

# HHV-6 is ubiquitously found using Western blot in tonsils and adenoid tissues of healthy people

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## SUMMARY

Few studies have examined the prevalence and cellular proclivity of latent human herpesvirus 6 (HHV-6) in healthy populations. Difficulties in detection of HHV-6 genome in different tissues using polymerase chain reaction (PCR) and immunohistochemistry (IHC) techniques have been reported by various researchers. We examined tonsils and adenoid tissues of 54 patients who had undergone tonsillectomy or adenoidectomy without any evidence of acute infection for the presence of latent HHV-6 infection. While we were investigating the prevalence of HHV-6, we tested the efficiency of PCR, IHC and Western Blot (WB) for detection of HHV-6 in tonsil tissues. We found that 100% of tonsil tissues were positive for HHV-6 with WB, 40% of tonsils were positive with PCR and no tonsil was positive with IHC. This result correlates well with most studies claiming HHV-6 is a ubiquitous organism in various populations and tissues. Western blot may be a good choice for detecting HHV-6 in tissues. Expression of the HHV-6 gp60/110 envelope protein disclosed by WB may indicate that HHV-6 does not have true latency. To our knowledge, this is the first report to use WB to test for HHV-6 in tissues.

**KEY WORDS:** Human herpes virus 6, Polymerase chain reaction, Western blot.

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## INTRODUCTION

Human herpesviruses-6 (HHV-6) is a widespread organism in all populations (De Bolle *et al.*, 2005). Primary infection with HHV-6 in young children may cause exanthem subitum and acute febrile illness (De Bolle *et al.*, 2005). In adults, HHV-6 has been associated with hepatitis, encephalitis, and a mononucleosis-like syndrome (Caselli and Di Luca, 2007). In some individuals HHV-6 is found integrated into human chromosomes, which results in a high viral load in blood (Ward *et al.*, 2006). Two variants of HHV-6 exist (A and

B), which have highly homologous genomes (Dominguez *et al.*, 1999). Several studies have also suggested an association between HHV-6 variants and certain diseases (Ahlqvist *et al.*, 2005; Moschettini *et al.*, 2006).

Like all other human herpesviruses, after primary infection HHV-6 remains latent in host cells (Zerr, 2006). Reactivation usually occurs in immunocompromised cases, and recently HHV-6 has been recognized as an opportunistic pathogen in patients with acquired immune deficiency syndrome and in transplant recipients (De Bolle *et al.*, 2005). This persistence involves both a true latent state (with no production of infectious virus) and a low-level chronic replication, each occurring at different anatomic sites (Kondo *et al.*, 2003). Whereas salivary glands and brain tissue are suspected to harbor persistent HHV-6 infection, monocytes and bone marrow progenitor cells are candidate sites for latency (Zerr, 2006). While viral DNA has been detected

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in these tissues by PCR, DNA hybridization and immunohistochemistry, none of these techniques have prove sensitive enough (Roush *et al.*, 2001; Berger *et al.*, 2007; Vrioni *et al.*, 2007). Due to their location, tonsils and adenoids can be infected acutely or chronically by a number of microorganisms (Brook *et al.*, 2000; Endo *et al.*, 2001). Some studies have shown a close relationship between certain viral infections and tonsillitis (Hudnall *et al.*, 2005). Recently, we searched for HSV-1, HSV-2, EBV and CMV using a new M-PCR technique and showed for the first time that tonsil tissues may harbor latent infections for HSV-1 in addition to EBV (Sahin *et al.*, 2007).

The purpose of this study was to verify whether these organs might harbor HHV-6 in addition to EBV and HSV-1. Fifty-four patients who had undergone tonsillectomy or adenoidectomy for chronic lymphoid hypertrophy without any evidence of acute infection were investigated for the presence of HHV-6. We used PCR, IHC and WB techniques to search for HHV-6 in the tonsil and adenoid tissues. The results of this study showed that all tonsil and adenoids presented the HHV-6 antigen and WB is the most sensitive technique for detecting of HHV-6.

## MATERIAL AND METHODS

### *Patients and clinical specimens*

Fifty-four tonsils ( $n=35$ ) and adenoids ( $n=19$ ) from patients were studied. Patients' ages ranged

from 2 to 45 years. Twenty-one were females and 33 were males. Indication for the resection of tonsils was chronic lymphoid hypertrophy. None of the patients showed any characteristics of acute tonsillitis due to primary virus infection.

### *Design of M-PCR primers*

The first set of primers was on the U90 gene previously described and used for detection of HHV-6 (Roush *et al.*, 2001). The second primer set was chosen from the HHV-6 U100 gene sequences, GenBank accession number U23467. Third sets of primers were chosen from the DNA pol gen of HHV-6, accession number M63804, all the primer sequences and expected sizes are shown in Table 1.

### *DNA extraction*

QIAamp DNA Mini Kit Qiagen (Qiagen Inc. Valencia, CA, USA) was used for DNA extraction of the tonsil and adenoid samples according to the protocol described in the kit.

### *DNA amplification*

DNA amplification was carried out in sterile ultra pure water with 5  $\mu$ l incubation buffer with 1.5 mM MgCl<sub>2</sub> (Gen Taq DNA polymerase-Hopegene biotechnology), 100  $\mu$ M each of dNTPs (Fermentas-Life Science Technologies, Lithuania), 0.5  $\mu$ M of each oligonucleotids, 2.5 units of Taq DNA polymerase (Fermentas-Life Science Technologies, Lithuania), in a 0.2 ml sterile thin-walled PCR tube, in a total volume of 50  $\mu$ l. Using an Eppendorf Mastercycler personal

TABLE 1 - *Primer names, genes and accession numbers in which primers were chosen, and expected length of PCR products.*

<i>Primer name</i>	<i>Gene</i>	<i>Primer sequence</i>	<i>Accession number</i>	<i>Length of PCR product</i>
HHV6-F	U90	TTCTCCAGATGTGCCAGGGAAATCC	ACCESSION AY245913	HHV-6A 325bp HHV-6B 553bp
HHV6-R		CACATTGTTATCGCTTTCCTCTC		
HHV-6abS	DNA pol gen	TGTATCCGAGCATTATGATG	ACCESSION M63804	293bp
HHV-6abR		CGTACACCGAGTTACATGTTG		
HHV6gpabS	U100	TGGATGGATTCAATCTAGTC	ACCESSION U23467.1	150bp
HHV6gpabR		CCGGAATAAATCCGATATAAACG		

thermocycler, template DNA was initially denatured at 94°C for 2 min. After this cycle, 39 thermocycles, each consisting of 30 s at 94°C, 45 s at 60°C and 45 s at 72°C, were performed. To ensure complete extension, reaction mixtures were further incubated for 10 min at 72°C. Amplified DNA was analyzed by gel electrophoresis using 2.0% (w/v) agarose in a Tris-Borate buffer containing ethidium bromide for detection of DNA under UV transillumination.

#### *PCR analysis of tonsil and adenoid samples*

The DNAs extracted from tonsils were tested for the PCR inhibitor and DNA quality with the internal control primer. In the next step, sample DNAs were tested for HHV-6 with the primer set previously described with PCR. Then the other primer sets specific for U100 and DNA pol gene sequences were tested in PCR.

#### *Restriction enzyme analysis of PCR products for confirmation of virus DNA*

To reconfirm their identity, the HHV-6 PCR products obtained from the samples were digested with appropriate restriction enzymes, HinfI (Gene Mark-Hopegene biotechnology), which were chosen after the individual virus DNA analyzed with the Restriction DNA analyzing program (Webcutter 2.0- Yale University). The digestion mixture consisted of 8 µl of PCR product, 2 µl of appropriate enzyme buffer, five units of enzyme and deionized water to give a final volume of 20 and 20 µl mineral oil also was added to prevent evaporation. The reaction mixture was incubated for 4 h at 37°C for HinfI. Digested products were separated by 20% PAGE and examined.

#### *DNA sequencing*

DNA sequencing of PCR products for reconfirmation of the restriction enzyme analysis was performed with Big Dye Terminator (PE Applied Biosystems) sequencing chemistry on an automatic DNA sequencer ABI 377 (Applied Biosystems).

#### *Preparation of positive control plasmids and PCR sensitivity assay*

To obtain well characterized positive controls and to determine PCR sensitivity, we cloned the PCR product belonging to HHV-6 proven with se-

quencing into the pBSK vector. For subcloning of the PCR product into pBSK vector plasmid; first, pBSK-T PCR cloning vector was prepared as described previously (Sahin *et al.*, 2007). Prepared pBSK-HHV-6 plasmids were used to define M-PCR sensitivity. To determine the sensitivity of the PCR assay, stock preparations at  $1 \times 10^6$  copies/µl were diluted in 100-fold steps to  $1 \times 10^{-2}$  copies/µl.

#### *Immunohistochemical analysis*

Four-micrometer tissue sections were deparaffinized and treated with Proteinase K. Sections were washed between each subsequent solution application with TBS, pH 7.6. Sections were treated with the vendor's concentrations of avidin/biotinBioGenex, San Ramon, CA, USA) and protein blocker (DAKO) before incubation with a monoclonal primary antibody for HHV-6 gp60/110 (Chemicon International, USA) at a dilution of 1:50 in 1% bovine serum albumin TBS, pH 7.6, for 60 minutes at 37°C. After washing, the sections were stained with the DAKO LSAB+ System, alkaline phosphatase, according to the manufacturer's protocol. The sections were examined after counterstaining with fuchsin (DAKO) and permanent mounting (Kannangai *et al.*, 2004).

#### **Western blot analysis**

Cell extracts were prepared in EBC buffer (50 mM Tris, pH 8, 120 mM NaCl, 0.5% Nonidet P-40 [NP-40], with the addition of 100 mM NaF, 0.2 mM sodium orthovanadate, 100 µg/ml polymethylsulfonil fluoride (PMSF), 2 µg/ml aprotinin, and 2 µg/ml leupeptin. Fifty micrograms of whole cell extract were loaded per lane by 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE). Transfer to nitrocellulose was performed using a semi dry transfer method (TRANS-BLOT, BIO-RAD), in 25 mM Tris base, 192 mM glycine, 10% methanol (for 1.5h at 15V) (Sahin *et al.*, 2004). After blocking with 1% nonfat dried milk in PBS/0.05% Tween 20, the primary antibody HHV-6 gp60/110 was added. For negative controls, protein extracted from human cell extracts including PancI cell (pancreatic cancer cell lines), Hep3B (hepatoma cell line), T98G (ganglioma cancer line) cell culture were used along with the tonsil samples and antisera omitted immunoblot was processed as described above.

## RESULTS

### Analysis of tonsil samples

The DNAs extracted from tonsils were tested for the PCR inhibitor and DNA quality with the internal control primer. All PCR reactions with the samples DNA and internal control primer gave expected size PCR product (data not shown). In the next step, sample DNAs were tested for HHV-6 PCR reactions. Initially we used the primer set described previously. We found that 15 of the 54 samples (27.7%) were positive for HHV-6. Since this result was not correlated with the previous results showing that all the tonsil samples were positive, we chose other primers specific to U100 gene and DNA pol gene of HHV-6 DNA and did PCR with these primer sets. We obtained the maximum positivity with the primer specific to the DNA pol gene (40%).

### Restriction enzyme analyses and sequencing of PCR products

To confirm the identity of HHV-6 PCR products using the most sensitive primer set, PCR products were cut with the restriction enzyme chosen after the individual virus DNA analyzed with the Restriction DNA analyzing program (Webcutter 2.0-Yale University). Restriction DNA analysis of PCR product showed the expected bands (data not shown). To reconfirm the identification of the PCR product, the first PCR product was cloned into the pBSK vector (pBSK-HHV-6) and sequenced. The sequence was analyzed with the n-blast program and identified as the HHV-6 virus genome. pBSK-HHV-6 plasmid was further used for control HHV-6 in PCR reactions and PCR sensitivity assay.

### PCR sensitivity assay

Since the prevalence of the HHV-6 in the tonsils was much lower than in previous reports, we tried to see the sensitivity of the PCR with the primer set giving the best result. PCR sensitivity assay showed that PCR with the primers chosen in the DNA pol gene was as sensitive as detecting 1 to 10 copies of plasmid (Figure 1).

### Immunohistochemistry and Western blot analysis

Analysis of all the stained samples did not give any sign of labeling of the tonsil tissue with IHC.

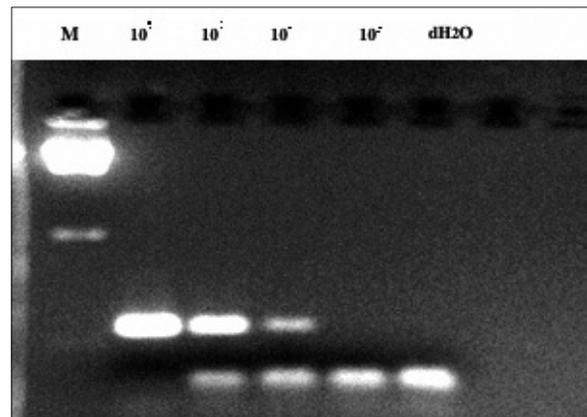


FIGURE 1 - To determine the sensitivity of the PCR assay, plasmid stock preparations at  $1 \times 10^5$  copies were diluted in 100-fold steps to  $1 \times 10^{-1}$  copies/50  $\mu$ l PCR reaction. PCR with plasmid-containing virus sequence was followed as described in material and methods.

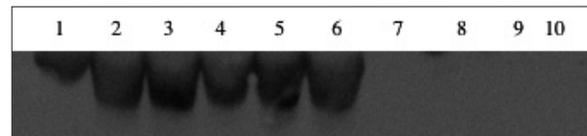


FIGURE 2 - Western blot of some of the samples. Lanes 1-6, samples of tonsils and adenoids. Lanes 7, 8, 9, protein extracts of human PancI, Hep3B, and T98G cell lines respectively and lane 10, primary antisera omitted.

Then we used Western blot to detect HHV-6 in tonsil tissue using the same monoclonal antibody used in immunohistostaining. Initially, we used five PCR positive extracted samples and five PCR negative extracted samples. Results of Western blot showed single bands in all samples including five PCR negative tonsil extracts at the expected size. Then we tested all the samples and found all samples positive for HHV-6 (Figure 2). For negative controls, primary antisera were omitted and the remaining WB processed as described above (data not shown). We also used human cell extracts including PancI, Hep3B, T98G cell extracts for negative controls. No band was seen when primary antisera were omitted or in the line fibroblast extract loaded (Figure 2).

## DISCUSSION

One of the most challenging problems in medicine concerns latent viral infections, in particular

the specific identification of tissues harboring these viruses, the ways viruses persist, and the mechanisms by which they are reactivated. Reactivation of latent HSV-1, HSV-2, EBV and CMV members of the herpesvirus family are responsible for significant morbidity and mortality in immunocompromised individuals, including recipients of solid organ and bone marrow transplants, as well as patients with AIDS (Duffus *et al.*, 2005; Razonable *et al.*, 2005). Similar to the other herpesvirus families, HHV-6 causes latent infection and acts as an opportunistic pathogen (Kondo *et al.*, 2003; De Bolle *et al.*, 2005).

The nasopharynx acts as the natural portal of entry for most herpesviridae family member (Hudnall *et al.*, 2005; Chagas *et al.*, 2006). The majority of primary infections are acquired by contact with contaminated droplets of respiratory secretions or saliva. EBV is well known for acute viral tonsillitis and adenoiditis and remains latent in the tonsils. Recently, we showed that HSV-1 is also involved in latent infection in tonsils (Sahin *et al.*, 2007).

Most HHV-6 infections are acquired mainly in early childhood or adolescence, and primary infection may be asymptomatic (Zerr, 2006). Almost all children in various parts of the world are HHV-6 seropositive by 2 years old, but the true prevalence of the virus in different tissues specifically considering latency is not clear (Yasukawa *et al.*, 1999; Ahlqvist *et al.*, 2005). We thought of the possibility of HHV-6 latency in the tonsils considering that HHV-6 primarily infects the lymphocytes, HHV-6 is ubiquitously present, and the anatomic location of tonsils and the presence of herpesviridae members in the tonsils.

Few papers have reported that tonsil tissues might harbor HHV-6 with different ratios. One of them showed that all the tonsil tissues presented HHV-6 DNA although its level was low (Roush *et al.*, 2001), the other paper showed a 77% positivity (Berger *et al.*, 2007). In this study we also tested the tonsil tissues of healthy individual for HHV-6 presence. Initially we used the same primer set in PCR as in the paper described (Roush *et al.*, 2001). However, we did not obtain more than 27% positivity. We tried to increase the PCR sensitivity by using different primer set belonging to different parts of the HHV-6 genome. Therefore, we chose different primer sets and searched for HHV-6 DNA in the tonsils and found 40% positivity. PCR

sensitivity test showed that PCR is sensitive for detecting as many as 1 to 10 copies of DNA. PCR with internal control also worked well. Therefore we assumed that difficulties for PCR might be related either to the HHV-6 genome or HHV-6 integration to the chromosome. We noticed that different primer sets gave positivity in different samples. Therefore we sensed that none of the primer sets yielded the true result. In the next step, we used immunohistochemistry with HHV-6 both A and B variant-specific monoclonal antibody. Immunostaining did not show any evidence of HHV-6 antigen in the tonsil tissue even with PCR positive cases. We tried WB with protein extracts from the tonsil tissues with the same monoclonal antibody used in immunostaining. We found that all the extracts except negative controls contained a single band of the expected size. Although immunostaining has great advantages in terms of showing tissue structure, it may not be sensitive enough to detect protein with the antibody, tissue preparation and technique-related problems.

The data from the present study, taken together with data published elsewhere, indicate that simple PCR detection of HHV-6 cannot be used as a diagnostic marker of acute infection (Roush *et al.*, 2001). In some studies it seems that PCR quantification of the viral burden may be an effective tool for differentiating acute infection from latency (Collot *et al.*, 2002; Berger *et al.*, 2007). Although we did not use true quantification, we noticed that PCR results and WB results are not correlated with some of the samples. For example, in some samples PCR product showed more intense bands but a weak WB band or in some others the opposite results were obtained, although we loaded equal amounts of DNA and protein extract added to each the PCR and WB respectively. Our study demonstrates the universal positivity for HHV-6 gp60/110 antigen in lymphoid tissue from patients between 23 months and 16 years of age. Interestingly, a high expression of gp60/110 protein which is the target of the monoclonal HHV-6 gp60/110 specific antibody used in IHC and WB may indicate that there is no true latency for HHV-6, if the gp60/110 is not a latency-related protein. Our literature search did not reveal any information on the relation between HHV-6 gp60/110 protein and latency. A comparison of our findings with other reports of lymphoid tissue detection is difficult

since few reports focus on tissue from persons without a pathologic diagnosis (i.e., "normal" subjects). In addition, to our knowledge this is the first report using Western blot to identify HHV-6 prevalence in tissue.

In conclusion, Western blot with the HHV-6 specific antibody may be a better choice for investigation of HHV6 in tonsil tissues. Our future plan is to establish the sensitivity of WB detection of HHV-6 for other tissues especially blood.

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