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Screening for *Chlamydia trachomatis* and *Ureaplasma urealyticum* infection in semen from asymptomatic male partners of infertile couples prior to in vitro fertilization

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Summary

In a prospective study, the prevalence of infection with *Chlamydia trachomatis* and *Ureaplasma urealyticum* was evaluated in the semen of 92 asymptomatic male partners of infertile couples using polymerase chain reaction and culture, respectively. The results were compared with the detection of serologically specific antibodies. *U. urealyticum* and *C. trachomatis* were detected in 12 (13%) and 10 (10.8%) of the tested ejaculates, respectively. One mixed infection was detected.

No correlation was found between detection of the pathogens in ejaculates and the presence of specific antibodies in serum. This study therefore confirms the limited diagnostic value of serological analysis to ascertain infection with *C. trachomatis* or *U. urealyticum*. The high frequency of detection of these pathogens among asymptomatic male partners of infertile couples emphasizes their potential role in the impairment of male fertility, and the need for sensitive and specific detection methods to prevent infection of the early embryo when using new reproductive techniques such as zona pellucida hatching or intracytoplasmic microinjection.

Keywords: *Chlamydia trachomatis*, *Ureaplasma urealyticum*, semen infection

Introduction

Infections with *Chlamydia trachomatis* and *Ureaplasma urealyticum* have been recognized as being common sexually transmitted diseases in industrial countries, and are both suspected to have a pathogenic role in human reproductive failure (Weström, 1994; Hill *et al.*, 1987). The role of *Chlamydia trachomatis* in male infertility has been intensively analyzed (Wolff *et al.*, 1991, 1994). A correlation has been found between the presence of an in situ humoral immune response and the development of an autoimmune response to spermatozoa (Witkin *et al.*, 1995) and electron microscopy has demonstrated that *C. trachomatis* can adhere to and even enter into human spermatozoa (Wolner-Hanssen *et al.*, 1984; Erbengi, 1993).

Less is known about the pathogenic role of ureaplasma. Busolo *et al.* (1984) have demonstrated its ability to bind to the sperm surface membrane and Rose & Scott (1994) have shown that ureaplasma can alter sperm concentration, sperm velocity and motility patterns, can impair capacitation, and produce peroxides which damage spermatozoa and therefore its ability to fertilize oocytes.

Recent assisted reproduction techniques (ART) such as in vitro fertilization (IVF) after zona pellucida (ZP) hatching or intracytoplasmic sperm injection (ICSI) through the zona of an oocyte have underlined the infectious risk. Veterinary studies have emphasized the importance of the ZP for disease control in livestock by embryo transfer (Shisong & Wrathall,

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1989). The most important route whereby pathogenic organisms gain access to the early (unhatched) embryo is *via* the gametes, in which case the organism infects either the preovulated oocyte or the spermatozoon (i.e. attached to the surface of the spermatozoa) before and/or at the time of fertilization.

Because *C. trachomatis* or *U. urealyticum* infections are frequent, and can be cured by antibiotics, reliable detection of these microorganisms is essential. *U. urealyticum* can cultivate in complex medium under 5% CO_2 at 35°C. Semen culture is not an applicable procedure for detection of *C. trachomatis* as culture is not a sensitive method due to cytotoxic seminal fluid components that alter McCoy cells (Mardh *et al.*, 1980). Urethral swabs may also be analyzed but this method is painful for the patient. The polymerase chain reaction (PCR) has been described as a sensitive method for the detection of *C. trachomatis* in semen (Van den Brule *et al.*, 1993).

In our ART centre, on the first visit, sera from infertile couples are investigated for antibodies to chlamydia and ureaplasma and a semen culture is performed prior to ART. If the pathogens are detected in semen, antibiotics are prescribed and the ART attempt delayed until the micro-organisms have been eliminated.

The present study has determined the prevalence of *Chlamydia trachomatis* and *Ureaplasma urealyticum* in the semen of asymptomatic male partners of infertile couples and compared this with serological results. We have used the polymerase chain reaction (PCR) for *C. trachomatis*-DNA amplification in semen samples. We detected *U. urealyticum* infection using broth and agar plate cultures.

Materials and methods

Patients and clinical specimens

A total of 92 asymptomatic male patients who entered the IVF programme and who also gave their informed consent for using excess semen for research or diagnostic purposes were included in this experimental, prospective protocol. A female factor (ovulatory disorder, tubal factor) was present in 89% of couples. All men were asked for their sexual history, past history of infections and were screened for chronic prostatitis or urethritis. All the 92 male patients were asymptomatic and did not present any clinical symptoms of infection of the lower genital tract. The mean age of the men was 35.3, with a range from 27 to 50 years.

Sampling was performed on the patient's first visit (D_0) and was repeated on the day of the IVF attempt (D_{IVF}). For each patient, semen was collected by masturbation after 3 days of abstinence. Semen samples were freshly inoculated onto culture and then stored at − 80 °C for a PCR assay. Couples with a positive semen samples (D_0) for chlamydia or ureaplasma (and their sexual partners) received antibiotic treatment for 3 weeks (Doxycyclin^R, Roxithromycin^R or Ofloxacin^R) and semen was subsequently re-tested (culture, PCR) (D_1). Among 92 infertile couples, 74 (80%) had an in vitro fertilization (IVF) attempt. On the day of the IVF attempt, only 63 (85%) patients had a sufficient ejaculate volume to have their semen re-tested (D_{IVF}). Prior to IVF, one mL of the raw semen sample was systematically submitted to centrifugation through a three layer (90%−70%−50%) discontinuous Percoll gradient with subsequent washing in BM1 culture medium (Ellios Bio Media, France). The semen samples and the prepared aliquots were used for IVF or ICSI and stored for further analysis ($D_{IVF\ Percoll}$).

Serological analysis

A blood sample was taken on the patient's first visit to detect *C. trachomatis* immunoglobulins G and A using the indirect immunoperoxidase assay (SeroELISA Chlamydia; Savyon Diagnostics, Beer-Sheva, Israel). According to the recommendations of the manufacturer, a serum sample was positive for *C. trachomatis* antibodies when the optical density value (OD at 450 nm) was 0.8 above the negative control. Since antibodies against *C. trachomatis* and *C. pneumoniae* can cross react, chlamydia antibodies in serum were determined by an ELISA species-specific test, the positive sera then being investigated for IgG and IgA antibodies with the species-specific microimmunofluorescence test (MIF) according to Wang & Grayston (1974). *Ureaplasma* antibodies were measured in serum samples by an enzyme-linked immuno-sorbent assay (ELISA) with the use of a cell lysate antigen from ureaplasma (Cassel & Brown, 1983).

Culture

Fresh semen samples were inoculated into U9 medium for ureaplasma (0.1 mL fluid per 0.9 mL of broth) and A8 Agar (Sanofi diagnostics Pasteur, France). Broth cultures were incubated at 35 °C under atmospheric conditions, and agar plates were incubated under 5% CO_2 and checked daily for 5 days. Growth in broth was detected by an alkaline shift due to the urease activity of ureaplasma, causing the phenol red indicator to turn from yellow to pink. The detection limit for ureaplasma was below 10 colony forming units (cfu)/mL.

PCR

A rapid DNA extraction procedure was used. Briefly, 100 µL semen was mixed with 100 µL PBS and 140 µL solution containing 0.5 g/L trypsin and 0.2 g/L EDTA. The mixture was homogenized for 10 s, then incubated for 15 min at room temperature. The suspension was pelleted (1 min at 12 000 × g) and the supernatant removed. The pellet was resuspended in 100 µL InstaGen Matrix (BioRad Laboratories, Richmond, CA, USA), incubated for 30 min at 56 °C, then for 10 min at 98 °C. The extract was stored at − 20 °C or immediately used for amplification.

Amplification of *C. trachomatis*-DNA by PCR was performed using the procedures of Loeffelholz *et al.* (1992). The target sequence for amplification was a 207-bp segment of the cryptic plasmid of *C. trachomatis*. The sensitivity of the PCR assay was 10–100 genomes/mL (unpublished data). PCR was performed for two concentrations of semen extract (undiluted and diluted 1/10 in distilled water), to avoid false negative PCR due to Taq inhibitors. Each PCR run included a positive control (*C. trachomatis* strain L2) and 2 negative controls (previously tested negative samples and distilled water).

PCR products (10 μL) were run at 200 V for 20 min through a 2% agarose gel (Sea Kem GTG agarose) in 1 × TBE buffer (Sigma, St Louis, USA) containing 10 μg/mL ethidium bromide (Fig. 1). The gels were photographed with a Polaroid camera (MP4 +) under UV light. The size of the PCR product was 207 bp.

Detection of amplified DNA by Gen-Eti-K-DNA enzyme immunoassay (DEIA) (Sorin Biomedica, Italy)

The Gen-Eti-K-DNA enzyme immunoassay is based on the immobilization of a capture probe/amplificate hybrid on a solid phase using a biotin avidin bridge. DNA duplexes were detected by an anti-DNA monoclonal antibody that specifically reacted with double-strand (ds) but not with single strand (ss) DNA. The specific ds-DNA/anti-DNA antibody complexes were visualized by a peroxidase-conjugated, antimouse antibody and a chromogen/substrate mixture (Sorin Biomedica, Italy). The detection of amplified *C. trachomatis* DNA with the DEIA was performed according to the manufacturer's recommendations (cut off at 0.2 OD at 450 nm).

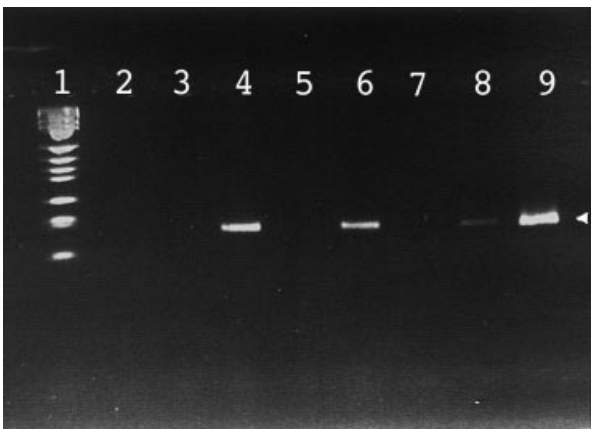

Figure 1. Ethidium bromide staining of a 2% agarose gel showing samples infected or not infected with *C. trachomatis* and the fractions. Lane 1: molecular weight markers (Boehringer, DNA V); lanes 2–3: negative controls; lane 4: positive sample $n = 1$; lane 5: 90% fraction obtained from a positive semen after a three layer discontinous Percoll gradient; lane 6: positive sample $n = 2$; lane 7: 100% fraction obtained from a positive semen after a six layer discontinuous Percoll gradient; lanes 8 and 9: positive controls (two dilutions).

Results

Chlamydial and ureaplasma serology

On the first visit, all 92 patients were investigated for serum antibodies. A total of 24 sera (26.1%) were positive for chlamydial antibodies. Of these, 8 patients (8.6%) were positive for IgA antibodies, 21 (22.8%) for IgG antibodies and 5 (5.4%) for both IgA and IgG.

Twenty-three sera (25%) were positive for ureaplasma antibodies. Amongst these, only two (2.1%) had ureaplasma IgM serum antibodies.

Outcome of the microbial screening in ejaculates and relationship of chlamydia and ureaplasma serologies with semen analysis

On analysis of semen taken on the first visit, *C. trachomatis* – DNA was detected by PCR in 10 of 92 (10.9%) ejaculates (D_0) (Fig. 1) with an OD ranging from 0.21 to 1.5.

U. urealyticum grew in 12 out of the 92 semen samples (13%) in culture with levels of infectious agent in the semen ranging from 10^3 to $>10^5$ cfu/mL. One semen sample was positive for both *C. trachomatis* (OD 0.301) and *U. urealyticum* ($>10^5$ cfu/mL).

The detection of chlamydia and ureaplasma in semen was not related to the presence or absence of serum antibodies (Table 1). Nine out of the 10 *C. trachomatis* – DNA positive semen samples corresponded to patients with negative chlamydia serologies, regardless of optical densities. Only one patient had both positive chlamydia serology (IgA and IgG antibodies) and DNA detected in his semen with a very high optical density (1.5). The majority (96%) of positive chlamydia serologies were observed in cases where no chlamydia DNA was detected in the ejaculate.

Table 1. Indirect methods (serum antibody titration) and direct diagnosis (PCR[5] and culture)

Chlamydia serology	PCR chlamydia +	PCR chlamydia –
[1] positive	1	23
[2] negative	9	59
total	10	82
Ureaplasma serology	Culture +	Culture –
[3] positive	3*	20
[4] negative	9	60
total	12	80

* one sample grew in *M. hominis* and in *U.urealyticum*
[1] IgA and/or IgG +
[2] IgG – and IgA –
[3] IgG and/or IgM +
[4] IgM – and IgG –
[5] Polymerase chain reaction

Nine out of the 12 (75%) patients with positive ureaplasma culture had no ureaplasma antibodies in their sera. Only three positive semen samples corresponded to patients with positive IgG serologies. In two cases only, positive serologies clearly corresponded to patients with a very high level of infectious agent in the semen ($>10^5$ cfu/mL) (Table 1). Furthermore, the two patients with IgM antibodies in their sera were negative in culture. No antibody was detected in the patient with both *U. urealyticum* and *C. trachomatis* in his semen.

Treatment and follow-up

Patients (and female partners) who tested positive for *U. urealyticum* or *C. trachomatis* in the first semen analysis (D_0) were subsequently treated (with Doxycycline^R, Roxithromycine^R or, Ofloxacine^R) for 21 days. Two weeks after completion of the treatment, semen culture control was performed (D_1): neither ureaplasma nor chlamydia was detected in their ejaculates. Two patients with very high levels of infectious agent remained positive for *U. Urealyticum* in their semen after three weeks of specific antibiotic treatment (Roxithromycine^R). This detection became negative after a second course of antibiotics.

Among the 92 infertile couples, 74 IVF attempts were performed during the course of this study. The 18 remaining couples had their ART either delayed (because of an inappropriate response to hormonal treatment) or cancelled (due to a spontaneous pregnancy or change of fertility centre). Only 63 of the 74 patients had an ejaculate volume sufficient for examination and subsequent testing (D_{IVF}). On the day of the IVF attempt, no ejaculate was positive for *U. urealyticum*, but 3 (4.8%) were positive for *C. trachomatis*. Amongst these, one had already been detected during the first screening and remained positive due to non-compliance with the antibiotic treatment. The two other positive ejaculates were detected in patients with previous negative DNA detection results. These patients had no antibodies against *Chlamydia trachomatis*. The ejaculates were systematically prepared using a discontinuous 3-layer (90%–70%–50%) Percoll gradient and subsequent washing in BM1 culture medium. The recovered aliquot containing washed spermatozoa was then tested negative by PCR ($D_{IVF\ Percoll}$).

Discussion

Ureaplasma urealyticum and *Chlamydia trachomatis* infections are considered as the most common sexually transmitted diseases in industrialized countries (Paavonen & Wolner-Hansen, 1989). However, their incidence among the whole population, and more specifically in infertile patients, is still a debatable issue (Wolff *et al.*, 1994; Wolff *et al.*, 1994). This is an important feature because transmission of *Chlamydia trachomatis* or *Ureaplasma urealyticum* by artificial insemination from an asymptomatic donor semen (AID) has been well demonstrated (Nagel *et al.*, 1986; Barwin, 1984). Invasive in vitro fertilization techniques – hatching or ICSI – might increase the risk of transmission to the early embryo. To avoid such infections, there is the need for an accurate means of detecting infectious agents in semen prior to IVF.

Using culture methods for *U. urealyticum* and PCR for *C. trachomatis*, our analysis of 92 semen specimens from asymptomatic male partners of infertile couples revealed that 10 (10.9%) were positive for *C. trachomatis* and 12 (13%) for *U. urealyticum*, in accordance with previous studies (Nagy *et al.*, 1989; Wolff *et al.*, 1991, 1994; Witkin *et al.*, 1993; Dieterle *et al.*, 1995). This is not in agreement with a recent report from Eggert-Kruse *et al.* (1997). They reported a surprisingly low percentage of *C. trachomatis* semen infection in a population of asymptomatic male partners of infertile couples (1/50), detected using a ligase chain reaction, a molecular technique with an equivalent sensitivity to PCR. They suggested that this low prevalence was related to the fact that asymptomatic patients suffering from long-standing infertility represent a low-risk population for ongoing chlamydia infection. There is no obvious explanation for such differences. Although our study was conducted on a similar population, we cannot confirm this low prevalence and our results suggest that there are occasional *C. trachomatis* and *U. urealyticum* infections in asymptomatic male partners of infertile couples.

The detection of chlamydia immunoglobulin IgG and IgA antibodies in serum is of no diagnostic value in male infertility (Wolff *et al.*, 1994; Dieterle *et al.*, 1995) and does not discriminate between a past or active current infection. Our study has confirmed the lack of correlation between serological results and direct detection of *C. trachomatis* and *U. urealyticum* in semen, as previously described by Dieterle *et al.* (1995) and confirmed by Eggert-Kruse *et al.* (1996, 1997). The bacterial level did not correlate to serology. Three patients had both positive ureaplasma serology and ureaplasma in their semen: $>10^5$ cfu/mL was noted for two of them, 10^2 for the other one. For *C. trachomatis*, only one patient had both chlamydia antibodies (IgA and IgG) in his serum and chlamydia DNA in his semen with a very high optical density (1.5), suggesting a chlamydia primary infection.

According to these results, considerable care should be taken in the management of male patients. We confirm that a sensitive direct detection method should systematically be used to check for chlamydia infection in semen. Discrepancies were observed with both pathogens. According to serological results, 25% of male partners of infertile couples showed evidence of past chlamydia or ureaplasma infections. The failure of *C. trachomatis* and *U. urealyticum* to elicit a systemic humoral immune response in patients with evidence of these organisms in the semen further indicates that the concentration of both organisms is probably very low. In addition, it has been postulated that some regions of the male genital tract are poorly accessible to the systemic immune

system (Byrne, 1996). This strongly suggests that the reported prevalence of asymptomatic male *C. trachomatis* infection may even be an underestimation of the real rate of past infection with *C. trachomatis*. Therefore, determination of serum immunoglobulin status is not informative in determining the potential risk of transmission of chlamydia or ureaplasma infection through semen.

In our study, chlamydia DNA was detected in three ejaculates on the day of the IVF attempt. Two out of the three positive semen samples had been negative previously and had had no specific antibodies in their serum. This intermittent detection of DNA *C. trachomatis* could correspond either to active or to non-active phases of chlamydia prostatitis, emphasizing the difficulty in completely eradicating these organisms from the prostate and therefore from the ejaculates, even after a course of antibiotic therapy. This persistence can be associated with abnormally large intracellular forms of the organisms in diseased tissue (Byrne, 1996).

The ejaculate of the patient must be analyzed at least twice before performing IVF or ICSI. If the analysis is positive, the couple must be treated according to the antibiogram and the IVF must be delayed until complete cure of the infection. After the treatment, the semen must be shown to be negative prior to starting an IVF or ICSI attempt.

Our study has shown that ejaculate preparation techniques, using for example a Percoll gradient centrifugation and subsequent washing as described above, not only separates motile spermatozoa from the ejaculate but also washes them to rid them of bacterial impurities. The evaluation of this washing technique is in progress. Preliminary results show that Percoll gradient centrifugation is able to clear *C. trachomatis* from the infected semen.

Our results emphasize that semen analysis of all male patients from infertile couples entering an IVF program should be systematically checked for ongoing *C. trachomatis* and *U. urealyticum* infection using direct sensitive methods. When a microorganism is found in semen during the first examination, a suitable course of antibiotic therapy should be given to both partners and a test of cure should be performed before starting IVF or ICSI. The monitoring of such patients cannot be reliably performed based on results of serological analysis.

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