not been firmly linked to any specific clinical scenarios, and presumably the most appropriate clinical scenarios requiring laboratory diagnosis would be similar to those for HHV-6 —

children with febrile illness and immunocompromised individuals.

(b) HHV-7 serologic assays must be carefully selected to avoid cross-reaction with antibodies to HHV-6 (e.g. the 89 KDa HHV-7 protein does not cross-react with antibodies to HHV-6).

(c) PCR-based assays can differentiate HHV-7 from HHV-6. Further studies are required to determine the most appropriate samples and the most appropriate PCR method format (i.e. qualitative or quantitative) for detecting an active HHV-7 infection.

Overview

Properties of HHV-6/7 and overview of the clinical spectrum of disease

Philip Pellett

Human herpesvirus (HHV) types 6 and 7 were first described in 1986 and 1990 respectively and belong to the beta-herpesvirus subfamily of HHVs. HHV-6 and 7 share a number of biological features, and the relation between them is as close as that between herpes simplex virus types 1 and 2 in the alpha-herpesvirus subfamily.

HHV-6

HHV-6 exhibits the following features:

- It grows primarily in CD4-positive T cells both *in vitro* and *in vivo.*
- It is present in most brains.
- Congenital transmission is possible.
- The seroprevalence is > 90%.
- It is associated with a wide range of diseases.
- There is possible pathogenic interaction with other viruses.
- It is frequently misdiagnosed or not diagnosed at all.
- There are two variants, HHV-6A and HHV-6B.

Although very closely related, HHV-6A and 6B variants can be differentiated on various parameters: cell tropism, reaction to monoclonal antibodies (MAb), restriction endonuclease profiles, nucleotide sequences, and epidemiologic features. There is no genetic gradient between 6A and 6B.

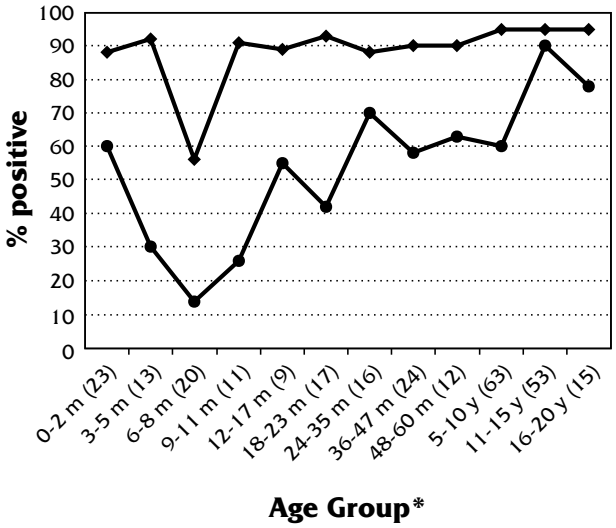
In the laboratory, both HHV-6A and 6B grow in CD4-positive stimulated PBMCs. The 6A strain replicates efficiently in the T-cell line HSB-2, and 6B is grown in the Molt-3 T-cell line. In the body, both variants are found in the central nervous system (CNS), skin, and lungs. HHV-6B is more likely than 6A to be detected in blood and saliva. Although HHV-6B is more common in cerebrospinal fluid (CSF), HHV-6A has greater relative neurotropism.

Southern blot hybridization with a probe of purified HHV-6 DNA and analysis of restriction endonuclease profiles have shown that HHV-6 hybridizes only to itself. There is no hybridization with human cytomegalovirus (CMV), also in the beta-herpesvirus subfamily, or with herpesviruses from other subfamilies, such as varicella-zoster virus (VZV) or EBV; with HHV-7 there is some limited cross-hybridization. Comparison of genomic HHV-6A and 6B sequences indicates over 90% shared nucleotide sequence identity in the middle portion of the genome, but rather less in the region of the termini and down to 50%-70% in the region that contains the immediate early gene. In a similar comparison of two HHV-6B strains there is very little sequence divergence, suggesting that variants 6A and 6B may, in fact, represent two distinct viruses.

The prevalence of HHV-6A and 6B as determined serologically is over 90% in adults. Primary infection with HHV-6 usually occurs early in infancy, possibly almost as soon as the level of protective

Figure 1

Cross-sectional study of the prevalence of HHV-6 (◆) and HHV-7 (●)



* Age groups are indicated in months (m) or years (y) with the total number of specimens analyzed per group shown in parentheses.

maternal antibodies has begun to wane (at around 6 months), and within a short time frame (Figure 1). HHV-6 appears to be transmitted mainly through saliva, but breast milk has not been found to be a vehicle of transmission. There is some evidence for congenital infection, and in rare cases chromosomal transmission has been reported. The main disease associated with primary infection in infancy is roseola (exanthum subitum), consisting of high fever for a few days followed by rash. Infection may also result in rash alone, fever without rash, or convulsions. Of the two variants, HHV-6B is the one almost exclusively involved in roseola. Febrile conditions in children due to HHV-6 may be misdiagnosed as sensitivity to an antibiotic or as measles or other childhood infection with rash.

In transplant recipients, HHV-6 has been associated with rash (occasionally mistaken for graft-versus-host disease), pneumonitis, graft rejection or failure, and marrow suppression or delayed engraftment in bone marrow transplant (BMT) recipients. *In vitro* studies have described a number of interactions between HHV-6 and HIV-1. HHV-6 can induce expression of CD4 cells, so that the potential number of cells susceptible to HIV-1 infection is increased. HIV load has been found to be higher in the presence of HHV-6. The role of HHV-6 in multiple sclerosis will be discussed by Dr. Jacobson. Other conditions that have been investigated for HHV-6 involvement are chronic fatigue syndrome, drug-induced hypersensitivity syndrome, and malignant disease.

With regard to laboratory diagnosis, there is a dearth of standardized, commercially available diagnostic kits. The options available to most laboratories — culture, antigen detection (with MAb), nucleic acid detection (from PCR on serum or whole blood, plus blot hybridization), and serologic methods (IFA, EIA [enzyme immunoassay]) — all have their own limitations, compounded by the high prevalence of HHV-6 in the general population: i.e. what does a positive result signify? There has been recent work on algorithms for diagnosis of primary infection in children, so that rapid point-of-care testing can be carried out. Multiplex assays will probably become important tools in this area. On the treatment front, HHV-6 has been shown to be sensitive to ganciclovir and foscarnet *in vitro*, and a reduction in HHV-6 activity in BMT patients given acyclovir has been reported.

HHV-7

HHV-7 is closely related both genetically and antigenically to HHV-6. It infects CD4-positive cells, but unlike HHV-6 (and like HIV) it uses CD4 as its receptor and then down-regulates CD4. The prevalence of HHV-7 is 85% among healthy adults, and the virus can be detected in the saliva of about 75% of people. Most seroconversions take place within the first 2 years of life, but the rate at which the virus is acquired after maternal antibodies have declined is not as steep as is the case with HHV-6, and acquisition may continue into later life (Figure 1).

The genome structure is very similar to that of HHV-6 in its high density of genes and their arrangement. Cross-hybridization between HHV-6 and 7 occurs only in one small area. In the laboratory, HHV-7 grows in CD4-positive cells; in the body it has been found in lymphocytes, salivary glands, and epithelial cells, but not in the brain. It does not co-localize with HHV-6.

HHV-7 is associated with roseola (5%-10% of cases), febrile convulsions (although the virus does not persist in the CNS), CMV disease in transplant recipients, and possibly pityriasis rosea. As with other herpesviruses, it is likely that HHV-7 acquired later in life leads to rather more severe disease.

The diagnostic tools available for HHV-7 are less well developed than for HHV-6, and serologic

Table 1
HHV-6A, HHV-6B and HHV-7 characteristics

	HHV-6A	HHV-6B	HHV-7
Infects CD4+ cells	yes	yes	yes
CD4 is receptor	no	no	yes
Virus in saliva	0/85	3/85	55/85
Virus in salivary glands	0/8	5/8	8/8
Virus in cervical swabs	0/72	14/72	2/72
Virus in PBL*	yes	yes	yes
Virus in CNS	yes	yes	no
Roseola	very few	most	some

* Peripheral blood lymphocytes

methods are complicated by the antigenic cross-reactivity between HHV-7 and HHV-6B. In the laboratory, HHV-7 has been found to reactivate latent HHV-6B. Primary infection with one of these two viruses in an individual already seropositive for the other results in a 2-4 fold increase in antibody titre to the other virus. Both HHV-6 and HHV-7 may co-infect the same cell, for example the CD68-positive cells in Kaposi's sarcoma (KS) lesions. How that interacts with KS pathogenesis is still to be determined.

Table 1 is a summary table comparing the features of HHV-6 and 7. For the future, closer investigation of the differences between the HHV-6 variants will likely be fruitful. For both HHV-6 and 7 there is a need to determine how the viruses persist and how they interact with other viruses, and to clarify their role in a number of diseases.

Laboratory Methods

● Overview of current HHV-6/7 diagnostic methods

Graham Tipples

The purpose of this short overview is to outline current diagnostic methods for HHV-6 and 7. Practical methods for HHV-6 and 7 diagnosis in the clinical laboratory context include serologic methods for antibody detection as well as molecular methods for virus detection. Virus isolation for HHV-6 and 7 is not discussed here as it is a fairly difficult and time-consuming method not currently suitable for rapid diagnostic testing in the clinical virology laboratory. It is very important for any method that is used in a clinical laboratory to be evaluated in the appropriate clinical context. Interpretation of results must be done with an understanding of the limitations of a particular test. Evaluation of diagnostic methods typically includes assessing sensitivity, specificity, and positive and negative predictive values, as shown in Figure 2. A more detailed discussion of method evaluation is provided by Dr. Campione-Piccardo (see page 8).

Serologic methods include, but are certainly not limited to, immunofluorescence assays (IFA) and enzyme immunoassays (EIA) for qualitative, quantitative, and avidity antibody testing. Both the IFA and EIA formats commonly use a human T-cell line such as HSB-2 infected with a strain such as GS (HHV-6A) for preparation of slides or preparation of antigen respectively. There are a number of difficulties with current serologic methods for HHV-6 and 7 and their interpretation. These difficulties include (1) the high prevalence of antibodies to HHV-6 and 7 in the general population (see Cermelli and Jacobson, page 10); (2) the inability to differentiate strains (HHV-6A versus HHV-6B) by serology; (3) the use of IgM as a general diagnostic marker of acute infection (5% of adults may be IgM positive at any given time yet not have any clinical disease symptoms); and (4) the likelihood of cross-reactivity between HHV-6 and 7.

There are numerous published studies using PCR-based methods for detection of HHV-6 and 7. These assays include both qualitative and quantitative PCR and RT-PCR, use numerous different viral genes as specific targets and include the use of serum, plasma, PBMCs, whole blood and saliva as sources of viral template.

In addition to the many in-house assays developed for HHV-6 and 7 detection, there are a number of commercial products for use in research. These include IFA and EIA assays for serology as well as molecular methods for detection of HHV-6 DNA.

There are only a few studies that have attempted to carefully evaluate the use of particular HHV-6 and

Figure 2

Specificity, sensitivity and positive and negative predictive values of a laboratory test

		true disease status: diseased	true disease status: non-diseased
test result	+	a	b
	–	c	d

sensitivity = a / (a + c)
specificity = d / (b + d)

PPV = a / (a + b)
NPV = d / (c + d)

7 methods in the clinical context. These key studies are briefly mentioned here. Norton et al[1] used virus isolation as the gold standard and examined the use of RT-PCR on PBMCs to differentiate latent and replicating virus. Ward et al[2] developed an IgG avidity assay to differentiate between low avidity primary infection and high avidity recurrent infection. Bland et al[3] used seroconversion or a significant rise in antibody titre as the gold standard to assess both IgM serology and serum PCR in the diagnosis of acute HHV-6 infection causing febrile illness in the pediatric population. They found that screening by both serum PCR and IgM is required for best sensitivity (i.e. IgM positive or PCR positive is indicative of acute HHV-6 infection). Chiu et al[4] also used IgG seroconversion in acute pediatric febrile illness cases as the gold standard and compared quantitative HHV-6 PCR on plasma and whole blood. They determined that positive PCR from whole blood and negative IgG in plasma collected within 5 days of disease onset is a good indicator of primary HHV-6 infection in children > 3 months and < 3 years of age. Black et al[5] compared EIA, immunoblot, and IFA HHV-7 serologic methods and determined that immunoblot was the most specific and EIA was the most sensitive of these methods. Using the immunoblot method, they also identified an 89 KDa protein as an HHV-7-specific serologic marker, which will likely prove useful in dealing with HHV-6/7 cross-reactivity problems.

In conclusion, further work needs to be done to clarify the most useful and interpretable methods for HHV-6 and 7 diagnosis in the clinical context.

● Standardization and QA/QC issues in laboratory virology

José Campione-Piccardo

The Reference Laboratory Capacity Program in Infectious Disease Surveillance (RLCIDS), LCDC, aims to provide an interface for laboratory and non-laboratory public health professionals to address laboratory investigative and developmental issues in public health, quality assurance and quality control, accreditation, and training.

Laboratory technologies are similar in both primary care and surveillance, and the same professionals and facilities are frequently involved. However, the different prevalence of disease in the populations under consideration (primary care vs. surveillance) call for different test sensitivities, specificities, and cut-off values, and the outcomes usually are also different.

An ideal diagnostic test will detect all individuals with the disease and no individuals without the disease. However, no test is perfect, and accordingly tests will exhibit some of the following characteristics:

- Test results and disease status are usually not in complete agreement.
- In some cases the disease status is difficult to evaluate.
- Inferences from test results are more important than the observed test values.
- Correlation among tests results is of no value.
- Because of biological factors, in some cases even correct test values may not correctly identify disease status.

Measures of test performance include sensitivity (Se) or the true positive rate, specificity (Sp), false positive rate (1 – Sp), efficiency (Ef) or correct identification of infected and non-infected individuals (a + d in Figure 2), positive predictive value (PPV), negative predictive value (NPV), likelihood ratio of a positive test result (LRP = Se/[1 – Sp]), likelihood ratio of a negative test result (LRN = Sp[1 – Se]), and odds ratios (OR = LRP/LRN), used mainly to combine results from different studies in meta-analysis.

Biases in test performance measures may arise because test sensitivity changes with the clinical stage of the disease, and the spectrum of disease is not proportionally represented in the sample. There may be work-up bias (the gold standard is evaluated after the fact, i.e. after use of the test and follow-up), sampling bias (i.e. the prevalence in the sample is pre-set), and selection bias. In order to improve the precision of test performance measures there are ways to pre-estimate sensitivity and specificity and calculate the sample size necessary to achieve optimal confidence intervals (e.g. $p < 0.05$) for Se and Sp. Variations in Se and Sp will also vary according to the host-pathogen relation being used, the study design, population and sampling strategy, and the weighing of Se and Sp by the cut-off value chosen.

Figure 3

ROC curves plotted for an assay with Se = 0.97 and Sp = 0.97 and for another with Se = 0.79 and Sp = 0.92 at optimal cut-off

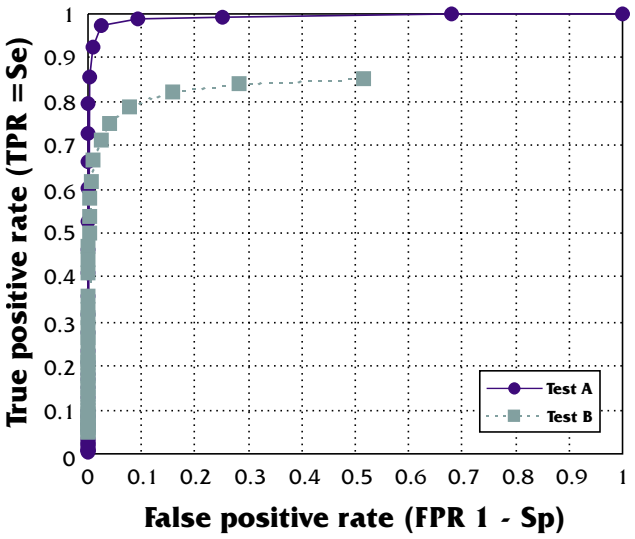
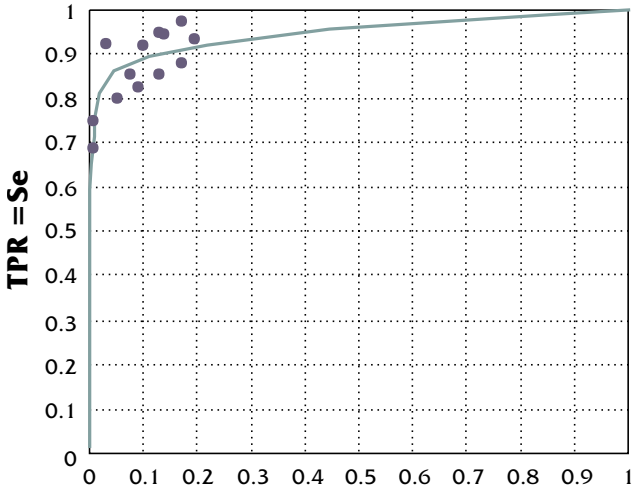


Figure 4

sROC (summary ROC) curve (plotted after fitting a linear regression to linear transforms of the individual points, each of which represents an independent evaluation of the same test)



The cut-off point for a positive test result will be determined by the true prevalence in the population, the cost of false positive and false negative results, and the decision strategy. One method for setting the cut-off is to use the point at which the mis-classification cost term is minimized. Another makes use of the receiver operator characteristic (ROC) curve. To obtain a ROC curve, the true positive rate (Se) is plotted against the false positive rate (1 – Sp) for a range of cut-off values. The closer the ROC curve is to the top left corner of the graph, the more accurate is the test, because the true positive rate is closer to 1 and the false positive rate is closer to 0. The "north-west" point furthest from the bisecting diagonal (Se = Sp) usually represents the best cut-off value (Figure 3). Different diagnostic tests can be compared by analysis of their ROC curves. This is particularly useful in meta-analysis of a number of independent validation studies, in which a summary ROC analysis (sROC) can be derived from the data from each study. The sROC curve will include one summary data point calculated for each of the different studies, through which a line of best fit can be drawn to show the average value obtained in the studies (Figure 4).

One of the advantages of ROC curves is that they can be used to compare tests without the need to rely on a gold standard. Depending on the definition of a gold standard can be a problem if not everyone agrees on what should be considered the gold standard or on when a new and better test should replace a previous acknowledged gold standard. A solution to such problems is to use maximum likelihood methods, one of which is the Latent Class Model. Two different tests are carried out in two populations with a different prevalence for the disease in question, both at two different laboratories. The Se, Sp, and the prevalence in each population are calculated by well-established iterative numerical methods using likelihood equations determined for each cell. The latent model, applied to at least two sets of data (each based on two tests and two populations), can be used to obtain a ROC curve for each test without the need for a gold standard.

There is a future role for the Reference Laboratory in Surveillance to determine appropriate cut-off values, validate tests by meta-analysis, and evaluate new tests on the basis of models with or without a gold standard.

International Research

● HHV-6 and 7 serology: comparison of epidemiologic findings from different countries and different assays

Claudio Cermelli and
Steven Jacobson

The seroepidemiology of HHV-6 and 7 has not progressed as rapidly as anticipated when these viruses were first described and investigated; diagnostic methods are not standardized, and there is still no gold standard. Even the commercially available tools seem to be no more reliable than home-made ones. A review of studies that have estimated the seroprevalence of HHV-6 at different ages (using IFA) shows a range of 39% to 100% among adults, and 80% to 100% among children. There is also a huge variation in the dilution cut-off value used, with a range from 1:2.5 to 1:160, and this greatly influences the percentage of seropositivity obtained. Other factors that may account for the discrepancies include geographic differences in the circulating virus, the HHV-6 variant and strain used, subjectivity in interpretation, and lack of standardization.

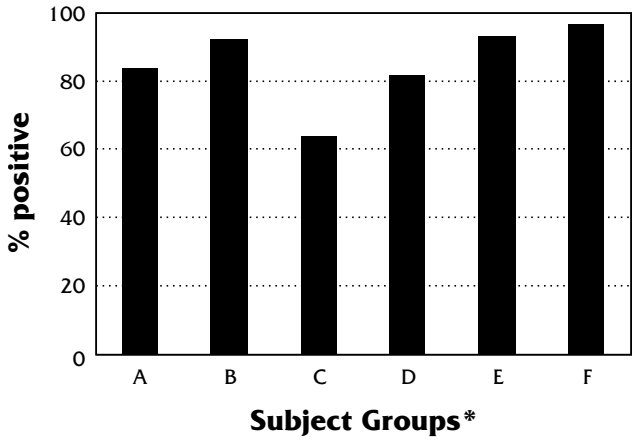
To evaluate how much the use of different variants and strains contributes to the widely divergent findings, IFA reactivity of 117 samples of sera to cord blood lymphocytes infected with either HHV-6A, U1102 strain, or HHV-6B, strain CV, was compared. All the sera were found to be IgG positive to the two virus variants. Only 14.7% of positive sera showed a difference of one dilution in the antibody titration with the two variants, and none differed by more than one dilution. Thus it seems clear that with these serologic assays it is impossible to distinguish antibodies against the two variants.

A study of HHV-6 seroprevalence in Italy was carried out using a home-made IFA with cord blood lymphocytes infected with a strain of HHV-6 from a child with exanthum subitum. The dilution cut-off value was 1:40. Serum specimens of 582 subjects of different ages and with various infections were tested for HHV-6 IgG. Figure 5 shows the sero-prevalence according to age and health status. The value drops with age, and for adults is highest among subjects with active CMV or HIV infection. In order to validate the results and the choice of 1:40 as the cut-off, an ELISA was developed using a crude lysate of cord blood lymphocytes infected with the same HHV-6 strain; part of the sera tested by IFA was used. The seroprevalence was found to be somewhat lower than with IFA: for healthy subjects 1-6 years old it was 87.1% versus 90.3%, for healthy subjects 6-18 years old 86.7% versus 90.0%, and for healthy adults 60.3% versus 63.6%. This is not a surprising finding, given the source of the antigen. The overall concordance was 79.7%. The choice of 1:40 cut-off was felt to be validated, but the concordance rates were not high enough to warrant a switch to ELISA as the preferred technique.

Detection of IgM is also problematic, particularly given that 5% of the population are positive for IgM at any one time. Modification of the IFA with an extra step can be used to assess antibody avidity, which is low just after primary infection and much higher in past or reactivated infection. The serum is prepared on two slides, one of which

Figure 5

Percentage of subjects having antibodies to HHV-6



* A = healthy subjects aged 1-6 years; B = healthy subjects aged 6-18 years; C = healthy subjects over 18 years; D = umbilical cord blood samples; E = subjects over 18 years with active CMV infection; F = subjects over 18 years who are HIV seropositive.

is washed with the normal phosphate buffered saline solution after serum incubation and the other with 8M urea. The urea elutes low-avidity but not high-avidity antibody from the antigen. Low avidity is considered to be present if the difference in titre between the two slides is equal to 3 or more dilutions. When this modified IFA was used to test the sera of a small sample of children with HHV-6 related pathologies, IgG avidity was found to be a more reliable indicator of recent primary infection than IgM. Figure 6 shows the results obtained when the assay was used to assess antibody avidity in an investigation of the seroprevalence of HHV-6 and 7 among children. Figure 7 presents the temporal sequence of HHV-6 and 7 acquisition at different stages of childhood, again using a measure of antibody avidity. For each age group, the number of children in whom HHV-6 infection had preceded HHV-7 infection was greater than the number in whom HHV-7 was acquired first. Overall, the results suggested that both viruses are widespread from infancy; HHV-6 infection occurs in the first 2 years of life, whereas primary infection with HHV-7 occurs later, between the ages of 3 and 6 years.

Current investigations, in collaboration with Dr. Steve Jacobson, at the National Institutes of Health (NIH), include the use of ELISA to detect IgM and IgG antibodies against the HHV-6 early

Figure 6

Percentage of children with low avidity antibody specific for HHV-6 (black bars) and HHV-7 (grey bars)

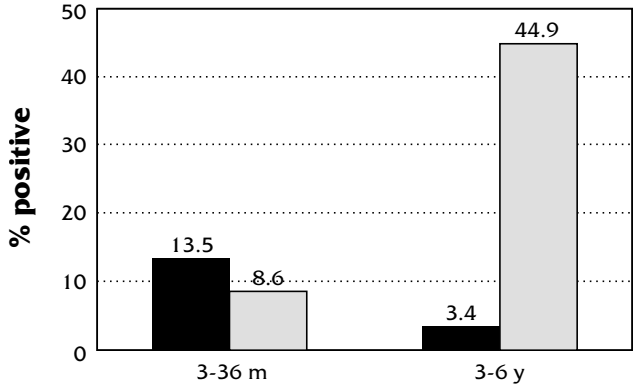
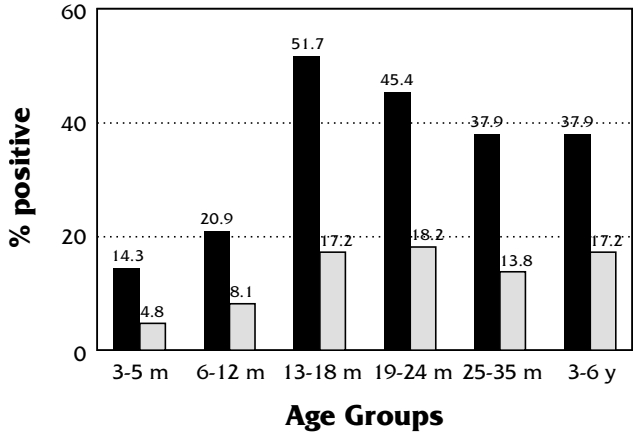


Figure 7

Temporal sequence of HHV-6 and HHV-7 infections in children (black bars: HHV-6 infection preceded HHV-7 infection; grey bars: HHV-7 infection occurred first)



antigen p41-38 in patients with multiple sclerosis (MS). The antigen is purified from an infected cell lysate by means of affinity columns prepared with the specific MAb. No difference in the IgG response was found between patients and normal control subjects (patients with other neurologic diseases, inflammatory diseases and healthy subjects), but there was a highly significant difference in IgM response, particularly for the patients with relapsing-remitting MS. It is hoped to expand this research.

● HHV-6 and multiple sclerosis

Steven Jacobson

Multiple sclerosis (MS) is a demyelinating disease affecting young adults (prevalence approximately 250,000-300,000 in the United States), the two main forms of which are the relapsing-remitting type and the chronic progressive type. Immunologic, genetic, and environmental factors have been implicated in its etiology. Many viruses have been linked over the past 50 years with MS, but it is not clear which, if any, actually play a role in the disease. In 1995, Challoner et al[6] used representational differential analysis on MS brain tissue and found a DNA sequence that matched HHV-6. Subsequent studies using MAb specific for the HHV-6 101K protein showed staining predominantly in the oligodendrocytes (the sites from which myelin is formed) of the MS brains but not in control material.

More recently, at the Viral Immunology Section, NIH, an ELISA assay has been used to detect IgM and IgG antibodies to the p41-38 early antigen of HHV-6 in about 130 samples from several groups: MS patients with the relapsing-remitting as well as the chronic progressive disorder, patients with other inflammatory diseases, patients with neurologic disorders, and healthy individuals. There were no differences in the IgG response, but a significantly greater IgM response was evident in the MS subjects. There were no differences among groups in IgM to EBV or CMV. Studies from other centres that have used this assay corroborate the findings of approximately 18% of normal subjects and 70%-80% of MS subjects with increased HHV-6 IgM. Review of the literature of studies using different assays on different MS populations throughout the world reveals a fairly consistent finding of increased IgM either in serum or CSF.

As well as serologic evidence, there is support from PCR techniques for HHV-6 DNA in MS patients. A nested PCR has been employed to detect DNA in acellular material in the serum of MS patients and control groups. By the first stage, typically, nothing is detectable, but differences emerge with the nested PCR. These studies are still in progress but, overall, HHV-6 DNA can be detected in about 25%-33% of MS patients and in none of the control groups. Of seven similar studies in the literature using different assays (with varying sensitivities) and different extraction methods, four support the association of HHV-6 in acellular material, either in the serum or CSF, of MS patients.

With this accumulating evidence, the question arises as to how the presence of HHV-6 is linked with clinical activity in MS patients. One profitable avenue of investigation has been to observe whether HHV-6 is associated with the occurrence of gadolinium-enhancing lesions, a hallmark of MS disease. These lesions can be detected by means of magnetic resonance imaging (MRI) and have been found to come and go in individual patients in a cyclical fashion over time. Observation of MRI results over several months together with PCR testing for HHV-6 in serum carried out at intervals over the same period (coded samples and investigators blind as to results) have shown a pattern of reduction in the number of lesions with the introduction of beta-interferon therapy and no evidence of HHV-6 DNA. In patients who start to fail therapy, the lesions are seen to return, and at that point the virus is once again detectable. As a result of these preliminary findings, a prospective study was carried out over 5 months of 67 MS patients, in order to follow exacerbations and remissions in the disease and correlate them with HHV-6 detected by PCR. Of the 59 patients for whom results were available, 10 patients showed an exacerbation, and in 5 of these the virus was detectable; of the 49 patients in remission, HHV-6 was found in only 9 patients. Using number of samples instead of number of patients, of 11 serum samples from patients experiencing exacerbation, 5 showed the presence of HHV-6; of 214 samples from patients in remission in only 11 was the virus detected. Follow-up of the exacerbations of two patients (one with gadolinium-enhancing lesions and one without) for longer periods (18 months and 23 months) revealed similar associations.

With regard to the particular variant of HHV-6 that might be involved in MS, this has been investigated in a fairly large study that aimed to detect HHV-6 DNA in serum, urine, saliva, and peripheral blood lymphocytes (PBLs) of MS patients and healthy controls by means of nested PCR. Two different primer sets were used, one to the MCP (major capsid protein) region and one to the LTP (large tegument protein) region; the latter, although less sensitive than the MCP primer, allowed restriction enzyme mapping to differentiate between HHV-6A and HHV-6B. In healthy controls, HHV-6

was not detected in serum or urine with either primer set, but it was found in 88% and 75% of samples of saliva and PBLs respectively. The results in MS patients were similar for the samples of saliva and PBLs; however, HHV-6 was detectable in the serum of 23% of MS patients and was also found in 26% of the urine samples. In the five patients with HHV-6 whose serum samples could be typed, four showed evidence of HHV-6A, and in the four urine samples that could be typed, three showed HHV-6A. Overall, none of the samples of saliva or PBLs showed evidence of HHV-6A.

From the immunologic point of view, PBL responses to HHV-6 and 7 in MS patients and healthy subjects have been investigated by means of older technology involving infected T-cell lysates that are used as antigens in a lymphoproliferative assay. No difference was found between MS patients and healthy controls in their proliferative response to the HHV-6B or HHV-7 lysate, but there was a difference in the cell mediated immune response to HHV-6A, with a higher CD4 T-cell response among the MS patients. Precursor frequency analysis showed a response to HHV-6A of 1 in 20,000 peripheral lymphocytes in healthy individuals, as compared with 1 in 2,000 in MS patients.

Immunohistochemical detection of HHV-6 in MS brains has been carried out and the results correlated with radiologic findings of MS lesion characteristics, for example, whether the lesion is active or chronic. In normal white matter and in the CNS material of control subjects there was no histologic evidence of HHV-6, whereas staining for gp116 was clear in the areas of demyelination of the MS material. More HHV-6 activity was evident in the active plaques. The cells involved were morphologically similar to oligodendrocytes, but also there was staining for GFAP (glial fibrillary acidic protein), a marker of astrocytes. It is hoped to amplify cells from the brain material and quantify the amount of HHV-6 in plaque and non-plaque regions.

There is good overall evidence that HHV-6 is implicated somehow in MS, although this could be an epiphenomenon if some other factor is involved in the disease process and is reactivating the virus. Possibly HHV-6 is one of the triggers of the disease, in that the immune response to the virus in a number of people is to generate antigen-specific cells that mediate through the release of chemokines and cytokines, which in turn play a part in the destruction of oligodendrocytes. In a recent study[7], it was reported that CD46 is the cellular receptor for HHV-6. CD46 is a membrane cofactor protein involved in complement regulation. It is expressed in all nucleated cells and is found in plasma, tears, and seminal fluid. CD46 is also the receptor for measles virus. These findings raise the question of whether MS is not a virus-specific disease but, rather, a condition in which a common virus receptor is involved. This shared receptor hypothesis is derived as follows:

- A number of viruses have been associated with MS, and other autoimmune and CNS diseases.
- No one virus has been definitively shown to be the etiologic agent in any of these disorders.
- Hypothesis: Multiple viruses utilize a common receptor (or family of receptors), whose expression and/or regulation and/or function is altered in disease.

More work on the virus and its receptor in CNS material may shed light on this new hypothesis.

Research in Canada

● HHV-6 in kidney transplantation

Philip Acott and Spencer Lee

The role of CMV and EBV in the renal transplant population is well established, either in rejection of the transplant or in post-transplantation disease, and herpesviruses may lead to certain types of interstitial nephritis such as mesangial proliferative nephritis. In 1993, it was decided to carry out a prospective serologic investigation of pediatric patients who were undergoing transplantation, receiving dialysis, or exhibiting rapidly progressing nephritis with renal insufficiency. Serologic testing was carried out at 6 monthly intervals and when there was clinical deterioration. Reactivation was defined as re-emergence of IgM with prior proven IgM negative status.

Examples of the types of patients showing HHV-6 reactivation include three children with interstitial nephritis and only mild symptoms (low platelet count, low-grade temperature, slight elevations on liver function tests [LFTs]); two children with confirmed viral myocarditis, in one child during treatment of acute nephritis and in the other after renal transplantation; and three children with rapidly progressing nephritis (two with lupus) who were receiving high doses of steroids and cytotoxic therapy, and showed a rash consistent with roseola, high fever, respiratory symptoms, and elevated liver function values together with IgM positivity for HHV-6.

More than 50% of the long-term dialysis and renal transplant population have shown HHV-6 IgM reactivation when followed over time. In the children undergoing dialysis, reactivation coincided with a period of fatigue and poor school performance; clinically, these children exhibited low-grade fever, mild liver function changes, and thrombocytopenia (at one time considered to be heparin induced). In the transplant group, most reactivations were early in the post-transplantation period and quite sustained (median duration 123 days, range 18-1,075). All of the three children with *de novo* infection (no prior immunity) showed IgM within 100 days after transplantation. It appears from the data that the earlier the reactivation occurs after transplantation the greater the probability of transplant rejection.

In a comparison of type of antibody induction therapy in transplant patients, it was found that in the group receiving polyclonal ATG/ALG (horse/rabbit) preparations (from 1993-1997) there was more fever, elevation of LFTs and thrombocytopenia than in the group given humanized monoclonal antibody to IL-2 (1977-1999). As well, HHV-6 reactivation occurred in 22 of 27 patients (81%) in the first group, as compared with 5 of 15 patients (33%) receiving the humanized antibody therapy (Table 2).

Several research questions arise in this pediatric population:

- Does HHV-6 reside in the kidney before transplantation?
  Liquid or *in situ* PCR or immunofluorescence staining for antigen expression on tissues from a donor seropositive for HHV-6 would indicate whether the donor's kidney harbors and disseminates the virus in an HHV-6 naïve host.

Table 2
Viral profile and renal transplant rejections

	Basiliximab (1997-99)	ATG/ALG (1993-97)
Total rejection	8/15	17/27
EBV infection	3	14
a. Reactivation	1	9
b. *de novo*	2	5
HHV-6 infection	5/15	22/27
a. Reactivation	5	21
b. *de novo*	0	1
Viral-associated rejection (1st year)	2	13
a. HHV-6	0	9
b. EBV	2	4
HHV-6 with no rejection on biopsy	4	7
HHV-6 with no renal deterioration	1	6

- Can active viral replication in the rejecting kidney be detected, particularly in children with serologic evidence of HHV-6 infection? Virus culture with PCR and antigen detection methods would help answer this question.
- Are the lymphoid cells in the rejecting kidney different when there is viral-associated rejection? Expression of activated lymphoid cell markers may clarify the role of immune cell involvement in kidney rejection.

The laboratory techniques in use for research purposes now include culture, IFA, and PCR. For culture, mononuclear cells from cord blood or peripheral blood are stimulated for 2 days with phytohemagglutinin and then cultured for up to 3 weeks in the presence of IL-2. Virus growth is monitored for syncytial cytopathic effect and viral antigens, detectable by IFA and MAb. Results from this method of virus culture serve as the positive controls in the assessment of IFA and PCR. The HAR-4 MAb recognizes the gp60-110 viral envelope protein and has been used to detect the presence of HHV-6 antigen in tubular epithelial cells of rejecting kidneys. The BO145 targets HHV-6 p41 and causes dense nuclear staining. Other MAb include BO150 (gp82) and BO151 (gp102). With regard to PCR, commercial oligonucleotide primers from Maxim Biotech were used. Dot blot may be needed in the interpretation of amplification patterns. Development has begun of an *in situ* PCR assay for HHV-6 infected or uninfected cell smears or paraffin-embedded biopsy sections.

● HHV-6, HHV-7, and CMV plasma viremia in bone marrow transplant recipients

Francisco Diaz-Mitoma

Positive PCR results for HHV-6 obtained at the Regional Virus Laboratory, Ottawa, have been observed in association with hepatitis, meningo-encephalitis, severe rash, and graft failure in bone marrow transplant (BMT) recipients. CMV is one of the main causes of complications in this group of patients and has been implicated in graft failure, pneumonitis, rash, fever, and ulcers. A retrospective laboratory study was carried out on a subgroup of BMT recipients, with the aim of assessing the clinical impact of plasma viremia in this population.

An effective diagnostic assay and the results it provides play an important part in the decision about whether potentially toxic antiviral drug therapy should be initiated and, if so, which agent would be the best choice. The laboratory method employed in the study was PCR for the detection of HHV-6, HHV-7 and CMV in plasma, since this finding is accepted as an indication of active virus replication. Blood samples were collected in EDTA every 1-3 weeks, and plasma was separated from cells; patient charts were reviewed every 2-3 weeks for clinical correlates of viremic infection. Over a period of 2 years, analysis has been completed on 34 BMT recipients, followed for a median duration of 5 months; 6 patients died during the 2 years. The total number of samples analyzed was 170.

In the laboratory, 1 mL of plasma was centrifuged at 17,000 rpm for 1 hour to obtain a viral pellet (if virus was present). DNA was extracted with Tri-reagent, and the DNA pellet was then dissolved in 25 µL of distilled water, ready for PCR testing. The PCR assays used were a PCR HHV-6 kit manufactured by Digene Diagnostics Inc., the CMV Amplicor PCR Assay by Roche Diagnostics, and an in-house nested PCR for the detection of HHV-7.

Of the 34 BMT patients, 15 (44%) were found to be positive for CMV and showed symptoms of fever, pneumonitis or gastrointestinal disturbance; 7 of the 34 (26%) were positive for HHV-6; and 5 patients (15%) were positive for HHV-7. (This high proportion of positivity relative to the results described later by Dr. Allen [see below] may be due, in part, to the fairly conservative approach taken in Ottawa to antiviral therapy before engraftment as well as to differences in laboratory protocol.) Five of the 34 patients had both HHV-6 and CMV viremia, and for 2 of these the HHV-6 preceded CMV infection. Of these 5, 2 patients had pneumonitis (both died), and 3 had fever, pancytopenia, and rash. These 3 responded to immunoglobulin and ganciclovir therapy.

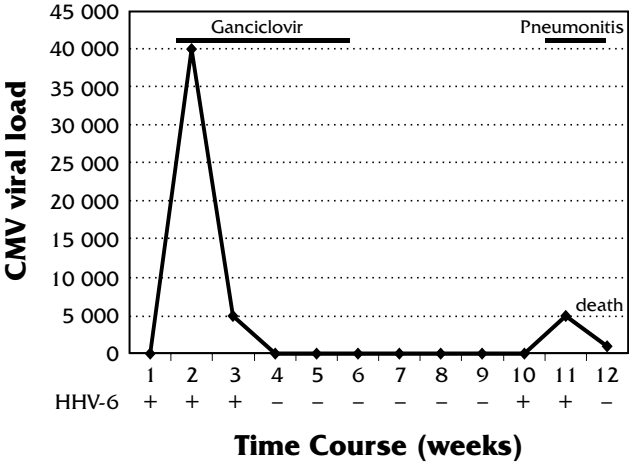
Figure 8 illustrates the viral profile and treatment of 1 patient with both HHV-6 and CMV infection. (CMV is measured quantitatively by the viral load in plasma, HHV-6 is a qualitative measure.) About 5 weeks after treatment with ganciclovir had been stopped, there was reactivation of HHV-6 together with CMV, and the patient died of pneumonia and bacteremia as complications of pneumonitis. Of the 5 patients with HHV-7 viremia, 4 had symptoms (fever, respiratory problems); 1 patient with both CMV and HHV-7 suffered severe headaches and pancytopenia.

In conclusion, the following clinical and virologic features were found:

- 70% of patients were or became symptomatic at the time of a positive plasma viremia with CMV, HHV-6, or HHV-7.
- The results of the assays were useful in deciding whether a patient required antiviral therapy.
- Negative results may avoid potentially toxic therapies.
- Patients who died had CMV viremia.
- Two patients had both CMV and HHV-6 viremia at the time of death (pneumonitis).
- One patient had HHV-6 in CSF (encephalopathy).
- Three patients had HHV-6 identified in skin lesions.

Other findings from the literature include the following: in 5 of 11 patients with CNS symptoms, HHV-6 DNA (detected by PCR in CSF) was associated with post-transplantation confusion, somnolence, or coma; HHV-6A and HHV-6B

Figure 8
Treatment and viral profile of a patient co-infected with HHV-6 and CMV (CMV viral load indicated by the line graph)



suppressed hematopoietic colony formation equally efficiently in BMT recipients, whereas HHV-7 had no such effect; HHV-7 DNA was associated with a significantly longer time to neutrophil engraftment in BMT recipients; and in 2 patients showing failure of engraftment, HHV-6 and HHV-7 DNA was detected.

● Diagnosis and management of HHV-6 infection among bone marrow transplant recipients in Toronto

Upton Allen

The objective of this study was to evaluate the utility of plasma PCR for surveillance of human HHV-6 infection among pediatric BMT recipients. This prospective study was non-interventional in design, involving a study group and control subjects.

Both BMT recipients and healthy control subjects were evaluated. For BMT subjects, HHV-6 PCR was carried out biweekly for 12 weeks after transplantation, and for controls a single PCR test was done. EDTA blood was collected for the PCR assay, and DNA was extracted from whole blood and cell-free plasma using standard procedures. The PCR was first performed on DNA from whole blood, and if a positive result was obtained, the test was repeated on the DNA from plasma. Detection of

HHV-6 by PCR was done using a primer pair that allowed the detection of HHV-6, HHV-7, and VZV. The species of the virus was determined by the restriction enzyme pattern obtained from the amplicon with the enzymes BamHI (Life Technologies) and BstUI (New England Biolabs). The restriction enzyme pattern also established whether the HHV-6 detected was variant A or variant B. The sensitivity of the PCR was estimated to be 1-10 genomic copies (Dr. Raymond Tellier, Hospital for Sick Children, Toronto, Ontario). Serology for HHV-6 was done using an ELISA.

Thirty BMT recipients were enrolled, 13 autologous and 17 allogeneic, and a total of 156 PCR tests were performed; six tests were performed on samples from six healthy control subjects. The median age of BMT subjects was 6.2 years (range 0.5-17.5 years), and of control subjects was 6.6 years (range 2-10 years). Sixty percent (18/30) had received ganciclovir prophylaxis, and 33% (10/30) had received acyclovir prophylaxis during the post-transplantation period. Eighty-seven percent (26/30) of the BMT recipients were seropositive for HHV-6 prior to transplantation. Among asymptomatic BMT patients who had PCR surveillance, the positivity rate was 3.3% (1/30) on whole blood and 0% (0/30) on plasma. None of the six healthy subjects had a positive PCR test on whole blood or plasma.

During the period of the surveillance study, HHV-6 PCR on plasma was used to assist in the diagnostic evaluation of 14 patients not included in the study. HHV-6 disease was diagnosed in 2 patients. In the absence of other explanations for these patients' illness, HHV-6 was regarded as the most likely cause given the following evidence. One patient had graft failure attributed to HHV-6, and the other had graft-versus-host disease, bone marrow suppression, encephalitis, and a generalized vesicular rash. In both patients, HHV-6B was detected by PCR on blood; the patient with graft failure only also had a positive test on plasma. In the other patient, HHV-6B was found by PCR in the cerebrospinal fluid in the presence of an encephalitic illness. In this latter patient, HHV-6B was detected by PCR in biopsy samples from the colon as part of the evaluation of possible colitis. HHV-6B DNA was detected in vesicular fluid aspirate from a skin lesion, although it was negative for VZV by electron microscopy and PCR. The presence of HHV-6 DNA in this sample was presumed to be due to trace amounts of blood in the sample.

In conclusion, despite the known fact that HHV-6 seropositivity rates are high among children, the frequency of HHV-6 plasma PCR positivity is low in this pediatric BMT population. A positive test on plasma is consistent with active infection. This increases the utility of the test as a diagnostic aid in evaluating syndromes presumed to be due to HHV-6 in pediatric BMT recipients. Although the study did not address therapy, it should be noted that data generated by others indicate that some strains of HHV-6A are resistant to ganciclovir. Thus foscarnet may be the preferred therapy when the variant is unknown. In other situations, such as ours, when the variant is known to be HHV-6B, either agent could be used. However, foscarnet is the preferred therapeutic agent in the setting of graft failure.

● HHV-6 in patients who are heterophile antibody negative with atypical lymphocytes and/or lymphocytosis

Patrick Doyle

Most cases of infectious mononucleosis (IM) are found to be heterophile antibody (Ab) positive. Of those who are Ab negative, the majority have atypical lymphocytes, and in these cases the differential diagnosis includes EBV, CMV, toxoplasmosis, HIV, viral hepatitis, rubella, HHV-6, mumps, and group A streptococcus. A prospective laboratory study was carried out from 1995 to 1996 to determine what proportion of a sample of patients who were heterophile Ab negative with atypical lymphocytes and/or lymphocytosis had an acute or a previous HHV-6 infection.

Most of the 70 specimens tested were obtained through mono spot requests from physicians, and a few (4/70) of the specimens included were those found to show atypical lymphocytes on the Stack-S (STK-S: a hematology analyzer). Lymphocytosis was defined as a level of $> 4.0 \times 10^9$/L, and atypical lymphocytes were defined by the STK-S criteria. Secondary objectives were to investigate the association between explanatory variables and HHV-6 infection using logistic regression analysis, and to determine the seroprevalence of other viruses (EBV, CMV, HIV).

IFA was used as the diagnostic test. A finding of IgM positivity was assumed to represent acute infection, and equivocal results were treated as negative; IgG testing was carried out to determine previous infection. Some of the problems with serologic tests that need to be addressed are as follows:

- EBV (for example) can non-specifically activate B lymphocytes, resulting in polyclonal response and increased antibodies to other agents.
- Another virus infection can result in reactivation of latent virus, with increases in antibody levels.
- Cross-reactivity (specific or nonspecific) can lead to false positive results and errors.

The mean age of the patients involved was 21.3 (median 10, range 1-71) years, and 30 were male. HHV-6 results are based on 65 specimens because of contamination of 5 of the 70. Of these 65, 16 (25%) were found to be HHV-6 IgM positive, indicating acute infection; 4 of the 16 were positive for HHV-6 only, 8 were positive for HHV-6 and EBV, 7 were positive for HHV-6 and CMV, and 1 was positive for HHV-6 and toxoplasmosis. There were no clinical data available for use in making a diagnosis from this complicated profile. Table 3 presents the proportion that were positive for various agents. With regard to IgG, 56 of 65 (86%) were positive, and 16/56 (29%) were both IgG and IgM positive. There were no cases of HHV-6 IgG negative with IgM positive.

Analysis of the Downey type of atypical lymphocytes revealed an association of CMV with Downey type I, EBV with Downey type II, and HHV-6 with Downey type III. The correlation between HHV-6 and Downey type III in the group tested for all viruses (n = 65) was significant (p = 0.013), as it was in the group excluding a positive result for multiple viruses (n = 45) (p = 0.039).

Table 3

Proportion of patients (heterophile antibody negative with atypical lymphocytes and/or lymphocytosis) with acute infections

	Number (proportion)	Number (proportion)*
HHV-6 IgM (+)	16/65 (25%)	4/45 (9%)
EBV IgM (+)	28/70 (40%)	9/45 (20%)
CMV IgM (+)	27/70 (39%)	10/45 (22%)
Toxo IgM (+)	2/70 (3%)	1/45 (2%)
HIV-1	0/70	–

* excluding positive results for multiple viruses

Logistic regression was carried out for hypothesis generation, and the variables included were Downey type, age, sex, and STK-S parameters. In the n = 65 group, the Downey type III was found to be significantly associated with HHV-6 IgM as the outcome variable (p = 0.0157); in the subset of n = 45, the association with Downey type was approaching significance (p = 0.0631).

The conclusions of the study are as follows:

- The proportion of patients with HHV-6 IgM (n = 65) was 25%.
- The proportion of patients with HHV-6 IgM in whom only one or no viral markers were positive (n = 45) was 9%.
- The prevalence of HHV-6 from current or past infection was 86%.
- Downey type III cells were significantly associated with HHV-6 IgM positivity in a hypothesis-generating logistic regression model.

● HHV-6 in the pediatric setting

Eva Thomas

HHV-6 has been demonstrated to be the causative agent in roseola infantum. We describe a case-control study to examine the hypothesis that acute HHV-6 infection occurs more commonly in children with febrile seizures than in controls. Eighty six pediatric patients with fever, with or without febrile seizures, were enrolled. A variety of other symptoms were observed, including otitis media, diarrhea, rash, and upper and lower respiratory tract infections.

With regard to whether HHV-6 is neurotropic, it has been detected in the brains of adults and in cerebrospinal fluid in children; it has also been linked with MS. It is known to grow in glial cells, glioblastoma cells, and human fetal astrocytes. Accordingly, the neurotropic properties of HHV-6 were evaluated in a case-control study of primary HHV-6 infection in children with febrile seizures. The study hypothesis was that primary HHV-6

infection occurs more commonly in children with febrile seizures than in control subjects.

Children between 6 months and 2 years of age were enrolled in the study between November 1992 and May 1995. The inclusion criteria were occurrence of a first or second febrile seizure, documented temperature of > 38° C, fever of < 4 days' duration, and agreement by a parent to clinical reassessment and specimen collection 2 weeks after the initial assessment. Control patients had the same inclusion criteria, but without the febrile seizures. Patients and controls were excluded if they had received diphtheria/pertussis/tetanus or *Haemophilus influenzae* type b vaccine < 48 hours before their visit or measles/mumps/rubella vaccine in the 10 days before; if they had an underlying immunologic disorder; if they showed CSF pleocytosis with positive bacterial gram stain or bacterial culture; or if there had been a previous afebrile seizure or evidence of other neurologic disorder or developmental delay.

Patients were examined at the time of presentation to the emergency room and 10-20 days later by a pediatric resident or pediatrician. At both visits samples were collected of acute serum, heparinized blood, saliva, urine, and stool. Parents were given a diary in which to record temperature and any rash or seizures.

The laboratory diagnostic methods employed were serology for HHV-6 by IFA and PCR. The IFA was carried out with HSB-2 cells infected with the U1102 strain and serum substitute instead of fetal calf serum. A nested PCR was used that detects about 10 copies. The criteria for primary infection were an HHV-6 IgM titre of > 1:40 in one or both specimens, a fourfold increase in HHV-6 IgG or seroconversion to an IgG titre of > 1:40, or a seronegative result for both specimens but a positive PCR result in saliva and/or PBMCs. The criteria for past infection were a finding of IgM negativity and IgG positivity in the first specimen, or less than a fourfold increase in IgG titre in the second serum sample. The laboratory criteria for absence of HHV-6 infection were a negative IgM and IgG in the first and second blood specimens, or negative PCR in saliva and PBMCs.

Of the 86 patients enrolled, HHV-6 status could be determined in 68 (35 with febrile seizures and 33 control patients). Table 4 shows the demographic and clinical data on patients enrolled. More of the patients than the control subjects had a family history of seizures. Table 5 shows that the addition of PCR to the serologic techniques increased the yield of HHV-6 infections from 19 to 30. The incidence of HHV-6 infection was found to be similar in the patients with febrile seizures and age-matched control groups (Table 6). Therefore, HHV-6 does not appear to play a role in the pathogenesis of first and second febrile seizures.

Table 4

Demographic and clinical data in case-control study of HHV-6 infection in children with febrile illness

	Seizure group *n* = 45	Control group *n* = 41
Mean age	15.9 years	12.27 years
Sex	21F/24M	17F/24M
Otitis	11	11
URT infection	15	21
LRT infection	3	6
UTI infection	4	5
Bacteremia	3	0
Diarrhea	10	10
Average temperature	36.69° C	39.55° C
< 2 day high temperature	100%	76%
Rash	17	16
Positive family history of seizure (febrile/afebrile)	9/9	2/6

F = female, M = male, URT = upper respiratory tract, LRT = lower respiratory tract, UTI = urinary tract infection

Table 5

HHV-6 infection status as determined by serology alone or serology and PCR

Infection	Serology alone	Serology and saliva PCR (+)
Primary	19	30
Past	21	21
Not HHV-6	28	17

Table 6
HHV-6 infection status in seizure patients and control patients

Group	*n*	Acute	Past	Not HHV-6
Seizure patients	35	15	13	7
Control patients	35	15	8	10

● Multiplex assay for semi-quantitative detection and distinction of human β-herpesviruses: prevalence during HIV infection and multiple sclerosis

Beni M. Sahai

Human -herpesviruses (CMV or HHV-5, HHV-6 and HHV-7) are slow-growing ubiquitous viruses commonly acquired during early childhood. Primary infection with these viruses leads to virus-specific antibody and cytotoxic T cell responses that effectively suppress viremia but fail to eliminate the virus completely. These viruses subsequently persist latently in PBMCs but apparently also in replicating form in some compartments of the body, as indicated by their occasional to frequent detection in urine and saliva. Such viral persistence does not a pose threat to the health of immuno-competent individuals. However, in immuno-compromised adults and previously unexposed naïve infants, the viruses may cause serious illnesses and have been implicated in a variety of neurologic, immunologic, and malignant diseases.

The diagnosis of these viruses as etiologic agent has been complicated by high seroprevalence, viral latency, and overlapping clinical symptoms, namely, skin rashes, encephalitis, retinitis, and bone marrow or organ disengraftment. Further, HHV-7, although not directly implicated in a disease, is involved in reactivation of HHV-6 and may therefore be seen during diseases caused by reactivation of HHV-6 without being the true etiologic agent. In addition to their role in direct pathogenesis, CMV and HHV-6 can transactivate HIV and induce surface molecules (such as C3b, Fc and CD4 receptors) that can mediate entry of HIV into certain CD4 cells. These viruses have therefore been suspected as co-factors in HIV pathogenesis.

In order to facilitate specific diagnosis of the viruses for clinical, epidemiologic, and pathogenesis studies, we developed a sensitive and specific PCR-based multiplex assay for simultaneous semi-quantitative detection and distinction of human -herpesviruses. The assay detects as low as 4 copies of CMV or HHV-6 and 12 copies of HHV-7, and monitors a conserved region of human genome as a control for template quality. The assay is applicable to a variety of clinical specimens such as PBMC, serum, CSF, urine, and saliva. The assay is readily amenable to automation, and the outputs can be either colorimetric or flow readings.

Individuals with HIV infection often exhibit CMV and HHV-6 replication in their PBMC, but a possible role of these viruses in HIV pathogenesis remains unknown. Further, it is unclear whether such viral replication is due to reactivation of latent virions or new infection resulting from prevailing immuno-deficiency. Recently, HHV-6 has also been implicated in the pathogenesis of MS, although an association between the virus and the disease has not been unequivocal, in part because of differences in virus detection methods. We therefore used the above multiplex assay to determine the prevalence of human -herpesviruses in HIV-infected patients who were either untreated or receiving mono or highly active antiretroviral therapy (HAART) and in MS patients.

The presence of human -herpesviruses in 1×10^5 PBMCs of each patient was examined; PBMC from healthy subjects served as the control. Our results indicate a varied but low prevalence of CMV (4%), HHV-6 (9%) and HHV-7 (7%) in healthy control subjects. In contrast, HIV-infected patients who were untreated or receiving mono antiretroviral therapy exhibited a much higher prevalence of CMV (25%, mostly with advanced HIV disease) and HHV-6 (37%) but a moderate prevalence of

HHV-7 (18%) than patients receiving HAART. Our study on MS patients revealed no significant association between human -herpesviruses and the incidence of MS.

● HHV-6 studies at the Central Laboratory of the Ontario Ministry of Health

Pauline George

A number of small HHV-6 serologic studies were performed in the Provincial Public Health Laboratory, Ontario, from the years 1994-2000. All studies used the HHV-6 serologic results from the Viral Exanthemata Laboratory of LCDC as reference values. The first study in 1994 was a retrospective study of HHV-6 seroprevalence in children < 4 years of age whose samples had been submitted for EBV testing. HHV-6 testing was performed using the Biotrin HHV 6 IgG immunofluorescence assay and Behring's IgM conjugate. The findings indicated 68% IgG positivity in random sera, and 62% and 100% in paired sera and transplant patients respectively. The results supported evidence of HHV-6 infection and were consistent with those reported in the literature.

The PanBio IgG ELISA for HHV-6 was evaluated in 1996. Samples were obtained from 89 patients, and for 86 of these (96%) information was available on age, sex, and clinical history. The sensitivity and specificity of the assay were 73.6% and 57.0% respectively. Review of the data suggested the cut-off values for the PanBio assay may be set too conservatively. IgM detection should be used to supplement IgG testing.

In 1998, an evaluation of the PanBio HHV6 IgM ELISA and Biotrin IgG IFA was performed. Retrospective ELISA IgM testing was performed on 81 samples and IFA IgG on 37 samples. The results for the ELISA IgM were sensitivity, specificity, PPV and NPV of 79.6%, 75.0%, 86.0% and 60.7% respectively. The results for the Biotrin IFA IgG assay indicated a sensitivity, specificity, PPV and NPV of 73.9%, 92.8%, 94.4% and 68.4% respectively. IgG seroconversions were detected by Biotrin and the reference assay (Table 7).

A summary of the reactive IgM and IgG results obtained for 77 sera sent to the reference laboratory for HHV testing in 1999 is presented in Table 8. Results suggest that there should be a re-evaluation of the cut-off parameters set by the reference laboratory.

Testing carried out for multiple viruses often shows a mixed set of results that the referring physician has to interpret in the light of the patient's clinical features. For example, one sample from a 37-year-old patient was found to be IgM positive for HHV-6, CMV and EBV, but the patient had no apparent symptoms. One sample from a 42-year-old was positive for HHV-6 and EBV and indeterminate for

Table 7
1998 evaluation of PanBio HHV-6 IgM ELISA and Biotrin IgG IFA: HHV-6 seroconversions

Patient	Sample	Age (yr)	LCDC EIA IgG	Biotrin IFA IgG	LCDC EIA IgM	PanBio ELISA IgM
A	1	3	–	–	–	–
	2		+	+	+	+
B	1	2	–	–	+ r = 0.27	+ r = 1.37
	2		+	+	+ r = 1	+ r = 1
C	1	27	+	+	–	+ r = 0.32
	2		+	+	+	+ r = 1

Table 8
Summary of IgM and IgG data (n = 77), 1999 (LCDC's HHV-6 EIA)

	IgG (+)	IgM (+)
Mean	1.084	0.26
Standard deviation	0.59	0.22
Maximum	2.36	1.58
Minimum	0.16	0.11

VZV and CMV. Samples positive for both HHV-6 and EBV were found from 3 patients, 1 with hepatosplenomegaly, 1 with fever and headache, and 1 a transplant recipient.

● HHV-6 testing at the Provincial Laboratory of Public Health for Southern Alberta

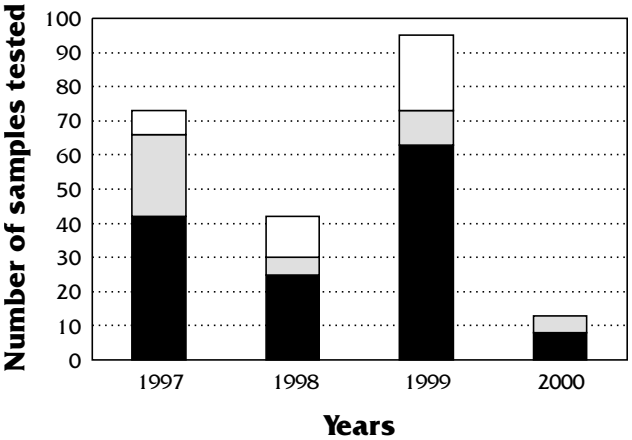
Kevin Fonseca

The results of HHV-6 serologic tests on samples received by the Provincial Laboratory of Public Health for Southern Alberta from 1997 to the present are described here. Of these tests, 95% were initiated by physicians in the community, and 5% of samples were sent for evaluation from other laboratories, along with the clinical history (including high fever and rash). Samples were forwarded to LCDC for HHV-6 IgG and IgM ELISA testing.

The number of samples tested for HHV-6 was 66 in 1997, 30 in 1998, 73 in 1999 and 13 so far in 2000. Figure 9 shows the proportion of samples that were found to be IgM positive and IgG positive and negative at the time of initial testing.

The acute phase of illness was defined as the first 5 to 7 days of the onset of symptoms, and the convalescent phase as 10 days or later after symptom onset. Samples were requested from the acute phase and at 2 weeks later, in order to detect seroconversions.

Figure 9
HHV-6 serology results,* 1997-2000



*White bar = IgM positive, grey bar = IgG negative, black bar = IgG positive/indeterminate

In 1997, the number of IgM positive samples was 7 (4 males and 3 females), in 1998 it was 12 (4 males and 8 females), and in 1999/2000 it was 22 (10 males and 12 females) with one indeterminate result. Positive IgM cases broken down according to origin showed that most (24/42 or 57%) were from the community; 15 (36%) were transplant patients; and 3 cases were of unknown origin. In analysis of the distribution of community cases by age and sex, males and females were equally represented at the earlier ages (from 0-2 and 2-4 years), whereas infection was found predominantly among males at the age of 4-6 years. Cases > 8 years appeared to be asymptomatic, in that the clinical information did not provide the same picture of rash and high fever as seen in the younger age groups; all the cases in this age group occurred among females.

The transplant patients were a mixed group. The majority of IgM positivity was found in renal transplantation patients (11 cases, 6 males and 5 females). Of the four remaining cases, a 15-year-old female died after bone marrow transplantation, a 44-year-old male had a diagnosis of multiple sclerosis, an 18-year-old female had acute lymphoblastic leukemia, and another 15-year-old female had a Wilms' tumour.

In many of the transplant patients the current HHV-6 infection represented reactivation of a previous infection. When the samples were tested for additional viral infection, about 30% showed low-level IgM responses to measles, parvovirus, CMV, or EBV, suggesting the possibility that these viruses play a role in stimulating HHV-6 reactivation. Further testing using PCR methods, performed at the Provincial Laboratory of Public Health for Southern Alberta, failed to provide a positive result for these viruses, and collection of another specimen may be needed to clarify such samples.

In a number of the transplant patients, there was a triad of HHV-6, CMV, and EBV infection, raising the question of whether immunosuppression or reactivation of one of these herpesviruses was the primary diagnostic factor.

In conclusion, mainly renal transplant rather than BMT patients were identified as being HHV-6 IgM positive, and it is possible that the virus differentially affects the clinical course in these two transplant populations. Whether HHV-6

reactivation is a measure of transplant rejection or an initiating factor in rejection has still to be determined, as has the possibility that other, concurrent herpesviruses act as co-factors.

● Pediatric Investigators Collaborative Network on Infections in Canada (PICNIC)

Joanne Embree

PICNIC is a network of pediatric infectious disease specialists in Canada that could be of value in any collaborative HHV-6/7 research project involving children. PICNIC has been involved in a number of surveillance studies carried out across the country, most of them hospital based but some involving work in the community and with outpatients. PICNIC can initiate its own projects or collaborate in research proposed by other, related bodies. A successful example of a PICNIC project is the study of respiratory syncytial virus, which investigated the presentation and epidemiology of the disease in Canada together with its diagnosis and management. Other organizations with which PICNIC members have been involved include the Canadian Immunization Monitoring Program (IMPACT) network, which monitors serious vaccine effects in pediatric hospitals, CNISP of the Canadian Hospital Epidemiology Committee (CHEC), which works with LCDC to monitor hospital-acquired infections, the Canadian Pediatric Society's surveillance programs, and the Canadian Pediatric AIDS Research Group.

Surveillance of the Blood Supply

● Surveillance for bloodborne pathogens in Canada

Antonio Giulivi

The role of the Bloodborne Pathogens Division, Bureau of Infectious Diseases, LCDC, is to establish the necessary surveillance systems and draw on the available expertise to ensure that the Canadian blood supply, blood transfusion, and transplantation (including xenotransplantation) are safe, as outlined in the Krever recommendations.

Routine surveillance for bloodborne pathogens is carried out through the existing notifiable diseases reporting system. The Transfusion Transmitted Injuries Surveillance System (TTISS) will monitor the risks of bloodborne pathogens in recipients of blood, tissue, and organs. Initially, four hospitals in four provinces will participate. The Enhanced Sentinel Health Unit Surveillance (ESHUS) system provides an overall risk assessment for those in the general population receiving blood. The Hospital-based Surveillance System (HSS) will evaluate medical practice and disease control measures as they apply to patients receiving blood or blood products; three centres (in Manitoba, Ottawa and Toronto) have been set up. The hepatitis surveillance system collects data from four enhanced centres in the community, and it is estimated that 20%-30% of viral hepatitis cases are currently caused by pathogens not yet identified. The Rapid Response Surveillance System (RRSS) is the look-out system for emerging pathogens – for instance, emergency surveillance has been established for a new pathogen similar to transfusion-transmitted virus (TTV), which may be a new virus or a subtype of TTV.

For 2 years there has been surveillance of Creutzfeld-Jakob disease (the CJD/Prion Surveillance System), which involves collection of the brains of patients with a possible diagnosis of this disease, pathology services in Toronto, and data collection from families of the patients. There are links with the U.K.

Public consultation on xenotransplantation will soon begin, and investigational drug submissions are expected this year. A surveillance framework and infection control protocol have to be in place before clinical trials begin.

There is a multicentre study, just completed, of G-CSF (granulocyte colony-stimulating factor) and stem cell factor in autologous BMT recipients. The results suggested that in some of the 40 patients or so in whom engraftment took overly long, there was evidence of HHV-6 and 7 infection, although the findings were not clear-cut. A proposal submitted under the auspices of the Canadian Blood and Marrow Transplant Group to the Medical Research Council (MRC) and Canadian Institutes for Health Research (CIHR) for a clinical trial in BMT recipients has been accepted. The 20 transplant centres (1,300 transplants per annum) across Canada will be involved, and they are currently setting up a common database. As part of the standardization of specimen collection, it has been decided that viruses suspected of influencing time to engraftment and relapse will be included. Testing will be carried out for HIV-1, HIV-2, HTLV-I (human T-cell lymphoma virus), HTLV-II, hepatitis B, hepatitis

C, CMV, parvovirus, HHV-6, HHV-7, possibly HHV-8, and a range of bacteria.

Preparations for the study will take about 2 years, but is important to have a consensus now on which laboratory methods will be appropriate for standardized testing for the herpesviruses.

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