Technics Used For The Demonstration of HHV-6 or HHV-7 Antigens in Tissues

Immunopathology Laboratory, The University of Cologne (G.R.F. Krueger)

Fixation: 4% Paraformaldehyde in 0.1 mol/L Buffer

4 g paraformaldehyde in 50 ml Aqua bidestillata
(dissolve by warming to 60°C -- NO COOKING!)
clear solution with 1n NaOH and let cool, then filtrate
mix with 50 ml 0.2 mol/L buffer and adjust pH to 7.3

Buffer: Phosphatbuffer (PBS), Stem Solution 0.2 mol/L

Disodium-hydrogen-phosphate dihydrate	28.8 g/L
Sodium-dihydrogen-phosphate-monohydrate	5.2 g/L
Sodium chloride	17.53 g/L

Fix small pieces (not more than 0.5 cm thick) for 24 hours, then embed in paraffin

Paraffin Embedding

Dihydration steps:	Ethanol 70%	1 hour all at 40°C
	Ethanol 80%	1 hour
	Ethanol 96%	1 hour
	Ethanol 96%	1 hour
	Ethanol 100%	1 hour
	Ethanol 100%	1 hour
	Ethanol 100%	1 hour
	Xylol	1 hour
	Xylol	1.5 hours
Paraffin embedding	Paraffin	1 hour all at 59°C
	Paraffin	1 hour
	Paraffin	1 hour

After cooling of tissue blocks, cut 5 micron sections at microtome, stretch sections in water bath at 37° and put on pre-coated glass slides (use "Superfrost Plus" slides or precoat with polylysine).

Deparafinize sections

2 x 15 min.	Xylol
2 min.	100% Ethanol
2 min.	96% Ethanol
2 min.	70% Ethanol
2 min.	distilled water
15 min.	TBS buffer, pH 7.4

Pre-digest sections before immunohistochemistry

Pre-heat vessels with citrate buffer (see below) in microwave to 100°C.
Put slides w sections into pre-heated citrate buffer and microwave at 750 W 2 x 5 minutes.
Cool slides w sections in citrate buffer for about 20 minutes in water bath at 20° and transfer slides w sections into TBS buffer for 5 minutes.

Citrate Buffer

0.1 M citric acid	9 ml
0.1 M sodium citrate sol.	41 ml
distilled water	450 ml
adjust with sodium citrate sol. to pH 6.0	

TBS Buffer

Tris(hydroxymethyl) aminomethan	6 g
NaCl	42 g
with HCl 25%age adjust at pH 7.4	(ca.5ml)
add distilled water to 5 L total volume	

Immunohistochemical APAAP method for HHV-6 & HHV-7 antigens

Antibodies used: p41/38 moab (ABI) for early antigen signaling active infection
gp116/64/54 moab (ABI, Advanced Biotechnologies Inc, Columbia, Maryland) for structural antigen signaling infection, previous or recent.
KR-4 moab (ABI) for HHV-7 antigens (positive

cytoplasmic reaction)

For positive controls served paraffin embedded cell blocks from HHV-6 or HHV-7 infected cultured cells (HSB2/HHV-6; SupT1/HHV-7). For negative controls sections processed according to APAAP technique w/o primary antibody.

APAAP Technic

Deparaffinized and rehydrated sections (see above) were incubated sequentially in following media:

1) Normal rabbit serum, 1:10 in TBS buffer 10 min.
2) 50 µL primary antibody (p41 etc) 1:50 diluted at 4° over night
   (other antibodies need pre-titration with known positive tissues)
   for antibody dilution use TBS buffer (30ml) with BSA (bovine serum albumin; 0.75g)
3) Wash slides in TBS buffer at room temperature 15 min
4) Block non-specific reactivity with pig serum,
   1:20 dilution in TBS buffer 10 min
   and remove buffer by blotting
5) Incubate in "bridging antibody" (rabbit anti mouse)
   1:50 dilution in TBS/BSA (see above) 45 min
6) Wash slides in TBS buffer at room temperature 10 min
7) Block non-specific reactivity with goat or rabbit serum
   1:10 dilution in TBS buffer 10 min
   remove by blotting
8) Incubate in APAAP complex,
   1:50 dilution in TBS/BSA 45 min
9) Wash slides in TBS buffer 10 min
10) Repeat steps 4-9 using only 15 min incubation for each bridging antibody and APAAP complex
11) Incubate in 50-100 µl Fast Red substrate solution
    (vol. according to size of section) 20-30 min
12) Wash in distilled water 15 min
13) Counterstain in Haemalum 2 min
14) Wash in tap water 2 min
15) Wash in distilled water 10 min
16) coverslip in water-soluble media (e.g. Aquatex)
    (caution: Fast Red is soluble in organic solvents)

Fast Red Substrate Solution

Tris-HCl buffer pH 9.5 (see below)	1 ml
Distilled water	8,8 ml
Levamisol solution	20 µL
Dissolve 2 mg Naphthol-AS-Biphosphate in 200 µL dimethylformamide and add	
ADD IMMEDIATELY BEFORE USE: Fast Red	10 mg

Levamisol solution:

Levamisol	2.41 g
Distilled water ad	10 ml

Tris-HCl buffer pH 9.5

Tris-HCl	121 g
Distilled water add to	1 L

Sources:

APAAP complex	Code # D651	Dakopatts A/S Denmark
"Bridging antibody"	Code # Z259	Dakopatts A/S Denmark
Dimethylformamide	Code # 10983	E.Merck, Darmstadt, Germany
Fast Red TR salt	Code # F1500	Sigma, Deisenhofen, Germany
Naphthol-AS-Biphosphat	Code # N2250	Sigma, Germany
Pig serum	Code # X901	Dakopatts A/S Denmark
Trishydroxymethylaminomehtan (TRIS)	Code # 8382	E.Merck, Germany
Goat serum	Code # X907	Dakopatts A/S Denmark

Comments:

1. Routinely paraffin-embedded tissue can be used. Disadvantage may be that routinely used paraffin has higher melting point (is cheaper) and higher temperatures used with it may destroy some antigens. Also, technics may need adjustment (e.g. omit additional microwaving). Results are less reliable.

2. Commonly, the routine immune-peroxidase technic is used as done in many immunochemical procedures. There are significant disadvantages of this technique over the APAAP technic as described here.

a) color contrast (for evaluation and photography) is less optimal
b) cell counting is more difficult including false positives and false negatives.
c) DAB as color reactant is carcinogenic
d) Technic is less sensitive

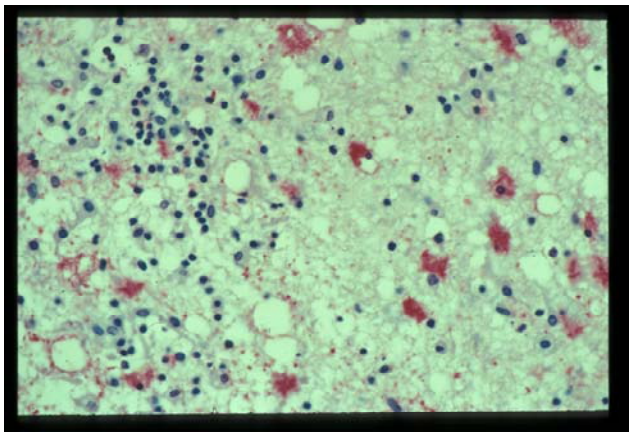
3. Addition of levamisole serves the blocking of endogenous alkaline phosphatase (AP) in cells & tissues. It thus may reduce non-specific background, yet may also decrease the intensity of the specific color reaction. It may be omitted in many tissues with low AP activity, yet must be used when studying blood cell infiltrates and bone marrow sections.

4. Whatever technical variations are used, control sections must be always treated in the same way and with every testing.

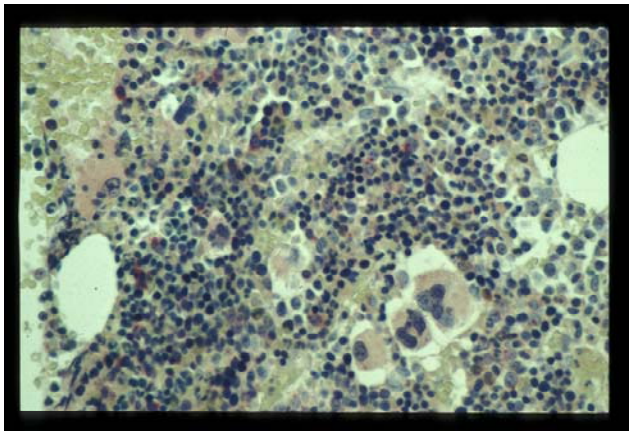
5. When data are used for a publication, we always apply two independent technics to demonstrate the presence of HHV-6/HHV-7 in tissues: e.g. APAAP for antigens and in situ hybridization for viral DNA. The former allows to distinguish active from previous infections (p41 antigen versus gp165), the latter does not distinguish active from latent or previous infections.
Also, viral antigen in cells may be shown even when low viral DNA copies are present in cells, which are below the sensitivity for detection by in situ hybridization.

6) Sensitive molecular technics as preferred by many virologists (e.g. PCR, nested PCR etc.) in body fluids may show the activity of infection, yet not that the virus is actually present in the diseased tissue. These technics are also used with DNA or RNA extracts from tissues kept in deep freeze. Again, they show the virus in these tissues, yet the association of viral activity and lesions itself can be demonstrated only by immunohistochemistry (APAAP method).

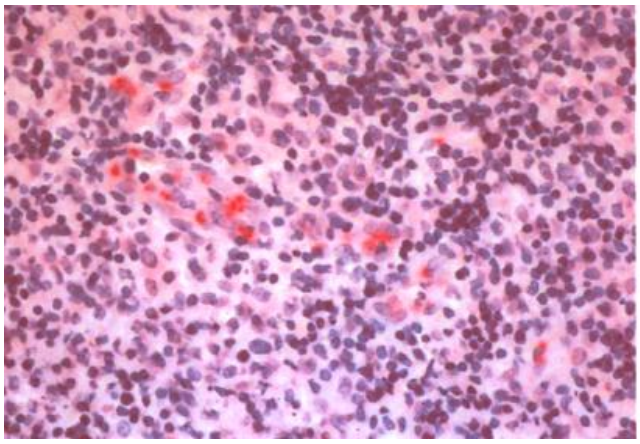
Examples of positive APAAP reactions for HHV-6 and HHV-7



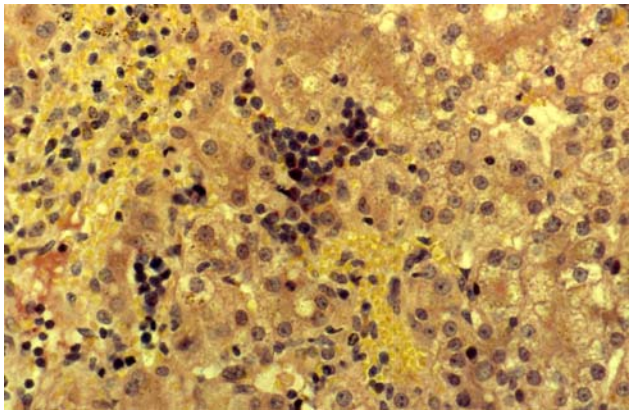
HHV-6 p41 in brain (necrotizing encephalitis)



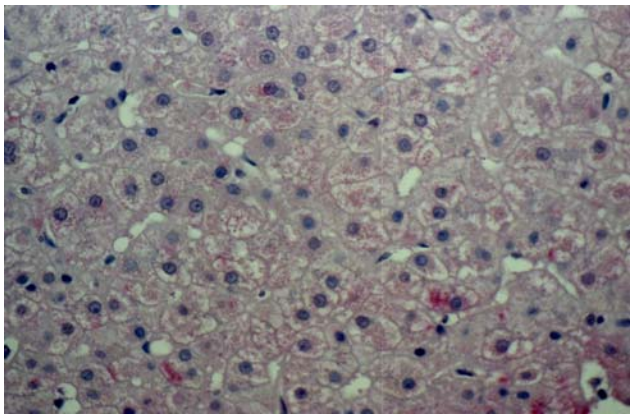
HHV-6 in bone marrow stem cells (red dots)



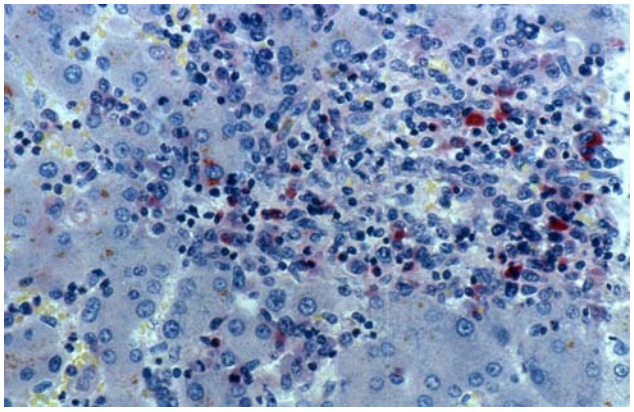
HHV-6 p41 in lymph node endothelial cells (hyperplastic lymph node)



HHV-6 p41 in hepatitis (see red dots in infiltrating lymphoid cells)



HHV-6 p41 in hepatocytes (patient with hepatitis, rare; may be also in bile duct cells similar to CMV)



HHV-7 in hepatitis (in infiltrating lymphoid cells & macrophages)