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Transmissible Kaposi's Sarcoma-Associated Herpesvirus (Human Herpesvirus 8) in Saliva of Men with a History of Kaposi's Sarcoma

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We have evaluated the physical state and infectious nature of Kaposi's sarcoma-associated herpesvirus (KSHV) in the saliva of nine persons with past or current Kaposi's sarcoma (KS). KSHV DNA in saliva had the physical characteristics of DNA present in virions. Inoculation of 293 cells with cell-free saliva fluid resulted in the persistence of KSHV DNA in culture for at least 13 passages of the cells. The addition of tetradecanoyl phorbol acetate to KSHV-infected 293 cells led to increased viral DNA. Two virus-specific RNAs were detected by reverse transcriptase PCR in 293 cells infected with cell-free saliva fluid and in cells present in saliva from subjects with KSHV salivary shedding. These results indicate that infectious KSHV can be present in saliva of patients with KS.

Epidemiological studies indicated that Kaposi's sarcoma (KS) has an infectious etiology (5), and this led to the identification of sequences of a new gammaherpesvirus in KS tumor tissue (9), termed Kaposi's sarcoma-associated herpesvirus (KSHV) or human herpesvirus 8. Molecular and serological studies have supported KSHV as an important etiologic factor in KS (7, 8, 12–14, 16, 19, 21, 30). Studies of KSHV exposure in the general population have found antibodies to latent antigens in 1 to 5% of persons at low risk for KS (13, 14). More recently, antibodies to KSHV lytic proteins were found in greater than 20% of the general U.S. population (16). Knowledge about the true epidemiology of KSHV is still evolving as sensitive serological assays for the virus become available.

The high frequency of KS in human immunodeficiency virus (HIV)-infected homosexual men, compared to other HIV-positive populations, suggested a sexually transmitted pathogen (3, 4). Some seroepidemiological results indicate that infection with KSHV has the pattern of a sexually transmitted disease (14). Reports of the frequency of KSHV DNA in semen have varied widely (2, 10, 17, 20, 28), but regardless of the frequencies of KSHV DNA detected in semen, the amount of DNA detected has been low and no evidence of infectious virus has been demonstrated to date. KSHV infection is found in elderly men of Mediterranean descent with KS, in persons with African endemic KS of all ages, and in transplant patients with KS (7, 8, 21). These are, for the most part, populations at low risk for sexually transmitted diseases, and would suggest that KSHV may have some other common route of transmission. Many herpesviruses are shed in saliva, and Epstein-Barr virus (EBV), the virus most closely related to KSHV infecting humans, has a replicative cycle in oropharyngeal epithelial cells with infectious salivary virus. The detection by PCR of KSHV DNA in saliva from HIV-positive subjects has been reported (6, 30). Studies in our laboratory have detected KSHV DNA in the saliva of HIV-positive/KS-positive, HIV-positive/KS-negative, and HIV-negative/KS-positive subjects (15). We have extended that work by examining the saliva of KS-positive subjects for the presence of transmissible KSHV.

Characterization of KSHV in saliva. We studied KSHV in saliva from nine subjects with KS (Table 1): eight HIV positive and one HIV negative (subject 10). Only three of the nine had any past or current oropharygneal KS lesions. All subjects had at least one positive detection by PCR of KSHV DNA in saliva; and except for subject 11, all had KSHV DNA detected in peripheral blood mononuclear cells (15). The analysis of KSHV DNA present in the saliva of subject 2, who had spontaneously resolved a KS lesion 7 years ago and who had no clinical evidence of cutaneous or oral KS at the time of study, is shown in Fig. 1A. KSHV DNA in saliva was amplified by PCR using primers KS-1 and KS-2 to open reading frame 26 (9), using PCR conditions, internal controls, and detection of the PCR products by liquid hybridization as previously described (15). KSHV DNA was present at approximately 10^6 copies per ml of whole saliva as determined by comparison of the PCR signal from 10 μl of saliva to the PCR signal from 10^4 copies of the positive control KSHV DNA that was generated by cloning the PCR product of primers KS-1 and KS-2 into the TA vector (Stratagene, San Diego, Calif.). Saliva was then fractionated into cells and cell-free saliva fluid by treatment with 1 mM dithiothreitol for 20 min at room temperature, centrifugation at 800 × *g* for 10 min to pellet the cells, and filtration (0.8 μM) of the supernatant. KSHV DNA was detected in both the cells and cell-free fluid (Fig. 1A). The cell-free saliva fluid was further fractionated by ultracentrifugation (90,000 × *g* for 1 h), which will pellet herpesvirus virions, and the majority of KSHV DNA was present in the pellet (Fig. 1A). Because DNA encapsulated within virions should be protected from DNase, the pelleted material was resuspended and treated with DNase (15). PCR amplification of KSHV DNA before and after DNase treatment demonstrated that it was protected (Fig. 1B). As an internal control for a herpesvirus shed in saliva, the same samples were analyzed for EBV DNA by PCR (15); EBV DNA was also pelleted by ultracentrifuga-

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TABLE 1. Clinical characteristics of subjects studied and the detection by PCR of KSHV DNA in whole saliva, in DNase-treated virus pelleted from cell-free saliva fluid, and in cell-associated DNA from cultures inoculated with cell-free saliva fluid

Subject[a]	Clinical characteristics		Detection by PCR of KSHV DNA		
	CD4 cells/μl	KS site	Saliva[d]	DNase	Cell culture
2[b]	528	Skin[c]	+	+	ND[e]
2			−	ND	−
2			+	+	+
2			+	ND	+
2			+	ND	+
3	33	Skin	−	ND	−
7	60	Skin	−	ND	ND
8	312	Skin	+	+	ND
10	667	Skin	+	ND	+
11	134	Skin	−	ND	ND
12	34	Skin, oral	+	+	+
15	86	Skin, oral	+	+	+
16	40	Skin, oral	+	+	+

[a] Subject numbers are equivalent to those in Koelle et al. (15).
[b] All samples from subject 2 were collected on separate days.
[c] No clinical KS at the time of saliva collection.
[d] KSHV DNA was approximately $10^{4.7}$ to 10^6 copies per ml in positive saliva samples (15, 29).
[e] ND, not determined.

tion and protected from DNase (Fig. 1B). Nonencapsulated varicella-zoster virus DNA, added as a DNase control, was amplified in the untreated sample but undetected in the DNase-treated sample (Fig. 1B). Saliva samples from subjects 8, 15, 12, and 16 (Table 1) were processed similarly, and the KSHV DNA was pelleted by ultracentrifugation and was DNase resistant (15, 29). The centrifugation and DNase results from all five subjects were consistent with KSHV DNA being contained within virions; therefore, saliva was tested for the presence of infectious KSHV.

Detection by PCR of KSHV DNA in cells inoculated with cell-free saliva fluid. 293 cells have been reported to be permissive for KSHV (11) and were therefore used as an assay for infectious virus. 293 cells were inoculated with pelleted virus from subject 2 and with cell-free saliva fluid from subject 15, as follows. 293 cells (ATCC CRL 1573), cultured in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, were inoculated with 0.4 ml of cell-free saliva fluid mixed with an equal volume of medium or with an equivalent amount of pelleted sample in 0.8 ml of medium in 22-mm-diameter dishes. KSHV DNA was subsequently detected in the cells 4 days postinoculation (Fig. 2A), as evidenced by the fact that following trypsinization the cells were washed four times with phosphate-buffered saline and the final wash of the cells was negative for KSHV by PCR (29). Heating (60°C for 20 min) of the pelleted virus, or the cell-free saliva fluid, prior to inoculation of 293 cells eliminated the detection of cell-associated KSHV DNA by PCR (Fig. 2A).

The persistence of KSHV in culture was then examined in 293 cells that had been inoculated with cell-free saliva fluid from subject 2. The culture was passaged when confluent (split 1:5), and KSHV DNA was detected by PCR in samples from cultures through 11 passages (Fig. 2B). While KSHV DNA was present in the culture, no cytopathic effect was evident. All cultures were also examined by PCR using a second set of primers, KS-A and KS-B, specific for open reading frame 25 of KSHV (15). All samples were positive with both sets of primers (29). The 293 cultures were also tested for EBV DNA, which was present in the original saliva sample at greater than 10-fold the level of KSHV, by PCR with primers specific for the EBER region (15). At the first passage, fewer than five copies of EBV DNA were detected and at the sixth passage EBV DNA was undetectable (Fig. 2C). A total of seven cultures inoculated with cell-free saliva fluid from four different subjects were found to have cell-associated KSHV DNA (Table 1).

The persistence of KSHV DNA, but not EBV DNA, in cell cultures indicated that there was some level of KSHV replication. Because phorbol esters have been shown to increase KSHV replication in cell lines harboring KSHV (23), the level of KSHV DNA in 293 cells inoculated with cell-free saliva fluid was tested with and without tetradecanoyl phorbol acetate (TPA). A culture initiated with saliva from subject 12 at passage 7 and two cultures initiated with independently collected samples from subject 2 at passages 13 (culture 2a) and 6 (culture 2b) were treated with TPA. For each experiment, one of two identical cultures was treated with 20 ng of TPA per ml and 4 days later both cultures were harvested for PCR analysis (Fig. 2D). With TPA, KSHV DNA was detected at approximately a 10-fold increase in the culture initiated from subject 7 and in culture 2a, and at an approximately 5-fold increase in culture 2b, as determined by comparison to the $\log_{10}$ dilution of standardized KSHV DNA (Fig. 2D) and by PCR analysis of $\log_{10}$ dilutions of the samples (29). The $\log_{10}$ dilutions of samples from 2a and 2b were also analyzed with a Molecular Dynamics PhosphorImager using the ImageQuant program, which indicated 11.7- and 4.2-fold increases in KSHV DNA with TPA in cultures 2a and 2b, respectively. A 10^{-3} dilution of each sample used for the detection of KSHV DNA was also analyzed for beta-globin DNA as a control for total cellular DNA of the treated and untreated samples (Fig. 2D). The reduced amount of beta-globin DNA in the samples from TPA-treated cultures was due to a cytopathic effect of TPA on 293 cells, regardless of the presence of KSHV, which resulted in a reduced number of cells in the treated cultures.

Detection of KSHV RNA transcripts. KSHV infection of 293 cells was further defined by examining a culture inoculated with a sample from subject 2 at passage three for two KSHV transcripts, a 1.1-kb RNA and a 0.7-kb RNA (31). To determine if KSHV gene expression occurred in 293 cells inoculated with cell-free saliva fluid, RNA was extracted from cultures

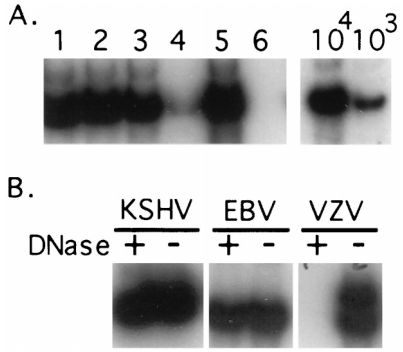

FIG. 1. (A) Identification of KSHV DNA by PCR in saliva from subject 2. Lanes: 1, whole saliva; 2, cells present in whole saliva; 3, cell-free saliva fluid; 4, supernatant following ultracentrifugation; 5, pellet from ultracentrifugation; 6, negative control with HSB-2 cells. (Right panel) Signal from 10^4 and 10^3 copies of cloned, quantified KSHV DNA that is the target for primers KS-1 and KS-2. (B) Detection by PCR of viral DNA before (+) and after (−) DNase treatment of material pelleted from cell-free saliva fluid by ultracentrifugation. KSHV and EBV were present in saliva; the nonencapsulated varicella-zoster virus (VZV) DNA was added as a DNase control.

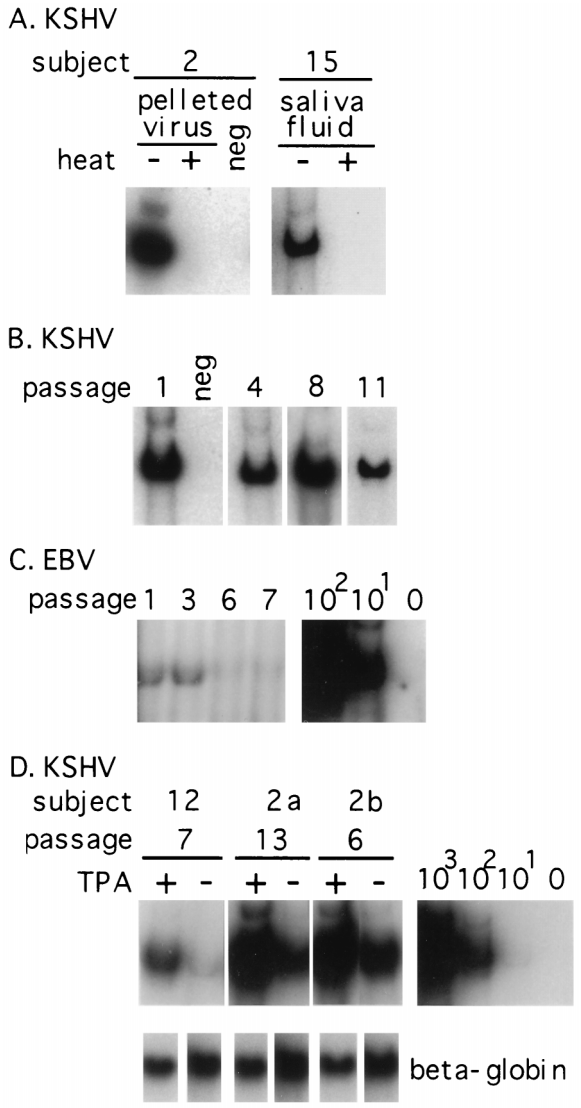

FIG. 2. Detection of viral DNA by PCR after inoculation of 293 cells with cell-free saliva fluid. (A) KSHV DNA present in 293 cell cultures inoculated with pelleted virus from subject 2 or with cell-free saliva fluid from subject 15. Samples were untreated (−) or heated (+) at 60°C for 20 min before inoculation, and cells were harvested at 4 days postinoculation. neg, 293 cells. (B) KSHV DNA in 293 cells inoculated with cell-free saliva fluid from subject 2 at passages 1, 4, 8, and 11. neg, 293 cells. (C) EBV DNA detected in passages 1, 3, 6, and 7 of the 293 cultures examined in panel B. (Right panel) EBV standard generated from DNA isolated from Raji cells containing an average of 50 genomes/cell (26) (D) KSHV DNA present in 293 cultures treated (+) and untreated (−) with 20 μg of TPA per ml for 4 days. Cultures were the seventh passage of cells inoculated with a sample from subject 12 and the thirteenth (2a) and sixth (2b) passages of cultures inoculated with samples from subject 2. (Lower panel) PCR analysis of beta-globin DNA present in samples used for KSHV detection. A 10^{-3} dilution of the sample used for KSHV DNA detection was analyzed. (Right panel) KSHV standard curve generated by a $\log_{10}$ dilution of the quantified cloned PCR product of the KS-1 and KS-2 primers.

untreated and treated with TPA and the RNA was examined by reverse transcriptase (RT) PCR for the presence of KSHV transcripts by the following procedure. RNA was extracted using standard guanidinium thiocyanate-acid phenol-chloroform procedures. One-third of each RNA sample was then treated with 6 U of RQ DNase (Promega, Madison, Wis.) for 90 min at 37°C. After the DNase was inactivated with an equal volume of 50 mM EDTA, RNA was reextracted twice with acid phenol-chloroform, precipitated with ethanol, and resuspended in 30 μl of diethyl pyrocarbonate-treated water. Five microliters of DNase-treated RNA was used to synthesize cDNA using primers P07-L (CCG GCC TTC TGA ACT GTG ACT GTG TTA), specific for the 0.7-kb RNA, and P11-L (AGC TTT TGT TCT GCG GGC TTA TGG AGA), specific for the 1.1-kb RNA. Each 25-μl RT reaction mixture contained 50 mM Tris, 75 mM KCl, 3 mM $MgCl_2$, 600 μM dithiothreitol, 150 μM each dinucleoside triphosphate, 66.4 μM primer, 25 U of RNAguard (Pharmacia Biotech), and 130 U of Moloney murine leukemia virus RT (Gibco BRL). Ten microliters of each cDNA and 2 μl of DNase-treated RNA were then analyzed by PCR using primers P07-L and P07-U (AGC TAG CGT GCC CTC CCA AAG AGT) or primers P11-L and P11-U (CTA TGG ATT TTG TGC TCG CTG CTT GC). After 35 cycles of 30 s at 96°C, 30 s at 56°C, and 30 s at 72°C, PCR products were analyzed by liquid hybridization with the ^{32}P-labeled probe P07-P (AAA GCA CAA TCA CGG TTG CAC CAA GCA) or P11-P (TTT CAT TGG TGC CGC CGA TTG TGG G). The 1.1-kb RNA and the 0.7-kb RNA were both detected in 293 cells, with or without TPA (Fig. 3). Although the 1.1-kb RNA was detected at approximately equivalent amounts with or without TPA and the 0.7-kb RNA was detected in increased amounts in the TPA-treated culture, a quantitative comparison of the RT PCR products in these reactions was not possible because a positive internal control was not available. As positive controls, analysis by RT PCR of RNA and analysis by PCR of DNA from BCBL-1 cells (23) were carried out by using the P07-U and P07-L and P11-U and P11-L primers (Fig. 3). Importantly, there was no signal detected without the addition of RT (Fig. 3), demonstrating that the RT PCR signal was due to viral RNA produced by the infection of 293 cells by KSHV present in saliva.

To determine if cells present in saliva may be productively infected by KSHV, RNA from cells present in saliva samples from subjects 2, 7, 10, and 11 were directly assayed by RT PCR for the 1.1- and 0.7-kb KSHV RNAs (31), as described above. The cells were approximately 10% viable by trypan blue exclusion and were epithelial cells upon microscopic inspection. The 1.1-kb RNA was detected in samples from subjects 2 and 10, and the 0.7-kb RNA was readily detected in cells from subject 2 and faintly detected in cells from subject 10 (Fig. 4A). Again, there was no signal detected without the addition of RT (Fig. 4A). The PCR analysis of KSHV DNA in whole saliva of these samples correlated with the RNA results, with greater than 10^5 copies of KSHV DNA per ml found in samples from subject 2 and 10, whereas KSHV DNA was undetectable in the samples from subjects 7 and 11 (Fig. 4B).

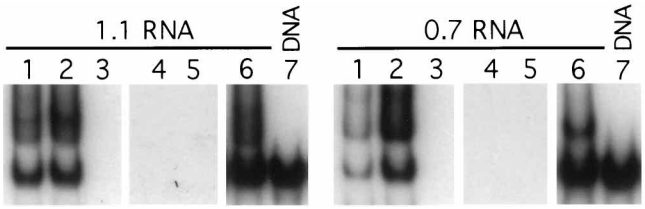

FIG. 3. Detection by RT PCR of the KSHV 1.1- and 0.7-kb RNAs in 293 cells inoculated with cell-free saliva fluid from subject 2 at the third passage and in BCBL-1 cells. Lanes: 1, 293 cells inoculated with cell-free saliva fluid; 2, 293 cells inoculated with cell-free saliva fluid treated with 20 ng of TPA per ml for 4 days; 3, 293 cells; 4, the same as lane 1 except without RT; 5, the same as lane 2 except without RT; 6, RT PCR analysis of BCBL-1 cells; 7, detection by PCR of DNA from BCBL-1 cells with the same primers used for RT PCR. The higher multiple bands in the RT reactions were due to the detection of RT PCR products by liquid hybridization.

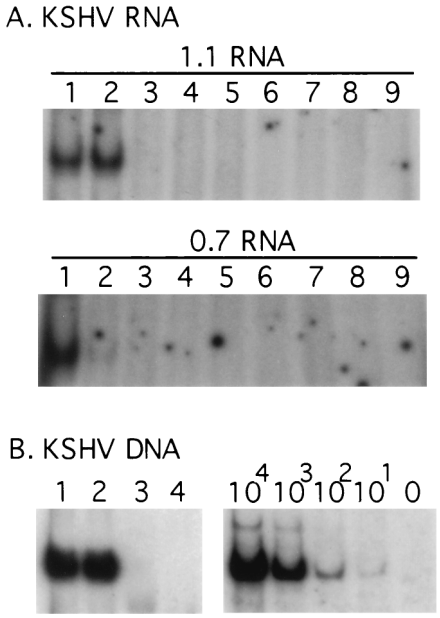

FIG. 4. Direct analysis of KSHV RNA and DNA in saliva. (A) Detection of the 1.1- and 0.7-kb RNAs in cells collected from saliva samples. Lanes: 1, subject 2; 2, subject 10; 3, subject 7; 4, subject 11; 5 to 8, the same as lanes 1 to 4 except without RT; 9, control RT PCR reaction mixture without RNA. (B, left panel) Detection by PCR of KSHV DNA in saliva samples used for detection by RT PCR of KSHV RNA. Lanes 1, subject 2; 2, subject 10; 3, subject 7; 4, subject 11. (Right panel) PCR signal from $\log_{10}$ dilutions of cloned, quantified KSHV DNA that is the target for primers KS-1 and KS-2.

Conclusions. Our results demonstrate that infectious KSHV can be present in saliva, as evidenced by the persistence of KSHV DNA in 293 cell cultures inoculated with cell-free saliva fluid, the induction of viral replication by TPA in 293 cells, and the expression of virus-specific RNAs in 293 cells inoculated with cell-free saliva fluid. Although we found that KSHV from saliva can infect 293 cells, we did not find a cytopathic effect due to KSHV infection or achieve the serial passage of cell-free KSHV in 293 cells (29). This contrasts with the report by Foreman et al. (11) of a productive infection in 293 cells with cytopathic effect evident and the serial passage of virus. Whether these differences are due to the cells used, viral isolate differences, or culture conditions is yet to be determined. The detection of the 1.1-kb RNA which has been indicated as a lytic RNA (31) of the early class (27) indicates that some level of viral gene expression involved in a productive infection occurs in 293 cells. Whether the virus persists by a low level of lytic replication or by a latent mode of replication with a subset of cells undergoing the lytic cycle requires further study.

In support of the data demonstrating infectious KSHV in saliva, virus-specific RNAs were directly detected in cells collected from the saliva of two subjects with salivary shedding of KSHV but without oral KS. The presence of these RNAs, particularly the lytic 1.1-kb RNA, suggests that productively infected cells are present in the oropharynx. EBV is the virus most closely related to KSHV that infects humans, and this similarity includes both genetic content (22, 24) and B-cell tropism (18). EBV DNA and RNA was found in oropharyngeal epithelial cells (25), which are considered the site of lytic replication accounting for infectious shedding of EBV in saliva. Although the cell type expressing the KSHV RNAs has not yet been determined, epithelial cells were the only cell type apparent on visual inspection. Whereas the presence of lymphoid cells that may harbor KSHV cannot be ruled out, KSHV DNA has been found in the saliva of subjects without detectable KSHV in peripheral blood mononuclear cells (15). Because oral KS is common it could also be considered that lesions are responsible for KSHV in saliva, but we have found infectious virus and viral RNAs in patients without oral KS. These data suggest that like EBV, KSHV undergoes lytic replication in oropharyngeal epithelial cells, but further studies using in situ hybridization or immunohistochemistry are needed to resolve this issue.

Although our studies demonstrate the potential infectivity of KSHV in saliva, the importance of salivary shedding of KSHV in the transmission of this virus remains to be determined. The role of immunosuppression in the amount and frequency of shedding of KSHV needs to be addressed. Increased shedding of EBV was reported in HIV-infected subjects (1), and immune suppression may contribute to shedding of KSHV. We have examined KSHV in detail only from the saliva of HIV-positive and HIV-negative/KS-positive individuals, and the presence or prevalence of salivary KSHV in other populations has not been determined. Saliva samples from 39 HIV-negative/KS-negative individuals were all negative for the detection of KSHV DNA (6). Longitudinal studies on subjects with known serostatus will be required to address shedding of KSHV in healthy individuals. Whether contact with infectious saliva plays a role in the transmission of this virus, or the stages at which shedding of KSHV saliva occurs, is unknown. This report, however, underscores the relationship of KSHV with other members of the herpesvirus group in that infectious virus can be present in saliva.

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