

## Assessment of *Chlamydia trachomatis* infection in asymptomatic male partners of infertile couples

Farida Hamdad-Daoudi,<sup>1</sup> Jacques Petit<sup>2</sup> and François Eb<sup>1</sup>

Bacteriology-Hygiene Laboratory<sup>1</sup> and Department of Urology<sup>2</sup>, University Hospital, Amiens, Place Victor Pauchet, 80054 Amiens Cedex 1, France

### Correspondence

Farida Hamdad-Daoudi  
Hamdad-Daoudi.Farida@  
chu-amiens.fr

Three specimens from 111 asymptomatic male partners of infertile couples attending the Department of Urology in Amiens, France, were examined by the PCR COBAS AMPLICOR test (Roche Molecular Diagnostics) for the presence of *Chlamydia trachomatis*. The specimens analysed were: first void urine (FVU), urine obtained after prostatic massage (UPM) and semen specimens. Serum from each patient was also obtained and analysed for the presence of IgG and IgA chlamydial antibodies by in-house microimmunofluorescence (MIF) and pELISA. *C. trachomatis* was detected by PCR in 5.4 % of FVU samples, 2.7 % of semen specimens and in 0.9 % of UPM samples. Two treatments for processing the samples (storage at –70 °C and heating to 95 °C) were routinely used before initial testing to reduce the effects of inhibitors of PCR. Despite these precautions, the PCR method revealed the presence of inhibitors in 7.3 % of semen specimens and 3.6 % of FVU samples. *C. trachomatis* was detected by PCR COBAS AMPLICOR in seven of 111 patients (6.3 %) and by serology in five of 111 patients (4.5 %). The detection of *C. trachomatis* in FVU, UPM and semen specimens can serve as a marker for the presence of this organism in the genital tract, and can be used as a reliable way of detecting asymptomatic carriers of infection.

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## INTRODUCTION

*Chlamydia trachomatis* is one of the most common sexually transmitted pathogens of humans, with an estimated 92 million new cases occurring worldwide each year (WHO, 2001). The number of cases is probably underestimated, particularly for men, who are less likely to seek diagnosis than women. *C. trachomatis* often causes asymptomatic genital tract infections in both men and women, and the high number of unrecognized infected individuals provides a reservoir for spreading the infection to men and women via sexual contact.

Chlamydial infection in the male urethra can be complicated by inflammation of the epididymis (Stamm *et al.*, 1984) and the prostate gland (Dan *et al.*, 1991), but the role that *C. trachomatis* infection plays in male infertility is controversial.

*C. trachomatis* can attach to spermatozoa (Hanssen & Mardh, 1984) and can be present in cytoplasmic droplets of spermatozoa (Villegas *et al.*, 1991). Previous studies have found a correlation between genital chlamydial infection and sperm quality (Custo *et al.*, 1989), although other studies have reported contradictory findings (Eggert-Kruse *et al.*, 1996; Gdoura *et al.*, 2001). It is important to resolve this

issue, since chlamydiae present in semen could be transmitted by adhering to spermatozoa, which may serve as vectors, spreading the pathogen to the uterus and Fallopian tubes. In fact, studies have reported finding spermatozoa with adhering chlamydial bodies in the peritoneal fluid of women with salpingitis at laparoscopy (Friberg *et al.*, 1987).

The presence of *C. trachomatis* in semen emphasizes the potential risk of this route of transmission, and underlines the need for sensitive direct detection methods in this group of patients (Dieterle *et al.*, 1995).

Screening with classical cell culture requires specimens from endo-urethral swabs, which are unacceptable to many asymptomatic men. The cell culture method cannot be used for semen and urine samples due to their cytotoxicity (Mardh *et al.*, 1980), and is not sufficiently sensitive to rule out infections of accessory glands (Berger *et al.*, 1978). Direct fluorescent antibody assays have been used to detect *C. trachomatis* in every type of specimen. These tests are sensitive and specific, but are time consuming and require highly skilled technicians; they are thus reserved for specialized research laboratories and have not been generally implemented as a routine diagnostic method (Taylor-Robinson, 1992).

The introduction of commercial PCR in the routine clinical microbiology laboratory is an important advance in our ability to diagnose asymptomatic chlamydial infections in male partners of infertile couples (Toye *et al.*, 1996; Gdoura

Abbreviations: FVU, first void urine; IC, internal control; MIF, microimmunofluorescence; MOMP, major outer-membrane protein; NAATs, nucleic acid amplification tests; UPM, urine after prostatic massage.

*et al.*, 2001). Nucleic acid amplification tests are considered to be a more suitable method for screening and diagnosing chlamydial infections, but one major problem with them is the presence of potential inhibitors in clinical specimens that can lead to false negative results.

The aim of this study was to determine the prevalence of asymptomatic *C. trachomatis* infections in male partners of infertile couples by PCR COBAS AMPLICOR (Roche Molecular Diagnostics) which includes an internal control (IC) able to detect inhibitors, and by serology using the in-house microimmunofluorescence (MIF) test and the pELISA (Medac, Hamburg, Germany) method.

## METHODS

**Patients.** A total of 111 randomly chosen males from infertile couples, who were tested for infertility in the Department of Urology at the University Hospital of Amiens, France, were enrolled in this study. None of the patients had clinical signs of genital tract infection and, apart from their infertility problem, were healthy individuals. These infertile couples are potential candidates for assisted reproductive techniques.

The median age of the whole study population ( $n = 111$ ) was 33 years old (range 24–44 years).

**Materials.** Three hundred and twenty-nine specimens were collected in our clinical microbiology laboratory. The specimens from each patient were: first void urine (FVU), urine obtained after prostatic massage (UPM) and semen samples. After FVU had been collected, a prostatic massage was performed and prostatic secretions were collected in 0.5 ml of urine. Semen specimens were then obtained after masturbation. Two patients refused prostatic massage and masturbation so UPM and semen were not collected for them.

For washing and reducing the presence of inhibitors in the semen, 200  $\mu$ l PBS was added to 100  $\mu$ l semen and vortexed. After centrifugation at 13 000  $g$  for 10 min, the supernatant was discarded and the cell pellet was frozen at  $-70^{\circ}\text{C}$ . Other samples were also stored frozen at  $-70^{\circ}\text{C}$  until further use.

All specimens were screened for *C. trachomatis* using the PCR COBAS AMPLICOR test according to the manufacturer's instructions.

Serum was obtained and analysed for the detection of species-specific *Chlamydia* antibodies by in-house MIF and by the pELISA method. It has been reported that the sensitivity of ELISA, especially for anti-*C. trachomatis* IgA, was higher than for MIF (Bourgoin *et al.*, 1998) so we used both of these methods.

**PCR COBAS AMPLICOR.** In the AMPLICOR test, primers target a 207 bp segment of the cryptic plasmid DNA present at 7–10 copies per genome of *C. trachomatis* strains. The plasmid is identical for all serovars of *C. trachomatis*.

All specimens were thawed, resuspended in 500  $\mu$ l wash buffer, vortexed vigorously and then incubated at  $37^{\circ}\text{C}$  for 30 min. After centrifugation at 13 000  $g$  for 15 min, the supernatant was discarded and 250  $\mu$ l CT/NG lysis buffer were added. After 15 min incubation at  $37^{\circ}\text{C}$ , 250  $\mu$ l CT/NG specimen diluent was added to the lysate. The contents were mixed by vortexing, and tubes were centrifuged at 13 000  $g$  for 10 min. The tubes were then incubated at  $95^{\circ}\text{C}$  for 10 min.

Fifty microlitres of the lysate was immediately transferred to A-rings

containing 50  $\mu$ l working master mix. An IC was included in the master mix to monitor inhibition and was amplified with the *C. trachomatis* target DNA.

The A-rings were placed in the COBAS AMPLICOR thermocycler system. Amplification and detection of *C. trachomatis* and IC DNA were automatically performed by the COBAS AMPLICOR system.

The PCR assay uses biotinylated oligonucleotide primers for target amplification. Amp Erase was incorporated to prevent carryover contamination by uracil-*N*-glycosylase. The COBAS AMPLICOR system automatically denatures the amplified DNA target, hybridizes the amplicon with the target-specific probes bound to magnetic microparticles, and detects the amplicon/probe complex by a colorimetric reaction.

The *C. trachomatis* target and IC were detected in separate reactions with specific oligonucleotide capture probes.

**Interpretation of results.** Specimens yielding *C. trachomatis* signals above the positive cut-off [absorbance (*A*) of 0.8] were interpreted as positive. Specimens in which the *C. trachomatis* signal was below the negative cut-off (*A* of 0.2) were interpreted as negative if the IC signal was above the assigned cut-off. Specimens yielding signals below the cut-off values ( $A < 0.2$ ) for both *C. trachomatis* and the IC were considered to contain inhibitory substances and were not interpretable. Specimens yielding *C. trachomatis* results between the negative and positive cut-off ( $0.2 < A < 0.8$ ) were considered ambiguous, regardless of the IC signal. Ambiguous results were resolved by retesting the processed specimen in duplicate. These specimens were interpreted as negative if at least one of the two repeat tests yielded a *C. trachomatis* signal of  $< 0.2 A$ .

**Chlamydial IgG and IgA antibodies.** The indirect, modified MIF assay was performed with three chlamydial strains prepared from infected egg yolk sacs (*C. trachomatis* LB1 serovar L2, *Chlamydia psittaci* Loth and *Chlamydia pneumoniae* IOL-207) (Treharne *et al.*, 1977; Orfila & Eb, 1985). Slides were fixed with acetone and could be stored frozen at this stage. Serial dilutions of patient serum were placed on the antigen dots and incubated for 30 min. The slides were then washed, dried and stained with a fluorescein-conjugated anti-human class-specific immunoglobulin (Ig) diluted at 1:100 (bioMérieux). Serum IgG antibodies were tested at dilutions of 1:16–1:128, while serum IgA antibodies were evaluated at a dilution of 1:12. A titre of  $\geq 16$  for IgG and titre of  $\geq 12$  for IgA were considered as positive. Qualitative *C. trachomatis* pELISA was performed for detection of species-specific antibodies using a synthetic peptide from the immunodominant region of the major outer-membrane protein (MOMP). Serum IgG and IgA antibodies were tested at dilutions of 1:50. The pELISA test used peroxidase-conjugated anti-human IgG and IgA antibodies to bind to *C. trachomatis* IgA and IgG antibodies. After incubation with tetramethylbenzidine substrate, the reaction was stopped by adding sulphuric acid. The absorbance was read photometrically at 450 nm. Cut-off values were calculated according to the manufacturer's instructions.

The IgG cut-off value equalled the mean *A* value of the negative control + 0.340. The IgA cut-off value equalled the mean *A* value of the negative control + 0.270. An absorbance greater than or equal to the cut-off value was defined as positive.

## RESULTS

*C. trachomatis* was detected by the PCR COBAS AMPLICOR test in seven of 111 patients (6.3%) and by serology in five of 111 patients (4.5%). Table 1 shows the distribution of positive and negative results in the specimens by either of the

**Table 1.** PCR and serology results for the presence of *C. trachomatis* in 111 male partners of infertile couples

+, Positive; -, negative; Inh+, detection of inhibitors; ND, not done.

PCR COBAS AMPLICOR			Serology				Totals
FVU	UPM	Semen	MIF		pELISA		
			IgG	IgA	IgG	IgA	
+	-	+	+	-	+	-	1
+	-	+	+	-	-	-	1
+	-	-	-	-	-	-	3
+	+	-	-	-	-	-	1
-	-	+	-	-	-	-	1
-	-	-	+	-	-	-	3
Inh+	-	Inh+	-	-	-	-	2
-	-	Inh+	-	-	-	-	6
Inh+	-	-	-	-	-	-	2
-	ND	ND	-	-	-	-	2
-	-	-	-	-	-	-	89

two methods. Inhibition occurred in 12 of 329 (3.6 %) specimens. PCR inhibition was detected in 7.3 % (8/109) of the semen specimens and 3.6 % (4/111) of the FVU.

#### Detection of *C. trachomatis* DNA in FVU and corresponding UPM and semen specimens by PCR

*C. trachomatis*-specific nucleic acid was detected in both the FVU and semen of two patients, and both the FVU and the UPM of one patient. *C. trachomatis* was detected in only the FVU of three patients and only in the semen specimen of one patient (Table 1).

To exclude false positives, the positive samples for *C. trachomatis* were retested and reported as positive if the reanalysis was also positive.

#### Chlamydial antibodies

Out of 111 patients, five (4.5 %) had chlamydial IgG serum antibody titres of 1 : 16 or greater by MIF, and in one of them (0.9 %) IgG *C. trachomatis* antibodies were also detected by pELISA. None of the patients had chlamydial IgA serum antibodies by the two methods tested (MIF and pELISA).

#### Correlation between the presence of *C. trachomatis* DNA and the presence of chlamydial antibodies in serum

*C. trachomatis* infection was detected in five men by PCR tests, for whom the serological tests were negative.

Two patients with both *C. trachomatis*-positive FVU and semen specimens by PCR had chlamydial IgG serum antibody titres of 1 : 16 or greater by MIF (two of seven; 28.5 %) and one of them also by pELISA.

Three patients without detectable DNA in specimens had *C. trachomatis* IgG serum antibodies only by the MIF test, but *C. pneumoniae* IgG antibodies were also detected.

## DISCUSSION

*C. trachomatis* is one of the most prevalent sexually transmitted pathogens, and it is thus important to determine its prevalence in asymptomatic male partners of infertile couples.

Because infected women are usually asymptomatic and because of the serious morbidity of these infections, *Chlamydia* programs have traditionally focused on screening women (Hocking & Fairley, 2003). Men are less likely to be infected than women, and most men with urethral chlamydial infection, like women, are free of symptoms. On the other hand, there are few data on the duration of chlamydial infection in men, it may be several months or years (Golden *et al.*, 2000). Transmission of *C. trachomatis* among infected men and their female sexual partners was examined using a design enhancing the likelihood that spread was directed from men to women (Lin *et al.*, 1998). Timely management of sexual partners is essential for decreasing the risk for reinfection.

Since the symptoms of *C. trachomatis* infection are not specific, laboratory methods are required for a definitive diagnosis of infection. These methods include several direct and indirect techniques.

Because asymptotically infected individuals may shed fewer organisms (Witkin, 2002), and in order to make tests easier and with higher specificity, nucleic acid amplification tests (NAATs) such as PCR may be the techniques of choice for *C. trachomatis* assessment in asymptomatic male partners

of infertile couples. Using the plasmid which is present at 7–10 copies per genome as target DNA therefore increases the sensitivity of the system and the specificity (the target is present only in *C. trachomatis*). One major problem with NAATs is the presence of potential inhibitors in clinical specimens that can lead to false-negative results. Genital and urine specimens are known to contain several factors that inhibit DNA polymerase and the exact nature of all of the inhibitors remains to be defined. Substances that inhibit PCR may be temperature sensitive or become inactive over time. Heating to greater than 95 °C or storage at –70 °C has been successfully used to remove inhibitors (Pasternack *et al.*, 1996; Verkooyen *et al.*, 1996). We have routinely incorporated both of these steps into the protocol for processing all the specimens before initial testing, to reduce the inhibition rate. Roche Molecular Diagnostics has developed a system that allows the detection of inhibitors, thus ensuring the reliability of the result.

Despite these precautions, the PCR COBAS AMPLICOR method revealed the presence of inhibitors in 7.3 % of the semen specimens and 3.6 % of the FVU. Inhibition was lower with FVU than semen specimens because of the higher dilution of inhibitory factors in the urine specimen. If the IC had not been used, these specimens would have been scored as false negative.

*C. trachomatis* was detected by PCR in 5.4 % of FVU samples, 2.7 % of semen specimens and 0.9 % of UPM samples. The presence of *C. trachomatis* DNA in FVU samples and its absence in other specimens may indicate an asymptomatic urethral infection.

The urine samples of three patients with a positive result for *C. trachomatis* PCR were also *Chlamydia*-positive by PCR in semen for two patients and UPM for one patient. This detection does not necessarily reflect an upper genital tract infection but may indicate an asymptomatic infection of the urethra. Contamination of semen and UPM with *C. trachomatis* colonizing the urethra is possible. On the other hand, the detection of *C. trachomatis* only in semen may indicate that these organisms are harboured in the epididymis or seminal vesicles.

For serological analysis, we routinely assessed the serological response to the chlamydial lipopolysaccharide (LPS) and MOMP; the MIF test and pELISA method were used to detect IgG and IgA antibodies.

The MIF assay of Wang and Grayston (1970) was developed for typing the *C. trachomatis* strains in serotypes and for seroepidemiological studies. Initially, antigens from elementary bodies of each of the 15 serotypes of *C. trachomatis* were included in the test, which provided serotype-specific testing. The test is technically difficult and impractical in a clinical laboratory, however, modifications have been introduced (Treharne *et al.*, 1977; Orfila & Eb, 1985). Until now, the MIF has been considered the 'gold standard' for the serological diagnosis of chlamydial infections.

The presence of chlamydial IgG antibodies in serum from

asymptomatic males does not allow a differentiation between past and present urogenital *C. trachomatis* infections. When individuals acquire a chlamydial infection, there is a lag period before the antibody response. Similarly, antibodies persist for a long time after an infection that is resolved by the cellular immune response (Greendale *et al.*, 1993). In a previous study, IgG antibodies were not reliable markers for acute chlamydial infection (Ochsendorf *et al.*, 1991), and high or persisting antibody titres in an individual does not necessarily correlate with current or persistent infection. Because the MIF test has been modified, and serotype-specific antigens are no longer used, the MIF test has been criticized for not being species-specific. Cross-reactivity between *C. trachomatis* and *C. pneumoniae* may occur due to the many common antigenic structures they share. Furthermore, the MIF test is laborious and a trained observer is required.

To eliminate cross-reactivity, ELISA tests for *C. trachomatis* have been developed based on synthetic peptides that do not share any sequence homology with *C. pneumoniae* MOMP. The *C. trachomatis* pELISA uses a synthetic peptide from the immunodominant region of MOMP. With this highly specific antigen, distinction of *C. trachomatis*-specific antibodies from the whole anti-chlamydiae antibody response is possible. Furthermore, enzyme immunoassays have many advantages over MIF assays, e.g. automation and objective reading of the results.

Of 111 patients, five had chlamydial IgG and two of the corresponding five specimens (1.8 %) were positive by PCR. None of these patients had chlamydial IgA serum antibodies. It was suggested that the presence of this class of antibodies may indicate active chlamydial infection (Dieterle *et al.*, 1995). An IgA antibody in serum has a half-life of about 5–7 days, and is regarded as indicator of active or recent infection (Tomasi *et al.*, 1972; Dieterle *et al.*, 1995; Su *et al.*, 1997)

In contrast, five males without chlamydial LPS and MOMP antibodies were found to be positive by the PCR COBAS AMPLICOR test. The failure to detect a systemic humoral immune response in the men with evidence of *C. trachomatis* in urogenital specimens may indicate further lower genital infection or that the concentration of organisms in the upper genital tract is very low (Witkin *et al.*, 1993). Furthermore, some regions of the male genital tract are inaccessible to the systemic immune system (Dym *et al.*, 1970) and *C. trachomatis* may therefore persist within the tract.

It has been shown *in vitro* that *C. trachomatis* can exist as aberrant reticulate bodies that have decreased levels of MOMP and LPS (Beatty *et al.*, 1993). Thus, a patient with persistent *Chlamydia* infection may have been missed by a routine serological examination detecting chlamydial LPS and MOMP. In contrast, the sensitivity of the PCR assay for *C. trachomatis* should not be affected by the formation of aberrant reticulate bodies.

Thus, it is not possible to exclude the possibility of chlamydial urogenital infections in asymptomatic men by

the absence of chlamydial antibodies in serum. The measurement of chlamydial antibodies in serum is therefore of limited use in diagnosis in male infertility cases, in contrast to its significance in female tubal infertility.

Discrepant results which are obtained may permit the localization of the *C. trachomatis* infection. However, the following suggestions must be confirmed by further studies. The presence of *C. trachomatis* only in FVU and the absence of an immune response may indicate an asymptomatic low genital infection. The presence of *C. trachomatis* in semen or in the UPM associated with the presence of serum antibodies may indicate an upper genital infection. The presence of *C. trachomatis* in semen or in the UPM and the absence of serum antibodies may indicate a persistent or chronic infection.

Thus, direct tests are the methods of choice for diagnosing *C. trachomatis* infections in male population.

The results of the present study show that, in our patient population, FVU and semen are the preferred specimens for diagnosing asymptomatic chlamydial infections in men. These specimens may prove to be useful because they can be obtained by non-invasive means and can be used to diagnose genital chlamydial infection. It has been suggested that testing for the presence of genital *C. trachomatis* in males is incomplete if semen samples are not included (Bornman *et al.*, 1998).

Results obtained by Pannekoek *et al.* (2003), who used the ligase chain reaction (LCR), are comparable with ours in the FVU but not in the semen specimens. This assay (LCR) may not be appropriate for the detection of *C. trachomatis* in semen, which may be due to the fact that semen might contain inhibitory components that interfere negatively with the LCR. However, a recent report by Hosseinzadeh *et al.* (2004) who used the LCR as a confirmatory test for in-house PCR, showed a good correlation between these methods of testing semen.

In conclusion, in this population, both FVU and semen samples permit the diagnosis of asymptomatic *C. trachomatis* infection in the lower and upper genito-urinary tracts by NAATs, which include an IC for detection of amplification inhibiting factors, validating the negative results.

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