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Prevalence of sexually transmissible pathogens in semen from asymptomatic male infertility patients with and without leukocytospermia

Guntram Bezold, M.D.[a], **Joseph A. Politch, Ph.D.**[a,b], **Nancy B. Kiviat, M.D.**[c], **Jane M. Kuypers, Ph.D.**[c], **Hans Wolff, M.D.**[d], and **Deborah J. Anderson, Ph.D.**[a,b]

a*Departments of Medicine and Obstetrics and Gynecology, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts*

b*Department of Obstetrics and Gynecology, Boston University School of Medicine, Boston, Massachusetts*

c*Department of Pathology, University of Washington, Seattle, Washington*

d*Department of Dermatology, Ludwig-Maximilians-University of Munich, Munich, Germany*

Abstract

Objective—To determine the prevalence of pathogens that cause sexually transmitted infections (STIs) in semen from asymptomatic male infertility patients with and without leukocytospermia (LCS), and associations between STIs, inflammatory markers and other semen variables.

Design—Retrospective, controlled study.

Setting—Center for Reproductive Medicine, Brigham and Women's Hospital, Boston, Massachusetts.

Patient(s)—241 male infertility patients undergoing routine semen analysis; 132 with LCS, and 109 without LCS.

Intervention(s)—None

Main Outcome Measure(s)—DNA from STI pathogens [human papillomavirus (HPV), cytomegalovirus (CMV), herpes simplex virus (HSV), human herpes virus type 6 (HHV-6), Epstein-Barr virus (EBV), hepatitis B virus (HBV) and *Chlamydia trachomatis* (CT)], routine semen parameters and markers of accessory gland and epididymal function and inflammation.

Results—STI DNA was detected in 45/241 (18.7%) of the samples (CMV 8.7%, HPV 4.5%, HHV-6 3.7%, HSV 3.7%, CT 2.5%, EBV 0.4%, and HBV 0%), with no difference in prevalence between LCS and non-LCS groups. STI DNA in semen was associated with a decrease in sperm concentration, motile sperm concentration, total sperm count and neutral α-glucosidase concentration, whereas LCS was associated with a decrease in total sperm count, % normal forms and fructose concentration.

Reprint requests: Deborah J. Anderson, Ph.D., Department of Obstetrics and Gynecology, Boston University School of Medicine, 670 Albany Street, Suite 516, Boston, MA 02118, (FAX: 617-414-8481; e-mail: E-mail: deborah.anderson@bmc.org).

Capsule: Sexually transmissible pathogens were detected in 19% of semen samples from infertility patients seeking routine semen analyses; their presence was not associated with leukocytospermia, but was associated with reduced semen quality.

Conclusion(s)—STI pathogen DNA was detected in semen from a high percentage of asymptomatic male infertility patients and was associated with poor semen quality. Efforts to diagnose and treat subclinical genital tract infections should be intensified.

Keywords

Infection; virus; Chlamydia trachomatis; PCR; infertility; semen; leukocytospermia; cytokines

Introduction

Sexually transmitted infections (STIs) are of major concern to clinicians and researchers in the field of reproductive medicine. Many STI pathogens cause incurable, often fatal diseases, and have been transmitted through insemination procedures (1-3). Furthermore, several of these pathogens can be transmitted from infected mothers to the fetus or newborn (2-5). Men can harbor subclinical infections in the genital tract over extended periods of time. Several viruses, including cytomegalovirus (CMV), Epstein-Barr virus (EBV), human papillomavirus (HPV), hepatitis B virus (HBV), hepatitis C virus (HCV), herpes simplex virus type 2 (HSV-2), human herpes virus type 6 (HHV-6), and human immunodeficiency virus type 1 (HIV-1), as well as the intracellular bacterium, *Chlamydia trachomatis* (CT), have been detected in semen from asymptomatic men (5-8).

Leukocytospermia (LCS), also known as leukospermia, pyospermia or pyosemia, is a term used to designate abnormally high concentrations of white blood cells (WBC) in semen (9) and has been defined by the World Health Organization (WHO) as greater than 10^6 white blood cells (WBC) per mL semen (10). The prevalence of LCS in male infertility patients varies from 2% to 40% in published reports (9,11-13), and elevated concentrations of WBC in semen have been associated with reduced sperm function and quality (9,13-23).

Although it is widely assumed that LCS is an indicator of genital tract infection, bacteria have been detected in only a minority of leukocytospermic samples (11,24,25). In addition, antibiotic therapy for the treatment of LCS has produced conflicting results (26-30). Thus, there has been little evidence for a strong relationship between bacterial infection in the male genital tract and LCS (11). However, CT, an intracellular parasite which is the most prevalent sexually transmitted bacterial pathogen in industrialized nations (31), has been associated with LCS. A recent study utilizing nested plasmid polymerase chain reaction (PCR) found both significantly higher seminal WBC concentrations and a greater prevalence of LCS in men who were positive for CT DNA (32).

Although viral STIs are also common and can persist for years often without symptoms, they have not been systematically studied as an etiologic factor in LCS. HIV-1 levels in semen have been associated with LCS (33,34). More recently, Krause and colleagues (12) compared the prevalence of CMV, EBV and HSV antibodies in serum of men with and without LCS. HSV IgM seropositivity was associated with LCS, but an additional smaller experiment reported by the investigators that utilized PCR to detect HSV DNA in semen failed to identify any positive samples (12).

The purpose of our study was to use powerful new molecular techniques to determine the prevalence and quantity of DNA from several common sexually transmitted viruses and CT in semen from asymptomatic infertility patients with and without LCS. In addition, we sought to determine whether detection of pathogen DNA is associated with other measures of genital tract inflammation, impaired accessory gland or epididymal function or reduced semen quality.

Materials and Methods

Patients

This study was approved by the Institutional Review Board of the Brigham & Women's Hospital. Male infertility patients from couples undergoing evaluation for infertility at the Center for Reproductive Medicine at Brigham and Women's Hospital, Boston, Massachusetts were screened for LCS ($\geq 10^6$ PMN/mL semen) by the Endtz assay (35). One hundred thirty-two men with LCS and 109 patients with $<10^6$ PMN/mL ("non-LCS" group) were included in the study. None of the men was symptomatic for genital tract infections or inflammation. The subjects ranged in age from 22 to 55.

Semen collection and analysis

Semen samples were obtained by masturbation into sterile containers after sexual abstinence of 48 to 72 hours. Samples were subjected to semen analysis within one hour of collection and processed for freezing within two hours of collection. Concentrations of sperm as well as sperm motility were determined using a Hamilton-Thorne Motility Analyzer (Hamilton Thorne Biosciences, Inc, Beverly, MA, USA). All settings used in this semen analysis have been described previously (28). Sperm with a velocity of >10 μm/sec were considered motile. Sperm morphology was assessed on methanol-fixed smears of fresh ejaculate stained with eosin and thiazine under a light microscope. PMNs were counted following peroxidase staining with Endtz-reagent (35). Whole semen was stored frozen at −70° C until used for DNA extraction and measurement of accessory organ markers, IL-1β and PMN-elastase.

Detection of pathogen DNA in semen

DNA extraction—All preparation work for the PCR assay was performed in a "clean room" (no post-PCR DNA products) under a laminar flow hood to minimize contamination. Frozen semen samples were thawed and centrifuged at 2,000 × g for 5 minutes. The seminal plasma was removed, and the cell pellet was resuspended in 1 mL of Trizol (Gibco BRL, Gaithersburg, MD, USA). This extraction system is based on acid guanidinium isothiocyanate-phenol-chloroform (36) which extracts DNA from somatic "round" cells and pathogens, but not from the condensed nuclei of sperm (37). If the total round cell number in the pellet exceeded 10^7, 1 mL of Trizol was used for any additional 10^7 round cells. The tubes were incubated at room temperature for 5 minutes. Then 0.2 mL of chloroform per mL Trizol was added. The tubes were mixed, incubated at room temperature for three minutes and centrifuged at 4° C and 12,000 × g for 15 minutes. After centrifugation, the interphase layer containing the DNA and the lower phenol phase containing protein were mixed with 0.3 mL of pure ethanol. After incubating for 3 minutes at room temperature, the tubes were centrifuged at 4° C and 2,000 × g for 5 minutes. The supernatant containing phenol and protein was removed, and the pellet was washed twice with 1 mL of 100 mM sodium citrate containing 10% ethanol, incubated at room temperature for 30 minutes and centrifuged at 4° C and 2,000 × g for 5 minutes. Then the pellet was washed once with 1 mL of 75% ethanol, incubated at room temperature for 20 minutes and centrifuged at 4° C and 2,000 × g for 5 minutes. The supernatant was removed, the pellet was air-dried and dissolved in sterile distilled water. To remove all insoluble material the solution was centrifuged at room temperature and 12,000 × g for 10 minutes. The supernatant was saved, and the DNA concentration was determined by OD reading at 260 nm.

DNA standards—CMV DNA of known concentration was obtained from the National Institutes of Health and DNA from HSV-1, HSV-2, HHV-6 and EBV with known concentration was obtained from Advanced Biotechnologies (Columbia, MD, USA). HBV was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). As the HBV concentration in this sample was not known, HBV PCR was performed, the product was

separated on a 2%-agarose gel containing ethidium bromide and made visible under UV-light. The specific HBV band was cut out, and the DNA was extracted using the Quiagen Gel Extraction Kit, a silicate-based method (Quiagen, Chatsworth, CA, USA). The fragment concentration was determined by OD reading at 260 nm. The fragment was used as the HBV standard. CT elementary bodies with known concentration were purchased from Advanced Biotechnologies (Columbia, MD, USA). The DNA of the CT elementary bodies was Trizol-extracted.

Semiquantitative PCR—PCR was performed as described by Saiki et al (38). Oligonucleotide sequences for CMV, HHV-6, HSV-1/2 (together) and EBV were obtained from the literature (39-43), the sequences for HBV were obtained from Boehringer Mannheim (Penzberg, Germany). The oligonucleotides were synthesized by Genosys (The Woodlands, TX, USA). Primers and a probe for β-actin were obtained from Genemed (South San Francisco, CA, USA). Sequences are given in Table 1.

Quantitation of the pre-PCR copy number was done using an external standard curve and internal controls of similar size (mimics). Internal controls were constructed using the PCR-MIMIC-Construction Kit (Clontech, Palo Alto, CA, USA).

During PCR, the synthesized fragments were labeled with digoxigenin (dig) using dig-labeled nucleotides provided in the PCR ELISA Dig Labeling Kit (Boehringer Mannheim, Indianapolis, IN, USA), in which 5% of dTTP are labeled with dig as dig-dUTP. The final PCR-mixture contained 12.5 mM TRIS pH 8.3, 62.5 mM KCL, 200 μM each of dATP, dCTP and dGTP, 190 μM dTTP and 10 μM dig- dUTP, 2.5 mM $MgCl_2$, 1 μM of both primers, 200 DNA-copies of internal controls and 1.0 unit of Taq polymerase in a total of 40 μL. For a negative control reference group, we used semen from twelve commercial donors that had tested negative for antibodies against CT in semen, for antibodies against HSV-1, HSV-2, CMV, HIV-1 and HIV-2 in blood, and for HBV surface antigen in blood. These specimens, provided as frozen aliquots, were a gift from the New England Cryogenic Center, Boston, MA, USA. Other negative controls for the PCR assay were: 0.4 μg of herring sperm DNA and sterile distilled water (reagent control). β-actin PCR was performed as a positive control to assure DNA quantity and quality.

PCR consisted of a first heating step (95° C for 5 minutes), 37 amplification cycles, and a final extension step at 72° C for 7.5 minutes. One amplification cycle consisted of these parameters: for CMV and CT, 95° C for 15 sec/60° C for 60 sec; for HSV1/2 and HHV6, 95° C for 15 sec/55°C for 60 sec; for EBV, 95°C for 15 sec/47°C for 30 sec/72°C for 30 sec; for HBV 95°C for 15 sec/52°C for 30 sec/72°C for 30 sec; for β-actin 95°C for 15 sec/55°C for 30 sec/72°C for 90 sec.

To decrease nonspecific amplification occurring during the first PCR-cycle, a hot start technique was used. A piece of wax (Ampliwax, Perkin-Elmer, Norwalk, CT, USA) was added to 13 μL of the initial PCR mix containing $MgCl_2$, primers, nucleotides and part of the buffer, and this solution was heated to melt the wax. After the wax had solidified, a second mix (7 μL) containing Taq polymerase (Boehringer), the same amount of internal controls per tube and the remaining amount of the buffer was added, and finally 20 μL of template DNA (standard).

An external standard curve was established from the DNA standards ranging from 4 to 40,000 copies of the specific DNA per PCR-tube.

PCR ELISA—The PCR ELISA Dig Detection Kit (Boehringer) was used to quantify the PCR products. Aliquots of the PCR-product were denatured with 20 μL denaturation solution in a

microcentrifuge tube. After 10 minutes 200 μL of the hybridization solution containing 7.5 pmol of biotinylated probe per mL were added. Of this solution, 200 μL were added to one well of an avidin-coated microplate and incubated at 55° C for 3 hours. Then the well was washed five times with 375 μL washing buffer, and 200 μL of a 1:100 diluted peroxidase labeled anti-dig-antibody were added. The plate was incubated at 37° C for 30 minutes, washed 5 times, and 200 μL of ABTS-solution were added and incubated at room temperature for 10 minutes. The reaction was stopped by adding 100 μL of 1 M hydrochloric acid. The OD was-measured immediately at a wavelength of 405 nm.

For every external DNA standard and sample, three different aliquots were processed: For CMV, CT, EBV, HSV-1/2 and HBV, aliquots 1 and 2 contained 0.25 and 10 μL of PCR-product, respectively, and were hybridized with the template-specific probe. Aliquot 3, containing 1.0 μL PCR-product, was hybridized with the mimic- specific probe. The corresponding volumes for HHV-6 were 1, 20 and 10 μL. For detection of β-actin 15 μL of PCR-product were hybridized with the β-actin-specific probe. To increase pipetting accuracy of these small volumes, the PCR-product was diluted appropriately with distilled water so that a minimum volume of 5 μL was pipetted.

Calculation—Net-ODs were calculated by subtracting the OD of the negative control DNA from the measured ODs of the external template standards as well as from template and mimic products from experimental samples. Next, a template-OD:mimic-OD ratio was obtained by dividing the net-OD from the well with aliquot 1 (low volume hybridized with the template-specific probe) by the net OD from the well with the corresponding aliquot 3 (hybridized with the mimic-specific probe). An additional ratio was obtained by dividing the net OD from the well with aliquot 2 (high volume hybridized with the template-specific probe) by the mimic net OD; this second value was utilized to improve the sensitivity of the PCR method and quantification in the lower end of the range.

Standard curve and quantification of samples—The template:mimic ratios of the standards were plotted against the log of the known template-copy number. The number of pre-PCR copies in experimental samples could be determined by calculating the template:mimic ratio, and comparing to values on the standard curve.

Definition of negative and positive samples—As the coefficient of variation of 10 negative controls was 10%, and huge differences were observed between negative and low-positive samples, a signal was considered positive if the OD was at least twice the OD of the negative control. A sample was considered negative when the OD-signal for the template was below the threshold, and there were positive signals for the mimics and β-actin. A sample was considered positive with a positive template signal (and the negative control being negative).

Detection of HPV—HPV was detected by a primer screening method (filter paper blot) followed by subtype analysis by RFLP as previously described (44).

Determination of IL-1β and biochemical markers

Interleukin-1β (IL-1β)—IL-1β, a proinflammatory cytokine primarily secreted by macrophages and epithelial cells, has been associated with LCS, accessory gland inflammation, CT infection, and decreased sperm parameters (45-49). We measured concentrations of IL-1β in seminal plasma using a commercially available ELISA kit (Genzyme, Cambridge, MA, USA) following the manufacturer's protocol. Ten microliters of seminal plasma were diluted with 90 μL of provided sample diluent. Two standard curves were run: 1) the usual IL-1β standards diluted in provided sample diluent, and 2) the same IL-1β standards diluted in

seminal plasma from a healthy non-LCS man. The standard curves were always the same, indicating that seminal plasma does not interfere with IL-1β detection in this assay system.

PMN-elastase—PMN-elastase, an enzyme secreted by activated PMNs (50), and a marker for male genital tract inflammation (51,52), was measured in seminal plasma using a commercial ELISA (PMN-Elastase ELISA, courtesy of Merck, Darmstadt, Germany) as previously described (49).

Fructose—Fructose, a marker for seminal vesicle function (10), was determined according to the WHO manual (10), using a modified hexokinase method (Boehringer Mannheim, Indianapolis, IN). Fructose levels above 13 μmol per ejaculate (2.34 mg/ejaculate) are considered normal (10).

Citrate—Citrate, a marker for prostatic function (53), was determined according to the ultraviolet method recommended by WHO (53) using a commercial test kit (Boehringer Mannheim, Indianapolis, IN). According to WHO (53), citrate levels above 52 μmol per ejaculate (9 mg/ejaculate) are considered normal.

Neutral α-glucosidase—Neutral α-glucosidase, a marker for epididymal function (10,54), was determined according to Chapdelaine (55), using the principle of a protocol developed by Cooper et al. (54). To facilitate the processing of a large number of samples, the whole assay was performed in uncoated microtiter plates. Eight μL seminal plasma and 100 μL p-nitrophenyl-glucopyranoside solution [5 mg/mL in potassium phosphate buffer (KH_2P0_4/K_2HP0_4) 100 mM pH 6.8 containing 1% sodium dodecyl sulfate] were incubated in the wells of a microtiter plate at 37°C for one hour as triplicates. The reaction was stopped by 200 μL sodium carbonate solution 0.5 M, and ODs were measured in a microtiter plate reader at 405nm. The OD of the blank (8 μL of a seminal plasma pool, 4 μL castanospermine 10 mM, 100 μL substrate solution) was subtracted. Glucosidase activity (mU/mL) was calculated by dividing the sample OD by the slope of a p-nitrophenol standard curve (expressed as OD per μM) and multiplying by 0.6417. Neutral α-glucosidase levels above 20 mU per ejaculate are considered normal (10).

Statistical Analysis

For continuous data, non-parametric statistical tests were utilized or data were logarithmically transformed prior to parametric analysis when the assumptions of normal distribution and/or homogeneity of variance were not fulfilled. Specifically, the Mann-Whitney U test was used for comparison of two groups and the Spearman Rank Correlation Coefficient was used to determine the correlation between two variables. One-factor analysis of variance (ANOVA) or the Kruskal-Wallis one-way ANOVA was used for comparisons of more than two groups. Two-factor analysis of variance was performed to examine the two independent variables, LCS (+/-) and STI pathogen DNA detection (+/-). Fisher's PLSD or Dunn's Multiple Comparison tests were utilized for post hoc pairwise comparisons. The Fisher Exact test and the Fisher-Freeman-Halton test were utilized for analysis of categorical variables. Data were analyzed by StatView (version 5.0.1, SAS Institute, Cary, NC) and StatXact (version 6, Cytel Software Corporation, Cambridge, MA) statistical software. In all cases, statistical significance was assumed when $P<0.05$.

Results

As shown in Table 2, the overall prevalence of STI DNA in semen of infertility patients was 18.7%. LCS and non-LCS groups had similar STI DNA detection rates (18.9% for LCS samples and 18.3% for non-LCS samples, $P>0.10$, Fisher Exact Test). CMV was the most prevalent

pathogen detected (8.7%), followed by HPV (4.5%), HSV (3.7%), HHV-6 (3.7%) and CT (2.5%). EBV DNA was detected in only one sample; HBV DNA was not detected in any semen sample. HPV was subtyped with a RFLP panel that differentiated 18 HPV subtypes: 3 HPV-positive samples were HPV-16, a subtype associated with cervical cancer; the remaining 5 samples were indeterminate. Eight of the 45 pathogen-positive samples contained DNA from more than one pathogen (4 in the LCS group and 4 in the non-LCS group). One of these samples, from the LCS group, was positive for 3 pathogens; the other seven co-infected samples were positive for 2 pathogens. Five of the eight co-infected samples were positive for HPV, 4 were positive for CMV, and 4 contained CT.

The copy numbers of pathogen DNA per ejaculate showed a large range (Table 2). Overall, CMV DNA copy numbers ranged from 110 to 12,000,000 copies per ejaculate, HSV from 300 to 22,000, and HHV-6 from 250 to 81,000,000. The only EBV-positive sample contained 210 copies. None of the twelve reference semen samples from seronegative sperm donors were positive for STI pathogens with the exception of one, a sample from a CMV-seronegative donor, which contained a low amount (950 copies) of CMV DNA.

Table 3 shows the results of two-factor ANOVA examining both LCS and STI pathogen DNA as independent variables. Samples with detectable DNA for any of the STI pathogens (n=45) had significantly decreased motile sperm concentration, total motile sperm count, and neutral α-glucosidase concentration ($P<0.05$ for all variables). Sperm concentration was also reduced in STI pathogen-positive samples but this effect was not statistically significant ($P<0.10$). LCS was associated with a statistically significant decrease in total sperm count, % normal forms and fructose concentration ($P<0.05$ for all variables). As expected, the three inflammation variables, PMN concentration, PMN elastase concentration and IL-β concentration in semen were significantly elevated in the LCS groups ($P<0.0001$ for all variables). A significant interaction between LCS and STI was revealed for motility; motility in the LCS-/STI+ group was significantly decreased in comparison to the other groups ($P<0.05$).

When individual STI pathogens were analyzed, most of the pathogens were associated with reduced sperm concentration, although this decrease was only statistically significant for the HSV+ group [HSV+ group=34.0 (7.0-109.0), median (range) $\times 10^6$/mL, vs. No Pathogen group=77.0 (0-379.0), $P<0.05$. Mann-Whitney U test]. Sperm motility was also significantly reduced in the HSV+ group [HSV+ group=39.0 (7.0-73.0), median (range) %, vs. No Pathogen group=58.0 (2.0-97.0), $P<0.05$. Mann-Whitney U test]. Paradoxically, motility was significantly increased in the HHV6+ group [HHV6+ group=79.0 (50.0-97.0), median (range) %, vs. No Pathogen group=58.0 (2.0-97.0), $P<0.01$. Mann-Whitney U test]. Most of the pathogens were also associated with reduced total sperm counts, although this decrease was only statistically significant for the HPV+ group [HPV+ group=139.3 (13.0-214.5), median (range) $\times 10^6$, vs. No Pathogen group=171.8 (0-1,340.0), $P<0.05$. Mann-Whitney U test]. Most pathogens were associated with reduced motile sperm concentrations, although this decrease was only statistically significant for the HSV+ group [HSV+ group=22.2 (0.6-43.7), median (range) $\times 10^6$/mL, vs. No Pathogen group=47.3 (0.2-306.2), $P<0.05$. Mann-Whitney U test]. Total motile sperm count was also reduced in samples with DNA for most of the pathogens, although this decrease was only statistically significant for the HSV+ group [HSV+ group=29.8 (0.9-153.1), median (range) $\times 10^6$, vs. No Pathogen group=108.0 (0.6-1,165.8), $P<0.05$. Mann-Whitney U test.

Neutral α-glucosidase concentration was significantly reduced in CT+ and HSV+ samples [CT + group=9.2 (1.7-28.5), median (range) mU/mL, HSV+ group=7.2 (1.7-44.9), vs. No Pathogen group=16.7 (1.2-86.6), $P<0.05$. Mann-Whitney U tests]. Similar reductions in neutral α-glucosidase concentration for CMV+ and HPV+ samples approached statistical significance ($P<0.10$, Mann-Whitney U test). Citrate concentration was significantly reduced in HSV+

samples [HSV+ group=2.8 (1.4-5.3), median (range) mg/mL, vs. No Pathogen group=3.8 (0.8-15.3), P<0.05. Mann-Whitney U test]. Mean values for the male sex accessory gland/epididymal markers in semen samples without any detectable pathogens exceeded their respective WHO reference values (10,53).

STI co-infection (samples containing DNA from more than one pathogen) and STI copy number were not associated with a further alteration in any of the semen variables (all P's>0.10, one-factor ANOVA).

Concentrations of accessory gland/epididymal function markers that were significantly correlated with semen parameters by Spearman Rank Correlation Coefficients included: neutral α-glucosidase with sperm concentration (rho=+0.53, P=0.0001), motile sperm concentration (rho=+0.46, P=0.0001), total motile sperm (rho=+0.34, P=0.0001), and total sperm count (rho=+0.35, P=0.0001); and citrate (mg/ejaculate) with total sperm count (rho=+0.29, P=0.0001).

By Spearman Rank Correlation Coefficients, all three inflammation variables were significantly correlated with one another: PMN-elastase and PMN concentration (rho=+0.78, P=0.0001), IL-1β and PMN concentration (rho=+0.71, P=0.0001) and IL-1β and PMN-elastase concentration (rho=+0.73, P=0.0001).

Detection of STI DNA and markers of inflammation were not associated with differences in semen volume or patient age.

Discussion

The importance of genital tract microorganisms as an etiologic factor in male infertility is still a controversial topic (5,11,31). The purpose of this study was to determine the prevalence of several common sexually transmitted pathogens in healthy, male, infertility patients, and study their relationship to LCS and other markers of genital tract inflammation, and to semen quality parameters. For this study, we chose 6 common STI viruses and 1 intracellular bacterium that can chronically infect the male genital tract, often without causing symptoms. Two other common sexually transmitted pathogens were not included in the study: HIV-1 was not studied because it is a retrovirus and requires a different processing and assay system; Neiserria gonorrhea was not studied because its infection is usually symptomatic in men.

CMV is a member of the herpesvirus family and can cause a variety of teratogenic effects in newborns as well as a clinical illness in adults resembling infectious mononucleosis (56). Its presence and persistence in semen has been reported previously (57-60). In the current study, CMV was the most frequently detected pathogen in semen of infertility patients (8.7%) with copy numbers ranging from 110 to 12 million. Detection of a low level of CMV DNA in semen from one of the CMV-seronegative commercial sperm donors could reflect a recent infection in this individual. We did not observe a significant association of CMV DNA with semen parameters in this study, although there was a trend for lower motile sperm and α-glucosidase concentrations in the CMV-infected group, indicating that CMV infection could have a modest effect on semen quality, perhaps by affecting epididymal function. Previous studies have not shown an association of CMV infection with a reduction in semen parameters (7,59).

Herpes simplex virus mainly affects epithelial surfaces and is a major cause of genital ulcers. Whereas primary infection usually occurs through direct or indirect contact with herpetic lesions (61,62), HSV-1 and -2 have been detected in semen (7,60,63) and on sperm (64), and transmission of HSV-2 has occurred through donor insemination (2). The detection rate of HSV-1/2 DNA in ejaculates from infertility patients in the current study was relatively low (3.7%). However, HSV was associated with the strongest effect of any of the pathogens on

semen parameters. HSV DNA+ samples had significantly reduced sperm concentration, sperm motility, motile sperm concentration, total motile sperm count, neutral α-glucosidase and citrate concentrations. This is consistent with earlier studies that found associations between HSV-1 or -2 in semen and low sperm count and poor motility (7,64), as well as infertility (65). Antiviral treatment of male infertility patients positive for HSV in semen has resulted in successful pregnancies (64,65). Thus, HSV-infection of the male genital tract could explain some cases of male infertility, due to its association with decreased semen quality. Our study showed decreased concentrations of neutral α-glucosidase and citrate in HSV-positive samples, suggesting that the effect on fertility may be due to impaired epididymal and prostate function.

Human papillomaviruses represent a group of small DNA viruses that induce epithelial cell proliferation. More than 35 types of HPV infect the genital tract. DNA from HPV 16, 18, 31 and 45 have been associated with invasive squamous cell cancers of the genital tract and anus (66,67). HPV is primarily transmitted through direct epithelial contact (62), but high risk HPV types have been detected in both semen and spermatozoa (68-71), as well as in the vas deferens (72). In the current report, 3 out of the 8 cases that tested positive for generic HPV DNA were subtyped as HPV-16 (the other 5 cases were indeterminate). HPV DNA was not associated with LCS but was associated with a significant decrease in total sperm count, and a statistically non-significant trend for lower total motile sperm count and neutral α-glucosidase concentrations. A previous study reported an association between HPV and reduced sperm motility (70), while another study did not find any affects on sperm quality (71). Thus, additional research is needed to determine whether HPV infection contributes to male infertility.

CT infects the genital tract and eye, and has been associated with conjunctivitis and pneumonia in infants infected via the birth canal (73,74). Our study found CT DNA in semen from 2.5% of infertility patients, with copy numbers ranging from 8,300 to 590,000. This prevalence rate is consistent with earlier publications (32,75,76). CT DNA was associated with a significant decrease in neutral α-glucosidase concentration (and a trend towards lower total sperm and total motile sperm counts). Other studies have shown that men with CT in semen have reduced sperm concentration, motility, velocity, viability, morphology, acrosome reaction and citrate (77-80). Spermatozoa incubated with elementary bodies of CT had decreased motility and increased cytotoxicity (81); these effects have been attributed to lipopolysaccharide (82). Thus, CT infection of the male genital tract may contribute to male infertility.

EBV, a member of the herpesvirus family, causes infectious mononucleosis and has been associated with Burkitt's Lymphoma (83). EBV is found in semen (7,60) and is thought to be sexually transmitted (84,85). In the current study, EBV was detected in only one sample. The low detection rate probably reflects the low prevalence of EBV infection in this country, and the fact that the virus' primary host cell, the B-lymphocyte, represents only a small minority of all WBC in semen (11,86). Previous studies have reported a higher prevalence of EBV in infertility patients in Europe, although no association of seminal EBV with reduced semen quality has been found (7,60).

HBV is the cause of hepatitis B, an infection of the liver often progressing into a chronic inflammatory condition associated with liver cell carcinoma (87). HBV is transmitted through infected bodily fluids such as blood and semen (87,88), and has been transmitted by artificial insemination (3). As HBV is not endemic in North America, and infertility patients as a group do not engage in high-risk behaviors such as intravenous drug use, a low prevalence of HBV was expected. Indeed, HBV DNA was not detected in any of the semen samples from our study. These data suggest that HBV does not contribute substantially to male infertility in North America, except perhaps in high-risk groups. However, HBV infection is endemic in other

areas of the world, and its potential contribution to male infertility should be studied in these regions.

HHV-6, another member of the herpesvirus family, has been detected in semen (60), and is causally associated with exanthema subitum (89), acute febrile diseases and seizures in young children (90,91), and EBV- and CMV-negative cases of mononucleosis in young adults (92, 93). In this study, HHV-6 DNA was detected in 3.8% of semen samples, and often occurred in high copy numbers (up to 81,000,000). None of the semen parameters measured in our study was adversely affected by HHV-6 infection; in fact, sperm motility was paradoxically higher in HHV-6-positive samples. Therefore, our data indicate that genital tract HHV-6 infection does not contribute significantly to male infertility.

Overall, STI DNA was detected in 45/241 (18.7%) of the samples, with no difference in prevalence between LCS and non-LCS groups. STI DNA and LCS were independently associated with poor semen parameters. STI DNA was associated with a significant decrease in sperm concentration, motile sperm concentration, total sperm count and neutral α-glucosidase concentration. Eight of the STI+ samples contained DNA from more than one pathogen, but STI co-infection was not associated with a further reduction in semen quality. LCS was associated with decreased sperm count, % normal forms and fructose concentration, a measure of seminal vesicle function. Thus, the present study confirms and extends earlier reports that STIs and LCS are associated with poor semen quality (7,13,45,48,49,51,64,65, 70,77-82).

LCS was strongly associated with elevated levels of the inflammatory markers, PMN-elastase and IL-1β, a proinflammatory cytokine produced by activated macrophages, epithelial cells and other cell types (94). Similarly, concentrations of all three inflammation markers (PMNs, PMN-elastase and IL-1β) were significantly correlated with one another, which was expected, as both PMNs and macrophages are important participants in inflammatory responses, and because numbers of PMNs and macrophages in semen are highly correlated (9,45,48,49,86). STI DNA was not associated with elevations in any of these inflammation markers.

The present study also revealed significant correlations between semen parameters and levels of accessory gland and epididymal function markers, which highlights the relationship between semen quality and normal genital tract function (10,53,95). LCS was associated with a reduction in fructose concentration, suggesting that LCS may affect seminal vesicle function. Pathogen DNA was associated with reduced levels of neutral α-glucosidase, suggesting that subclinical infections affect epididymal function. In the case of one pathogen, HSV, citrate concentrations were also significantly reduced, suggesting an effect of this pathogenic virus on prostate function. Of all the pathogens studied, HSV was associated with the strongest effects on both semen quality and levels of accessory gland/epididymal function markers.

In conclusion, using sensitive molecular assays, we detected an unexpectedly high prevalence of sexually transmitted pathogens in semen from asymptomatic infertility patients. Pathogen DNA was not associated with LCS or other markers of inflammation, but was associated with decreased semen parameters and signs of reduced epididymal and prostate function. These findings indicate that asymptomatic infections of the male genital tract are common and may contribute to male infertility. Because they also threaten the health of partners and offspring, efforts to diagnose and treat subclinical genital tract infections should be intensified.

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References

1. Wortley PM, Hammett TA, Fleming PL. Donor insemination and human immunodeficiency virus transmission. Obstet Gynecol 1998;91:515–8. [PubMed: 9540933]
2. Moore DE, Ashley RL, Zarutskie PW, Coombs RW, Soules MR, Corey L. Transmission of genital herpes by donor insemination. Jama 1989;261:3441–3. [PubMed: 2542632]
3. Berry WR, Gottesfeld RL, Alter HJ, Vierling JM. Transmission of hepatitis B virus by artificial insemination. Jama 1987;257:1079–81. [PubMed: 3806899]
4. Politch, J.; Anderson, DJ. Use of assisted reproduction technology to prevent the transmission of HIV-1 in HIV-discordant couples desiring children. In: Arici, A., editor. Immunology and allergy clinics of north america, vol 22. Philadelphia: W. B. Saunders; 2002. p. 663-79.
5. Dejucq N, Jegou B. Viruses in the mammalian male genital tract and their effects on the reproductive system. Microbiol Mol Biol Rev 2001;65:208–31. [PubMed: 11381100]
6. Pekler VA, Robbins WA, Nyamathi A, Yashina TL, Leak B, Robins TA. Use of versant TMA and bDNA 3.0 assays to detect and quantify hepatitis C virus in semen. J Clin Lab Anal 2003;17:264–70. [PubMed: 14614751]
7. Kapranos N, Petrakou E, Anastasiadou C, Kotronias D. Detection of herpes simplex virus, cytomegalovirus, and Epstein-Barr virus in the semen of men attending an infertility clinic. Fertil Steril 2003;79:1566–70. [PubMed: 12801561]
8. Hamdad-Daoudi F, Petit J, Eb F. Assessment of Chlamydia trachomatis infection in asymptomatic male partners of infertile couples. J Med Microbiol 2004;53:985–90. [PubMed: 15358820]
9. Anderson, DJ.; Politch, JA. White blood cells in semen and their impact on fertility. In: Centola, GM.; Ginsburg, KA., editors. Evaluation and treatment of the infertile male. Cambridge: Cambridge University Press; 1996. p. 263-76.
10. WHO. WHO laboratory manual for the examination of human semen and sperm-cervical interaction. Cambridge: Cambridge University Press; 1999.
11. Wolff H. The biologic significance of white blood cells in semen. Fertil Steril 1995;63:1143–57. [PubMed: 7750580]
12. Krause W, Herbstreit F, Slenzka W. Are viral infections the cause of leukocytospermia? Andrologia 2002;34:87–90. [PubMed: 11966574]
13. Wolff H, Politch JA, Martinez A, Haimovici F, Hill JA, Anderson DJ. Leukocytospermia is associated with poor semen quality. Fertil Steril 1990;53:528–36. [PubMed: 2407566]
14. Berger RE, Karp LE, Williamson RA, Koehler J, Moore DE, Holmes KK. The relationship of pyospermia and seminal fluid bacteriology to sperm function as reflected in the sperm penetration assay. Fertil Steril 1982;37:557–64. [PubMed: 6896033]
15. Chan PJ, Su BC, Tredway DR, Whitney EA, Pang SC, Corselli J, Jacobson JD. White blood cells in semen affect hyperactivation but not sperm membrane integrity in the head and tail regions. Fertil Steril 1994;61:986–9. [PubMed: 8174744]
16. Maruyama DK Jr, Hale RW, Rogers BJ. Effects of white blood cells on the in vitro penetration of zona-free hamster eggs by human spermatozoa. J Androl 1985;6:127–35. [PubMed: 3838741]
17. Cohen J, Edwards R, Fehilly C, Fishel S, Hewitt J, Purdy J, Rowland G, Steptoe P, Webster J. In vitro fertilization: a treatment for male infertility. Fertil Steril 1985;43:422–32. [PubMed: 3979581]
18. Talbert LM, Hammond MG, Halme J, O'Rand M, Fryer JG, Ekstrom RD. Semen parameters and fertilization of human oocytes in vitro: a multivariable analysis. Fertil Steril 1987;48:270–7. [PubMed: 3609338]
19. Eggert-Kruse W, Bellmann A, Rohr G, Tilgen W, Runnebaum B. Differentiation of round cells in semen by means of monoclonal antibodies and relationship with male fertility. Fertil Steril 1992;58:1046–55. [PubMed: 1426357]

20. Gonzales GF, Kortebani G, Mazzolli AB. Leukocytospermia and function of the seminal vesicles on seminal quality. Fertil Steril 1992;57:1058–65. [PubMed: 1572474]
21. Omu AE, Al-Qattan F, Al-Abdul-Hadi FM, Fatinikun MT, Fernandes S. Seminal immune response in infertile men with leukocytospermia: effect on antioxidant activity. Eur J Obstet Gynecol Reprod Biol 1999;86:195–202. [PubMed: 10509791]
22. Vicino M, Loverro G, Simonetti S, Mei L, Selvaggi L. The correlation between idiopathic leukocytospermia, embryo quality and outcome in the FIVET and ICSI procedures. Minerva Ginecol 1999;51:413–20. [PubMed: 10726440]
23. Thomas J, Fishel SB, Hall JA, Green S, Newton TA, Thornton SJ. Increased polymorphonuclear granulocytes in seminal plasma in relation to sperm morphology. Hum Reprod 1997;12:2418–21. [PubMed: 9436676]
24. Cottell E, Harrison RF, McCaffrey M, Walsh T, Mallon E, Barry-Kinsella C. Are seminal fluid microorganisms of significance or merely contaminants? Fertil Steril 2000;74:465–70. [PubMed: 10973639]
25. Trum JW, Mol BW, Pannekoek Y, Spanjaard L, Wertheim P, Bleker OP, van der Veen F. Value of detecting leukocytospermia in the diagnosis of genital tract infection in subfertile men. Fertil Steril 1998;70:315–9. [PubMed: 9696227]
26. Erel CT, Senturk LM, Demir F, Irez T, Ertungealp E. Antibiotic therapy in men with leukocytospermia. Int J Fertil Womens Med 1997;42:206–10. [PubMed: 9222805]
27. Branigan EF, Spadoni LR, Muller CH. Identification and treatment of leukocytospermia in couples with unexplained infertility. J Reprod Med 1995;40:625–9. [PubMed: 8576877]
28. Yanushpolsky EH, Politch JA, Hill JA, Anderson DJ. Antibiotic therapy and leukocytospermia: a prospective, randomized, controlled study. Fertil Steril 1995;63:142–7. [PubMed: 7805903]
29. Zorn B, Virant-Klun I, Meden-Vrtovec H. Semen granulocyte elastase: its relevance for the diagnosis and prognosis of silent genital tract inflammation. Hum Reprod 2000;15:1978–84. [PubMed: 10966999]
30. Branigan EF, Muller CH. Efficacy of treatment and recurrence rate of leukocytospermia in infertile men with prostatitis. Fertil Steril 1994;62:580–4. [PubMed: 7520396]
31. Keck C, Gerber-Schafer C, Clad A, Wilhelm C, Breckwoldt M. Seminal tract infections: impact on male fertility and treatment options. Hum Reprod Update 1998;4:891–903. [PubMed: 10098479]
32. Hosseinzadeh S, Eley A, Pacey AA. Semen quality of men with asymptomatic chlamydial infection. J Androl 2004;25:104–9. [PubMed: 14662792]
33. Anderson DJ, O'Brien TR, Politch JA, Martinez A, Seage GR 3rd, Padian N, Horsburgh CR Jr, Mayer KH. Effects of disease stage and zidovudine therapy on the detection of human immunodeficiency virus type 1 in semen. Jama 1992;267:2769–74. [PubMed: 1349654]
34. Xu C, Politch JA, Tucker L, Mayer KH, Seage GR 3rd, Anderson DJ. Factors associated with increased levels of human immunodeficiency virus type 1 DNA in semen. J Infect Dis 1997;176:941–7. [PubMed: 9333152]
35. Politch JA, Wolff H, Hill JA, Anderson DJ. Comparison of methods to enumerate white blood cells in semen. Fertil Steril 1993;60:372–5. [PubMed: 8393411]
36. Chomczynski P. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. Biotechniques 1993;15:532–534. 536–7. [PubMed: 7692896]
37. Giusti A, Baird M, Pasquale S, Balazs I, Glassberg J. Application of deoxyribonucleic acid (DNA) polymorphisms to the analysis of DNA recovered from sperm. J Forensic Sci 1986;31:409–17. [PubMed: 3011955]
38. Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 1988;239:487–91. [PubMed: 2448875]
39. Holland SM, Gaydos CA, Quinn TC. Detection and differentiation of Chlamydia trachomatis, Chlamydia psittaci, and Chlamydia pneumoniae by DNA amplification. J Infect Dis 1990;162:984–7. [PubMed: 2401796]
40. Saito I, Servenius B, Compton T, Fox RI. Detection of Epstein-Barr virus DNA by polymerase chain reaction in blood and tissue biopsies from patients with Sjogren's syndrome. J Exp Med 1989;169:2191–8. [PubMed: 2543732]

41. Shibata D, Martin WJ, Appleman MD, Causey DM, Leedom JM, Arnheim N. Detection of cytomegalovirus DNA in peripheral blood of patients infected with human immunodeficiency virus. J Infect Dis 1988;158:1185–92. [PubMed: 2848898]
42. Cao M, Xiao X, Egbert B, Darragh TM, Yen TS. Rapid detection of cutaneous herpes simplex virus infection with the polymerase chain reaction. J Invest Dermatol 1989;92:391–2. [PubMed: 2918243]
43. Cone RW, Huang ML, Ashley R, Corey L. Human herpesvirus 6 DNA in peripheral blood cells and saliva from immunocompetent individuals. J Clin Microbiol 1993;31:1262–7. [PubMed: 8388889]
44. Kuypers JM, Critchlow CW, Gravitt PE, Vernon DA, Sayer JB, Manos MM, Kiviat NB. Comparison of dot filter hybridization, Southern transfer hybridization, and polymerase chain reaction amplification for diagnosis of anal human papillomavirus infection. J Clin Microbiol 1993;31:1003–6. [PubMed: 8385147]
45. Comhaire F, Bosmans E, Ombelet W, Punjabi U, Schoonjans F. Cytokines in semen of normal men and of patients with andrological diseases. Am J Reprod Immunol 1994;31:99–103. [PubMed: 8049031]
46. Dousset B, Hussenet F, Daudin M, Bujan L, Foliguet B, Nabet P. Seminal cytokine concentrations (IL-1beta, IL-2, IL-6, sR IL-2, sR IL-6), semen parameters and blood hormonal status in male infertility. Hum Reprod 1997;12:1476–9. [PubMed: 9262280]
47. Gruschwitz MS, Brezinschek R, Brezinschek HP. Cytokine levels in the seminal plasma of infertile males. J Androl 1996;17:158–63. [PubMed: 8723440]
48. Sanocka D, Jedrzejczak P, Szumala-Kaekol A, Fraczek M, Kurpisz M. Male genital tract inflammation: The role of selected interleukins in regulation of pro-oxidant and antioxidant enzymatic substances in seminal plasma. J Androl 2003;24:448–55. [PubMed: 12721221]
49. Sanocka D, Fraczek M, Jedrzejczak P, Szumala-Kakol A, Kurpisz M. Male genital tract infection: an influence of leukocytes and bacteria on semen. J Reprod Immunol 2004;62:111–124. [PubMed: 15288187]
50. Fritz, H.; Jochum, M.; Duswald, KH.; Dittmer, H.; Kortmann, H.; Neumann, S. Granulocyte proteinases as mediators of unspecific proteolysis in inflammation: a review. In: Goldberg, DM.; Werner, M., editors. Selected topics in clinical enzymology. New York: deGruyter-Verlag; 1984. p. 305-28.
51. Wolff H, Anderson DJ. Evaluation of granulocyte elastase as a seminal plasma marker for leukocytospermia. Fertil Steril 1988;50:129–32. [PubMed: 3384106]
52. Jochum M, Pabst W, Schill WB. Granulocyte elastase as a sensitive diagnostic parameter of silent male genital tract inflammation. Andrologia 1986;18:413–9. [PubMed: 3530047]
53. WHO. WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction. Cambridge: Cambridge University Press; 1992.
54. Cooper TG, Weidner W, Nieschlag E. The influence of inflammation of the human male genital tract on secretion of the seminal markers alpha-glucosidase, glycerophosphocholine, carnitine, fructose and citric acid. Int J Androl 1990;13:329–36. [PubMed: 2283178]
55. Chapdelaine P, Tremblay RR, Dube JY. P-Nitrophenol-alpha-D-glucopyranoside as substrate for measurement of maltase activity in human semen. Clin Chem 1978;24:208–11. [PubMed: 23909]
56. Drew, WL.; Bates, MP. Cytomegalovirus infection. In: Holmes, KK.; Mardh, PA.; Sparling, PF.; Lemon, SM.; Stamm, WE.; Piot, P.; Wasserheit, JN., editors. Sexually transmitted diseases. Vol. 3. New York: McGraw-Hill; 1999. p. 313-26.
57. Aynaud O, Poveda JD, Huynh B, Guillemotonia A, Barrasso R. Frequency of herpes simplex virus, cytomegalovirus and human papillomavirus DNA in semen. Int J STD AIDS 2002;13:547–50. [PubMed: 12194737]
58. Lang DJ, Kummer JF, Hartley DP. Cytomegalovirus in semen. Persistence and demonstration in extracellular fluids. N Engl J Med 1974;291:121–3. [PubMed: 4365667]
59. Yang YS, Ho HN, Chen HF, Chen SU, Shen CY, Chang SF, Huang ES, Wu CW. Cytomegalovirus infection and viral shedding in the genital tract of infertile couples. J Med Virol 1995;45:179–82. [PubMed: 7775936]
60. Bezold G, Schuster-Grusser A, Lange M, Gall H, Wolff H, Peter RU. Prevalence of human herpesvirus types 1-8 in the semen of infertility patients and correlation with semen parameters. Fertil Steril 2001;76:416–8. [PubMed: 11476803]

61. Corey, L.; Wald, A. Genital herpes. In: Holmes, KK.; Mardh, PA.; Sparling, PF.; Lemon, SM.; Stamm, WE.; Piot, P.; Wasserheit, JN., editors. Sexually transmitted diseases. Vol. 3rd. New York: McGraw-Hill; 1999. p. 285-312.
62. Elder, K.; Baker, DJ.; Ribes, JA. Infections, infertility, and assisted reproduction. Cambridge: Cambridge University Press; 2005.
63. el Borai N, Inoue M, Lefevre C, Naumova EN, Sato B, Yamamura M. Detection of herpes simplex DNA in semen and menstrual blood of individuals attending an infertility clinic. J Obstet Gynaecol Res 1997;23:17–24. [PubMed: 9094812]
64. Kotronias D, Kapranos N. Detection of herpes simplex virus DNA in human spermatozoa by in situ hybridization technique. In Vivo 1998;12:391–4. [PubMed: 9706490]
65. El Borai N, LeFevre C, Inoue M, Naumova EN, Sato K, Suzuki S, Tsuji K, Yamamura M. Presence of HSV-1 DNA in semen and menstrual blood. J Reprod Immunol 1998;41:137–47. [PubMed: 10213306]
66. Galloway, DA. Biology of genital human papillomaviruses. In: Holmes, KK.; Mardh, PA.; Sparling, PF.; Lemon, SM.; Stamm, WE.; Piot, P.; Wasserheit, JN., editors. Sexually transmitted diseases. Vol. 3rd. New York: McGraw-Hill; 1999. p. 335-46.
67. Koutsky, LA.; Kiviat, NB. Genital human papillomavirus. In: Holmes, KK.; Mardh, PA.; Sparling, PF.; Lemon, SM.; Stamm, WE.; Piot, P.; Wasserheit, JN., editors. Sexually transmitted diseases. Vol. 3rd. New York: McGraw-Hill; 1999. p. 347-59.
68. Kyo S, Inoue M, Koyama M, Fujita M, Tanizawa O, Hakura A. Detection of high-risk human papillomavirus in the cervix and semen of sex partners. J Infect Dis 1994;170:682–5. [PubMed: 8077728]
69. Lai YM, Yang FP, Pao CC. Human papillomavirus deoxyribonucleic acid and ribonucleic acid in seminal plasma and sperm cells. Fertil Steril 1996;65:1026–30. [PubMed: 8612829]
70. 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. [PubMed: 9176459]
71. Rintala MA, Grenman SE, Pollanen PP, Suominen JJ, Syrjanen SM. Detection of high-risk HPV DNA in semen and its association with the quality of semen. Int J STD AIDS 2004;15:740–3. [PubMed: 15537460]
72. Rintala MA, Pollanen PP, Nikkanen VP, Grenman SE, Syrjanen SM. Human papillomavirus DNA is found in the vas deferens. J Infect Dis 2002;185:1664–7. [PubMed: 12023775]
73. Stamm, WE. Chlamydia trachomatis infections of the adult. In: Holmes, KK.; Mardh, PA.; Sparling, PF.; Lemon, SM.; Stamm, WE.; Piot, P.; Wasserheit, JN., editors. Sexually transmitted diseases. Vol. 3rd. New York: McGraw-Hill; 1999. p. 407-22.
74. Schacher, J. Biology of Chlamydia trachomatis. In: Holmes, KK.; Mardh, PA.; Sparling, PF.; Lemon, SM.; Stamm, WE.; Piot, P.; Wasserheit, JN., editors. Sexually transmitted diseases. Vol. 3rd. New York: McGraw-Hill; 1999. p. 391-405.
75. Eggert-Kruse W, Rohr G, Kunt B, Meyer A, Wondra J, Strowitzki T, Petzoldt D. Prevalence of Chlamydia trachomatis in subfertile couples. Fertil Steril 2003;80:660–3. [PubMed: 12969722]
76. Wolff H, Neubert U, Volkenandt M, Zochling N, Schlupen EM, Bezold G, Meurer M. Detection of Chlamydia trachomatis in semen by antibody-enzyme immunoassay compared with polymerase chain reaction, antigen-enzyme immunoassay, and urethral cell culture. Fertil Steril 1994;62:1250–4. [PubMed: 7957993]
77. Jungwirth A, Straberger A, Esterbauer B, Fink K, Schmeller N. Acrosome reaction in Chlamydia-positive and negative patients. Andrologia 2003;35:314–6. [PubMed: 14535862]
78. Veznik Z, Pospisil L, Svecova D, Zajicova A, Unzeitig V. Chlamydiae in the ejaculate: their influence on the quality and morphology of sperm. Acta Obstet Gynecol Scand 2004;83:656–660. [PubMed: 15225191]
79. Cengiz T, Aydoganli L, Baykam M, Mungan NA, Tuncbilek E, Dincer M, Yakupoglu K, Akalin Z. Chlamydial infections and male infertility. Int Urol Nephrol 1997;29:687–693. [PubMed: 9477368]
80. Wolff H, Neubert U, Zebhauser M, Bezold G, Korting HC, Meurer M. Chlamydia trachomatis induces an inflammatory response in the male genital tract and is associated with altered semen quality. Fertil Steril 1991;55:1017–9. [PubMed: 2022260]

81. Hosseinzadeh S, Brewis IA, Eley A, Pacey AA. Co-incubation of human spermatozoa with Chlamydia trachomatis serovar E causes premature sperm death. Hum Reprod 2001;16:293–9. [PubMed: 11157823]
82. Hosseinzadeh S, Pacey AA, Eley A. Chlamydia trachomatis-induced death of human spermatozoa is caused primarily by lipopolysaccharide. J Med Microbiol 2003;52:193–200. [PubMed: 12621083]
83. Slobod, KS.; Sixbey, JW. Epstein-Barr virus infection. In: Holmes, KK.; Mardh, PA.; Sparling, PF.; Lemon, SM.; Stamm, WE.; Piot, P.; Wasserheit, JN., editors. Sexually transmitted diseases. Vol. 3rd. New York: McGraw-Hill; 1999. p. 327-33.
84. van Baarle D, Hovenkamp E, Dukers NH, Renwick N, Kersten MJ, Goudsmit J, Coutinho RA, Miedema F, van Oers MH. High prevalence of Epstein-Barr virus type 2 among homosexual men is caused by sexual transmission. J Infect Dis 2000;181:2045–9. [PubMed: 10837190]
85. Crawford DH, Swerdlow AJ, Higgins C, McAulay K, Harrison N, Williams H, Britton K, Macsween KF. Sexual history and Epstein-Barr virus infection. J Infect Dis 2002;186:731–6. [PubMed: 12198605]
86. Wolff H, Anderson DJ. Immunohistologic characterization and quantitation of leukocyte subpopulations in human semen. Fertil Steril 1988;49:497–504. [PubMed: 3342902]
87. Lemon, SM.; Alter, MJ. Viral hepatitis. In: Holmes, KK.; Mardh, PA.; Sparling, PF.; Lemon, SM.; Stamm, WE.; Piot, P.; Wasserheit, JN., editors. Sexually transmitted diseases. Vol. 3rd. New York: McGraw-Hill; 1999. p. 361-84.
88. Jenison SA, Lemon SM, Baker LN, Newbold JE. Quantitative analysis of hepatitis B virus DNA in saliva and semen of chronically infected homosexual men. J Infect Dis 1987;156:299–307. [PubMed: 3598231]
89. Yamanishi K, Okuno T, Shiraki K, Takahashi M, Kondo T, Asano Y, Kurata T. Identification of human herpesvirus-6 as a causal agent for exanthem subitum. Lancet 1988;1:1065–7. [PubMed: 2896909]
90. Asano Y, Yoshikawa T. Human herpesvirus-6 and parvovirus B19 infections in children. Curr Opin Pediatr 1993;5:14–20. [PubMed: 8397041]
91. Hall CB, Long CE, Schnabel KC, Caserta MT, McIntyre KM, Costanzo MA, Knott A, Dewhurst S, Insel RA, Epstein LG. Human herpesvirus-6 infection in children. A prospective study of complications and reactivation. N Engl J Med 1994;331:432–8. [PubMed: 8035839]
92. Krueger GR, Klueppelberg U, Hoffmann A, Ablashi DV. Clinical correlates of infection with human herpesvirus-6. In Vivo 1994;8:457–85. [PubMed: 7893974]
93. Pellett PE, Black JB, Yamamoto M. Human herpesvirus 6: the virus and the search for its role as a human pathogen. Adv Virus Res 1992;41:1–52. [PubMed: 1315478]
94. Thomson, AW.; Lotze, MT. The cytokine handbook. Amsterdam; Boston: Academic Press; 2003.
95. Grudzinskas, JG.; Yovich, J. Gametes : the spermatozoon. Cambridge; New York: Cambridge University Press; 1995.

Table 1

Oligonucleotide Sequences

Name	Sequence (5′-3′)
Chlamydia-upstream	CCTGTGGGGA ATCCTGCTGA A
Chlamydia-downstream	GTCGAAAACA AAGTCWCCRT AGTA
Chlamydia-probe	AAACCTTCCC AYAGAATTCC GTCGATCATA AGGCTTGG
CMV-upstream	CCGCAACCTG GTGCCCATGG
CMV-downstream	CGTTTGGGTT GCGCAGCGGG
CMV-probe	TTCTTCTGGG ACGCCAACGA CATCTACCGC ATCTTCGCCG
HSV-upstream	CATCACCGAC CCGGAGAGGG AC
HSV-downstream	GGGCCAGGCG CTTGTTGGTG TA
HSV-probe	CTTTGTCCTC ACCGCCGAAC TGAGCAGACA CCCGCGCGCG
HHV6-upstream	GTATCCCGAC GGCAGAGGTT
HHV6-downstream	TTAAACAGCC GTTGTCAGGG
HHV6-probe	GAGATTCTGG AATCGAGCAC
EBV-upstream	CCAGAGGTAA GTGGACTT
EBV-downstream	GACCGGTGCC TTCTTAGG
EBV-probe	TTCTGCTAAG CCCAAC
Actin-up stream	ATGGATGATG ATATCGCCGC G
Actin-downstream	CTAGAAGCAT TTGCGGTGGA CGATGGAGGG GCC
Actin-probe	GGCTGGGGTG TTGAAGGTCT CAAACATGAT
MIMIC-probe	CAATCAGCTC ACGAAACTTG

All probes were 5′-biotinylated. The oligonucleotide sequences for HBV were obtained from Boehringer Mannheim, Germany.

Table 2

Pathogen prevalence and amount in semen samples

Pathogen	LCS[a]	Non-LCS[b]	Total
Any Pathogen			
Prevalence[c]	25/132 (18.9)	20/109 (18.3)	45/241 (18.7)
HPV			
Prevalence	3/70 (4.3)	5/109 (4.6)	8/179 (4.5)
C. trachomatis			
Prevalence	3/132 (2.3)	3/108 (2.8)	6/240 (2.5)
Range[d]	10,000-460,000	8,300-590,000	8,300-590,000
CMV			
Prevalence	14/132 (10.6)	7/109 (6.4)	21/241 (8.7)
Range	110-580,000	3,200-12,000,000	110-12,000,000
HSV-1/2			
Prevalence	4/132 (3.0)	5/109 (4.6)	9/241 (3.7)
Range	510-15,000	300-22,000	300-22,000
HHV-6			
Prevalence	5/132 (3.8)	4/109 (3.7)	9/241 (3.7)
Range	250-81,000,000	4,400-1,500,000	250-81,000,000
EBV			
Prevalence	1/132 (0.8)	0/108 (0)	1/240 (0.4)
Range	210	----------	210
HBV			
Prevalence	0/132 (0)	0/109 (0)	0/241 (0)

[a] Leukocytospermic ($\geq 10^6$ PMN/mL)

[b] Non-leukocytospermic

[c] number of positive samples/total number (percentage) of analyzed samples

[d] DNA copy number per ejaculate

Table 3

Semen parameters in infertility patients with leukocytospermia and/or STI pathogens in semen

Semen Variable	1). LCS-[a]/STI-[b] N=89	2). LCS-/STI+ N=20	3). LCS+/STI- N=107	4). LCS+/STI+ N=25	Significance
Sperm/mL × 10^6	107.0[c] 1.0-363.0[d]	73.5 3.0-314.0	64.0 0-379.0	34 1.0-261.0	STI↓†
Motility (%)	59.0 8.0-90.0	39.5 5.0-88.0	54.0 2.0-97.0	53.0 16.0-97.0	STI × LCS**
Total Sperm × 10^6	236.0 2.5-1,340.0	253.5 9.8-519.2	162.0 0-1,124.5	85.0 4.0-965.7	LCS↓**
Motile Sperm/mL × 10^6	66.9 0.8-300.6	36.0 0.2-276.3	40.3 0.2-306.2	20.0 0.5-240.1	STI↓**
Total Motile Sperm × 10^6	135.4 2.1-1,165.8	96.8 0.6-442.1	88.9 0.6-787.2	43.3 1.8-888.4	STI↓**
% Normal Forms	52.0 7.0-90.0	53.5 22.0-96.0	48.0 3.0-76.0	48.0 10.0-74.0	LCS↓**
PMN/mL × 10^6	0 0-0.8	0 0-0.8	2.0 1.0-19.0	1.6 1.0-8.8	LCS↑***
PMN-Elastase (ng/mL)	195.0 0-15,126.0	175.5 0-5,885.0	3,515.0 0-38,802.0	3,246.0 482.0-18,396.0	LCS↑***
IL-1β (pg/mL)	30.0 0-310.0	40.0 0-230.0	203.0 0-9,650.0	130.0 0-1,130.0	LCS↑***
Neutral α-Glucosidase (mU/mL)	16.4 1.6-49.6	10.9 5.9-49.1	16.8 1.2-86.6	12.5 1.7-44.9	STI↓**
Citrate (mg/mL)	4.3 1.3-15.3	3.6 2.0-8.8	3.5 0.8-12.1	3.5 1.4-8.7	NS[e]
Fructose (mg/mL)	2.2 0.3-5.5	2.3 0.8-5.8	2.1 0.1-4.48	1.9 0.1-4.7	LCS↓**

[a] LCS=Leukocytospermic (≥10^6 PMN/mL)

[b] STI=sexually transmissible pathogen detected in semen

[c] Median

[d] Range

[e] NS=Not Significant

F-test significance for main effects and interactions:

† P<0.10 (NS)

** P<0.05

*** P<0.0001