Cytomegalovirus detection in cryopreserved semen samples collected for therapeutic donor insemination

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The presence of cytomegalovirus (CMV) in semen was retrospectively evaluated in 178 cryopreserved semen samples obtained from 97 donors recruited by two sperm banks. Thirty-three (34.0%) donors were CMV-seronegative, 51 (52.6%) were CMV-seropositive and 13 (13.4%) had an unknown serological status. CMV was detected in sperm specimens by cell culture and polymerase chain reaction (PCR). CMV was detected in five (2.8%) samples from two (2.0%) donors by cell culture and in 10 samples (5.6%) from five donors (5.1%) by PCR; all the samples which were positive by cell culture were also positive by PCR. Our data indicate that cryopreserved semen specimens obtained from healthy volunteer donors represent a potential source of CMV infection through therapeutic donor insemination. PCR, which is more sensitive than cell culture, should be used for direct and rapid identification of CMV in semen samples collected from volunteer donors. CMV detection in semen by PCR could be advantageously compared to CMV-antibody tests in serum which are only indicative of a former CMV infection.

Key words: cell culture/cytomegalovirus/PCR/semen/therapeutic donor insemination

Introduction

Cytomegalovirus (CMV) is a ubiquitous virus which frequently infects humans without any clinical signs. After the primary infection, the virus remains in a latent state in the host and subsequent reactivations of latent virus can occur, leading to asymptomatic viral excretion. CMV is frequently recovered from genital secretions and, therefore, CMV infection may be sexually transmitted (Handsfield et al., 1985). Pregnant women with CMV infection may transmit the virus to their fetuses and CMV is the leading cause of congenital viral infection. Primary CMV infection during pregnancy may result in fetal death, severe disease at birth or subsequent development of neurological or sensory impairment (Pass et al., 1980; Stagno et al., 1986). A maternal secondary CMV infection also may result in a symptomatic congenital infection (Fowler et al., 1992).

Several infectious agents, including human immunodeficiency virus (HIV), hepatitis B virus (HBV) or Chlamydia trachomatis, may be present in semen and their transmission to women through therapeutic donor insemination (TDI) has been reported (Steward et al., 1985; Nagel et al., 1986; Berry et al., 1987). The risk of CMV transmission through TDI has also been established (Prior et al., 1994). Therefore, the American Fertility Society (1993) recommended the determination of CMV serological status of semen donors by a CMV-specific immunoglobulin G (IgG) assay and stated that semen from CMV-seropositive donors may only be used to inseminate CMV-seropositive recipients. The French legislation is more restrictive, since only semen specimens from CMV-seronegative donors can be used for TDI; these specimens must be cryopreserved and quarantined for at least 180 days before use. On this basis, specimens from ~50% of French volunteer donors are rejected.

To date, the frequency of CMV secretion into semen specimens obtained from healthy volunteer donors has not been evaluated. Therefore, we wondered whether direct detection of CMV in semen could constitute a more informative marker of semen infectivity than the CMV-antibody status. This study was thus designed to establish the frequency of CMV infection in semen collected from healthy volunteer donors recruited by our sperm banks in two French university hospitals.

Materials and methods

Study population and collection of specimens

A retrospective study was conducted on 178 semen samples obtained from 97 healthy volunteer semen donors recruited between March 1986 and November 1995 by two French sperm banks located in the university hospitals of Toulouse and Montpellier. These donors were aged from 25 to 48 years, they lived in stable couples and had previously fathered at least one child. One semen donation was obtained from 52 donors, whereas two to five consecutive donations were obtained from each of the remaining 45 donors.

Potential donors were examined for current genital infection or heritable disease. In addition to CMV antibody tests, serological tests were routinely performed for HIV, human T cell leukaemia virus (HTLV), HBV, hepatitis C virus (HCV) and Treponema pallidum infections. Basic semen analysis, including sperm count, motility and morphology, was carried out at the time of collection. Bacteria in semen were detected by standard culture procedures. Bacteriospermia higher than either $1 \times 10^3$ or $1 \times 10^5$ colony forming units (CFU)/ml, according to the bacterium identified, was considered as significant. Semen specimens from patients who had significant bacteriospermia...
were not cryopreserved and thus were not included in this study. Ejaculates were aliquotted into 0.25 ml straws and cryopreserved in liquid nitrogen according to a previously described procedure (Behrmann and Ackerman, 1969) which does not affect CMV viability. Transport to the laboratory and conservation until testing were carried out in liquid nitrogen.

**CMV antibody tests**

Sera were collected at the time of the ejaculate collection. They were screened for the presence of anti-CMV IgG ([IMX CMV IgG; Abbott, Rungis, Toulouse, France (Toulouse) or Vidas CMV IgG; Biomérieux, Marcy l’Étoile, France (Montpellier)]) and IgM ([Wellcozyme anti-CMV IgM; Wélcozyme]t) in Montpellier, Toulouse, France (Toulouse) or Captia CMV-M; Malvern, PA, USA (Montpellier)).

**Detection of CMV by cell culture**

Semen samples were tested for the presence of CMV by shell vial culture (SVC) in both centres and additionally, by conventional cell culture (CCC) in Toulouse. SVC was performed on MRC-5 monolayer cell cultures in 24-well tissue culture plates. A randomly selected aliquot from each ejaculate was diluted 1:10 and 1:100 in Eagle’s minimum essential medium (MEM) supplemented with 2% fetal calf serum (FCS) and dilutions were inoculated onto cells. The aim of the sample dilutions was to suppress the potential toxicity of semen. Plates were then centrifuged at 980 g for 45 min at 37°C. After removal of the inoculum and addition of 2 ml of MEM–2% FCS, plates were incubated for 18 h at 37°C in 5% CO2. Identification of CMV-immediate–early antigen was performed by an immunoperoxidase procedure, using monoclonal antibody E-13 (Argène, Varilhes, France) directed against the products of the major immediate–early gene (Mazeron et al., 1992), antinucleus immunoglobulin G peroxidase–labelled conjugate (Argène) and 3,3-diaminobenzidine plus H2O2 as substrate. A culture was defined as positive based on the observation of intranuclear staining.

For CCC, semen samples diluted 1:10 were inoculated onto MRC-5 cell monolayers as described above. Cells were examined for CMV-specific cytopathic effects (CPE) twice a week. Cultures were maintained for 6 weeks before being considered as negative. The AD169 CMV-strain was used as a positive control.

**Detection of CMV DNA by PCR**

A randomly selected aliquot from each ejaculate was tested by PCR. Each centre used its own ‘in house’ PCR procedure. In Toulouse, 200 µl of semen was inoculated in lysis buffer (10 mM Tris–HCl pH 8.2, 50 mM KCl, 2.5 mM MgCl2, 0.45% Nonidet P40, 0.45% Tween 20 and 60 µg/ml proteinase K) for 2 h at 36°C. Total DNA was extracted according to standard procedures (Maniatis et al., 1982). PCR was performed according to previously published conditions (Hisia et al., 1989) by using primers located in the highly conserved immediate–early gene region 1 (IE1). The PCR product was detected by a commercial enzyme immunoassay, according to the manufacturer’s instructions (Gen-Eti-K; Sorin Biomedica, Antony, France). In Montpellier, semen samples were incubated for 1 h at 56°C in lysis buffer. Lysates were then heated for 10 min at 95°C to inactivate proteinase K. After centrifugation (10 min at 10 000 r.p.m.), 10 µl of supernatant was directly used for DNA amplification in a 50 µl reaction mixture. Nested PCR was performed by using outer and inner primer sets located in the IE1 region, delimiting a 242 bp sequence and a 148 bp sequence, respectively (Brytting et al., 1992). The final concentration of each deoxyribonucleotide triphosphate and each primer was 200 µM and 0.2 µM, respectively. Each tube contained 0.5 IU Taq DNA polymerase (Appligene, Illkirch, France); the incubation buffer was supplied by the manufacturer. For amplification, tubes were pre-heated for 5 min at 94°C and subjected to 30 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for 1 min; the extension was 7 min in the last cycle. After the first amplification with outer primers, 5 µl of the amplification product was amplified under the same conditions by using the inner primers. The PCR products were electrophoresed on a 2% agarose gel containing ethidium bromide and the gel was photographed with short wave ultraviolet illumination.

Both PCR procedures were performed under stringent conditions (Kwok and Higuchi, 1989). All specimens were tested at least twice in separate runs; positive, negative and blank controls were included in each run. DNA extracted from CMV-infected or mock-infected MRC5 cells were used as positive and negative controls respectively; distilled water was used as blank control. Samples from the patients who had CMV detected in semen were exchanged between the two centres in order to be tested by each PCR procedure. In addition, 28 randomly selected samples (14 from each centre) were also exchanged and tested by each procedure. In our experience, the limit of detection of both PCR procedures was <10 CMV DNA copies/ml.

**Results**

The donors included in this study had a sperm count (mean ± SD) of 89.6 ± 76.5 × 106/ml, the progressive motility was 51.4 ± 8.3% and the normal morphology was 58.8 ± 12.0%. All the donors were clinically healthy. They were free of sexually transmitted or heritable diseases and they were seronegative for HIV, HTLV, HBC, HCV and T. pallidum. Thirty-three donors (34.0%) were CMV-seronegative, 51 (52.6%) were CMV-seropositive and the remaining 13 donors (13.4%) had an unknown CMV serological status. Forty-eight ejaculates were collected from the CMV-seronegative donors, 111 were collected from the CMV-seropositive donors and 19 were obtained from the donors who had unknown serological status. All the donors tested were negative for CMV-specific IgM antibodies.

As shown in Table I, CMV was detected in five (2.8%) ejaculates from two (2.1%) donors by cell culture and in 10 (5.6%) ejaculates from five (5.1%) donors by PCR. Among the CMV-seropositive donors, four (7.8%) showed CMV shedding in semen and CMV was detected in five (4.5%) and nine (8.1%) of the samples collected from the CMV-seropositive donors by cell culture and PCR respectively.

Three of the donors were found positive for CMV seminal shedding by both PCR procedures, whereas two samples from one donor were found positive only in Toulouse and one sample from another donor was found positive only in Montpellier. All the ejaculates which were positive by cell culture were also positive by PCR in both centres. Concordant negative results were obtained by both PCR procedures in the 28 semen samples exchanged between both centres.

**Discussion**

Microorganisms are often detected in ejaculates of asymptomatic individuals, which could influence in-vitro fertilization success (Shalika et al., 1996). Moreover, CMV shedding in semen has been previously reported in studies conducted in HIV-infected patients (Rinaldo et al., 1992; Krieger et al., 1995) and in patients with sexually transmitted diseases.
CMV in cryopreserved donor semen samples

Table I. Data obtained in donors who had at least one semen sample positive for CMV detection

<table>
<thead>
<tr>
<th>Centre</th>
<th>Donor identification</th>
<th>CMV serological status</th>
<th>Ejaculate collection date</th>
<th>Cell culture</th>
<th>Toulouse</th>
<th>Montpellier</th>
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NT = not tested.

(Handsfield et al., 1985). CMV seminal shedding has also been investigated in men seeking fertility evaluation and controversial results have been reported. Indeed, Bantel-Shaal et al. (1993) concluded that CMV was not present in the semen from 63 men tested in Germany, whereas in Taiwan, Shen et al. (1994) and Yang et al. (1995) found a high prevalence (32.7 and 33.5% respectively) of CMV shedding in semen. This discrepancy might be explained by marked differences in CMV seroprevalence and by the different procedures used for CMV detection (DNA hybridization and PCR respectively). To our knowledge, studies on CMV seminal shedding in healthy semen donors have not been reported until now. Such a study could be of interest, since CMV in semen represents a source of infection for women undergoing a TDI and could be a cause of congenital CMV disease in their offspring. Therefore, we conducted a retrospective study on cryopreserved semen samples collected from healthy volunteer donors in our sperm banks. Detection of infectious CMV was performed by cell culture; samples were also tested by PCR in consideration of the high sensitivity of this technique.

Our results clearly indicate that CMV is present in the semen from healthy volunteer donors, since CMV was detected in 5.6% of the specimens tested. Moreover, the infectious character of semen was assessed in 2.8% of the specimens by isolation of CMV in cell culture. Indeed CMV was detected in 8.1% of the semen specimens collected from the CMV-seropositive donors and 4.5% of these specimens were found to be infectious by cell culture. Among the five donors who had CMV in semen, three excreted CMV transiently, whereas the remaining two donors presented protracted CMV shedding, since CMV was detected by cell culture and PCR in consecutive samples.

Among the donors who had CMV detected by PCR, discrepancies between the two PCR procedures were observed in four samples. Since PCR was repeatedly performed for each sample under stringent conditions, with inclusion of negative and positive controls for each run, the possibility of false-positive results is unlikely. Moreover, concordant negative results obtained in the 28 randomly selected specimens exchanged between the two centres indicated that both PCR procedures have a good specificity. Therefore the discrepancy results are probably due to very low levels of CMV DNA in the semen specimens examined. Indeed, if the level of DNA is around the detection limit, its detection by PCR is linked to the chance of obtaining sufficient numbers of DNA copies in the volume of semen sampled for analysis. Moreover, each PCR procedure was performed on different ejaculate aliquots and this difference also could explain the discrepancies observed. These discrepancies could also result from different sensitivities of the two PCR procedures to inhibitors present in the semen specimens.

Unlike cell culture, which detects only infectious virions, the PCR procedure only detects viral DNA and thus cannot prove the infectious character of a semen specimen. However PCR is a highly sensitive technique which could constitute an effective screening method to exclude those potentially infectious specimens. The detection of CMV-specific IgG antibodies in serum is indicative only of a past infection and cannot substantiate CMV shedding in semen. Moreover, our results show that most CMV-seropositive donors do not excrete CMV in their semen. Our results also indicate that CMV-specific IgM antibody status is not predictive of CMV shedding in semen, since all the donors were negative for this marker. Since, in France, all the semen specimens collected from CMV-seropositive subjects are currently excluded from donation, a systematic testing by PCR may lead to reconsideration of the criteria for donor exclusion.

A CMV infection cannot be completely excluded in CMV-seronegative donors. Indeed CMV serological tests are not 100% sensitive and, moreover, it has been reported that CMV nucleic acids can be detected in a subset of CMV-seronegative subjects (Schrier et al., 1985; Zhang et al., 1995). Therefore,
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although the risk of CMV transmission by a CMV-seronegative donor is probably extremely low, this risk could be eliminated by testing the semen specimens by a very sensitive method such as PCR.

In conclusion, our results indicate that cryopreserved semen specimens collected from healthy volunteer donors constitute a possible source of CMV infection through TDI. Detection of CMV in semen by a highly sensitive method such as PCR could exclude the potentially infectious specimens. However, since discrepant results may be obtained between laboratories that use current ‘in house’ PCR procedures (Defer et al., 1992), the availability of a standardized commercial test is now requisite.

References


