

Absence of human herpes virus 8 in semen from healthy Danish donors

Jens Kelsen¹, Britta Tarp and Niels Obel

Department of Infectious Diseases, Marselisborg Hospital, Aarhus University Hospital, P.P. Oerumsgade 11, DK-8000 Aarhus, Denmark

¹To whom correspondence should be addressed

Epidemiological data indicate a sexual route of transmission of acquired immune deficiency syndrome (AIDS) associated Kaposi's sarcoma. Recently human herpes virus 8 (HHV-8) has been proposed as the aetiological agent for development of Kaposi's sarcoma. Further the virus has been reported in semen obtained from healthy men. In Denmark strict biochemical and microbiological criteria are used in combination with an intensive interview to select semen donors. Despite these strict criteria, HHV-8 may be transmitted to a recipient and even the child by the use of donor semen. We used four different polymerase chain reaction (PCR) and one nested PCR to test semen from 100 Danish donors for the presence of HHV-8 DNA. All 100 samples were consistently negative for HHV-8 DNA, while only one sample (1%) was positive for cytomegalovirus DNA. As HHV-8 was not demonstrated in any of the semen samples, we conclude that the frequency of HHV-8 in semen from Danish donors is very low.

Key words: CMV/donor/human herpes virus 8/PCR/semen

Introduction

Epidemiological data indicate a sexual route of transmission of Kaposi's sarcoma. In support of this it has been reported (Martin *et al.*, 1998) that acquired immune deficiency syndrome (AIDS)-associated Kaposi's sarcoma is linked to the number of male intercourse partners. Also, a higher incidence of Kaposi's sarcoma was observed in human immunodeficiency virus (HIV)-infected homosexuals compared with haemophiliacs and drug abusers (Beral *et al.*, 1992). In 1994 human herpes virus 8 (HHV-8) was demonstrated in Kaposi's sarcoma and was identified as the potentially transmissible agent (Chang *et al.*, 1994; Moore and Chang, 1995). Ever since, efforts have been made to determine the primary route of HHV-8 acquisition. Knowing that Kaposi's sarcoma is linked to sexual practices like oro-anal contact (Beral *et al.*, 1992), focus has been on detecting HHV-8 in body fluids and faeces (LaDuca *et al.*, 1998).

The prevalence of HHV-8 in the general population remains controversial (Blackbourn and Levy, 1997). The frequency of HHV-8 detection in semen from healthy individuals ranges from non-detectable (Corbellino *et al.*, 1996; Howard *et al.*,

1997) to 23% (Lin *et al.*, 1995) and 91% (Monini *et al.*, 1996a). This might in part be explained by geographical and methodological differences, although the divergence seems overwhelming.

If HHV-8 is ubiquitous, it may share other characteristics with the known herpes viruses, such as establishment of a latent infection and reactivation with intermittent shedding. This raises important implications as to the use of donor semen in assisted conception procedures. HHV-8 may be transmitted to the mother and even vertically to the unborn child (He *et al.*, 1998).

The purpose of the present study was to determine if HHV-8 is present in semen from Danish donors.

Materials and methods

Semen samples were obtained from 100 healthy men who regularly donated semen for assisted conception procedures. All donors in this study fulfilled the criteria for selection of semen donors outlined by the Danish National Health Service (1997). Donors were recruited from different social classes. Donation was anonymous and consequently no information about marital status was available. All donors signed a declaration denying any previous sexual contact with men, prostitutes, or women from areas with high HIV prevalence.

Donors with a history of syphilis or treatment with blood products were excluded. Ninety-eight were white, native Danish males while two donors were immigrants from Iran.

Donors were tested for: syphilis using Wasserman's reaction, a serological test; gonorrhoea by culturing from the semen and serological test if the donor had received antibiotic treatment recently; *Chlamydia trachomatis* by culturing from urethral swabs or polymerase chain reaction (PCR) from urine; HIV-I and -II, human T-cell lymphotropic virus (HTLV)-I and II, hepatitis B surface-antigen (HbsAg) and anti-hepatitis C virus (HCV) every 3 months. Semen was released for use 6 months after the first tests. Blood type and male karyotype were determined.

Semen was stored in liquid nitrogen and for shorter periods at -70°C . DNA was extracted from 250 μl semen, equivalent to 5×10^6 highly motile spermatozoa, according to the description for Puregene[®] DNA Isolation Kit (Gentra Systems Inc, Minneapolis, MN, USA). The concentration and purity of DNA were estimated by measuring optical densities at 260 and 280 nm, using a Gene Quant II spectrophotometer (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany).

No attempts were made to separate semen into fractions since HHV-8 might be present preferentially in the fraction containing urothelial cells and to a lesser degree in sperm heads (Monini *et al.*, 1996a).

All primers used in our study were manufactured by DNA Technology ApS, Aarhus, Denmark. To assure the quality of the extracted DNA, all samples were analysed using PCR with a primer pair specific for the human gene glyceraldehyde-3-phosphate dehydro-

genase (*GADPH*): *GADPH1* (5′GAGTCAACGGATTTGGTCGT3′) and *GADPH2* (5′GGTGCCATGGAATTTGCCAT 3′). These primers amplified a 157 bp fragment of human DNA.

To detect HHV-8 DNA, simple as well as nested PCR was done. The primers used included: the inner pair of primers KS1 (5′AGCCGAAAGGATTCACCAT3′) and KS2 (5′TCCGTGTTGTCTACGTCCAG 3′) amplifying a 233 bp fragment of ORF 26 (Chang *et al.*, 1994); and the outer primers KS4 (5′GACTCTTCGCTGATGAACTGG 3′) and KS5 (5′AGCACTCGCAGGGCAGTACG 3′) amplifying a 700 bp fragment of HHV-8 DNA (Boshoff *et al.*, 1995).

Furthermore two sets of non-overlapping primers were used: KS10 (5′AGATGTTCTGTATGTTGTCTGCAG3′) and KS11 (5′ATGTAATCCAAATGCTCTGTCGATG 3′) amplifying a 270 bp hyper-variable region of ORF K1 (Kasolo *et al.*, 1998), and KS12 (5′TCGTGGGATCCAC-GGAGCATACACC3′) and KS13 (5′ATAGGATACGCTGGCAGGTGGGCG 3′) from ORF 75, a putative tegument protein of HHV-8 (Russo *et al.*, 1996). PCR was performed with 100 ng to 500 ng of DNA (equivalent to DNA extracted from 17 µl of semen) in a 21µl PCR-mixture containing 0.5U AmpliTaq Gold® (Perkin Elmer, Applied Biosystems Division, Foster City, CA, USA) 2 µl MgCl₂ 25 mmol/l (Perkin Elmer), 2 µl dNTP 500 µmol/l, 2 µl PCR Buffer II (Perkin Elmer) and 20 pmol of each primer. The amplification conditions were as follows: ‘Hot start’ 10 min at 95°C followed by 39 cycles of alternating denaturation for 1 min at 94°C, primer annealing for 1 min at 55°C and primer extension for 2 min at 72°C, terminated by a 10 min extension at 72°C. Higher annealing temperatures could have been used (and were used in some control experiments) but to ensure a high sensitivity we used a relatively low annealing temperature. In the case of nested PCR, 1 µl of the PCR product was further amplified with the inner primers under the same conditions as mentioned above. One positive and one negative sample were included for every nine semen samples. The positive controls were derived from paraffin-embedded biopsies of Kaposi’s sarcoma and from the cell line BCP-1, a commercially available (ATCC) body cavity-based lymphoma (BCBL) cell line, each cell harbouring 150 copies of HHV-8.

The PCR products were visualised on 2% agarose gels containing ethidium bromide.

In addition we tested the samples for cytomegalovirus (CMV) DNA with an un-nested PCR procedure using the primer pairs CMV1 (5′CAAGCGGCCTCTGATAACCAAGC′3) and CMV2 (5′CTCTTCTCTGGGGCAACTTCCTC′3) amplifying a 421 bp fragment. The amplification was performed with 35 cycles of alternating denaturation for 1 min at 94°C, primer annealing for 1 min at 65°C, and primer extension for 1 min at 72°C. Each extension time was increased by 10 s on each cycle (Hansen *et al.*, 1994).

Results

All the extracted semen samples were positive for the human gene *GADPH*, confirming the integrity of the extracted DNA. To evaluate the sensitivity of the PCR assay, we used purified DNA from the HHV-8 containing cell line, BCP-1. The BCP-1 cell line contains 150 genome copies of HHV-8/cell. We calculated that the nested PCR reaction (ORF 26) could have detected less than 5 copies of HHV-8 as could the inside primers alone.

The four primer sets for HHV-8 gave strong bands on ethidium bromide gels using DNA extracted from Kaposi’s sarcoma and BCP-1. But the 100 semen samples were consistently negative for HHV-8 DNA by the simple as well as the nested PCR procedures. As to detection of CMV DNA, we

found only one CMV-positive donor. The samples were tested twice for each primer set by different operators.

In a control experiment HHV-8 negative semen was spiked with HHV-8 from BCP-1 cells and serially diluted in semen. When compared to dilution series of HHV-8 in water, there was no indication that semen inhibited the PCR reactions.

The negative outcome concerning HHV-8 was not altered either by increasing the number of amplification cycles to 45 (Monini *et al.*, 1996a), nor by doubling the amount of DNA, decreasing the annealing temperature to 48°C or varying the concentration of MgCl₂ in the interval 1.25–3.5 mmol/l.

Discussion

The prevalence of HHV-8 in semen from healthy individuals is still a matter of debate. The problems related to possible HHV-8 transmission by assisted conception have recently been reviewed (Howard and Bahadur 1997b), who asked for regional studies to establish the local frequency of HHV-8 in semen donors. The first reported presence of HHV-8 was in 1995, in semen from healthy HIV-negative American citizens, with a prevalence of 23% (7/30), (Lin *et al.*, 1995). HHV-8 was surprisingly detected in 91% (30/33) of semen specimens from HIV-negative Italian patients undergoing surgery for varicocele (Monini *et al.*, 1996a). In a later and smaller control study this high frequency was not substantiated and instead a frequency of 23% was recorded (3/13) (Monini *et al.*, 1996b).

These findings were supported by studies in which HHV-8 was detected in 13% (6/45) of Sicilian men, thereby showing a roughly uniform picture of the prevalence of HHV-8 (Viviano *et al.*, 1997). All the above mentioned studies used a nested PCR procedure, only yielding positive signals after the second step of amplification.

These findings were challenged (Corbellino *et al.*, 1996; Howard *et al.*, 1997a) by studies which failed to confirm the presence of HHV-8 in semen from 115 English semen donors and 20 Italian semen donors respectively. Nested PCR was used in both of the studies.

In our study all 100 semen specimens remained negative for HHV-8 DNA, after having used both nested and un-nested PCR procedures. According to our experience, different primer sets may lead to unexplained differences in PCR sensitivity. We therefore used two pairs of non-overlapping primers in excess of the nested primers.

No study detected HHV-8 DNA in semen from healthy men by the use of a simple un-nested PCR procedure. The nested PCR presumably improves the detection level to a few HHV-8 DNA copies per µg of DNA. Several authors have argued that HHV-8 may establish a latent infection in otherwise healthy individuals, shedding virus in a low number of copies. It is still a matter of debate, whether the comparatively high detection rates of HHV-8 in some studies may be due to contamination of the samples, a problem often observed when using nested PCR (Howard and Bahadur 1997b; Moore, 1998).

At present the most sensitive method for detection of CMV in semen is PCR (Mansat *et al.*, 1997). We only detected CMV in one out of 100 donors. This frequency corresponds to earlier studies of donor populations 4/115 (Howard *et al.*,

1997a) and 5/97(Mansat *et al.*, 1997). As CMV was detectable in semen from one donor, herpes viruses seem to be recovered by the extraction method used in this study. The handling of samples prior to PCR is of unquestionable importance. In our study, semen samples were put into liquid nitrogen within the first hour after ejaculation and only stored at -70°C for shorter periods. As shown in previous studies (Howard *et al.*, 1997a) cryopreservation of semen does not affect PCR sensitivity of HHV-8 detection.

Geographical differences may have contributed to the different detection rates but it is more likely that the variation is due to the obvious differences in enrolment criteria.

Based on lifestyle questionnaires, the semen donors in our study had an exclusively heterosexual background and a rather limited number of sexual partners. This rules out most of the significant epidemiological risk factors for acquisition of HHV-8. The two studies (Corbellino *et al.*, 1996; Howard *et al.*, 1997) reporting negative results used authorized semen donors from fertility clinics while the three studies with positive results seem to have recruited semen donors from a potentially more promiscuous part of the male population, e.g. men with a known history of sexually transmitted disease.

In Denmark strict biochemical and microbiological criteria are used in combination with an intensive interview to select semen donors. As HHV-8 was not demonstrated in any of the semen samples analysed, we conclude that the frequency of HHV-8 in semen from Danish donors is very low.

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References

- Beral, V., Bull, D., Darby, S. *et al.* (1992) Risk of Kaposi's sarcoma and sexual practices associated with fecal contact in homosexual or bisexual men with AIDS. *Lancet*, **339**, 632–635.
- Blackbourn, D.J. and Levy, J.A. (1997) Human herpesvirus 8 in semen and prostate. Editorial comment. *AIDS*, **11**, 249–250.
- Boshoff, C., Whitby, D., Hatzioannou, T. *et al.* (1995) Kaposi's-sarcoma-associated herpesvirus in HIV-negative Kaposi's sarcoma. *Lancet*, **45**, 1043–1044.
- Chang, Y., Cesarman, E., Pessin, M.S. *et al.* (1994) Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science*, **266**, 1865–1869.
- Corbellino, M., Bestetti, G., Galli, M. *et al.* (1996) Absence of HHV-8 in prostate and semen. Letter. *N. Engl. J. Med.*, **335**, 1237.
- Danish National Health Service (1997) Guidelines for Assisted Conception Procedures.
- Hansen, K.K., Ricksten, A., Hofmann B. *et al.* (1994) Detection of cytomegalovirus DNA in serum correlates with clinical cytomegalovirus retinitis in AIDS. *JID*, **170**, 1271–1274.
- He, J., Bhat, G., Kankasa, C. *et al.* (1998) Seroprevalence of human herpesvirus 8 among Zambian women of childbearing age without Kaposi's Sarcoma (KS) and mother child pairs with KS. *JID.*, **78**, 1787–1790.
- Howard, M.R., and Bahadur G. (1997) Kaposi's sarcoma-associated herpes virus – a new concern for human reproduction? *Hum. Reprod.*, **12**, 2567–2571.
- Howard, M.R., Whitby, D., Bahadur, G. *et al.* (1997) Detection of human herpes virus 8 DNA in semen from HIV-infected individuals but not healthy semen donors. *AIDS*, **11**, F15–19.

- Kasolo, F.C., Monze, M., Obel, N. *et al.* (1998) Analyses of variability of human herpesvirus 8 (HHV-8) strains identifies a new group within both febrile infants and children with endemic Kaposi's sarcoma in Africa. *J. Gen. Virol.*, **79**, 3055–3065.
- LaDuca, J.R., Love, J.L., Abbott, L.Z. *et al.* (1998) Detection of human herpesvirus 8 DNA sequences in tissues and bodily fluids. *JID*, **178**, 1610–1615.
- Lin, J.C., Lin, S.C., Mar, E.C. *et al.* (1995) Is Kaposi's-sarcoma-associated herpesvirus detectable in semen of HIV-infected homosexual men?. *Lancet*, **346**, 1601–1602.
- Mansat, A., Mengelle, C., Chalet, M., *et al.* (1997) Cytomegalovirus detection in cryopreserved semen samples collected for therapeutic donor insemination. *Hum. Reprod.*, **2**, 1663–1666.
- Martin, J.N., Ganam, D.E., Osmond, D.H., *et al.* (1998) Sexual transmission and the natural history of human herpesvirus 8 infection. *N. Engl. J. Med.*, **338**, 948–954.
- Monini, P., De Lellis, L., Fabris, M. *et al.* (1996a) Kaposi's sarcoma-associated herpesvirus DNA sequences in prostate tissue and human semen. *N. Engl. J. Med.*, **334**, 1168–72.
- Monini, P., De Lellis, L., Cassai, E. (1996b) Letter. *N. Engl. J. Med.*, **335**, 1238–1239.
- Moore, P.S., Chang, Y. (1995) Detection of herpes-like DNA sequences in Kaposi's sarcoma patients with and without HIV infection. *N. Engl. J. Med.*, **332**, 1181–1185.
- Moore, P. (1998) Human herpesvirus 8 variants. *Lancet*, **351**, 679–680.
- Russo, J.J., Bohenzky, R.A., Chien, M.-C. *et al.* (1996) Nucleotide sequence of Kaposi' sarcoma-associated herpesvirus (HHV-8). *Proc. Natl. Acad. Sci., USA*, **93**, 14862–14868.
- Viviano, E., Vitale, F., Ajello, F. *et al.* (1997) Human herpesvirus type 8 DNA sequences in biological samples of HIV-positive and negative individuals in Sicily. *AIDS*, **11**, 607–612.

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