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Detection of Human *Betaherpesvirinae* in Saliva and Urine from Immunocompromised and Immunocompetent Subjects

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Human cytomegalovirus (HCMV) is a well-known opportunistic agent that reactivates in human immunodeficiency virus (HIV)-seropositive subjects. Human herpesvirus 6 (HHV-6) and HHV-7 were discovered recently and, like HCMV, belong to the *Betaherpesvirinae* subfamily. We looked for the presence of HCMV, HHV-6, and HHV-7 by PCR with saliva and urine samples from 125 HIV-seropositive patients at different stages of HIV infection and with saliva and urine samples from 29 HIV-seronegative subjects. All three viruses were frequently detected in the saliva (overall rates of detection, 61, 43, and 63% for HCMV, HHV-6, and HHV-7, respectively) with no correlation with the stage of immune deficiency. In contrast, HCMV was detected in urine much more frequently than the two other herpesviruses (overall rates of detection, 37, 2, and 6.5% for HCMV, HHV-6, and HHV-7, respectively) and was associated with immune deficiency. This suggests that these three genetically related viruses differ from each other with regard to replication in the urinary tract.

Human cytomegalovirus (HCMV), human herpesvirus 6 (HHV-6), and HHV-7 are genetically related and have been classified as members of the *Betaherpesvirinae* subfamily (3, 13). In human immunodeficiency virus (HIV)-seropositive subjects, HCMV is a major opportunistic agent that causes retinitis (18) as well as gastrointestinal diseases (7), and HHV-6 has also been associated with retinitis (20). Both viruses have been proposed as cofactors for the progression to AIDS (16, 22), but the role of HHV-6 remains a matter of discussion (8). By contrast, HHV-7 epidemiology in HIV-seropositive and immunocompetent subjects remains unclear. All three viruses are present in saliva, which could play a role in their transmission (5, 6). HCMV can be readily detected in urine, which may be a source of viral transmission, whereas the presence of HHV-6 and HHV-7 in urine has not been well studied. In particular, this question has not been addressed for HIV-seropositive subjects, and the effect of immune deficiency on viral excretion remains unclear. We studied the presence of HCMV, HHV-6, and HHV-7 in saliva and urine from HIV-seropositive patients at different stages of infection and in saliva and urine from immunocompetent subjects. Our first goal was to investigate the role of urine as a source of HHV-6 and HHV-7 for interhuman transmission. A second objective was to analyze the possible influence of HIV infection on viral secretion in saliva and urine.

One hundred twenty-five HIV-seropositive and 29 HIV-seronegative healthy subjects were studied. As previously reported (8), the 125 HIV-seropositive subjects were classified into four groups, as follows, according to CD4-positive ($CD4^+$) T-cell count per microliter and antiretroviral therapy: $CD4^+$-cell count of $\leq$200/μl with antiretroviral therapy except in four patients, $n = 33$; $CD4^+$-cell count of >200 to <450/μl with antiretroviral therapy, $n = 31$; $CD4^+$-cell count of >200 to <450/μl without antiretroviral therapy, $n = 33$; and $CD4^+$-cell count of $\geq$450/μl without antiretroviral therapy, $n = 28$. The $CD4^+$-cell counts for the healthy HIV-seronegative subjects were not known. Nucleic acids from 1 ml of whole-saliva samples were purified as described previously (8) and were resuspended in 100 μl of distilled water. One milliliter of whole urine was first clarified by centrifugation at 180 × *g* for 5 min at 20°C in order to pellet cells, cell debris, and insoluble material. Cell-free viruses present in the supernatant were then pelleted by ultracentrifugation at 356,160 × *g* for 10 min at 4°C. The two pellets obtained by the first centrifugation (cell pellet) and the ultracentrifugation (supernatant), were lysed overnight at 37°C in 350 μl of lysis buffer (10 mM Tris hydrochloride [pH 7.5], 1 mM EDTA) containing 0.2 mg of proteinase K per ml and 0.5% sodium dodecyl sulfate. Nucleic acids were phenol-chloroform extracted, ethanol precipitated with the addition of 20 μg of glycogen, and resuspended in 100 μl of distilled water.

Ten microliters of each DNA sample, corresponding to 1/10 of the initial sample, was subjected to PCR. The primer pairs used to search for HCMV, HHV-6, and HHV-7 sequences were 625 and 461, LP1 and LP2, and HV10 and HV11, respectively, as described previously (3, 9, 12). The HHV-6 primers detected HHV-6 variants A and B. The cross-reactivity of each system was tested with strains of HCMV, HHV-6 variant A, HHV-6 variant B, and HHV-7. HCMV AD 169, kindly provided by D. Lang, was cultured on MRC5 cells. HHV-6 variant A strain TAN and HHV-6 variant B strain MAR were obtained by coculture of peripheral blood mononuclear cells (PBMCs) from healthy blood donors (2). HHV-7 IM was obtained in our laboratory by culture of saliva from a healthy subject on PBMCs from healthy blood donors (19). Amplification of beta-globin gene sequences was performed with the primers previously described (21) to test for the presence of nucleated cells and to verify the absence of major *Taq* polymerase inhibitors. For each PCR run including 15 to 20 samples, one positive control (consisting of HCMV, HHV-6 variant A, HHV-6 variant B, or HHV-7 DNA) and one negative control consisting of distilled water instead of a DNA sample were tested. The positive controls were DNA extracts obtained from cell cultures by extraction from the cells for HCMV and by extraction from the culture supernatant for HHV-6 variants A and B and

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TABLE 1. Detection of HCMV, HHV-6, and HHV-7 in saliva

Virus (no. of samples tested)	No. (%) of PCR-positive saliva samples from different subject groups					
	HIV-seropositive group with $CD4^+$-cell count					HIV seronegative group
	Total	≤200/μl	201 to 449/μl (treated[a])	201 to 449/μl (untreated[b])	≥450/μl	
HCMV (123)	64 (65)	20 (67)	19 (68)	16 (59)	9 (64)	11 (46)
HHV-6 (154)[c]	48 (38)	12 (36)	14 (45)	12 (36)	10 (36)	18 (62)[d]
HHV-7 (123)	64 (65)	18 (60)	20 (71)	15 (56)	11 (79)	14 (58)

[a] Patients were treated with antiretroviral drugs.
[b] Patients were not treated with antiretroviral drugs.
[c] Results concerning HHV-6 detection in saliva were previously reported (8).
[d] $P = 0.023$ compared with result for HIV-positive patients.

for HHV-7. All steps of sample preparation, DNA extraction, PCR amplification, and PCR product analysis were done in different areas to prevent PCR carryover.

Fifteen microliters of PCR products were detected and characterized by Southern blot hybridization with ^{32}P-labelled oligonucleotide probes, 626 for HCMV (12), LP3 for HHV-6 (9), and HV12 for HHV-7 (3), according to the method described by Aubin et al. (2). Statistical comparisons of the results were done by chi-square analysis and Fisher's exact test. A P value of <0.05 was considered significant.

Of all the saliva samples tested, 75 of 123 (61%) were positive for HCMV, 66 of 154 (43%) were positive for HHV-6, and 78 of 123 (63%) were positive for HHV-7 (Table 1). Twenty-eight percent of the saliva specimens that were tested for all three viruses were positive for all three, and 18% were negative for all three viruses but positive for beta-globin gene sequences. Statistical analysis demonstrated a significant association between the detection of HHV-7 and that of HCMV (<0.0001). Some saliva samples were positive for HCMV, HHV-6, and HHV-7 but negative for the beta-globin gene, indicating the presence of cell-free viruses, which might correspond to active infections. The analysis of a possible relationship between HIV infection and the rate of *Betaherpesvirinae* detection in saliva led to contrasting results. Firstly, the rates of HCMV and HHV-7 detection in saliva did not differ significantly for HIV-seropositive and HIV-seronegative subjects, while, as previously reported (8), the rate of HHV-6 detection was significantly higher for HIV-seronegative subjects than for HIV-seropositive subjects taken together ($P = 0.023$). Secondly, the rate of detection did not differ according to $CD4^+$-cell count among HIV-positive patients for any of the three herpesviruses tested.

In urine cell pellets and supernatants (Table 2), overall detection rates for HCMV, HHV-6, and HHV-7 were 37, 2, and 6.5%, respectively. Twenty percent of the urine samples were positive for HCMV both in the cell pellet and the supernatant, whereas 19% were positive for HCMV only in the cell pellet. HHV-6 was detected both in the cell pellet and in the supernatant, whereas HHV-7 was detected only in the cell pellet. A comparison between the presence of HCMV in urine (cell pellet and/or supernatant) and its presence in saliva did not evidence any significant association. In contrast, seven of eight urine samples positive for HHV-7 corresponded to subjects with saliva positive for HHV-7. Similarly, the three urine samples positive for HHV-6 corresponded to subjects positive for HHV-6 in saliva and/or PBMCs (8). Eight urine supernatants were positive for HCMV and negative for the beta-globin gene, strongly suggesting that HCMV was present as a cell-free virus in these cases. In contrast, the only urine supernatant that was positive for HHV-6 was positive for the beta-globin gene. The rate of HHV-6 and HHV-7 detection in urine did not differ according to the presence of HIV infection or to $CD4^+$-cell count for HIV-seropositive subjects. In contrast, the rate of HCMV detection in urine supernatant as well as in urine cell pellets was significantly higher for subjects with $CD4^+$-cell

TABLE 2. Detection of HCMV, HHV-6, and HHV-7 in urine cell pellets and supernatant

Virus (no. of samples tested)	Urine fraction	No. (%) of PCR-positive urine samples from different subject groups					
		HIV-seropositive group with $CD4^+$-cell count					HIV seronegative group
		Total	≤200/μl	201 to 449/μl (treated[a])	201 to 449/μl (untreated[b])	≥450/μl	
HCMV (123)	Cell pellet	38 (38)	17 (57)	8 (29)[c]	11 (41)	2 (14)[d]	8 (33)
	Supernatant	23 (23)	13 (43)	5 (18)[e]	5 (23)[f]	0 (0)[g]	2 (8)[h]
HHV-6 (123)	Cell pellet	3 (3)	1 (3)	1 (3.5)	1 (4)	0 (0)	0 (0)
	Supernatant	1 (1)	1 (3)	0 (0)	0 (0)	0 (0)	0 (0)
HHV-7 (123)	Cell pellet	5 (5)	0 (0)	3 (11)	2 (7)	0 (0)	3 (12.5)
	Supernatant	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

[a] Patients were treated with antiretroviral drugs.
[b] Patients were not treated with antiretroviral drugs.
[c] $P = 0.031$ compared with value in "≤200 μl" column.
[d] $P = 0.008$ compared with value in "≤200 μl" column.
[e] $P = 0.036$ compared with value in "≤200 μl" column.
[f] $P = 0.044$ compared with value in "≤200 μl" column.
[g] $P = 0.003$ compared with value in "≤200 μl" column.
[h] $P = 0.004$ compared with value in "≤200 μl" column.

counts of ≤200/μl than for subjects with higher $CD4^+$-cell counts (Table 2).

In our study, all three human betaherpesviruses were highly prevalent in saliva from HIV-seropositive and immunocompetent subjects, as previously reported for HCMV and HHV-7 in the case of HIV-seropositive subjects (6, 15) and for HHV-6 and HHV-7 in the case of immunocompetent subjects (5, 6). When the study groups were compared with one another, the only significant finding for saliva was the higher detection rate of HHV-6 in HIV-seronegative subjects than in HIV-seropositive subjects. As discussed elsewhere (8), this result might be considered paradoxical in view of the possible cofactor role of HHV-6 in HIV infection, but it is in agreement with previous preliminary reports (10). Other reports have shown that HHV-6 and HHV-7 could be isolated from saliva and therefore were present as infectious viruses (4, 14). As previously reported (8), all 71 HHV-6 strains identified corresponded to HHV-6 variant B, except one HHV-6 variant A strain in the saliva of a healthy HIV-seronegative subject. These results are in agreement with those of other studies (1, 6). From previous reports and from our present results, we infer that saliva could play a major role in the interhuman transmission of HHV-6 and HHV-7.

In urine, HCMV was detected much more frequently than HHV-6 and HHV-7, with respect to both cell pellets and supernatants. HCMV was cell free as well as cell associated, whereas HHV-6 and HHV-7 were cell associated in all cases. The difference in excretion rate as well as the difference in the state of the excreted virus may reflect distinct patterns of replication for these viruses in the urinary tract. HCMV has been found in kidneys from healthy subjects (11), whereas HHV-6 antigens have been detected in rejected kidneys from kidney transplant recipients but not in normal kidneys (17). HHV-7 has been previously detected in urine (23), but to our knowledge, the presence of HHV-7 antigens or DNA in renal tissue has never been studied. In our study, two of the three subjects whose urine was HHV-6 positive also tested positive for HHV-6 in PBMCs, suggesting that the presence of HHV-6 in urine might be due to the presence of infected blood cells. Similarly, the very low rate of HHV-7 detection in urine does not favor the hypothesis of an active renal HHV-7 infection leading to high level of urinary excretion. It is possible that in contrast to HCMV, the presence of HHV-6 and HHV-7 in urine does not occur through the active replication of these two viruses within the urinary tract. The analysis of virus excretion in urine according to the level of immune deficiency in HIV-seropositive subjects strengthens the differences between HCMV on the one hand and HHV-6 and HHV-7 on the other. The presence of HCMV in urine supernatants was indicative of immune deficiency, whereas the presence of HHV-6 or HHV-7 was not. Accordingly, other authors found that the rate of positive HCMV culture from urine was inversely correlated to $CD4^+$-cell count (24).

Taken together, these results led us to postulate two distinct scenarios for the multiplication of *Betaherpesvirinae* subfamily members in the human urinary tract. HCMV is present in the human urinary tract and is actively replicated, this replication being increased in the case of immune deficiency, and working as a source of virus for possible interhuman transmission. Conversely, HHV-6 and HHV-7 are not normally present in the urinary tract. Their presence in urine could reflect contamination from blood or other tissues rather than active replication in kidney cells and does not appear to be influenced by immune deficiency, at least not at a detectable level. According to this scenario, urine does not appear to be a common route of HHV-6 and HHV-7 transmission in adults. This finding is in contrast with that for saliva, which appears to be the most common route of transmission for the three human betaherpesviruses.

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