PCR Assessment of *Chlamydia trachomatis* Infection of Semen Specimens Processed for Artificial Insemination

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In order to ascertain the microbiological quality of stored semen specimens processed for artificial insemination by a donor (AID), we developed a PCR assay targeting the chlamydial plasmid to detect *Chlamydia trachomatis* in semen. The lower limit of detection of this assay corresponded to 2.5 to 5 elementary bodies per μl of semen. A total of 669 cryopreserved ejaculates from 97 asymptomatic donors were tested for *C. trachomatis* infection. Twelve ejaculates, originating from four donors, were found to be positive, indicating a 4% prevalence of *C. trachomatis* infection among the donor population studied. Cross-contamination between the cryopreserved specimens in the storage container was studied by typing using sequence analysis of PCR-amplified omp1 genes of the strains. Two donors were infected with serovar E, one was infected with serovar F, and one was infected with serovar K. For two donors, the duration of *C. trachomatis* positivity could be assessed. One donor donated *C. trachomatis*-positive semen for 4 successive months, and the other did so for at least 16 months. The occurrence of *C. trachomatis* infection in cryopreserved donor semen indicates that ejaculates from donors not tested for a *C. trachomatis* infection just prior to donation should be tested for infection by a direct test such as the PCR described here. Direct testing of semen specimens will detect not only donors with an active infection but also *C. trachomatis*-infected ejaculates already stored and will thus improve the microbiological quality of AID, since discrepancies in the presence of *C. trachomatis* in urine and semen specimens have been reported.

*Chlamydia trachomatis* infections are the most prevalent bacterial sexually transmitted diseases worldwide (11). It has been estimated that there are approximately 4 million new *C. trachomatis* infections each year in the United States (7). A *C. trachomatis* infection, if not treated in an early stage, can lead to severe sequelae, such as pelvic inflammatory disease, ectopic pregnancy, and tubal infertility (16). However, 50 to 80% of infected men and women are asymptomatic (9, 16, 32). This high number of unrecognized infected individuals provides the reservoir for spreading the infection to men and women via sexual contact. In addition, women may also contract *C. trachomatis* infection after artificial insemination by a donor (20, 26). Considering the high prevalence of *C. trachomatis* infections worldwide, guidelines have been formulated to prevent the transmission of *C. trachomatis* infections through AID (2, 3). According to the recommendations of the American Society for Reproductive Medicine (2) and the European Society for Human Reproduction and Embryology (3), semen donors should be screened by testing of either urethral, urine, or semen specimens for *C. trachomatis*.

A variety of DNA amplification methods to detect *C. trachomatis* in urethral, urine, and cervical specimens are currently available and have appeared to be far more sensitive than cell culturing or antigen detection (4). These methods are now widely used in diagnostic laboratories for screening high-risk populations for *C. trachomatis* infection and donors providing semen for AID (4).

However, it has been reported that *C. trachomatis* was not detected in urinary or urethral specimens despite its presence in testicular specimens (10, 14). In addition, our semen bank contains large numbers of cryopreserved ejaculates from donors for whom no corresponding urethral or urine specimens have been tested.

As part of the quality requirements for human tissues processed for therapeutic purposes, we developed a PCR assay to detect *C. trachomatis* in semen specimens. This PCR was used to detect and to type *C. trachomatis* in stored semen specimens. (The results of this study were presented in part at the 39th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, Calif., September 1999 [abstr. no. 1739, 1999], and at the annual meeting of the American Society for Reproductive Medicine, Toronto, Ontario, Canada, September 1999 [abstr. no. 227, 1999].)

**MATERIALS AND METHODS**

**Semen samples.** Semen samples collected between 1984 and 1999 are stored in the Center for Reproductive Medicine, Academic Medical Center. In total, 669 ejaculates, donated by 97 men between 20 and 45 years old, were divided into aliquots in 0.25-ml straws with egg yolk, human sperm preservation medium (15), or SpermFreeze (Fertipro NV, Breemeng, Belgium). Straws were cryopreserved in liquid nitrogen and stored in a single container. *C. trachomatis* EB preparation. *C. trachomatis* biogroup Lymphogranuloma venereum L2/434/Bu was obtained from P. B. Wyrick, East Tennessee State University, Johnson City (originally provided by C. C. Kuo and S. P. Wang, University of Washington, Seattle). Bacteria were propagated in McCoy cells (CRL 1696; American Type Culture Collection) according to standard procedures (21), and elementary bodies (EBs) were released from the cells by sonication in a water bath at 60 W for 5 min (Branson 2200; Bransonics, Shelton, Conn.). Host cell debris was removed by centrifugation at 500 × g for 10 min, and EBs were concentrated by centrifugation at 12,000 × g for 30 min. Pellets were washed twice with 0.02 M phosphate buffer (pH 7.2) containing 0.2 M sucrose and 0.73 mg of glutamine per ml (SPG), resuspended in SPG, divided into aliquots, and stored at −70°C. The number of EBs was quantified using direct immunofluorescence (Microtrak; Syva, San Jose, Calif.).

**Purification of DNA from semen specimens.** Duplicate semen samples of 50 μl, derived from one straw, were processed. Prior to DNA extraction, 250 EBs (strain L2/434/Bu; Advanced Biotechnologies Inc., Columbia, Md.) (5 EBs per μl

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of semen) were added to one of the two samples as a positive control for lysis, DNA extraction efficiency, and inhibition of the PCR. Then, both samples were subjected to DNA extraction by the silica-GuSCN procedure using buffer L6 and 20 μl of size-fractionated silica particles (6). DNA was eluted in 100 μl of sterile water. For comparison, DNA of some semen samples was also extracted in the presence of buffer L7A (5).

**PCR to assess *C. trachomatis* infection in semen (C-PCR).** Primers, purified by high-pressure liquid chromatography, were obtained from Perkin-Elmer (PE; Nieuwekerk a/d IJssel, The Netherlands). The primers used for amplification of a 201-bp fragment of the chlamydial plasmid membrane were CTPI and CTTP2 (Table 1); this primer pair has been described previously (12). The final reaction mixture (50 μl) contained 20 μM dNTPs (PE), 2.5 μM of each primer, 1.25 U of Taq polymerase; 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 2.0 mM MgCl₂; 250 μM dATP, dGTP, and dCTP; and 400 μM dTTP (PE). PCRs were performed with a Trio thermoblock (Biometra, Goettingen, Germany) as follows: 5 min at 95°C, 1 min at 59°C, 40 cycles each consisting of 1 min at 95°C, 1 min at 55°C, and 2 min at 72°C; and a final elongation step of 10 min at 72°C. Semen samples previously testing negative were simultaneously processed as follows: 5 min at 50°C; 5 min at 95°C; 40 cycles each consisting of 1 min at 72°C; and a final elongation step of 10 min at 72°C. The nested PCR product (50 μl) was subjected to DNA extraction by the silica-guanidinium thiocyanate (GuSCN) procedure (6). The amplicons obtained from the *C. trachomatis*-positive semen samples were loaded on a 1% agarose gel, gel purified (Qiagen Inc., Chatsworth, Calif.), cloned into the TA vector pCR2.1, and transformed into Escherichia coli Top10F⁺ cells (Invitrogen, Groningen, The Netherlands). Recombinants were selected by blue-white screening and ampicillin resistance. Insert sizes were checked by performing a standard colony PCR with the universal ~21M13 and M13Reverse primers (PE). In brief, a portion of one colony was resuspended in 100 μl of distilled water, boiled for 10 min, and centrifuged for 1 min at maximum speed in an Eppendorf table centrifuge; 2.5 μl of the supernatant was used as a template in a 25-μl PCR mixture. Two independent recombinant clones from each transformation were subjected to sequence analysis.

**Nucleotide sequence accession numbers.** The nucleotide sequence data have been deposited in the EMBL/GenBank/DDBJ nucleotide sequence databases under the accession numbers AF265237, AF265238, AF265239, and AF265240.

**RESULTS**

Detection limit of the C-PCR. Using the silica-GuSCN procedure with buffer L6 for lysis and DNA extraction, it was possible to detect the equivalent of 10 to 20 EBs on the blot (Fig. 1). Since the equivalent of 4 μl of semen was loaded on the gel, a detection limit of 2.5 to 5 EBs per μl of semen was achieved. Some variation in the strength of the signals obtained between experiments was observed (Fig. 1). Using buffer L7A instead of buffer L6 in the silica-GuSCN procedure (5) did not improve the detection limit of the C-PCR (Fig. 1).

Assessment of *C. trachomatis* in semen samples present in the semen bank by the C-PCR. A total of 669 semen samples, derived from 97 donors, were blindly assessed for the presence of *C. trachomatis* by the C-PCR. Duplicate semen samples, to one of which EBs were added as a positive control, were processed. The semen samples supplied with EBs yielded a positive signal on the blot, indicating successful DNA extraction and amplification. Again, some variation in the strength of the signals obtained was observed (data not shown).

Twelve (1.8%) of the 669 nonspermic semen samples yielded a positive signal on the blot, indicating the presence of *C. trachomatis* DNA. At least two or more additional semen samples from these 12 C-PCR-positive ejaculats were also positive. Decoding of the *C. trachomatis*-positive ejaculate speci-
moms revealed that the *C. trachomatis*-positive samples were obtained from four donors (encoded K23, H38, V12, and B38).

**Duration of *C. trachomatis* infection.** The results of C-PCR testing of all of the ejaculates obtained from the four positive donors over time were used to study the persistence of infection. Donor K23 donated between November 1989 and February 1997 six ejaculates. Only two ejaculates, donated in October 1990 and January 1991, were C-PCR positive, demonstrating that donor K23 carried *C. trachomatis* for at least four successive months (Fig. 2). Donor H38 provided 13 ejaculates between June 1995 and May 1999, of which 8, donated between January 1997 and April 1998, were found *C. trachomatis* positive. Therefore, the duration of the infection was at least 16 months (Fig. 2). From donor V12, only two ejaculates were left in the semen bank. The first ejaculate, donated in July 1990, represented the positive ejaculate. The last ejaculate, donated in March 1999, was negative. From donor B38, only one ejaculate was still present; it was found *C. trachomatis* positive by the C-PCR (Fig. 2).

**DISCUSSION**

We developed and evaluated a PCR assay (C-PCR) to assess *C. trachomatis* infection in semen specimens processed for artificial insemination. A total of 669 semen samples, donated by 97 donors participating in an AID program, were examined for *C. trachomatis* infection by the C-PCR. Twelve positive ejaculates originating from four donors were identified, indicating a prevalence of 4% among the donor population studied.

By using semen samples spiked with a quantified amount of purified *C. trachomatis* EBs, the lower limit of detection of the assay was found to range between 2.5 and 5 EBs per μl of semen. The observed range of the detection limit and the variation in the strength of the signals obtained between experiments are most likely not due to variation in the DNA extraction efficiency. We used the silica-GuSCN procedure (6) to extract DNA from semen specimens. This procedure has been acknowledged for its potency in the removal of inhibitory substances in a variety of clinical specimens interfereing with PCR (5, 17, 30). All spiked samples were clearly positive, indicating that the silica-GuSCN procedure (6) can also be successfully used to extract *C. trachomatis* DNA from semen specimens. Spiking with purified EBs may have caused the variation in the strength of the signals obtained. It is well known that EBs have a strong tendency to form aggregates (21). Although stocks were subjected to sonication in order to reduce the number of aggregates, new aggregates could have been formed during the time span between sonication and addition to the semen samples, leading to an uneven distribution of EBs in the SPG stock solutions used. This problem could have easily been avoided by using naked plasmid DNA instead of EBs, but we chose to use EBs to control the efficiency of lysis of EB particles. With this approach, the detection limit obtained was similar to that previously reported by Van den Brule et al. (26). These investigators used cloned *C. trachomatis* plasmid DNA to spike semen samples and obtained a detection limit of between 10 and 100 copies of target plasmid DNA, corresponding to 1 to 10 EBs per μl of semen, assuming a plasmid copy number of 10 (24). Therefore, the
Results of C-PCR tests of semen donated in 1995 as well as done. In 1995, donor H38 tested negative by the urine LCx. Then. Donors V12, B38, and K23 were not tested in 1995. All actively participating in the AID program in 1995, tested positive.

North Chicago, Ill.). None of these 40 donors, who were active and dilution methods on the preservation of human spermatozoa. Andrology. 2000. 16:355–366.


