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Omar Bagasra
Claflin University, obagasra@claflin.edu

Deepa Patel
Claflin University

Lisa Bobroski
Claflin University

Jamil A. Abbasi
Claflin University

Alex U. Bagasra
Claflin University

See next page for additional authors

Authors

Omar Bagasra, Deepa Patel, Lisa Bobroski, Jamil A. Abbasi, Alex U. Bagasra, Hasna Baidouri, Twaina Harris, Albert El-Roeiy, Zsolt Lengvarszky, Homayoon Farzadegan, and Charles Wood

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Locatization of Human Herpesvirus Type 8 in Human Sperms By *In situ* PCR

Omar Bagasra[1,*], Deepa Patel[1], Lisa Bobroski[1], Jamil A. Abbasi[1], Alex U. Bagasra[1], Hasna Baidouri[1], Twaina Harris[1], Albert El-Roeiy[2], Zsolt Lengvarszky[3], Homayoon Farzadegan[4] and Charles Wood[5]

[1]South Carolina Center for Biotechnology, Department of Biology, Claflin University, 400 Magnolia Street, Orangeburg, SC, 29115, USA
[2]Crozer-Chester Medical Center, One Medical Center Boulevard, Upland, PA, 19013, USA
[3]Department of Mathematics & Computer Science, Claflin University, Orangeburg, SC, 29115, USA
[4]Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA
[5]University of Nebraska, Nebraska Center for Virology, School of Biological Sciences, E249 Beadle Center, Lincoln, NE 68588, USA
*Author for correspondence: obagasra@claflin.edu

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Summary
Objectives: Defining the mechanism of infection with human herpesvirus-8 (HHV-8) or Kaposi's sarcoma associated herpesvirus (KSHV) is an important clinical issue. HHV-8 has been linked to Kaposi's sarcoma (KS) development in HIV-1-infected individuals, and KS develops in 40% of those infected with both viruses. A series of epidemiological data suggest that sexual transmission is one of the routes of transmission for HHV-8. In our studies, we sought to assess the cellular reservoirs of HHV-8 DNA in the semen of HIV-1-infected men and the potential role of HHV-8 infected spermatozoa in horizontal transmission. *Design and methods:* A nested polymerase chain reaction (PCR), *in situ* PCR (ISPCR) and a sodium iodide (NaI) DNA isolation technique that extracts both nuclear and episomal DNA were utilized to amplify specific genes *in vitro* and within intact cells to evaluate the types of seminal cells infected with HHV-8 in HIV-1-infected and uninfected men. *Results:* HHV-8 was present in the spermatozoa and mononuclear cells of the semen in 64 of 73 (88%) HIV-1 infected individuals. Both the sperms as well as the mononuclear cells of the semen specimens of HIV-1 infected men were found to be infected with HHV-8. Multiplex ISPCR revealed that a significantly higher percentage of semen cells were infected with HHV-8 than HIV-1 ($p > 0.001$). Rare (less than one in a 100,000) sperm cells were co-infected with both viruses. A co-culture of HHV-8 infected sperm with uninfected 293 or Sup-T1 cell lines resulted in an abortive infection of these cells with HHV-8. DNA isolation by NaI yielded 73% of the positive sperm, whereas the standard phenol/chloroform method resulted in significantly lower positives (45%) from the same specimens. *Conclusions:* Design and methods: Our data strongly suggest a potential sexual/horizontal route of transmission of HHV-8, via the HHV-8 infected sperm and other semen cells, where a large percentage of HIV-1 infected men's sperm and other semen cells are infected with HHV-8. Co-culture studies have further supported the observations that HHV-8 in the sperm cells is infectious and capable of transmission of the virus to uninfected cells.

Introduction

Long before the HHV-8 or KSHV was implicated as the likely cause of Kaposi's sarcoma, epidemiological studies suggested the involvement of a sexually transmitted agent as the etiology (reviewed in Berel *et al.* 1990, Chang *et al.* 1994). However, the detection of HHV-8 sequences in semen has been variable. For example, several investigators have been able to detect HHV-8 in significant number of semen specimens from HIV-1 seropositive men and in a low percentage of HIV-1 uninfected men (Gupta *et al.* 1996, Huang *et al.* 1997, Howard *et al.* 1997, Viviano *et al.* 1997, Bobroski *et al.* 1998, Diamond *et al.* 1998, LaDuca *et al.* 1998, Belec *et al.* 1998). Others have been unable to detect any HHV-8 in semen specimens (Tasaka *et al.* 1996, Diamond *et al.* 1997, Kelsen *et al.* 1999, Antman and Chang, 2000). In part, different detection rates among various investigators could be secondary to different risk factors among HIV-1 infected groups or different geographic locations or ethnic makeup of the study population (Lacoste *et al.* 2000, Goedert, 2000). Several studies have shown varying frequencies of

HHV-8 in different groups and different geographical areas. For example, HHV-8 infection appears to have low seroprevalence in the general population in the U.S., northern Europe and Asia, but is more prevalent in parts of the Amazon basin and Italy (reviewed in Gardella *et al.* 1984, Lacoste *et al.* 2000, Goedert, 2000). However, it is highly prevalent in the general population in sub-Sahara Africa (reviewed in Gardella *et al.* 1984, Lacoste *et al.* 2000, Goedert, 2000). In addition, similar to other herpes viruses, the life cycle of HHV-8 consists of latent and lytic replication phases. Following primary infection, HHV-8 establishes a lifelong latent infection in host cells, which can be reactivated into lytic replication upon induction by intracellular or environmental factors. In KS lesions and other HHV-8-related malignancies, the tumor cells are latently infected by the virus and express viral latent antigens (Gardella *et al.* 1984). Latent infection with HHV-8 is important in the development of KS. In HHV-8 latent infection, viral genomes persist as nonintegrated circular episomes. To sustain latent infection, HHV-8 episomes have to be stably maintained in cells, efficiently replicated before cell division, and then are segregated into daughter cells during mitosis. The majority of the investigators utilize DNA isolation techniques that isolate nuclear DNA, but HHV-8 is also found as episomes, and the standard DNA isolation technique may not be optimal for isolation of episomal virions (Bagasra *et al.* 1992, El Borai *et al.* 1997, Pellett *et al.* 1999). Therefore, in this study we utilized an isolation method that can isolate episomal as well as nuclear DNA.

In order to determine if HHV-8 is present in the mononuclear cells and spermatozoa of semen specimens from HIV-1-infected and uninfected healthy men, we have utilized a highly sensitive ISPCR technique (Bagasra *et al.* 1992, 1994, Patterson *et al.* 1993, Embretson *et al.* 1993, Harrington *et al.* 1996, Bobroski *et al.* 1998) and by NaI DNA isolation method that extract episomal and nuclear DNA (El Borai *et al.* 1997).

Previously, we have utilized *in situ* polymerase chain reaction (ISPCR) to examine the presence of HIV-1provirus in the blood as well as in the seminal specimens (Bagasra *et al.* 1992, 1994) and HHV-8 in the KS skin lesions and peripheral blood mononuclear cells (PBMC) (Bobroski *et al.* 1998, Harrington *et al.* 1996). This technique allowed the amplification of a single copy of specific genetic elements within intact cells, even in their subcellular compartments (Bobroski *et al.* 1998, Bagasra *et al.* 1992, Bagasra *et al.* 1994, Harrington *et al.* 1996, Patterson *et al.* 1993, Embretson *et al.* 1993). Utilizing primers and labeled probes to the conserved regions of the HIV-1 gag gene or HHV-8 Bam330 segment, ISPCR have demonstrated significantly higher levels of HIV-1 provirus and HHV-8 episomal DNA in unfractionated PBMC, peripheral blood monocytes, and peripheral blood CD4-positive lymphocytes in HIV1 and HHV-8 infected-individuals, respectively, as compared to many standard PCR techniques (Bagasra O. Bagasra *et al. et al.* 1992, 1994, Patterson *et al.* 1993, Embretson *et al.* 1993, Harrington *et al.* 1996, Bobroski *et al.* 1998). In the present report, we further utilized the ISPCR technique to evaluate the presence of HHV-8 in the semen cells of HIV-1 infected men. In several cases, we have utilized a multiplex ISPCR technique (MP-ISPCR), where the DNA sequences for HIV-1 and HHV-8 could be amplified simultaneously, *in situ*, and then detected by utilizing specific probes labeled with different chromogens (Bagasra *et al.* 1992, Patterson *et al.* 1993, Embretson *et al.* 1993, 1994, Harrington *et al.* 1996, Bobroski *et al.* 1998).

HHV-8 DNA was demonstrated by ISPCR in sperms of a significantly high proportion of HIV-1 infected men; however, co-culture studies resulted in abortive infection, suggesting that sexual transmission of HHV-8 may not be a major route of infection.

Materials and methods

Sperm specimens from HIV-1-infected and uninfected homosexual men

Seventy-three semen samples were utilized from homosexual men who participated in the Multicenter AIDS Cohort Study (MACS), before the routine use of antiretroviral therapy, the HIV/AIDS Clinic, the International Medical Center of Japan (a gift from Dr. Natsuo Tachikawa), the fertility clinic of Crozer Chester Medical Center in Pennsylvania. Whenever it was possible, the freshly obtained semen was frozen at 70°C, and was evaluated for HHV-8 and in some cases for both HHV-8/HIV-1 by MP-ISPCR.

Semen isolation and preparation

Semen samples either freshly obtained within 2 h of ejaculation or frozen at)70°C, from HIV-1-seropositive or HIV-1-seronegative individuals, were evaluated for HHV-8 DNA using the ISPCR. Freshly obtained semen samples were evaluated unfractionated, and active sperm cells were also separated from inactive sperms, seminal mononuclear cells and debris using a modified 'swim up' technique described previously (Bagasra *et al.* 1994). Briefly, to separate motile sperm from seminal mononuclear cells and immotile sperm cells, one milliliter of the semen specimen was placed at the base of a sterile plastic transfer pipette (Falcon), which was carefully overlaid with 1 ml of serum-free RPMI-1640 media supplemented with 1% lactose. The transfer pipette was incubated in the vertical position, with the bulb portion at the base and the tip at the top. Fractions of motile sperm were collected at the 'top layer' of the pipette after overnight incubation, by slowly squeezing the bulb and collecting the top one ml layer (Bagasra *et al.* 1994). This simple procedure yields over 98% of live sperms and

leaves mononuclear cells and the dead sperm debris at the bottom of the bulb (Bagasra *et al.* 1994).

Positive control for HHV-8 and HIV-1

BCBL cell line, which carries HHV-8 (Arvanitakis *et al.* 1996, Renne *et al.* 1998), was cultured in a T-25 flask for 48 hours. The cells were washed in serum-free media, and seeded on specially designed glass slides overnight, and then fixed in 4% paraformaldehyde–phosphate-buffered saline (PFA-PBS). Similarly, U1 and ACH-2 cell lines, which carry low copy numbers of HIV-1 were used as positive controls for HIV-1, as described previously (Bagasra *et al.* 1992, 1994, Harrington *et al.* 1996). Following fixation, the cells were processed for DNA-ISPCR using primer pair for HHV-8 and HIV-1, respectively (Bagasra *et al.* 1992, 1994, Harrington *et al.* 1996, Bobroski *et al.* 1998).

In vitro *infection of cell lines with HHV-8 by seminal cells*

293 cell line, a primary human embryonic kidney cell line transformed by sheared human adenovirus type 5 (Ad 5) DNA, and Sup-T1, a T-lymphoid cell line, were utilized to determine if these cell lines can be infected by cell-free HHV-8 virion or infected sperms. For this purpose, either cell-free supernatant from the BCBL cell line was used in 1:10 ratio in complete culture media to infect the cells or by fractionated motile sperm isolated by the modified 'swim-up' technique. The motile sperms were co-cultured overnight with Sup-T1 or 293 cells at 1 cell to 200 motile sperm ratio. After overnight incubation, the cells were washed in serum-free RPMI-1640. Cells were cultured for as long as 8 months, and after every passage, a portion of the cells were placed in the specially designed ISPCR slides and processed as described below. These cells labeled as '293-HHV-8' or 'Sup-T1-HHV-8' and were maintained for over 14 months and each passage was given a sequential Roman number, representing one week. Each passage was examined for the presence of HHV-8 by ISPCR and solution-based PCR to determine the presence of the virus. As controls, BCBL cells with known HHV-8 infection, uninfected Sup-T1 and 293 cells were used to prepare slides. After amplification, HHV-8 specific FITC-labeled probe was utilized and counterstained with propidium iodide (Sigma) at 0.5 µg/ml for 3 minutes which stains nucleic red so that HHV-8 positive sperms can be distinguished from the cellular elements.

In situ *polymerase chain reaction*

To perform ISPCR, for the presence of HHV-8/HIV-1 provirus, seminal samples (to a maximum of 5×10^3 sperm cells per well) were seeded into clean glass slides. Slides were air dried and then placed sequentially; first, on a heat block at 104°C for 30 seconds, and then fixed in 4% PFA-PBS solution (pH 7.4) for 2 hours. Paraformaldehyde was inactivated by washing the slides twice in 3×PBS, then the slides were washed 3 times in 1×PBS. Endogenous peroxidase activity was removed by quenching the specimens with a 0.3% solution of hydrogen peroxide, overnight at room temperature. The slides were then treated with Proteinase K 6 (µg/ml in PBS) for exactly 12 minutes at room temperature. Proteinase K was critical, as longer treatment resulted in diffusion of the viral signals from the subcellular compartments of the sperm. Proteinase K was inactivated by placing slides on a heat block at 96°C for 2 minutes. Slides were then washed in distilled water and air-dried (Gardella *et al.* 1984, Bagasra *et al.* 1992, 1994, Harrington *et al.* 1996, El Borai *et al.* 1997, Lacoste *et al.* 2000, Goedert, 2000).

The cells were then subjected to dual amplifications as described previously (Patterson *et al.* 1993, Embretson *et al.* 1993, Bagasra *et al.* 1994, Harrington *et al.* 1996, Bobroski *et al.* 1998). A primer pair complementary to a conserved region of HIV-1 gag (SK38; nucleotides 1551– 1578; SK39; nucleotides 1638–1665, Synthetic Genetics) for HIV-1 DNA and HHV-8-specific primers KS330 (5'-AGC, CGA, AAG, GAT, TCC, ACC, ATT-3' and 5'-TCC, GTG, TTG, TCT, ACG, TCC, AGA-3') were utilized. The multiplex amplifications were carried out by touchdown PCR: denaturing at 92°C for 1 minute, annealing sequentially at 58–57–56–55–53–52–51–50°C for 20 seconds each and extension at 72°C for 2 minutes, for 30 cycles. Each semen sample was also evaluated using solely *in situ* hybridization, without amplification procedures.

After amplification, all slides were washed in a 2×SSC buffer (0.3 M NaCl and 0.03 M Na-citrate) and amplification products were detected utilizing a Cy3-conjugated oligonucleotide (SK 19, nucleotides 1595–1635: Synthetic Genetics) probe for HIV-1, and either a biotinylated probe or FITC-labeled probe for HHV-8, by *in situ* hybridization (Bagasra *et al.* 1992, 1994, Patterson *et al.* 1993, Embretson *et al.* 1993, Harrington *et al.* 1996, Pellett *et al.* 1999). After incubation, the slides were thoroughly washed with PBS. In cases where a biotinylated probe was used, the color was developed with 3' amino 9' ethylene carbozone (AEC) in the presence of 0.03% hydrogen peroxide in 50-mM acetate buffer for 10 minutes at 37°C. The slides were then washed with water to stop the reaction and cover slips were applied with a 50% solution of glycerol/PBS, and observed under optical microscope. In cases where fluorescent labeled probes were used, the slides were analyzed using an UV-Epiflourescence.

PCR amplification of semen specimens

The method of detecting HHV-8 DNA by PCR assay has been described previously (Bagasra *et al.* 1994,

Pellett *et al.* 1999). We utilized the standard phenol/chloroform method as well as a modified sodium iodide method described by El Borai *et al.* (1997) designed to isolate episomal herpesvirus as well as nuclear DNA (WAKO BioProducts, Richmond, VA, USA). DNAs were extracted from 10,000 sperm cells by both methods from 11 randomly chosen semen specimens. The same primer pair as well as identical conditions was used to detect HHV-8 DNAs isolated by both methods with solution-based PCR.

Statistical Analyses

The specimens were divided into three groups based on their CD4 count.

Group A: >800
Group B: 400–800
Group C: <400

Two dependent variables were investigated: the percent of HHV8+ sperm cells in the semen and HIV-1 sperms in the semen. At least 10,000 sperm cells were counted in each slide prepared for evaluation. All the slides were analyzed in a blinded fashion by at least two trained investigators. We have used SAS® for the data analysis.

Descriptive statistics (mean, standard deviation, minimum, maximum) by the MEANS procedure for both HHV-8 and HIV1 are shown in the Table 1.

Results

HHV-8 DNA in the semen of men at various stages of clinical disease

To determine the cellular reservoirs and the levels of HHV-8 in the semen, various specimens from different sources were collected and analyzed for the presence of HHV-8 by ISPCR. Semen samples from 63 homosexual men who participated in the Multi-Center AIDS Cohort Study (MACS) (Harrington *et al.* 1996), 10 heterosexual HIV-1-infected men and 45 specimens from HIV-1 sero-negative men were analyzed for HHV-8 DNA by *in situ* PCR. Semen samples from 73 HIV-1-infected men were evaluated separately for HHV-8. As shown in Table 1, out of 73 semen specimens (63 from the U.S. and 10 from Japanese donors) from the HIV-1 seropositive individuals evaluated, 64 (88%) specimens were positive for HHV-8 (Figure 1a–c).

Table 1. *In situ* polymerase chain reaction for HHV-8 and HIV-1 in semen of HIV-1-infected Men

Total peripheral blood CD4-positive Lymphocyte Count	Total semen samples positive for HHV-8 (%)* (cells/mm^3)	Total semen samples positive for HIV-1 (%)*
>800	12/17 (70)	5/17 (29)
400–800	32/34 (94)	12/34 (35)
<400	20/22 (90)	13/22 (59)
Total	64/73 (88)	30/73 (41)

*Positive semen samples for HHV-8 or HIV-1 provirus, by in situ PCR, are broken down into groupings of samples which demonstrated HHV-8 or HIV-1 DNA within sperms.

Forty-five semen specimens from HIV-1 seronegative individuals were also evaluated for the presence of HHV-8. Two out of the 45 specimens (4.4%) tested showed definite positive signals for HHV-8 by ISPCR. In addition, 6 other exhibited faint signals that were attributed to the residual endogenous peroxidase activity. Solution based PCR by NaI method (see below) also showed only two out of 45 specimens to be positive. In the two positive cases the number of sperm or mononuclear cells positive for HHV-8 was very low (less than 1 in 10,000), (data not shown) As shown in Table 1, each category of HIV-1-infected seminal cells within each clinical group of HIV-1-infected men was stratified by total peripheral blood CD4+ lymphocyte count (cells/mm3). The percentages of HHV-8 infected sperm or mononuclear cells in the semen specimens varied from 0.01% to 22%. The One-Way ANOVA (SAS GLM procedure) was utilized to test whether groups A, B, and C differ with respect to either of the two dependent variables. The three groups did not have the same means either in the case of HHV-8 or in the case of HIV-1. The results for pair wise comparisons showed that the mean percentage was higher for Group C than both for Groups A and B. The difference between Group A and Group B did not appear to be statistically significant for HHV-8. The highest percentage of HHV-8-positive seminal samples, by ISPCR, was in the groups of HIV-1-infected men with total peripheral blood CD4+ lymphocyte counts between <400/mm^3 and 400–800/mm^3 (90–94%). There were statistically significant differences (p>0.0087) in semen samples harboring HHV-8 between men with total CD4+ lymphocyte counts of greater than 800/mm^3 versus those with CD4 counts less than 400/mm^3. Of note, all studies were performed coded and blinded, with HIV-1-seronegative control samples added to each experiment.

Validation of DNA-ISPCR was carried out by utilizing HHV-8 chronically infected cell-line, BCBL and HIV-1 infected cell lines, ACH-2 and U1, as reported previously (Patterson *et al.* 1993, Embretson *et al.* 1993, Bagasra *et al.* 1992, 1994, Harrington *et al.* 1996, Bobroski *et al.* 1998, Pellett *et al.* 1999) (data not shown).

Multiplex DNA-ISPCR for HHV-8 and HIV-1

The semen specimens obtained fresh from 7 HIV-1 seropositive individuals who previously had shown relatively higher percentage of HIV-1 infected sperms (Bagasra *et al.* 1994) were subjected to multiplex amplifications. After amplification, the products were

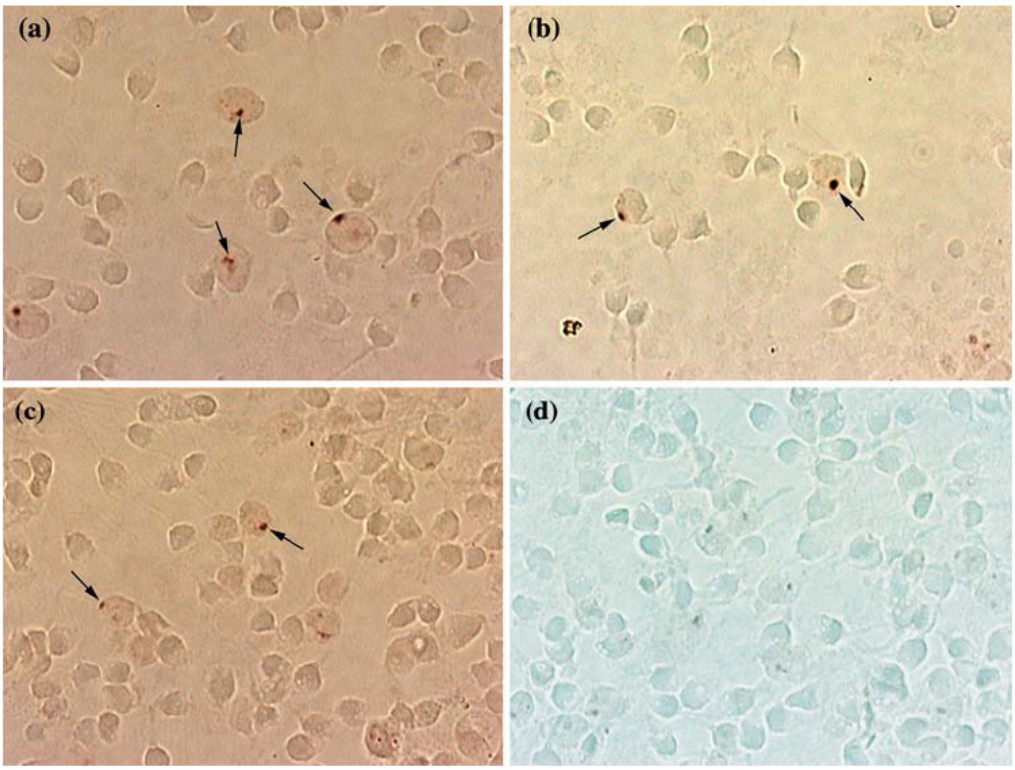

Figure 1. Detection of HHV-8 DNA in human sperm and seminal mononuclear cells by *in situ* polymerase chain reaction (ISPCR). Panel a–c, DNA ISPCR of semen from five HIV-1-seropositive men demonstrating positive staining of the semen cells for HHV-8 DNA (1000 x magnification); panel d, DNA ISPCR of semen from a HIV-1-seronegative man demonstrating rare and weak positive staining of the sperm cells for HHV8 DNA (1000 x magnification).

detected utilizing a HIV-1-specific Cy3-labeled oligoprobe and a FITC-labeled-probe specific for HHV-8 (Bagasra *et al.* 1994, Chang *et al.* 1994, Pellett *et al.* 1999). Out of seven semen specimens analyzed for dual infection, only three specimens showed rare (<1 in 100,000 sperm cells to be positive for both HIV-1 and HHV-8.

As shown in Figure 2a and b, the percentage of sperm infected with HHV-8 (arrows: green signals) was significantly higher than the sperm infected with HIV-1 (red signals) (Patterson *et al.* 1993, Embretson *et al.* 1993, Harrington *et al.* 1996). As with HIV-1 infection of sperm, HHV-8 was also present in these individuals' semen in all stages of HIV-1-infection. HHV-8 was predominantly located in the heads and rarely in the mid-piece of the sperm (Figure 1a–c).

In vitro *infection of Sup-T1 cells by co-culturing with HHV-8 infected sperm cells*

To further evaluate the potential infectivity of HHV-8 in sperm cells, *in vitro* infection studies were performed. Isolated sperm cells from HHV-8-infected and uninfected specimens were incubated, *in vitro*, with Sup-T1 cells. Samples were co-cultured for various period of times ranging from 1 to 24 h. Representative microphotographs are shown from the overnight-incubated specimens, which were analyzed by ISPCR for the presence or absence of HHV-8 in Sup-T1 cells. Figure 3a–c represents co-infections with HHV-8 infected sperm with Sup-T1 cells that resulted in the transfer of infection to Sup-T1 cells. Figure 3a represents results 1 h post-infection (arrows), whereas, Figure 3b–c shows the results after 24 h of co-cultures, utilizing sperms from three different individuals with relatively higher percentages of HHV-8-infected sperms (see Figure 1b–c).

In vitro *infection of 293 cells with HHV-8 and long-term cultures*

In order to determine the potential infectibility of 293 cell line with HHV-8 *in vitro*, we infected these cells with cell-free supernatants from BCBL cultures as well with HHV-8-infected motile sperm and determined by the presence of HHV-8 by solution-based PCR as well as by *in situ* PCR. We maintained the cells for over 24-months. Figure 4 shows the results of infection with cell-free BCBL cell-free cultures. Figure 4a shows negative control (uninfected 293 cell line). Figure 4b shows the BCBL cell line infected with HHV-8. One can see multiple virions in the cytoplasmic compartment. Figure 4c 1 h post-infection, we were able to detect the amplified episomal signals in the infected cells. Approximately, 80% of the cells showed episomes (red dots). After 3 weeks, the percentages of the cells infected with HHV-8, as

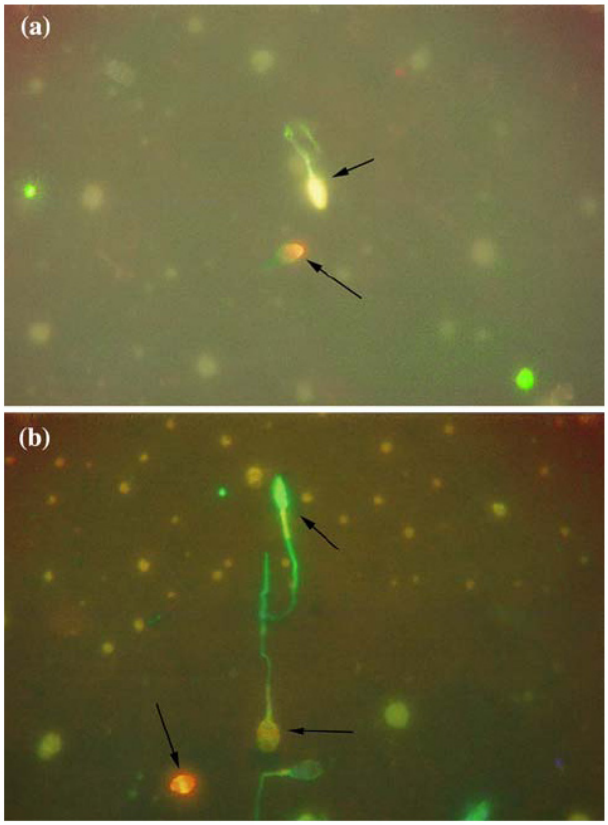

Figure 2. Multiplex DNA-ISPCRs for HIV-1 and HHV-8. High magnification view (2000 x) showing sperm cells, analyzed by multiplex DNA-ISPCR, for HIV-1 (red) and HHV-8 (green) DNA; sperm cells positive for HIV-1, utilizing flouresent (FITC) labeled probes. (a) Shows one possibly dually infected sperm (central: arrow) and one HIV-1 sperm head detected by cy3-labeled probe and HHV-8 (FITC-labeled probe) after *in situ* PCR. In the background, one can decent the shadows of many apparently uninfected sperms. Similarly, in (b) one can see a dually infected sperm cell at the central bottom section. Three additional sperm cells appear to be infected with only HHV-8 and one HIV-1 infected sperm (arrow; bottom-left). Again, the background shows many unstained sperms.

determined by *in situ* PCR, were 25–35% (Figure 4d), and the numbers of episomes per cells were also significantly lower – 20–30 virion dots per cell in certain cells. Twelve weeks post-infection, the number of cells as well as number of episomal virion particles per cell dramatically declined (data not shown). After 24 months, 293 cells appeared to be free from HHV-8 as determined by *in situ* PCR and by solution-based PCR (data not shown). It appears that 293 cells were unable to support the long-term replication of HHV-8 and resulted in an abortive infection, perhaps in the absence of certain undetermined factors that are found in HIV-1 infected individuals. We conclude that our initial observations regarding the presence of HHV-8 immediately post-infection was the result of entry of a large number of virions from the cell–cell supernatant during the primary infection. After multiple cell divisions, the cells infected with virions either were diluted due to slower replication rate of the infected cells or preferentially died due to viral patho-

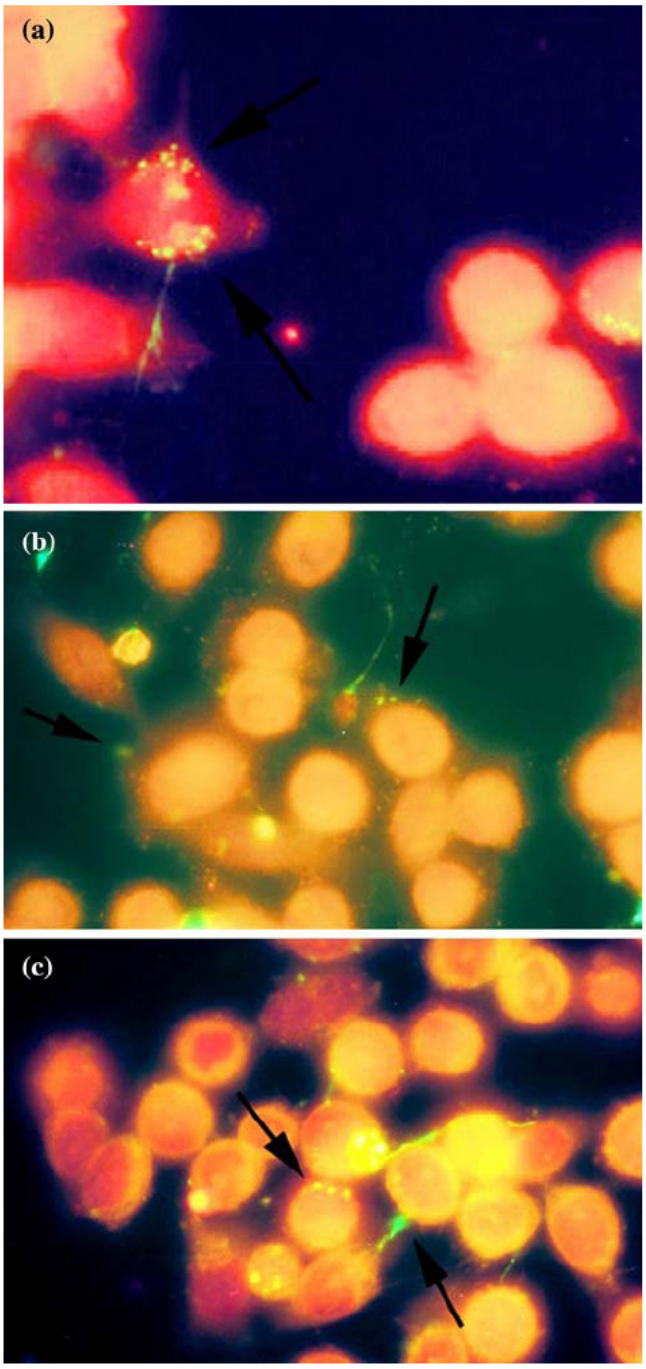

Figure 3. *In vitro* infection of the SupT1 T-cells by HHV-8 infected sperm. DNA-ISPCR of sperm separated by the 'modified swim up' technique, using a transfer pipette, demonstrating HHV-8-positive sperm (2000 x) penetrating lymphocytes. FITC-labeled HHV-8 probe was used post-amplification to detect HHV-8 signals. After *in situ* hybridization, cells were counterstained with Propidium iodide to stain nucleic acid that would appear red. Figure (a–c) represents co-infections with HHV-8 infected sperm with SupT1 cells that resulted in the transfer of infection to Sup-T1 cells, (a) represents results from a 1 h post-infection experiment. One can see that uninfected as well as infected sperms have penetrated the cells, but the infection has not taken a root and episomes appear to be still latent, (c–e) shows the results after 24 h of co-cultures, utilizing sperms from three different individuals with relatively higher percentages of HHV-8-infected sperms (see Figure 1b–c). In these cells episomal virions appear yellow inside the cells behind the reddish background.

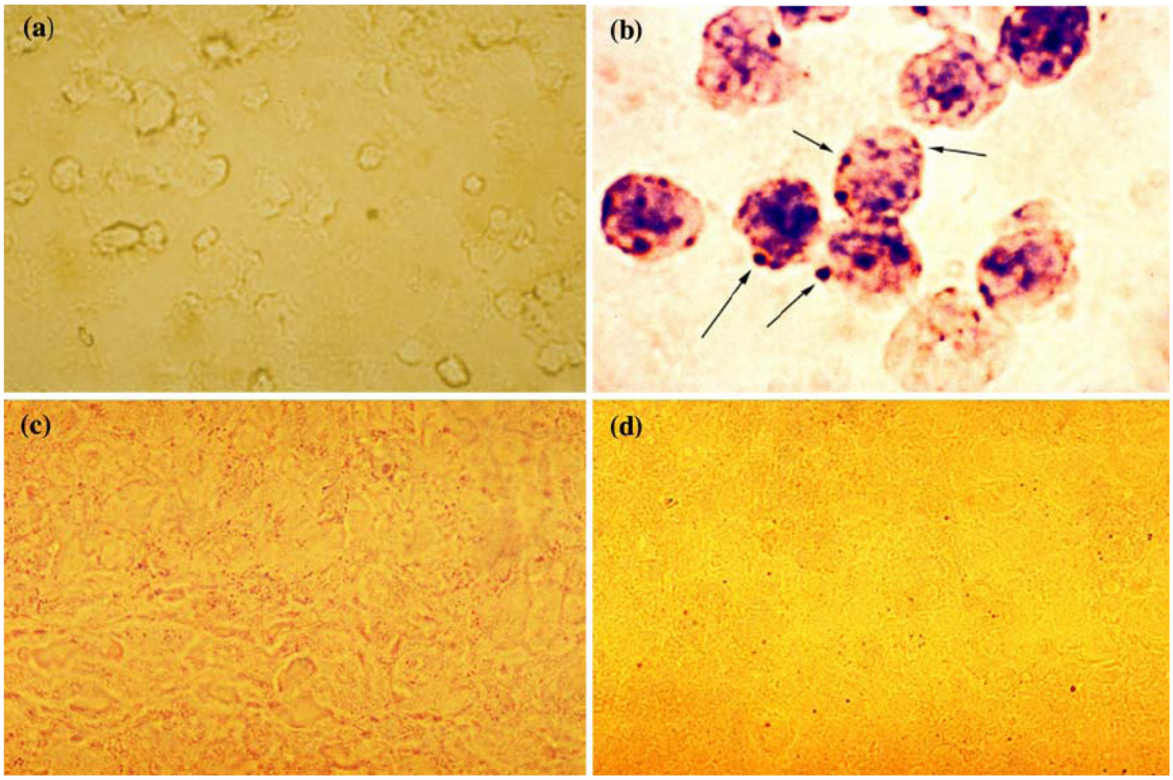

Figure 4. DNA-ISPCR analyses of 293 Cell Line Infected with HHV-8 from BCBL Cell-Free Supernatants *in vitro*: Figure 4a shows the complete absence of HHV-8 signals in the uninfected 293 cell line, (b) exhibits a positive control, BCBL cell line that is known to carry both episomal HHV-8 sequences. One can easily discern HHV-8 in the cytoplasmic compartment. Figure (c) shows HHV-8 virions in 293 cells infected with cell-free supernatant from BCBL cell line 1-h post-infection. In this case, episomal tiny virions could be visualized by DNA-ISPCR for HHV-8 in about 50% of the cells. Figure (d) shows 3 weeks post-infected cells, going through viral replication and death. In these cells one can visualize episomal virions signals. The spread of newly produced virions are not rampant as one would aspect, indicating some sort of intracellular defense mechamism. In the productively infected cells, number of virions per cells were also very low-varying from 20–30 virions per cells.

genicity, resulting in an apparent purge of HHV-8 from 293 cell line. Others have reported similar findings (Cesarman *et al.* 1995, Blackbourn *et al.* 2000, Bechtel *et al.* 2003). However, these findings were not consistent and in certain rare subculture cell lines we were able to observe HHV-8 episomes 3 years after the initial infection (data not shown).

Isolation methods and frequency of detection of HHV-8

In order to determine whether the variable results in detecting HHV-8 DNA in semen specimens could be attributed to the difference in methodologies used in DNA extraction, we utilized two different methods to isolate HHV-8 from 11 randomly selected semen specimens from HIV-1 infected men. As shown in Figure 5a, by utilizing the NaI DNA extraction method, which is generally utilized to isolate episomal HSV as well nuclear DNA in the cervical lavage, we were able to isolate HHV-8 in 8/11 (73%) specimens from HIV-1infected patient. However by using the standard DNA extraction after standard chloroform/phenol extraction, we were able to detect a HHV-8 signal in only 5/11 (45%) specimens (Figure 5b). The same specimens with the same amount of the starting materials were used in the both cases. The most striking observations were the much higher intensity of the signals of amplified products detected in the DNA utilizing the NaI extraction method, suggesting much more viral DNA was recovered in the infected cells using this method (El Borai *et al.* 1997). It is likely that in an event where the initial viral load was very low one would get a false negative result (El Borai *et al.* 1997).

Discussion

Nearly all KS patients and a significant number of HIV1 infected homosexual men (70–90%) show antibodies to HHV-8 (Ambroziak *et al.* 1995, Dupin *et al.* 1999, Rezza *et al.* 1999, Hayward, 1999, Chatlynne and Ablashi, 1999). In addition, in the general U.S. population the HHV-8-seropositive rate was between 5–20% in adults and 0–8% among children (Miller *et al.* 1996, Kedes *et al.* 1996, He *et al.* 1998, Martin *et al.* 1998, Olsen *et al.* 1999, O'Brien *et al.* 1999, Serraino *et al.* 2001). Therefore, our study, which showed that 88% of semen specimens from HIV-1-infected men were positive for HHV-8, is in agreement

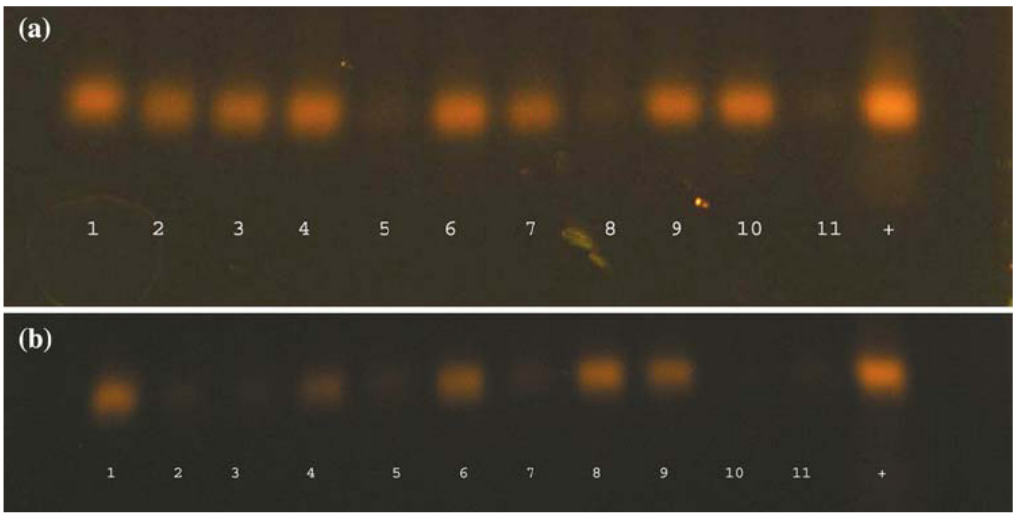

Figure 5. Ethidium bromide gel electrophores of PCR bands: Example of KS330230 PCR Amplifications from eight semen specimens: (a) showing amplifications after DNA extraction by the modified sodium iodide method; (b) showing DNA extraction after standard chloroform/phenol method. The degree of amplifications is significantly lower by the standard extraction method, as compared to NaI method extraction method.

with the majority of seroepidemiological data (Ambroziak *et al.* 1995, Miller *et al.* 1996, Kedes *et al.* 1996, Dupin *et al.* 1999, Rezza *et al.* 1999, Hayward, 1999, Chatlynne and Ablashi, 1999). Moreover, the presence of HHV-8 in the 17% of HIV-1 seronegative healthy individuals is also in agreement with the previously published serological data, suggesting that between 5 and over 20% of HIV-1 seronegative individuals were seropositive, depending on the population studied and the methods used for detection (Ambroziak *et al.* 1995, Miller *et al.* 1996, Kedes *et al.* 1996, Dupin *et al.* 1999, Rezza *et al.* 1999, Hayward, 1999, Chatlynne and Ablashi, 1999). Of note, we found that out of 10 semen specimens from Japanese HIV-1 infected patients, only two were positive for HHV-8 (data not shown), and the percentage of HHV-8 infected sperm were very low, as compared to the U.S. semen specimens. These data are also in agreement with the previously published data, where prevalence of HHV-8 has been shown to be low (Fujii *et al.* 1999). Of note, all of the semen studies we studied were collected pre-HAART (highly active anti-retroviral therapy) era (1986–1991). As it is reported by several recent studies, the prevalence of KS has decreased post-HAART therapy (Serraino *et al.* 2001).

With the detection of HHV-8 in semen specimens, even though the amounts of infected cells were low, our data nevertheless suggest a potential route of sexual transmission of HHV-8, via the infected seminal cells. The ability of the motile sperm to penetrate lymphocytes and most probably epithelial cells (*i.e.* HEK-293 cells) that may encounter during the oral or rectal sex, points towards the sexual transmission of HHV-8 (Ambroziak *et al.* 1995, Dupin *et al.* 1999, Rezza *et al.* 1999, Hayward, 1999, Chatlynne and Ablashi, 1999). However, this may not be the only route of transmission, since in certain African countries the seropositivity rate is over 50% among their adult population, regardless of their HIV-1 status (Ambroziak *et al.* 1995, Rezza *et al.* 1999, Hayward, 1999, Chatlynne and Ablashi, 1999). This suggests that more than one route of transmission may be responsible for its prevalence in Africa and sexual contacts may not be the only route of transmission. This notion is further supported by epidemiological studies, which suggested HHV-8 is prevalent in children in sub-Sahara Africa and between 20% and 40% of the children less than 14 years of age may be seropositive for HHV-8. These findings indicate that besides sexual transmission, vertical as well as horizontal transmission may be taking place (He *et al.* 1998, Martin *et al.* 1998).

The detection of HHV-8 sequences in the semen has been infrequent and controversial (Gupta *et al.* 1996, Huang *et al.* 1997, Howard *et al.* 1997, Viviano *et al.* 1997, LaDuca *et al.* 1998, Belec *et al.* 1998). This variation in detection could be due to the sensitivity of different detection methods, and *in situ* PCR has been demonstrated to be more sensitive and was able to detect a low copy of viral DNA in very few infected cells, as was shown in the case of HIV-1 (Bagasra *et al.* 1992, 1994, Patterson *et al.* 1993, Embretson *et al.* 1993, Harrington *et al.* 1996). In addition, this variability could be due to the lost of the episomal viral DNA during the routine DNA isolation methods (Pellett *et al.* 1999). The episomal DNA may be lost in the phenol layer and may give false negative results, as has been shown with human herpesvirus type 1 (El Borai *et al.* 1997). For example, several investigators have been able to detect HHV-8 in the significant number of the semen specimens from HIV-1 seropositive men, and in a low percentage of HIV-1 uninfected men (Gupta *et al.* 1996, Huang *et al.* 1997, LaDuca *et al.* 1998, Belec *et al.* 1998). Others have been unable to detect any HHV-8 in semen specimens (Tasaka *et al.* 1996, Kelsen *et al.* 1999, Antman and Chang, 2000). One of the reasons of such variability

may be due to the HHV-8 latency infection that persists as nonintegrated circular episomes. To sustain latent infection, HHV-8 episomes have to be stably maintained in the infected cells.

HHV-8 remained as an episome in the majority of the cells it infects. (Harrington *et al.* 1996, Arvanitakis *et al.* 1996, Bobroski *et al.* 1998, Renne *et al.* 1998; Blackbourn *et al.* 2000). Our experiments also confirmed this notion as we have shown in Figure 5, that by utilizing the sodium iodide method, we were more successful in isolating episomal viral DNA than by the standard DNA extraction, which is designed to preferentially extract nuclear DNA (El Borai *et al.* 1997). This point was further reinforced by a double blind study involving multiple laboratories testing the same specimens, revealing significant variations in the detection efficiency of HHV-8 by solution-based PCR (Pellett *et al.* 1999). In part, varying prevalent rates among various studies could be secondary to different risk factors among HIV1 infected groups or different geographic locations or ethnic makeup of the study population. Several studies have shown that unlike HSV, HHV-8 is not globally ubiquitous (Ambroziak *et al.* 1995, Miller *et al.* 1996, Kedes *et al.* 1996, He *et al.* 1998, Martin *et al.* 1998, Dupin *et al.* 1999, Rezza *et al.* 1999, Hayward, 1999, Chatlynne & Ablashi, 1999, Olsen *et al.* 1999, O'Brien *et al.* 1999, Serraino *et al.* 2001). For example, HHV-8 infection appears to be uncommon in most of Asia, North American and northern Europe (Gardella *et al.* 1984, Chang *et al.* 1994, Lacoste *et al.* 2000, Goedert, 2000). The rates of infection are intermediate in the Mediterranean, Eastern Europe and Amazon Basin. However, it is highly prevalent in the general population in sub-Sahara, and Northern Africa (Chang *et al.* 1994). Pauk et al (2000) has shown that mucosal cells are infected with HHV-8 and may be a source of transmission.

Our study showed that, as with HIV-1 infection of seminal mononuclear and sperm cells, the HHV-8 infected semen cells are found in HIV-1-infected individuals within all stages of HIV-1-induced clinical disease and within a wide range of blood CD4+ cell count. This data is also in agreement with previously published seroepidemiological data (Berel *et al.* 1990, Chang *et al.* 1994, Huang *et al.* 1997, Viviano *et al.* 1997, Bobroski *et al.* 1998, Diamond *et al.* 1998, Lacoste *et al.* 2000, Jacobson *et al.* 2000, Renwick *et al.* 2001). This may imply that seminal cells can be co-infected with HIV-1 and HHV-8. However, in the 20 patients we examined, only rare dually infected cells were found. This indicates that HIV-1 and HHV-8 may be infecting cells at different stages of spermatozoa differentiation (Diamond *et al.* 1998; Howard *et al.* 1997, Antman & Chang, 2000), and needs to be investigated further. In addition, there are still a number of important questions that need to be addressed; they include factors that may contribute to the higher HHV-8 infection rate in the high-risk population.

In order to determine the potential of HHV-8 positive semen to infect other cells, we evaluated two separate potential modes of infection. First, we co-cultured the cell-free HHV-8 with HEK 293 cells. We were unequivocally able to show that 293 cells can be infected with cell-free HHV-8. These cells were able to carry the infection for several months, but subsequently became viral free. In a second series of experiments, we were able to show that motile HHV-8-infected sperm can directly infect T-lymphocytic cells and transmit the virions. We utilized these two co-culture methods to exhibit that semen from infected men can be infectious by either of these routes-cellular and cell-free virions. In the *in vitro* condition, neither of the two cell types – endothelial or lymphocytic cells – were able to maintain HHV-8 infection, however, in vivo these are the most commonly infected cell types found to be infected and more particularly the endothelial cells appear to be the origin of KS (Staskus *et al.* 1997, Bagasra and Pomerantz, 1997). It appears that certain cofactors play a pivotal role in transforming HHV-8 infected endothelial cells into malignant form. In addition, it is also probable that lack of certain cytokines or abnormal levels of certain T-lymphocyte cytokines may promote an environment that results in KS transformation (Mercader *et al.* 2000, Nickoloff & Foreman, 2002).

Previous studies by different groups to infect 293 cells with HHV-8 *in vitro* have shown variable results (Cesarman *et al.* 1995, Renne *et al.* 1998, Dupin *et al.* 1999, Blackbourn *et al.* 2000, Bechtel *et al.* 2003). Three large studies have examined the infection of several cell lines, including HEK 293, in culture. For example, Blackbourn *et al.* (2000) examined 25 lines for infectibility by HHV-8, by utilizing DNA-PCR and RT-PCR methods and found seven of them supported HHV-8 entry and gene expression, but infections can only be established in one cell line (t-DMVECs). Renne *et al.* (1998) examined 39 cell lines for infectibility by HHV-8, using RT-PCR and found that 11 of these 39 lines could support at least low-level spontaneous lytic replication, including 293 cells. Renne *et al.* (1998) also found that 293 cells only inefficiently supported a serial transfer of a virus. Most recently, Bechtel *et al.* (2003) have shown that several cell lines including 293 can be infected with HHV-8 *in vitro*. Our studies are in agreement with several of these recent studies where infectibility of 293 cell line with cell-free virions is confirmed. In addition, our data are in agreement with the observations that serial transfer of HHV-8 in 293 cell line is inefficient and may result in abortive infection after long-term culture. Our data are unique in regards to the direct infectibilty of Sup-T1 lymphocytic cells by HHV-8-infected motile sperm. This may be an important route of infection in the areas of the globe where HHV-8 is highly prevalent and male-to-female rate of infection is possible (Gardella *et al.* 1984, Bagasra *et al.* 1992, 1994, El Borai *et al.* 1997, Pellett *et al.* 1999, Lacoste *et al.* 2000, Goedert, 2000). Previously, Akula *et al.* (2003) have

demonstrated that human fibroblast cells are infected with HHV-8 via endocytosis. Others, and we, have also shown that both B- and T-lymphocytes carry HHV-8 in human blood (Bagasra and Pomerantz, 1997).

One can assume that semen can get infected via HHV-8 infected lymphocytes which can infect the urogenital tract. Previously Diamond *et al.* (1998) have utilized *in situ* PCR to analyze the serial sections of prostate for HHV-8 and have shown that the infection could be localized to discrete areas of the prostate. They detected HHV-8 DNA in the nuclei of >90% of the glandular epithelial cells. They suggested that the intermittent replication of HHV-8 in the prostate and subsequent shedding of the virus in semen might be crucial factors for determining whether HHV-8 can be transmitted through sexual activity (Diamond *et al.* 1998).

Previously, several investigators have shown infection of sperm with other human herpesviruses, including human herpes simplex virus-1 (HSV-1), HVS-2, Cytomegalovirus (CMV) and perhaps Epstein-Barr virus (Kapranos *et al.* 2004). For example, Kotronias and Kapranos (1998) investigated the localization of HSV in 80 sperm samples from men attending a maternity center because of fertility problems and analyzed for the presence of HSV-1 and HSV-2 DNA using digoxigeninlabelled DNA probes. They detected HSV DNA in the nuclei of spermatozoa in 37 semen samples (46%), HSV1 specifically in 21 cases (26%) and HSV-2 in 16 cases (20%). They showed that the number of HSV positive sperm cells ranged from 2 cells per specimen to 60% of the sperm cells. They determined that the mean number of HSV-2 positive sperm cells per sample was 3.7% and that of HSV-1 1.5%. They also determined that the percentage of HSV positive sperm cells was also inversely correlated with sperm count and motility.

Similar observations have been reported concerning CMV (Torino *et al.* 1987). A high concentration of CMV was detected in the semen of a patient with CMV mononucleosis, and this was associated with a transient decrease in sperm motility (Lang & Kummer, 1972, Lang *et al.* 1974). Numerous HSV2 receptors have been identified, including heparan sulphate-like glycosaminoglycans (Trybala *et al.* 2000), nectins and a receptor belonging to the tumour-necrosis factor family (Campadelli-Fiume *et al.* 2000). Therefore, it appears that human herpesviruses may have developed receptor mediated mechanisms to infect human sperms and infection of sperm with HHV-8 may not be unique.

In summary, our data strongly suggest a potential sexual/horizontal route of transmission of HHV-8, via the HHV-8 infected sperm and other semen cells, where a large percentage of HIV-1 infected men's sperm and other semen cells are infected with HHV-8. Co-culture studies have further supported the observations that O. Bagasra *et al.* HHV-8 in the sperm cells is infectious and capable of transmission of the virus to uninfected cells.

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