2001

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Published in the *International Journal of Cancer* (2001) 94: 749-752. Publication of the International Union Against Cancer.

Short Report: Vertical Transmission of Kaposi's Sarcoma-Associated Herpesvirus

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Written informed consent was obtained from all the study participants. The Zambian Ministry of Health, Research and Ethics Committee of the University Teaching Hospital in Lusaka, Zambia and Institutional Review Boards of the University of Miami, Miami, FL, USA and University of Nebraska, Lincoln, NE, USA approved the study. Grant sponsor: PHS; Grant nos.: CA 75903, CA 76958, RR15635; Grant sponsor: Fogarty International Training; Grant nos.: TW01429, TW98-002.

Received March 1, 2001; Revised May 10, June 28, 2001; Accepted June 29, 2001.

Little is presently known about the specific routes of transmission of Kaposi's sarcoma-associated herpesvirus (KSHV) or human herpesvirus-8 (HHV-8). To investigate whether this agent might be transmitted vertically from mother to infant, we conducted a study on 89 KSHV seropositive mothers and their newborn infants. Thirteen mothers (14.6%) had KSHV DNA detected in their peripheral blood mononuclear cells (PBMC). Two of 89 samples drawn at birth from infants born to KSHV seropositive mothers had KSHV DNA detectable within their PBMC. These findings suggest that KSHV can be transmitted perinatally, but infrequently. Other routes of transmission such as horizontal transmission remain the most likely means of KSHV transmission.
Key words: KSHV; HHV-8; vertical transmission

Kaposi's sarcoma-associated herpesvirus is a recently isolated gamma herpesvirus[1] that has been found in all forms of Kaposi Sarcoma (KS),[2] and is now thought to play an important role in the development of this tumor.[3] Current knowledge about the routes of transmission of KSHV is limited. Serological evidence has suggested that there may be several routes of KSHV transmission, including sexual contact, as well as nonsexual contact *via* saliva.[4-7] In Africa, cross-sectional serological studies of children in different age groups have shown that infection with KSHV begins during early childhood and its frequency increases with age.[8-12] This suggests that non-sexual modes of transmission of the virus may be occurring in Africa. The role of vertical transmission of this virus remains poorly understood. Some studies have reported serological data disputing the occurrence of vertical transmission of KSHV[13,14] whereas others have presented contrasting serological data suggesting that vertical transmission does occur.[15,16] A case report of KS in a 6-day-old child[17] strongly suggests that KSHV may indeed be transmitted from a mother to her infant *in utero*. In our study, we present evidence showing that KSHV can be transmitted vertically from mother to infant.

Material and Methods

Patient recruitment

A subgroup of 89 KSHV seropositive women and their infants were selected from a cohort of over 2,000 mothers participating in a prospective study at the University Teaching Hospital (UTH) in Lusaka, Zambia. In this cohort, pregnant women in the early stages of labor were recruited for the study at the time of admission to the labor ward of the University Teaching Hospital (UTH), Lusaka, Zambia. Women who were in active labor or who had more than 5cm cervical dilation were not recruited. Before giving written consent to participate in the study, the women were informed about the purpose of the study and counseled on HIV and KSHV. All women were enrolled before giving birth and their newborns were enrolled immediately after delivery. In case of a multiple delivery, only the first sibling was enrolled into the study.

Sample collection

Maternal and infant blood was collected by venipuncture into acid citrate dextrose tubes and processed within 6 hours of being drawn. The blood samples from the mothers were drawn before delivery and those from the infants were drawn during their first 24 hours of life.

Isolation of peripheral blood mononuclear cells

The blood was centrifuged at 1,800 rpm for 10 minutes and the plasma was collected then frozen at -20°C. The PBMC were isolated from whole blood using a ficol density gradient (Nycomed Pharma AS, Oslo, Norway). The PBMC were then suspended in fresh freezing medium (70% RMPI 1640, 20% fetal bovine serum, 10% DMSO) and stored at -80°C.

Screening for syphilis

Plasma was screened for *Treponema pallidum* antibodies using the rapid plasma reagin (RPR) testing kit (Arlington Scientific Inc, Springville, UT). All the RPR reactive samples were then confirmed by *Treponema pallidum* hemagglutination using the Serodia®-TP.PA kit (Fujirebio Inc., Tokyo, Japan).

Immunofluorescence assay (IFA) for KSHV

Plasma was tested for KSHV antibodies by indirect IFA. BC-3 cells (kindly provided by Dr. Ethel Cesarman, Weill Medical College, Cornell University), a KSHV positive and Epstein-Barr virus (EBV) negative B-cell lymphoma cell line, were used for the IFA. The IFA was performed using the procedure described by Lennette *et al.*[18] with minor modifications. Briefly, 5 x 10^5 cells/ml were stimulated with tetra decanoyl phorbol acetate (TPA, 20 ng/ml, Sigma, St. Louis, MO) for 72 hours in culture medium (90% RMPI 1640, 10% fetal calf serum, 100 U/ml penicillin G and 100 mg/ml streptomycin). Two different lab workers tested all samples at 1:40 dilution. To exclude false positive results due to background staining, all positive sera were retested with BJAB cells (KSHV negative B lymphoma cell line). Only sera found to be negative with uninfected cells were considered positive.

Testing for HIV-1

Plasma was screened for HIV-1 antibodies using 2 rapid assays, Capillus (Trinity Biotech Plc, Ireland) and Determine (Abbott Laboratories, Abbott Park, IL). The assays were done according to the manufacturers' instructions. IFA was then used to confirm the HIV-1 result. The HIV IFA was performed as described above using a chronically HIV-1 infected T-cell line, HUT-78/ARV (given to us by Dr. Cecilia Cheng-Mayer). Uninfected HUT-78 cells were used for background control. Plasma were considered HIV-1 positive if they tested positive by at least one rapid test and confirmed by IFA.

Western blot analysis for antibodies to KSHV ORF65

An ORF65 bacterial clone, ORF65.2 (kindly provided by Dr. T. Schulz), expressing the carboxylterminal half of ORF65 with a 6xHis tag was used for Western blot analysis of sera. ORF65.2 protein was expressed then purified using immobilized metal affinity chromatography according to the manufacturers' procedure (Qiagen Ni-NTA Superflow, Qiagen, Chatsworth, CA). Western blot was performed as follows. Ten microliters of each test serum was blocked with 10 µl of normal *E. coli* bacterial lysate for two hours at room temperature then diluted 1:50 in blocking solution (20 mM Tris-HCl, pH 7.5; 500 mM NaCl; 0.1% Tween 20; 5% skim milk; 5% normal goat serum). The membranes were incubated with the test serum for 1 hour at room temperature then washed with TBST (20 mM Tris-HCl, pH 7.5; 500 mM NaCl; 0.1% Tween 20). The membrane was incubated with alkaline phosphatase conjugated goat anti-human IgG (Bio-Rad, Richmond, CA) and detection was performed using nitroblue tetrazolium and 5-bromo-4-chloro-3indolyphosphate colorimetric substrate.[19]

Extraction of DNA from PBMC

Each cell pellet was incubated in 400 µl digestion buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 25 mM EDTA, 0.5% SDS, 0.1 mg/ml Proteinase K) for 2 hours. Longer incubation times, up to 15 hours, were used for larger cell pellets. The DNA was extracted by phenol/chloroform/isoamyl alcohol (25:24:1) extraction followed by ethanol precipitation.

PCR Analysis

PCR was performed using 2 sets of primers amplifying different genes of the KSHV genome. The KS-1 (5′-AGCCGAAAGATTCCACCAT-3′, genomic locus 47586-47605) and KS-2 (5′-TCCGTGTTGTCTACGTCCAG-3′, locus 47818-47799) primers, amplifying a 233 bp region of the ORF26 gene were used for screening the DNA for KSHV sequences because they were previously found to be most sensitive for detecting genomic KSHV DNA.[1] An extra pair of primers KS-4 (5′-CGAATCCAACGGATTTGACCTC-3′, locus 47611-47633) and KS-5 (5′-CCCATAAATGACACATTGGTGGTA-3′, locus 47785-47761) for the ORF26 gene was also used for the nested PCR of the gene. Another set of primers, gBN-3 (5′-GCCACCCTGGGGACTGTCAT-3′, locus 8701-8720) and gBN-4 (5′-TTGGTGATGGCGGACTCTGTC-3′, locus 9083-9063) amplifying a 382 bp fragment of the N-terminal end of the glycoprotein B gene (gBN), was used for screening in addition to the KS-1 and KS-2 primers. All the primer sets were designed from the genomic sequence U93872 (GenBank accession number U93872). The PCR reactions were carried out in a PE 2400 thermal cycler (Perkin-Elmer, Oak Brook, IL). Each reaction mixture (25 µl) contained 0.8 µM primers, 0.25 mM dNTP, 1.5 mM $MgCl_2$, 20 mM Tris-HCl, pH 8.4, 50 mM KCl and 1 U Taq polymerase (GIBCO BRL, Gaithersburg, MD). In every case, 300–400 ng of genomic DNA template was used. Fifty nanograms of BC-3 DNA were used as the positive control whereas 400 ng of BJAB DNA in one reaction tube and 2 µl deionized water in a second tube were used as the negative controls. The cycling procedure for the KS-1, KS-2 primer pair was 94°C for five minutes, then 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds and one final extension cycle of 7 minutes at 72°C. For the KS-4 and 5 and gBN primers, the amplification conditions were similar except the annealing temperature was at 53°C and 62°C, respectively.

Southern blot analysis

DNA was transferred onto a Zeta-Probe® nylon membrane (Bio-Rad Laboratories, Hercules, CA) by vacuum transfer for 90 minutes and then cross-linked by ultra-violet irradiation. Digoxigenin labeled PCR products from ORF26 and glycoprotein B were then used as probes, following a standard protocol for hybridization and detection using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyphosphate colorimetric substrate.[19]

Cloning and sequencing of PCR products

The amplified product of the ORF26 nested reaction was cloned into pGEM-T vector (Promega, Madison, WI) and the cloned fragment was sequenced using ABI PRISM Big-dye terminator sequencing kit on an ABI 373S DNA sequencer (Applied Biosystems; San Jose, CA).

Results

Medical history and clinical examination of the study participants

To document the occurrence of vertical transmission of KSHV from seropositive mothers to their newborns, 89 women and their infants in which DNA was available, were selected from the group of HHV-8 seropositive women in an ongoing cohort study in Zambia. The medical histories of the mothers are summarized in Table I. Seven mothers had a positive history of tuberculosis. One of the 89 women had a history of gonorrhea. Thirteen mothers had histories of ulcerative genital diseases. Clinical examination at the time of recruitment documented that ten mothers had genital warts. Five mothers had non-genital skin ulcers. Two women had Kaposi Sarcoma-like skin lesions but could not be confirmed upon histological examination.

Serological analysis for KSHV antibodies in the mothers and their infants

Of the 89 mothers testing positive for KSHV by IFA, 72 (81%) were also seropositive by ORF65 Western blot (Table II). Fifty-one women (57%) had a KSHV antibody titer greater than 1:40 (ranging from 1:80 to 1:1,280). With regards to HIV-1, 32 (36%) of the 89 women in the cohort were HIV-1 seropositive with 8 of them (9%) also positive for syphilis (Table I). The infants born to these 89 women were also characterized. The male:female ratio of the infants was 1:1. The mean birth weight was 2,852 g (range 1,300–4,100 g). All the infants were born healthy and were breast fed within the first 24 hours of their life. Most of the infants (74/89, 83.1%) were found to have passively acquired anti-KSHV antibodies at birth by IFA (Table II). The acquisition of maternal antibodies, however, did not correlate with high maternal antibody titers (data not shown).

PCR analysis of infant PBMC DNA for KSHV sequences

To determine if any of the infants born to these 89 KSHV seropositive women had acquired KSHV infection from their mothers, PCR was first performed on the infant PBMC DNA obtained

TABLE I – MATERNAL CHARACTERISTICS[1]

Mean age (in years)	24 (range:16–36)
Mean no. of pregnancies	3 (range:1–7)
Married/co-habiting	81 (91%)
Completed more than 7 years in school	45 (51%)
History of ulcerative STDs	23 (26%)
Diagnosed with skin ulcers	9 (10%)
History of non-ulcerative STDs	1 (1%)
History of tuberculosis	7 (8%)
Reactive RPR for syphilis	8 (9%)
HIV-1 positive status	32 (36%)

[1] $n = 89$.

at birth using the ORF26 primers. Of the 89 infant DNA samples analyzed, KSHV DNA sequences were detected in two infants after Southern blot analysis using a probe specific for the ORF26 gene. To confirm that KSHV DNA was indeed present in the infant PBMC, a second PCR assay was performed using primers specific for the N-terminus of glycoprotein B. The same two infants were also positive using these primers.

PCR analysis of maternal PBMC DNA for KSHV sequences

The PBMC DNA of the 89 women was also analyzed for the presence of KSHV DNA by PCR using primers specific for ORF26. KSHV DNA was detected in 13 (14.6%) mothers (Table II). The PCR signal was quite weak and only detectable after Southern blotting. Nine of the 13 KSHV DNA positive mothers were HIV-1 seropositive ($p < 0.001$) indicating a possible correlation between HIV-1 infection and the presence of KSHV DNA in this group of women. The number of specimens analyzed was extremely small, however, and needs to be substantiated. Furthermore, we observed that five of the 13 KSHV DNA positive mothers had a history of genital ulcers. The significance of such an observation or whether there is a correlation between having KSHV DNA and a history of genital ulcers is not clear at this point. The mothers of the two KSHV DNA positive infants were also positive for KSHV DNA by PCR, but only one of the two mothers was positive with both ORF26 and gBN primers. Both of these mothers had KSHV antibody titers of 1:160, were HIV-1 seronegative and did not have any other conditions that may have been risk factors for vertical transmission. The two mothers that had unconfirmed KS-like lesions at the time of recruitment were not found to be PCR positive for KSHV DNA in their PBMC.

KSHV DNA sequence analysis

The amplified ORF26 gene product from one of the infants was cloned and sequenced. The cloned sequence is closely related to recently published Zambian KSHV and other African sequences with a conserved C at position 47738 of the ORF.[17,18] This point mutation is conserved amongst all Zambian KSHV ORF26 genes sequenced so far and is distinct from BCBL KSHV sequences.[20] The infant sequence also had a unique point mutation (C to A) at position 47702 of the ORF26 sequence. This mutation was distinct from the other known Zambian KSHV sequences (Figure 1). Several attempts were made to clone the KSHV PCR product from the mother of the infant in which we had KSHV sequence information on. The maternal signal has consistently been weak and only detectable by Southern blot. Subsequent cloning and analysis of the KSHV sequence from this patient was unsuccessful.

TABLE II – KSHV TEST RESULTS[1]

	Positive mothers (%)	Positive infants (%)
KSHV IFA	89 (100)	74 (83)
KSHV WB[3]	72 (81)	ND[2]
KSHV PCR	13 (15)	2 (3)

[1]n = 89 pairs.–[2]ND, not determined.–[3]WB, Western blot.

Discussion

Since the discovery of KSHV, the question of whether this agent can be vertically transmitted has been intensely debated. Previous reports have presented serologic evidence disputing the possibility of vertical transmission of this virus.[13,14] In these studies, the clearance of maternal KSHV antibodies among infants followed longitudinally was demonstrated and KSHV DNA was not detected in any of the infants tested. Plancoulaine *et al.*[22] recently demonstrated in a French Guyana population of African descent that KSHV transmission mainly occurs postnatally from mother to child and from sibling to sibling. The low seroprevalence of KSHV among children less than 5 was cited as evidence against vertical transmission being a major route of transmission. It has also previously been documented in Africa that primary infection with KSHV can occur during childhood.[8-12] Kasolo *et al.*[20] reported the finding of KSHV sequences in PBMC of infants with febrile illnesses. The reported cases of KS in infants less than 6 months of age,[17,23] however, suggest the potential of vertical transmission of KSHV. Our hypothesis that KSHV might be transmittable from mother to infant perinatally was based upon two previous observations: first that KS has become one of the most common tumors among infants and children in Zambia and second, that KS is closely associated with KSHV infection. We have now identified two neonates who have tested positive for KSHV DNA at birth. This finding in these two infants provides direct evidence of vertical transmission of KSHV. Interestingly, both infants were born to HIV-1 negative mothers. We think that this is a chance finding. One would expect that HIV-1 positive mothers would be more likely to vertically transmit KSHV to their babies because of higher viral copy numbers as a result of their immunosuppression. We do not believe that these results were due to contamination because KSHV DNA was detected in only two of the 89 infants that we tested. The fact that PCR was done on the infants before DNA extraction and PCR on the mothers PBMC DNA supports the claim that it is not a contamination from maternal sequences. Separate laboratories were used for the extraction of DNA and PCR and the results were confirmed by different researchers. Also, these same two infants were positive with two different sets of non-nested PCR primers. Furthermore, sequence analysis of the ORF26 gene from one of the infants revealed that it is closely related to previously published Zambian sequences of the same gene. Therefore contamination

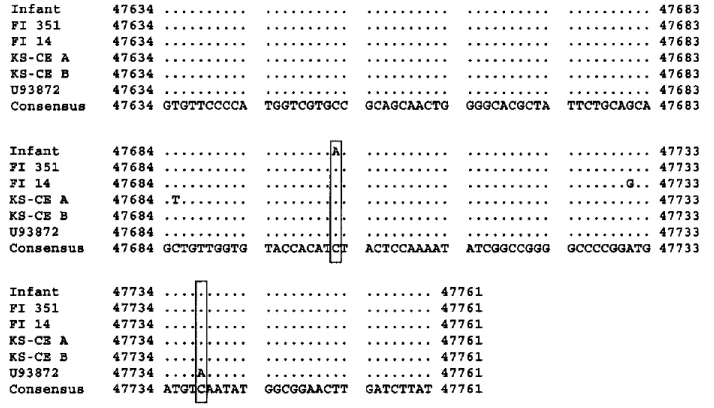

FIGURE 1 – Sequence analysis of the PCR positive infant. The nested PCR product of ORF26 encompassing nucleotide positions 47634–47761 was sequenced and aligned with published Zambian ORF26 KSHV sequences and to a published BCBL KSHV sequence. The boxed region at position 47702 shows the mutation unique to our PCR positive infant and the boxed region at position 47738 shows the conserved African sequence. Sequences FI 14, FI 315, KS-CE A, KS-CE B are previously analyzed specimens from Zambia.[17] Sequence U93872 is a prototype BCBL sequence from the GenBank accession number U93872. The consensus sequence was derived from the nucleotide sequence present in the majority of the isolates analyzed. The sequences shown exclude the primer sequences.

from maternal sequences or the laboratory BCBL strain is unlikely. The cloned portion of the ORF26 sequence, however, is known to be relatively conserved and in our infant sequence, we detected only two nucleotide changes with one of the changes being conserved amongst Zambian KSHV isolates. We were unable to clone other KSHV genes that show more variability such as the ORF K1, or KSHV sequences from the mothers of the two PCR-positive infants due to weak signals and insufficient DNA. Although KSHV viral DNA in the PBMC of two infants has been detected, it is difficult to determine whether infection occurred *in utero* or *intrapartum*. *Intrapartum* or *postnatal* exposure as a result of breast feeding is unlikely because KSHV DNA would not have been detected in the immediate *postnatal* period.

KSHV DNA has consistently been detected in KS tissues and the viral copy numbers in these tissues are usually high.[24] Other tissues and body fluids in which KSHV DNA has been detected include saliva, semen, skin, cervical secretions and PBMC.[24-26] KSHV DNA is more easily detected in the bodily fluids of individuals with KS than in those without KS.[24] This may be due to lower copy numbers of KSHV DNA in individuals without KS. In our study, we detected KSHV DNA in the PBMC of approximately 15% of the mothers. The KSHV copy numbers in the PBMC DNA seem to be very low as we were only able to detect the viral DNA by Southern blot analysis and not by direct gel examination of PCR-amplified products. In our hand, the limits of detection of KSHV by the ORF26 and glycoprotein B PCR were 3 and 20 copies of viral DNA respectively. An increase in the detection of KSHV DNA in HIV-1 positive mothers (9/13) compared to the HIV-1 negative mothers (4/13) was found to be statistically significant. This was based on a small number of PBMC analyzed, however, and will need to be substantiated by testing a larger number of samples. This finding nevertheless suggests that HIV-1 may play a role in activating replication of KSHV and thereby increasing the viral load of KSHV in the blood.

Several reports have documented that not all the children born to KSHV infected mothers become KSHV seropositive at birth.[8-10,13] Our results here indicate that only 83% of the children born to KSHV positive mothers acquired antibodies to KSHV. Considering that only 57% of the mothers had a KSHV antibody titer greater than 1:40 in our study, there seemed to be no correlation between the maternal KSHV antibody titer and the presence of KSHV antibodies in the infants.

In conclusion, we believe that our findings of two neonates who at their birth have tested positive (with two different sets of non-nested PCR primers) for KSHV DNA provide direct evidence of vertical transmission of KSHV. Although our findings do suggest that the vertical transmission of KSHV may occur, this is unlikely to be a major route of transmission of this virus to children. In endemic areas, horizontal transmission remains the most likely means by which infants become infected.

Acknowledgments

This study was supported by PHS grants CA75903, CA76958, RR15635 & Fogarty International Training grants TW01429 to C.W. & TW98-002 to C.M. We would also like to acknowledge the Fogarty International Fellowship for their support to Dr. H. Mantina. We would like to thank Profs. L. Mukonge, Y. Numazaki & Dr. E.M. Chomba for their unwavering support. We would also like to thank the Zambia KS/KSHV study group for their tireless effort to keep the study going.

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