

# Detection of herpes simplex virus, cytomegalovirus, and Epstein-Barr virus in the semen of men attending an infertility clinic

Nikiforos Kapranos, M.D., Ph.D.,<sup>a</sup> Eftichia Petrakou, Ph.D.,<sup>a</sup>  
Cathrin Anastasiadou, M.Sc.,<sup>a</sup> and Dimosthenis Kotronias, M.D.<sup>b</sup>

Amalia Fleming Hospital and Mitera Maternity and Surgical Center, Athens, Greece

**Objective:** To investigate the prevalence of herpes simplex virus (HSV), cytomegalovirus (CMV), and Epstein-Barr virus (EBV) in the semen of men with fertility problems.

**Design:** A descriptive clinical study.

**Setting:** Outpatient infertility clinic of a private hospital.

**Patient(s):** One hundred thirteen men attending an infertility clinic in Athens.

**Intervention(s):** Semen samples were collected by masturbation.

**Main Outcome Measure(s):** Detection of HSV, CMV, and EBV in semen by a nested polymerase chain reaction technique. Complete spermogram including sperm count, motility, pH, viscosity, and morphology.

**Result(s):** Viral DNA was detected by the nested polymerase chain reaction technique in 64 (56.6%) of 113 semen samples. Specifically, HSV DNA was detected in 56 (49.5%) semen samples, EBV DNA in 19 (16.8%) semen samples, and CMV DNA in 8 (7.1%) semen samples. HSV was significantly related to low sperm count and poor motility. In contrast, CMV and EBV did not show any association with sperm concentration and motility.

**Conclusion(s):** Herpes simplex virus seems to play a significant role in male infertility, and its early detection by the nested polymerase chain reaction technique will permit successful antiviral therapy to increase the possibility for fertility restoration and long-term protection of the sperm quality. Finally, the detection of herpes viruses within semen will allow better control of the transmission of these viruses. (*Fertil Steril*® 2003; 79(Suppl 3):1566–70. ©2003 by American Society for Reproductive Medicine.)

**Key Words:** HSV, CMV, EBV, male sperm, infertility, PCR

Infertility is a major problem of modern medicine as it affects almost 20% of reproductive-aged couples. The cause of this problem is attributed to the male partner in nearly 40%–50% of these cases (1, 2). The most common disorder associated with male infertility is varicocele, which accounts for 35% of the cases. Endocrine disorders, spermatic duct obstruction, antisperm antibodies, gonadotoxins, drugs, cryptorchidism, infection, sexual dysfunction, and ejaculatory failure are less common causes, accounting for <5% of male infertility cases (3). In >50% of male infertility cases, the etiology remains unknown, and the infertility is classified as idiopathic. Idiopathic infertility represents a serious situation as it affects a high percentage of infertile men who

cannot be successfully treated by the empirical therapeutic modalities currently applied (3, 4).

In a few studies, the relation of viruses such as human papilloma virus and adeno-associated virus to male infertility has been investigated (5, 6). Herpes simplex virus (HSV) DNA has been detected by polymerase chain reaction (PCR) technique in the sperm of men with genital HSV infection (7, 8), and in one study, it was also associated with infertility (9). In a previous study (10) we examined HSV in semen of infertile men by the *in situ* hybridization technique, and we localized HSV DNA within spermatozoa. Consequently, we thought it would be interesting to apply the sensitive nested PCR technique for the detection of HSV

Received March 29, 2002;  
revised and accepted  
January 7, 2003.

Reprint requests: Nikiforos  
Kapranos, M.D., Megalou  
Spileou 11, 115 22,  
Athens, Greece (FAX:  
+301-6462686; E-mail:  
nkapran@otenet.gr).

<sup>a</sup> Division of Molecular  
Pathology, Department of  
Pathology, Amalia Fleming  
Hospital.

<sup>b</sup> Department of Obstetrics  
and Gynaecology, Mitera  
Maternity and Surgical  
Center.

0015-0282/03/\$30.00  
doi:10.1016/S0015-0282(03)  
00370-4

TABLE 1

List of primers used in the detection of HSV, CMV, and EBV.

Virus	Gene	Sequence	PCR product (base pairs)
HSV	HSV-1 DNA pol gene (UL30 gene)	Outer primers: 5'-CCC GTG GTG GTG TTC GAC TTT GCC-3' 5'-GCA CAA AGA TGG AGT CCG TGT CCC-3'	544
		Inner primers: 5'-TTC TTC GTC AAG GCT CAC GTG CG-3' 5'-CCG AGT TAC ACA CGA CCT TGA TGG C-3'	154
CMV	Major immediate-early protein (IE) gene	Outer primers: 5'-GCA ACG AGA ACC CCG AGA AAG-3' 5'-AAG CCA TAA TCT CAT CAG GG-3'	698
		Inner Primers: 5'-GCG GCA TAG AAT CAA GGA GCA CAT G-3' 5'-CAA GCC ATC CAC ATC TCC CGC-3'	222
EBV	EBNA-2 gene	Outer primers: 5'-ATC CTT GCA CTT AGC CAA GC-3' 5'-TCC AGA TGT GTC TCC CTT CT-3'	556
		Inner primers: 5'-CCA GTA GCA TCT CTG TCT GG-3' 5'-GAA CCA TCC TCG TCC TCA TC-3'	190

Kapranos. HSV, CMV, and EBV in semen. *Fertil Steril* 2003.

as well as other members of the herpesvirus family, such as cytomegalovirus (CMV) and Epstein-Barr virus (EBV), in the semen of men with fertility problems.

## MATERIALS AND METHODS

### Samples

Semen samples were collected by masturbation from 113 men who were attending the infertility clinic at the Mitera Maternity and Surgical Center of Athens because of couple fertility problems. From those men, an informed consent was obtained to search for HSV, CMV, and EBV. None of the studied men or their wives had reported any clinically confirmed genital herpetic infection in their medical history. In all cases, complete spermogram was performed, including sperm count, motility, pH, viscosity, and morphology.

### DNA Extraction From Semen Samples

After collection each semen sample was processed by centrifuge at 2,500 rpm for 10 minutes. The supernatant was removed and the pellet was transferred to an Eppendorf tube. DNA extraction was performed using the protocol of EPI-CENTRE DNA extraction kit (MasterPure DNA Purification kit, Madison, WI).

### PCR Amplification Reactions

All the samples were examined for the presence of HSV, CMV, and EBV DNA by the nested PCR technique using one set of outer primers for primary PCR and one set of inner primers for nested PCR (Table 1). About 3–5  $\mu$ L of extracted DNA of each sample was initially amplified in a reaction solution of 50  $\mu$ L containing 25 mM MgCl<sub>2</sub>, 0.25

$\mu$ M of each primer, 200  $\mu$ M of each dNTP, 1 $\times$  PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, at 22°C), and 2.5 U Taq Polymerase (EPICENTRE MasterAmp Taq DNA polymerase). The same reaction mixtures were applied for nested PCR using 2  $\mu$ L of the primary PCR product as template.

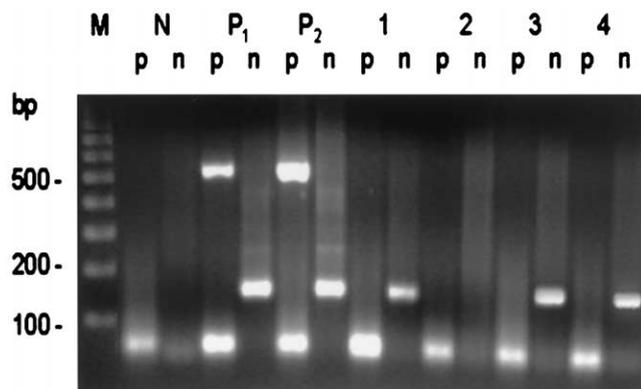
For HSV, the primary PCR mixture was heated for 5 minutes at 95°C and then subjected to 40 cycles of amplification in the following conditions: denaturation at 95°C for 45 seconds, annealing at 60°C for 45 seconds, and elongation at 72°C for 1 minute. For nested PCR, the mixture was heated for 5 minutes at 95°C and then subjected to 40 cycles of amplification in the following conditions: 95°C for 45 seconds, 57°C for 45 seconds, and 72°C for 45 seconds.

For CMV, the primary PCR mixture was heated for 5 minutes at 95°C and then subjected to 40 cycles of amplification in the following conditions: denaturation at 95°C for 45 seconds, annealing at 50°C for 45 seconds, and elongation at 72°C for 75 seconds. For nested PCR, the mixture was heated for 5 minutes at 95°C and then subjected to 40 cycles of amplification in the following conditions: 95°C for 45 seconds, 58°C for 45 seconds, and 72°C for 45 seconds.

For EBV, the primary PCR mixture was heated for 5 minutes at 95°C and then subjected to 40 cycles of amplification in the following conditions: 95°C for 45 seconds, 53.5°C for 45 seconds, and 72°C for 60 seconds. For nested PCR, the mixture was heated at 95°C for 5 minutes and then subjected to 35 cycles of amplification in the following conditions: 95°C for 45 seconds, 54°C for 45 seconds, and 72°C for 45 seconds. All the primary PCR and nested PCR

**FIGURE 1**

Nested PCR detection of HSV in semen samples. The amplified products were visualized in 2% agarose gel. M = molecular weight marker; p = primary PCR; n = nested PCR; N = negative control (no DNA); P<sub>1</sub>, P<sub>2</sub> = PCR product from HSV1-positive and HSV2-positive controls (both primary PCR and nested PCR products are shown); 1, 3, 4 = PCR product from 3 HSV-positive semen samples (only nested PCR product is evident); 2 = HSV-negative semen sample.



*Kapranos. HSV, CMV, and EBV in semen. Fertil Steril 2003.*

reactions ended with an elongation step at 72°C for 10 minutes.

The PCR products were examined by electrophoresis in a 2% agarose gel and photographed on an ultraviolet light transilluminator.

### Positive Controls

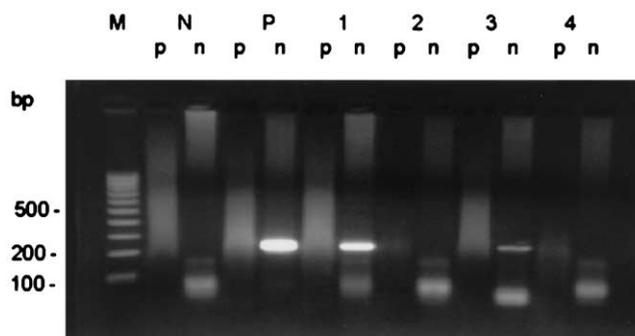
As positive controls, we used DNA extracted from HSV-1-infected and HSV-2-infected Vero cells, from CMV-infected MRC-5 cells, and from EBV-infected  $\beta$ 95.8 cells.

### Statistical Analysis

Statistical analysis was performed by using the SPSS statistical software package (SPSS Inc., Chicago, IL). Comparison of the mean sperm count and motility between vi-

**FIGURE 2**

Nested PCR detection of CMV in semen samples. M = molecular weight marker; p = primary PCR; n = nested PCR; N = negative control (no DNA); P = PCR product from positive control; 1, 3 = two CMV-positive semen samples; 2, 4 = two CMV-negative semen samples.



*Kapranos. HSV, CMV, and EBV in semen. Fertil Steril 2003.*

rally infected and noninfected samples was performed by analysis of variance (ANOVA). A *P* value of <.05 was accepted as statistically significant.

## RESULTS

Viral DNA was detected by the nested PCR technique in 64 (56.6%) of 113 semen samples. Specifically, HSV was detected in 56 (49.5%), EBV in 19 (16.8%), and CMV in 8 (7.1%) semen samples, respectively (Figs. 1–3). The simultaneous presence of two DNA viruses was detected in 18 (16%) semen samples. Specifically, HSV and EBV DNA were detected in 14 (12.4%) samples, and HSV and CMV DNA, in 4 (3.6%) samples. DNA of all three viruses was found in only 1 (0.9%) semen sample.

By the first PCR, only four cases were positive for HSV and none for CMV and EBV, whereas all viral cultures were positive for the first and second PCR. Regarding HSV, the

**TABLE 2**

Mean sperm count in virally infected and noninfected semen samples.

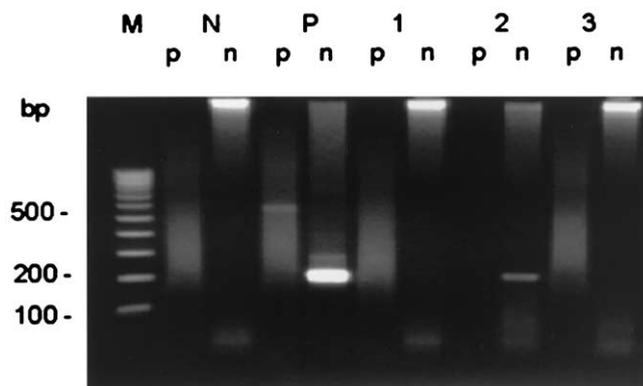
Viral DNA	N (%)	Mean sperm count (million/mL)	SD	<i>P</i> <sup>a</sup>
HSV+	56 (49.5%)	19.8	25.6	.000003
HSV-	57 (50.5%)	54.5	47.3	
EBV+	19 (16.8%)	34.5	35.6	.85
EBV-	94 (83.2%)	36.4	42.2	
CMV+	8 (7.1%)	46.9	45.9	.43
CMV-	105 (92.9%)	35.3	40.7	

<sup>a</sup> Analysis of variance.

*Kapranos. HSV, CMV, and EBV in semen. Fertil Steril 2003.*

**FIGURE 3**

Nested PCR detection of EBV in semen samples. M = molecular weight marker; p = primary PCR; n = nested PCR; N = negative control (no DNA); P = primary PCR and nested PCR product from positive control; 2 = EBV-positive semen sample; 1, 3 = two EBV-negative semen samples.



Kapranos. HSV, CMV, and EBV in semen. *Fertil Steril* 2003.

PCR method was thought to be unable to discriminate between HSV1 and HSV2, as both viral cultures were strongly positive using the HSV1 DNA polymerase gene primers.

Table 2 shows the mean sperm count of virally infected and non-infected semen samples. According to the results, the presence of HSV was significantly associated with low sperm count. More specifically, the mean sperm count of HSV-positive semen samples was 19.8 million per milliliter, and that of HSV-negative ones was 54.5 million per milliliter ( $P=.000003$ ). In contrast, EBV and CMV infection did not show any influence on sperm count.

Table 3 presents the mean sperm motility and HSV, CMV, or EBV infection. The mean sperm motility of HSV-positive semen samples was significantly lower (39.1%) compared with that of HSV-negative semen samples (48.6%) ( $P=.0054$ ). In contrast, the presence of CMV or EBV did not show any association with poor motility.

**TABLE 3**

Mean sperm motility in virally infected and noninfected semen samples.

Viral DNA	N (%)	Mean sperm motility (%)	SD	$P^a$
HSV+	56 (49.5%)	39.1	19.1	.0054
HSV-	57 (50.5%)	48.6	13.9	
EBV+	19 (16.8%)	42.3	12.2	.74
EBV-	94 (83.2%)	43.9	18.4	
CMV+	8 (7.1%)	47.7	13.8	.52
CMV-	105 (92.9%)	43.3	17.7	

<sup>a</sup> Analysis of variance (ANOVA).

Kapranos. HSV, CMV, and EBV in semen. *Fertil Steril* 2003.

## DISCUSSION

At the onset of the new millennium, despite improvements in assisted reproduction technology, infertility continues to be a serious social problem (11). In an effort to investigate the causes of male factor infertility, which in about half of the cases are unknown, we explored the role of HSV, CMV, and EBV using PCR technology. According to our findings, 56.6% of the examined semen samples from males with fertility problems harbored at least one of the investigated viruses. However, HSV seemed to be the most important as it was found to be detected much more commonly than CMV and EBV and was significantly related to both parameters of poor sperm quality, namely low count and motility.

A significant association between HSV and infertility was also observed by El Borai et al. (9), who detected HSV DNA in 24% of semen samples from infertile men using a nested PCR technique. Moreover, Wald et al. (8), using PCR, found HSV DNA in the semen samples of 7 (47%) of 15 men with genital HSV-2 infection, whereas none of the samples was positive by culture. The authors attributed the presence of the virus to potential contamination of the semen sample during collection. However, according to our findings (10), by applying the in situ hybridization technique, we found HSV to be detected within the spermatozoa themselves, which is evidence against contamination as a cause.

Experimental studies with transgenic mice elucidate the relation between HSV and infertility. According to Huttner et al. (12), the expression of HSV thymidine kinase in testis of transgenic mice was associated with defects in spermatogenesis characterized by acrosomal aberrations, structural abnormalities in the neck, and flagellum of the spermatozoa and developmental arrest. Moreover, in revertant mouse males, the decrease of HSV thymidine kinase levels resulted in marked reduction of spermatozoa abnormalities and restoration of fertility (12, 13).

The implication of HSV in male infertility and the facility of its detection by the use of nested PCR technique allow important therapeutic interventions, as was confirmed in our preliminary trial of three men that resulted in successful pregnancies

after antiviral therapy (10). Similar findings had been published by Kundsins et al. (14), who reported successful pregnancies in 5 of the 12 infertile couples after acyclovir treatment. The above clinical observations, although few in number, offer further support to the role of HSV in male infertility.

The significance of herpes viruses infection for IVF techniques is obvious, as their presence could probably be responsible for the high rate of fertilization failure observed by the use of low-quality semen from infertile males (15, 16). Moreover, CMV DNA has been detected by PCR in 5.1% of cryopreserved semen samples (17), with a serious risk for transmission and developmental abnormalities of the fetus because of elimination of natural spermatozoa selection. Consequently, it would be useful to analyze semen specimens for herpes viruses, and especially HSV, by PCR, before any intervention by assisted reproduction techniques is planned.

It is important to emphasize that herpes viruses may long persist within the semen in a very low copy number, without causing symptoms and without yielding positive cultures. This has been found even in the case of men with genital herpes in whom the virus failed to be isolated in the semen by tissue culture (8, 18, 19). Nevertheless, the acquisition of HSV through artificial insemination from an asymptomatic semen donor has been reported (20). Consequently, we cannot be sure about the critical viral load for HSV transmission, because it has been found that PCR-positive and culture-negative women can infect neonates during delivery (21). It is evident that the sensitive nested PCR technique is more suitable than viral culture for the detection of asymptomatic chronic HSV infection.

In conclusion, the findings of the study indicate that HSV, by affecting the most important factors of semen quality, plays an important role in male infertility. The early detection of this virus by the nested PCR technique will permit the suitable antiviral therapy to increase the possibility of fertility restoration and long-term protection of sperm quality. Finally, the detection of herpes viruses within semen will provide better control of the transmission of these viruses.

---

*Acknowledgments:* HSV-1-infected and HSV-2-infected Vero cells, CMV-infected MRC-5 cells, and EBV-infected  $\beta$ 95.8 cells were kindly provided by Panagiotis Markoulatos, M.D., Pasteur Institute of Athens.

## References

1. Mosher WD. Reproductive impairments in the United States, 1965–1982. *Demography* 1985;22:415–30.
2. Dawson C, Whitfield H. Subfertility and male sexual dysfunction. *BMJ* 1996;312:902–5.
3. Greenberg SH, Lipschultz LI, Wein AJ. Experience with 425 subfertile male patients. *J Urol* 1978;119:507–10.
4. Skakkaebæk NE, Giwercman A, de Kretser D. Pathogenesis and management of male infertility. *Lancet* 1994;343:1473–9.
5. Lai YM, Lee JF, Huang HY, Soong YK, Yang FP, Pao CC. The effect of human papillomavirus infection on sperm cell motility. *Fertil Steril* 1997;67:1152–5.
6. Erles K, Rohde V, Thaele M, Roth S, Edler L, Schlehofer JR. DNA of adeno-associated virus (AAV) in testicular tissue and in abnormal semen samples. *Hum Reprod* 2001;16:2333–7.
7. Aynaud O, Bijaoui G, Ionesco M, Corgette O, Poveda JD, Zummer K. Genital herpes simplex virus infection among men screened for genital papillomavirus. *Ann Dermatol Venerol* 1994;121:376–81.
8. Wald A, Matson P, Ryncarz A, Corey L. Detection of herpes simplex virus DNA in semen of men with genital HSV-2 infection. *Sex Transm Dis* 1999;26:1–3.
9. El Borai N, Inoue M, Lefevre C, Naumova EN, Sato B, Yamamura M. Detection of herpes simplex virus DNA in semen and menstrual blood of individuals attending an infertility clinic. *J Obstet Gynaecol Res* 1997;23:17–24.
10. Kotronias D, Kapranos N. Detection of herpes simplex virus DNA in human spermatozoa by in situ hybridization technique. *In Vivo* 1998;12:391–4.
11. Mortimer D. The future of male infertility management and assisted reproduction technology. *Hum Reprod* 2000;5(Suppl 5):98–110.
12. Huttner KM, Pudney J, Milstone DS, Ladd D, Seidman JG. Flagellar and acrosomal abnormalities associated with testicular HSV-tk expression in mouse. *Biol Reprod* 1993;49:251–61.
13. Wilkie TM, Braun RE, Ehrman WJ, Palmiter RD, Hammer RE. Germ-line intrachromosomal recombination restores fertility in transgenic Myk-103 male mice. *Genes Dev* 1991;5:38–48.
14. Kundsins RB, Falk L, Hertig AT, Horne HW Jr. Acyclovir treatment of twelve unexplained infertile couples. *Int J Fertil* 1987;32:200–4.
15. Aydin S, Inci O, Alagol B, Huseyin I, Kaya E, Dellalloglu G. Failure of artificial insemination of husband's semen in the treatment of male infertility. *Int J Urol Nephrol* 1996;28:117–22.
16. Pilikian S, Guerin JF, Czyba JC, Pinatel MC. Study of different components in spermatid function after failure of in vitro fertilization. *Contracept Fertil Sex* 1993;21:865–8.
17. Mansat A, Mengelle C, Chalet M, Boumzebra A, Mieuxset R, Puel J, et al. Cytomegalovirus detection in cryopreserved semen samples collected for therapeutic donor insemination. *Hum Reprod* 1997;12:1663–6.
18. Douglas JM Jr, Davis LG, Remington ML, Paulsen CA, Perrin EB, Goodman P, et al. A double-blind, placebo-controlled trial of the effect of chronically administered oral acyclovir on sperm production in men with frequently recurrent genital herpes. *J Infect Dis* 1988;157:588–93.
19. McGowan MP, Hayes K, Kovacs GT, Leydon JA. Prevalence of cytomegalovirus and herpes simplex virus in human semen. *Int J Androl* 1983;6:331–6.
20. Moore DE, Ashley RL, Zarutskie PW, Coombs RW, Soules MR, Corey L. Transmission of genital herpes by donor insemination. *JAMA* 1989;261:3441–3.
21. Cone RW, Hobson AC, Brown Z, Ashley R, Berry S, Winter C, et al. Frequent detection of genital herpes simplex virus DNA by polymerase chain reaction among pregnant women. *JAMA* 1994;272:792–6.