Molecular Detection of *Chlamydia trachomatis* and Other Sexually Transmitted Bacteria in Semen of Male Partners of Infertile Couples in Tunisia: The Effect on Semen Parameters and Spermatozoa Apoptosis Markers

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Abstract

This study was undertaken to determine the prevalence of *Chlamydia trachomatis*, *Mycoplasmas*, and *Ureaplasmata* in semen samples of the male partners of infertile couples and to investigate whether *Chlamydia trachomatis* could initiate apoptosis in human spermatozoa. A total of 85 males partners of infertile couples undergoing routine semen analysis according to World Health Organization guidelines were included. Specimens were examined for the presence of *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Mycoplasma hominis*, *Mycoplasma genitalium*, *Ureaplasma urealyticum* and *Ureaplasma parvum* by Real time PCR (qPCR). Semen specimens were analysed for the appearance of apoptotic markers (sperm DNA fragmentation, activated caspase 3 levels, mitochondrial membrane potential (ΔΨm)) using flow cytometry. *C. trachomatis*, *N. gonorrhoeae*, *U. urealyticum*, *M. genitalium* were detected in semen samples of 13 (15.2%), 5 (5.8%), 5 (5.8%) and 3 (3.5%) male partners of infertile couples, respectively. *M. hominis* and *U. parvum* were detected in semen sample of only one patient (1.1%). The semen of infertile men positive for *C. trachomatis* showed lower mean of semen count and lower rapid progressive motility (category [a]) of spermatozoa compared to uninfected men with statistically significances (p = 0.02 and p = 0.04, respectively). Flow cytometry analyses demonstrated a significant increase of the mean rate of semen with low ΔΨm and caspase 3 activation of infertile men positive for *C. trachomatis* compared to uninfected men (p = 0.006 and p = 0.001, respectively). DNA fragmentation was also increased in sperm of infertile men positive for *C. trachomatis* compared to uninfected men but without statistical significances (p = 0.62). *Chlamydia* infection was associated to loss of ΔΨm and caspase 3 activation. Thus, *C. trachomatis* infection could be incriminated in apoptosis induction of spermatozoa. These effects may explain the negative direct impact of *C. trachomatis* infection on sperm fertilizing ability.

Introduction

Sexually transmitted infections are of major concern to researchers and clinicians in the field of reproductive medicine. It is estimated that 15% of male infertility is related to genital tract infection [1]. Men can harbor subclinical infections in the genital tract over extended periods of time and several sexually transmitted infection pathogens, such as *C. trachomatis* have been detected in semen from asymptomatic men [2]. According to a World Health Organization (WHO) [3] report, *C. trachomatis* is responsible for the most common sexually transmitted bacterial infection worldwide, affecting more than 90 million people and has been known for some time to have a significant effect on human reproduction [4]. The role of *C. trachomatis* infections in male infertility is controversial [5–6]. A number of studies have specifically looked at the relationship between *Chlamydia* infection and semen quality. While some authors have shown that *C. trachomatis* infection is associated with poor semen quality [7–8], others have claimed that it does not [9–10]. Some reports indicated that *C. trachomatis* infection is associated with a decrease in sperm concentration and motility and also with altered semen pH and reduced volume of the ejaculate [11,12,13–14]. Conversely, other studies have revealed no association between *C. trachomatis* infection of the male genital tract and altered sperm quality [9,15,16,17,18,19,20–21]. In summary, the available evidence is conflicting and still makes it impossible to establish a clear relationship between *C. trachomatis* infection and semen quality.

The apoptotic mode of cell death is an active and defined process which plays an important role in the development of multicellular organisms and in the regulation and maintenance of
the cell populations in tissues upon physiological and pathological conditions [22]. Apoptosis markers characterized in somatic cells were noted in human spermatozoa in several studies. These include, principally, plasma membrane externalization of phosphatidylserine (PS) and DNA fragmentation. Such markers are observed with higher frequency in ejaculates of infertile men compared with fertile controls [23–24]. In addition, key components of the somatic cell apoptosis pathways, such as presence and activation of caspases, have been described in purified populations of ejaculated sperm from the high and low-mobility fractions [24–25]. Moreover, mitochondria play a major role in the control of apoptosis [26]. Marchetti et al. (2002) demonstrated that analysis of ΔΨm is a sensitive test to determine sperm quality when compared with the analysis of the basic sperm parameters, generation of reactive oxygen species, and presence of DNA fragmentation [27].

Several in vitro and in vivo studies tried to establish a relationship between apoptosis markers in spermatozoa and Chlamydia infection. In vitro, some authors have demonstrated that C. trachomatis is able to interact with sperm cells, affecting their function and inducing apoptosis [28,29–30]. Apoptosis of human sperm can be induced by in vitro incubation of human sperm cells with Chlamydia LPS, which has a 550 fold greater spermicidal activity than Escherichia coli LPS [31–32]. In addition, C. trachomatis serovar E can attach to human spermatozoa and influence its function leading to premature capacitation [33]. It has been shown that Chlamydia LPS interact with CD14 on the sperm surface, thus leading to increased production of reactive oxygen species and resulting in caspase-mediated apoptosis [29]. Despite all this in vitro studies, a clear association between C. trachomatis and sperm damage has not yet been corroborated by in vivo studies. Gallegos et al. (2008) reported that patients with C. trachomatis and Mycoplasmas genitourinary infections have increased sperm DNA fragmentation in comparison with fertile controls [34]. Lastly, we showed that inoculation of fertile male Swiss mice in the uterine urethra with C. trachomatis could lead to alteration of semen parameters, induction of apoptosis in spermatozoa, and decrease of the reproductive performance of male mice [35]. Taken together, these data support a role of C. trachomatis in sperm apoptosis induction. However, most studies indicate that apoptosis-inducing mechanism is unknown.

In the present Study, we aimed to determine the prevalence of C. trachomatis, Mycoplasmas, and Ureaplasmas in semen samples of the male partners of infertile couples and mainly to investigate whether C. trachomatis could initiate apoptosis in human spermatozoa.

Materials and Methods

Subjects

A total of 85 infertile men attending obstetrics and gynecology clinics in Sfax (South of Tunisia) for diagnostic semen analysis were selected to the study. All men were undergoing semen analysis as part of a work-up for infertility investigations after failing to conceive with their partner after one year of unprotected intercourse. The mean duration of infertility was 4 years (range 1–15). The mean age of patients was 36.7 years (range 23–57). This study was approved by our institutional review board “Habib Bourguiba University hospital ethics committee” with the given number 8–12. All subjects signed a written informed consent. Consent form was also approved by our ethic committee

Sperm seminological variables

Prior to semen analysis, the men were asked to abstain from sexual intercourse or masturbation for 3–5 days before attending the clinic. All samples for analysis were produced on site and collected into standard containers that had previously been shown not to have any cytotoxic effects on human spermatozoa according to the methods outlined by WHO. Immediately after semen production, samples were placed in an incubator and liquefied at 37°C for up to 30 minutes before analysis. Semen analysis was performed according to the WHO criteria [36] to determine the following variables: sperm concentration, vitality, total progressive motility (category [a+b]), rapid progressive motility (category [a]) Peroxidase staining, a practical and reliable method recommended by WHO [36] for determining leukocytes in the semen, was employed to count and differentiate leukocytes (white blood cells) from immature germ cells. Leukocytospermia was indicated by a concentration of leukocytes ≥10⁶/ml.

Spermiocultures analysis

Samples were seeded quantitatively using a calibrated loop on agar plates: blood agar, chocolate agar with isovitalex (1%) incubated in 5% CO2 at 37°C for 48 hours. Microorganisms were identified by Gram staining and Bio-Mérieux Api systems (Bio-Mérieux, Marcy l’ETOILE, France).

Spermiocultures were considered positive when the number of colonies was ≥10⁴ CFU ml⁻¹ in case of Gram positive cocci and ≥10⁵ CFU ml⁻¹ in case of Gram negative rods.

Bacterial quantification in semen specimens by qPCR

For each male patient, 200 μl of semen specimens were used for bacterial quantification by Real time PCR.

Extraction of DNA by Cetyltrimethylammonium bromide (CTAB)-phenol-chloroform/isoamyl alcohol method. The precipitates from each 200 μl of semen specimens were harvested by centrifugation at 14,000 g for 20 minutes. The precipitates were treated with 5 μl of proteinase K (20 mg/ml) at 55°C for 2 h in 600 μl of digestion buffer (30 μl of 10% sodium dodecyl sulphate and 570 μl of TE buffer [10 mM Tris-HCl (pH: 8); 1 mM EDTA]).

After homogenisation, the samples were incubated in a solution of CTAB-NaCl (100 μl of 5 M NaCl and 80 μl of 10% CTAB) for 10 minutes at 65°C, and then mixed with 750 μl of chloroform-isoamyl alcohol [24:1 [vol/vol]] and centrifuged for 15 minutes at 14,000 g in an Eppendorf centrifuge. The aqueous phase was separated, mixed with 750 μl of phenol chloroform/isoamyl alcohol [25:24:1 [vol/vol/vol]] and centrifuged for 15 minutes at 14,000 g in an Eppendorf centrifuge. The obtained aqueous phase was mixed with an equal volume of isopropanol.

The samples were left at −80°C for 1 h and then centrifuged for 15 minutes at 14,000 g. The DNA pellet was washed up once with 70% ethanol, air dried, and dissolved in a final volume of 100 μl of TE buffer.

Primers and probes for QPCR. Initially, the extracted DNA was tested for human β-globin gene to check that there were no PCR inhibitors in the samples. Primers β-GPCO (5'-ACA-CAACTGTGTTGACTAGC-3') and β-GPPO (5'-GACAGGATGCTTGTCT-3') were used to amplify a 209-bp fragment of the human β-globin gene [37]. Samples found to be negative by PCR for β-globin were retested after dilution 10-fold in distilled water. Samples shown to be β-globin positive were then examined for bacterial quantification by Real time PCR.

The real-time PCR assay was performed on a CFX96™ real-time PCR cycler (BioRad, USA) in a 20 μl final volume with Ex Taq Premix Tli RNaseH Plus (Takara, Japan). A pair of primers and a labeled probe in the TaqMan format was used to amplify: 149 bp region of Cryptic plasmid for C. trachomatis, 80 bp of MgPa region of Adhesin gene for M. genitalium, 101 bp region of the 16 S rRNA-encoding gene for M. hominis, 101 bp region of...
controls were run in duplicate. To detect any possible contaminating DNA, samples and Diethylpyrocarbonate (DEPC) treated H2O instead of DNA were amplified with the same primers in (Table 1).

**Positive recombinant plasmid control.** To facilitate bacterial quantification, a plasmid containing the target gene for all bacteria was constructed.

DNA was extracted from *C. trachomatis*, *N. gonorrhoeae*, *M. genitalium*, *M. hominis*, *U. parvum* and *U. urealyticum* reference strains and the target sequence for all genes selected for Real Time PCR was amplified with the same primers in (Table 1).

The final 25 μl reaction mixture contained 1X PCR buffer (Promega, Lyon, France), 0.2 mM each primer, 0.2 mM each dNTP, 2.5 mM MgCl2, 1.25 U Go Taq DNA polymerase (Promega), and 5 μl of DNA extract. PCR was performed in GeneAmp PCR System 9700 (Applied Biosystems, Foster City, California) according to the following procedure: 4 min at 95 °C for 1 min, 72 °C for 20 min. PCR products were then purified with QiAquick Gel Extraction Kit (Qiagen) and cloned into a vector using a cloning kit (pGEM-T Vector; Promega, Madison, WI, USA), in accordance with the manufacturer's instructions. Isolation of recombinant plasmid DNA was performed using the QIAprepSpin Miniprep kit (Qiagen) and references strains were amplified and quantified with a NanoDrop ND-1000 Spectrophotometer. Copy numbers of the cloned gene was calculated using the following equation reported by [38] to generate standards ranging from 1 to 106 molecules and stored at −20°C.

### Evaluation of Viability of sperm using 7-amino-actinomycin-D Dye

The percentage of dead sperms cells (cells with 7-AAD positive) and viable sperm cells (cells with 7-AAD negative) were assessed using 7-AAD Dye. 7-AAD penetrates only dead cells. From each sperm sample, 1 ml of a sperm solution in PBS containing 2×10^6 cells/ml was stained with 10 μl of 7-amino-actinomycin-D (7-AAD) (Immunotech, a Beckman Coulter Company, Marseille–France). The samples were incubated in the dark at room temperature for 20 minutes before flow cytometric analysis. After the incubation period, 1 ml PBS was added and the sample was analyzed by flow cytometry.

### Evaluation of Mitochondrial Membrane Potential (ΔΨm)

JC-1 possesses the unique ability to differentially label mitochondria with low and high ΔΨm. In mitochondria with high ΔΨm, JC-1 forms multimeric aggregates that emit in the high orange wavelength of 590 nm when excited at 488 nm. In mitochondria with low ΔΨm, JC-1 forms monomers; these monomers emit in the green wavelength (525–530 nm) when excited at 488 nm.

The ΔΨm was analyzed using MitoProbe JC-1 Assay kit (Molecular Probes, Eugene, OR). For staining, 2 μM stock

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**Table 1. Primers and probes used for detection and quantification of C. trachomatis, N. gonorrhoeae, U. urealyticum, M. genitalium, U. parvum and M. hominis by qPCR.**

| Bacteria          | Primers and probes | Oligonucleotide sequence (5’→3’) | Target gene | Product size (bp) | Ref |
|-------------------|--------------------|---------------------------------|-------------|-------------------|-----|
| *C. trachomatis*  | Forward            | ACCAAAGTGTGCATGGTATAG           | Cryptic plasmid | 149               | [73]|
|                   | Reverse            | TCAGATAATTGGCCTATCTT            |             |                   |     |
|                   | Probe              | ROX-CGAACCTCATGGCCTATAAGG-BHQ2  |             |                   |     |
| *N. gonorrhoeae*  | Forward            | CCGGAAGCTGGTGTCATCTGATT         | PorA        | 101               | [74]|
|                   | Reverse            | GTTCAGGCGCAGATTC                |             |                   |     |
|                   | Probe              | FAM-CCGTGAAAGTGCTAGCCGCTTAGAC-T-BHQ1 |             |                   |     |
| *M. genitalium*   | Forward            | GAGAAATACCTTGTACGTGCA          | MgPA        | 80                | [75]|
|                   | Reverse            | GTTAATACATATAACGGCTTGAGTCATTAC  |             |                   |     |
|                   | Probe              | HEX-ACCTTTGAATTTTGGAAGG-BHQ1   |             |                   |     |
| *M. hominis*      | Forward            | TTTGTCGAATCTGCCCAACCA          | 165 rRNA-encoding gene | 101               | [76]|
|                   | Reverse            | CCCCCCTGCCTTCGCAAGTA           |             |                   |     |
|                   | Probe              | ROX-TACTAAACATTTAGTGGACTCTA-BHQ1 |             |                   |     |
| *U. urealyticum*  | Forward            | CATGGATGTTGCAACAGG            | Urease (UreD Subunit) | 146               | [77]|
|                   | Reverse            | CGATTTAATGATTTGGCCCTTC        |             |                   |     |
|                   | Probe              | FAM-TTGCACCTTAACTTGCAG-BHQ1   |             |                   |     |
| *U. parvum*       | Forward            | CATGGATGTTGCAACAGG            | Urease (UreD Subunit) | 147               | [77]|
|                   | Reverse            | CGATTTAATGATTTGGCCCTTC        |             |                   |     |
|                   | Probe              | Hex-TTGGCCCTTACGAG-BHQ1       |             |                   |     |

doi:10.1371/journal.pone.0098903.t001
solution of JC-1 in dimethylsulphoxide (DMSO) was prepared. From each sperm sample, 1 ml of a sperm solution in PBS containing 2×10^6 cells/ml was stained with 10 μl of JC-1 stock solution. The samples were incubated at 37°C in the dark for 20 minutes before flow cytometric analysis. In this way, 2 sperm subpopulations were identified:

1) Represented spermatozoa with high ΔΨm (orange fluorescence),
2) Represented spermatozoa with low ΔΨm (green fluorescence).

As suggested by the protocol, in order to confirm the JC-1 sensitivity to changes in membrane potential, carbonyl cyanide 3-chlorophenylhydrazone (CCCP = 50 μM final concentration) was used as membrane potential disruptor (negative control).

**Flow cytometric detection of activated caspase 3**

Activated Caspase 3 levels were detected in spermatozoa using fluorescein-labeled inhibitor of caspases (FLICA), which is cell permeable, non cytotoxic, and binds covalently to active Caspase 3. The inhibitor was used with the appropriate controls according to the kit instructions provided by the manufacturer (Carboxyfluorescein FLICA Apoptosis Detection Kit, AbCys, France). Briefly, 3.10^6 sperm were resuspended in 300 μl PBS. A 150-fold stock solution of the inhibitor was prepared by dissolving the lyophilized caspase-inhibitor in 50 μl dimethyl sulfoxide (DMSO) and was further diluted 1:5 in PBS to yield a 30-fold working solution (per aliquot: 2 μl of the stock solution plus 8 μl PBS). All test aliquots and controls (with 300 μl PBS) were incubated at 37°C in the dark for 1 h with 10 μl of the working solution. Sperm samples were then washed resuspended in 400 μl of Wash Buffer and kept in ice until flow cytometry analysis.

A negative control (sample with 300 μl PBS) and a positive control (sample treated with 10 μM H₂O₂ for 1 h at 37°C) were used in all experiments.

**TUNEL assay**

For the evaluation of DNA fragmentation, a commercial kit [In situ Cell Death Detection Kit, Fluorescin, Takara, Japan] based on an enzymatic reaction of labelling free 3'-OH termini was used. In brief, 3.10^6 cells were washed with phosphate-buffered saline (1xPBS, pH 7.4) then fixed with 200 μl of 4% paraformaldehyde for 1 h at room temperature in the dark. After wards, sperm cells were washed with 1xPBS and permeabilised using 0.1% Triton X-100 in 0.1% sodium citrate for 15 min on ice. After washing with PBS, sperm DNA was labelled by incubating spermatozoa with 50 μl of the TUNEL reaction mixture (TdT enzyme and FITC-labelled nucleotides) in a humidified atmosphere for 60 min at 37°C in the dark, with mixing each 15 min. Washed and labelled sperm cells were then resuspended in 1xPBS for flow cytometry analysis. A negative control (sample without the addition of TdT enzyme) and a positive control (sample treated with DNase I [3 U/ml, Invitrogen] for 10 min at room temperature to generate DNA strand breaks) were also assessed by TUNEL assay.

**Flow Cytometry and data analyses**

Flow cytometric analysis was carried out using an EPICS XL flow cytometer (Beckman Coulter) equipped with a 15mW argon-ion laser for excitation at 488 nm. At least 10,000 events per sample were analysed. Light-scattering and fluorescence data were obtained at a flexed gain setting in logarithmic mode. Debris was excluded by establishing a region around the population of interest on the basis of light scatter characteristics (forward-angle light scatter (FSC) vs. side-angle light scatter (SSC). The percentage of labelled sperm was characterized by identifying a region that included >90% of events in the frequency histogram of the positive controls both in the assessments of Viability, ΔΨm, Caspase 3 activation and DNA fragmentation. Data were expressed as percentage of stained cells from histograms using System II software. Typical examples of histograms obtained by flow cytometry for the detection of fluorescence are shown in Figure 1 (sperm viability), Figure 2 (ΔΨm), Figure 3 (Caspase 3 activation) and Figure 4 (TUNEL assay).

**Statistical analysis**

The SPSS 18.0 software (SPSS Inc, Chicago, Ill) was used for statistical analysis. Test χ² was used to compare frequencies. Non-parametric test (Mann-Whitney) from SPSS software was used to compare distribution sperm parameters and flow cytometry data of infected and uninfected men. Correlation between semen parameters means, ΔΨm, DNA fragmentation and caspase 3 activation and C. trachomatis infection was assessed using T-test. All tests were considered statistically significant when p<0.05.

**Results**

**Spermiocultures analysis**

Spermioculture analysis was positive in 6 cases (7%). Group B Streptococcus (GBS) was found in 3 samples (3.5%), Enterococcus spp in 1 sample (1.1%), Staphylococcus aureus in 1 sample (1.1%) and Corynebacterium spp in 1 sample (1.1%).

**Frequency of urogenital bacteria in semen samples using qPCR**

Among 85 semen samples, 13 (15.2%) were positive for C. trachomatis and 5 (5.8%) for N. gonorrhoeae. U. urealyticum, U. parvum and M. hominis were detected in 5 patients (5.8%), 3 patients (3.5%), 1 patient (1.1%) and 1 patient (1.1%) respectively. The distribution of detected species in patients is shown in table 2.

**C. trachomatis infection and semen quality**

The mean values (±SD) for semen parameters of the 85 included patients are shown in Table 3. The sperm vitality and total motility of spermatozoa in the male partners of infertile couples with C. trachomatis DNA in semen specimens were lower but not significantly that those of uninfected male partners (71.3% vs 73.3%, p = 0.65 and 41.1% vs 43.9 %, p = 0.39, respectively) (Table 3). The sperm concentration and rapid progressive motility category a of spermatozoa in C. trachomatis DNA positive semen were significantly lower than those of uninfected semen (41.4×10^9/ml vs 84.4×10^9/ml, p = 0.02 and 8.8% vs 12.6%, p = 0.04, respectively) (Table 3). The leukocyte count in the male partners of infertile couples with C. trachomatis DNA in semen specimens was higher but not significantly than those uninfected semen (0.8×10^9/ml vs 0.4×10^9/ml, p = 0.36) (Table 3).

**Sperm viability using 7-aminooactinomycin-D Dye**

Figure 1 presents frequency distribution histograms of negative control (Fig. 1A), positive control (Fig. 1B), and one semen of male partners of infertile couples positive for C. trachomatis qPCR (Fig. 1C). The percentages of viable sperm cells (cells with 7-AAD negative) were assessed in semen specimens of male partners of infertile couples positive for C. trachomatis qPCR and uninfected men. The mean proportion of viable spermatozoa (±SD) in uninfected patients was 63.2±13.9%, while it decreased to...
51.3±21.13% in patients positive for *C. trachomatis* qPCR with a statistically significant difference (*p* = 0.014) (Table 4).

**Mitochondrial Membrane Potential (ΔΨm)**

Analysis of the state of mitochondrial respiration in human spermatozoa was assessed using JC-1 to determine the ΔΨm as shown in Figure 2. Flow cytometry results are expressed as percentage of sperm cells with low ΔΨm (green fluorescence). Figure 2 presents frequency distribution histograms of negative control (Fig. 2A), positive control (Fig. 2B), and one semen of male partners of infertile couples positive for *C. trachomatis* qPCR (Fig. 2C). The mean percentage of spermatozoa with low ΔΨm (±SD) was higher in male partners of infertile couples positive for *C. trachomatis* qPCR than those of uninfected patients (33.7±13.3% vs. 24.5±9.7%) and the difference was statistically significant (*p* = 0.006) (Table 4).

**Caspase 3 activation**

The results of flow cytometry are expressed as percentage of activated caspase 3 sperm cells. Figure 3 presents frequency distribution histograms of negative control (Fig. 3A), positive control (Fig. 3B), and one semen of male partners of infertile couples positive for *C. trachomatis* qPCR (Fig. 3C). Mean percentage of spermatozoa with activated caspase 3 (±SD) was higher in male partners of infertile couples positive for *C. trachomatis* qPCR than those of uninfected patients (54.5±18.1% vs. 20.8±14%) and the difference was statistically significant (*p* < 0.001) (Table 4).

**DNA fragmentation**

TUNEL coupled flow cytometry results are expressed as percentage of DNA fragmented sperm cells. Figure 4 presents frequency distribution histograms of negative control (Fig. 4A), positive control (Fig. 4B), and one semen of male partners of infertile couples positive for *C. trachomatis* qPCR (Fig. 4C). Mean percentage of spermatozoa with DNA fragmentation (±SD) was higher in male partners of infertile couples positive for *C. trachomatis* qPCR than those of uninfected patients (29.2±17.2% vs. 25.1±14.3%). But the increase in sperm DNA fragmentation remains statistically not significant (*p* = 0.62) (Table 4).

**Discussion**

The importance of genital tract microorganisms as an etiologic factor in male infertility is still a controversial topic [39]. The purpose of this study was to determine the prevalence of several common sexually transmitted pathogens among male members of infertile couples. Asymptomatically infected individuals may carry lower amount of organisms [40]. Besides, real time PCR is easier and has higher sensitivity and specificity. Thus, real time PCR may be the technique of choice for bacterial detection and quantification in semen specimens of asymptomatic male partners. Our study demonstrated that *C. trachomatis* seems to be the most widespread sexually transmitted pathogen among male partners of infertile couples.

Figure 1. Flow cytometric of sperm viability using 7-amino-actinomycin-D Dye. Histograms show: (A) Negative control with 10% 7-AAD positive cells. (B) Positive control with 98.5% 7-AAD positive cells. (C) Semen sample of one male partner of infertile couples positive for *C. trachomatis* qPCR with 56.5% 7-AAD negative cells and 43.5% 7-AAD positive cells. B: window adjusted to detect the percentage of cells with 7-AAD positive.

doi:10.1371/journal.pone.0098903.g001
infertile couples in Sfax (South of Tunisia), as shown by its high prevalence. Our findings confirm previous reports among male partners of infertile couples in Tunisia [41], with lower frequency (15.2% vs 43.3%). This difference might be explained by the use of different methods for the detection of this bacterium. We have used a quantitative real time PCR, which is more specific than in-house PCR-microtiter plate hybridization method. The prevalence of *N. gonorrhoeae* in our study was (5.8%) among male partners of infertile couples. This prevalence was higher than that previously reported in recent studies conducted in other country such as in Canada [42] and in our country [41]. This prevalence of *N. gonorrhoeae* (3.8%) was nearly similar to that reported in other recent studies [43] in Jordan (6.5%). In addition, the qPCR used in our study was reported to be highly sensitive and specific by two authors [44–45]. The results of this study, also revealed that the prevalence of *M. genitalium* (3.5%) in infertile men is nearly similar with that reported by Gdoura et al (2008) (4.8%) in our country and Al-Sweih et al (2012) in Jordan (3.2%) [41–46]. Surprisingly, the prevalence of *U. urealyticum* (3.8%) found in our study was considerably lower than previously reported in our country by Gdoura et al (2008) [41]. In the literature, the prevalence of *U. urealyticum* in the semen samples of male infertile patients varies from 5% to 42% [47,48–49]. This wide range might be explained by the diversity of detection methods used for characterizing the studied populations. Most of the previous reported studies have discussed the role of *Ureaplasma* in male infertility without discriminating between *U. urealyticum* and *U. parvum* [47–50]. In our study, we used a quantitative real time PCR for facilitating the detection and quantification of *U. urealyticum*, *U. parvum*, *M. hominis*, and *M. genitalium* in semen specimens. By this method, *U. parvum* was detected in only one patient (1.1%). The prevalence of this species in our study was lower than that reported by Knox et al (2003) (19.2%) and was nearly similar to that reported by Gdoura et al (2008) in our country (2.9%) [41–48]. In the literature, *M. hominis* has been associated with bacterial vaginosis, pelvic inflammatory disease in women [51]. However, its role in nongonococcal urethritis and in infertility was rarely investigated [52]. The prevalence of *M. hominis* in our study was (1.1%) comparable to that reported by Rosemond et al (2006) (0%) but less than that found by Gdoura et al (2008) (9.6%) [41–53]. The role of *C. trachomatis* infection on semen parameters in male infertility is controversial. In fact, a large number of studies have suggested that positive markers for *Chlamydia* infection are not associated with altered sperm parameters [18,19,46,54–55]. Others, however, have found that *Chlamydia* infection correlates with reduced sperm motility [34–56], increased proportion of sperm abnormalities [57], significant reductions in semen density, sperm morphology, and viability [58] and increased likelihood of leukocytospermia [34]. In addition, Veznik et al (2004) reported decreases in seminal plasma, sperm motility, velocity, and normal morphology in *C. trachomatis*-infected infertile patients compared with those without infection [59]. Mazzoli et al (2010) found that *C. trachomatis* affects sperm concentration, percentage of motile sperm and normal morphological forms in patients with prostatitis [12].

Figure 2. Flow cytometric of changes in the mitochondrial membrane potential (∆Ψm). Histograms show: (A) Negative control with 95.8% low ∆Ψm cells. (B) Positive control with 9.6% low ∆Ψm cells. (C) Semen sample of one male partner of infertile couples positive for *C. trachomatis* qPCR with 32.5% low ∆Ψm cells and 67.5% with high ∆Ψm cells. C: window adjusted to detect the percentage of cells with low ∆Ψm.

doi:10.1371/journal.pone.0098903.g002
A final conclusion from all studies is difficult to establish due to the diversity of population on one hand and variability in sensitivity and specificity of used techniques on the other hand. Moreover, during infertility assessment, infertile couples are not systematically screened for this infection, hence clinically silent C. trachomatis infection may be revealed by complications. In fact, the mean duration of infertility in our study was 4 years and patients consulted at different stages of the infection. Lastly, we showed that inoculation of fertile male Swiss mice in the meatus urethra with C. trachomatis could lead to alteration of semen parameters (the sperm motility, viability, morphology and sperm concentration) [35]. Our study are concordant with our latter experimental study, the sperm concentration and rapid progressive motility (category a) of spermatozoa in the male partners of infertile couples with C. trachomatis DNA in semen specimens showed a significant decrease in comparison with those without infection. Moreover, the sperm vitality and total motility of spermatozoa in the male partners of infertile couples with C. trachomatis DNA in semen specimens was lower but without significances compared to patients without infection. The leukocytes count in the male partners of infertile couples with C. trachomatis DNA in semen specimens was higher but without significances compared with those without infection. Thus, C. trachomatis infection could lead to a decrease in sperm quality.

Apoptosis is a mode of programmed cellular death based on a genetic mechanism that induces a series of cellular, morphological and biochemical alterations, leading the cell to suicide without eliciting an inflammatory response. Mature sperm cells have been reported to express distinct markers of apoptosis-related cell damage [60–61]. Externalization of PS to the sperm outer membrane brochure is considered to mark terminal apoptosis. Activated caspase-3, loss of the integrity of the ΔΨm and DNA fragmentation are other markers of terminal apoptosis expressed by a varying proportion of ejaculated sperm [25–62]. It has been hypothesized that sperm cell death is associated with male infertility [63–64]; however, the exact mechanisms of its involvement remain to be elucidated [65]. Sperm apoptosis and dysfunction have also been reported after sperm exposure to C. trachomatis both in vivo and in vitro. In vitro studies have shown that the coincubation of human sperm with C. trachomatis serovar E causes a significant decline in the percentage of motile sperm and results in premature sperm death [33]. This sperm death has been demonstrated to be primarily caused by LPS [32]. Moreover, it has been shown that Chlamydial LPS interact with CD14 on the sperm surface, leading to increased production of reactive oxygen species and resulting in caspase-mediated apoptosis by using a fluorogenic substrate [29]. Lastly, Satta et al (2006) observed that the experimental C. trachomatis infection causes sperm PS externalization and DNA fragmentation [30]. In vivo studies have reported a higher frequency of sperm cells with fragmented DNA in infertile subjects with C. trachomatis genitourinary infection than in control fertile subjects, using the sperm chromatin dispersion test [34]. Moreover, our experimental mouse model has also showed a significant increase of apoptotic and necrotic spermatozoa percentages in infected mice when compared with the control group [35]. In line with these findings, our data demonstrated a direct role of C. trachomatis in apoptosis. In order to elucidate the implication of apoptosis in infected semen with C. trachomatis DNA, we studied in the first part of our study the viability of spermatozoa using 7-AAD vital stain dye. We found a

Figure 3. Flow cytometric caspase 3 detection histograms. (A) Negative control with 0.85% FITC labelled cells. (B) Positive control with 95.8% FITC labelled cells. (C) Semen sample of one male partner of infertile couples positive for C. trachomatis qPCR with 32.5% FITC labelled cells. D: window adjusted to detect the percentage of cells exhibiting caspase 3 activation. doi:10.1371/journal.pone.0098903.g003
significant decrease of the mean percentage of viable spermatozoa (7-AAD negative) in male partners of infertile couples with C. trachomatis DNA in comparison with uninfected male partners of infertile couples. C. trachomatis infection was more correlated negatively with the viability measured using 7-AAD dye than with the viability measured using eosin staining. 7-AAD Dye is more objective than eosin staining. In the second part of our study we studied the state of mitochondrial membrane potential in semen using the lipophilic fluorescent probe JC-1. JC-1 probe has been validated in the assessment of stallion and bull spermatozoa using Flow Cytometry [66–67] and provides a more rigorous estimate of metabolic function than Mito Tracker or Rhodamine 123 [67]. In

![Figure 4](image)

**Figure 4. TdT (terminal deoxynucleotidyl transferase)-mediated dUTP nick-end labeling (TUNEL) assay of spermatozoa.** Histograms show: (A) negative control with 2.35% TUNEL positive cells. (B) Positive control (spermatozoa treated with DNaseI) with 90.5% TUNEL positive cells. (C) Semen sample of one male partner of infertile couples positive for C. trachomatis qPCR with 20.5% TUNEL positive cells. B: window adjusted to detect the percentage of TUNEL positive cells.

doi:10.1371/journal.pone.0098903.g004

| Table 2. Frequency of urogenital bacteria detected by qPCR and spermiocultures analysis in semen samples of 85 infertile male patients. |
|---------------------------------|-------------------------------------------------|-------------------------------------------------|
| Species                        | Patients N = 85                                  | Frequency (%)                                   |
| **qPCR**                       |                                                 |                                                 |
| C. trachomatis                 | 13                                              | 15.2                                            |
| N. gonorrhoeae                 | 5                                               | 5.8                                             |
| M. genitalium                  | 3                                               | 3.5                                             |
| M. hominis                     | 1                                               | 1.1                                             |
| U. urealyticum                 | 5                                               | 5.8                                             |
| U. parvum                      | 1                                               | 1.1                                             |
| **Spermiocultures**            |                                                 |                                                 |
| Group B Streptococcus          | 3                                               | 3.5                                             |
| Staphylococcus aureus          | 1                                               | 1.1                                             |
| Enterococcus spp               | 1                                               | 1.1                                             |
| Corynebacterium spp            | 1                                               | 1.1                                             |

doi:10.1371/journal.pone.0098903.t002
In our study, we found a significant increase of the mean percentage of spermatozoa with low $\Delta \Psi_m$ in male partners of infertile couples with *C. trachomatis* DNA in semen specimens compared to male partners of infertile couples without *C. trachomatis* DNA in semen specimens. At our knowledge, our study represents the first study to characterize the state of $\Delta \Psi_m$ in spermatozoa of infertile couples with *C. trachomatis* infection. In line with this study, we noticed a slight increase in sperm DNA damage in male partners of infertile couples with *C. trachomatis* DNA in semen specimens in comparison with male partners of infertile couples without *C. trachomatis* infection had significantly greater sperm DNA fragmentation than fertile control subjects [34]. These results suggest that *C. trachomatis* and *Mycoplasma* infections had significantly greater sperm DNA fragmentation than fertile control subjects [34]. These results indicate that *C. trachomatis* and *Mycoplasma* infections may affect sperm DNA in vitro. In line with this study, we noticed a slight increase in sperm DNA damage in male partners of infertile couples with *C. trachomatis* DNA in semen specimens in comparison with male partners of infertile couples without *C. trachomatis* DNA in semen specimens. The limitations of our study were firstly the low number of our population (only 85 infertile men) and secondly the absence of a control groups composed of fertile men. Thus, we have limited our comparison between semen from infected and uninfected infertile men with *C. trachomatis*.

In conclusion, using a quantitative Real time PCR our study indicated that this PCR provides a sensitive measure to detect human *C. trachomatis*, genital *Mycoplasmas*, and genital *Ureaplasmas* DNA, which is useful for epidemiologic studies of these pathogens. Our results also demonstrated that *C. trachomatis* seems to be widespread among male partners of infertile couples in Sfax (South of Tunisia). This study supports that *C. trachomatis* infection could lead to a decrease in sperm quality and apoptosis induction. In fact, *C. trachomatis* infection was found to increase the $\Delta \Psi_m$.

### Table 3. Seminological variables of semen of *C. trachomatis* positive patients compared to uninfected patients.

| Variables                        | Total Semen | Uninfected Semen | *C. trachomatis* positive semen | $p$ value $^a$ |
|---------------------------------|-------------|------------------|---------------------------------|----------------|
| Sperm concentration (x10^6/ml)  | 71.1 ± 60.1 | 84.4 ± 64.6      | 41.4 ± 42.7                     | 0.02           |
| Vitality (%)                    | 72.4 ± 14.7 | 73.3 ± 14.7      | 71.3 ± 16.4                     | 0.65           |
| Total progressive motility (category [a+b]) (%) | 41.2 ± 12.2 | 43.9 ± 10.6      | 41.1 ± 10.4                     | 0.39           |
| Rapid progressive motility (category [a]) (%) | 10.8 ± 7.4 | 12.6 ± 7         | 8.8 ± 5.4                       | 0.04           |
| Leukocyte count (x10^5/ml)      | 0.55 ± 1.4  | 0.4 ± 1.5        | 0.8 ± 1.2                       | 0.36           |

Note: Values are means (# Standard Error).

$^a$Unless indicated, variables were tested T-Test.

doi:10.1371/journal.pone.0098903.t003

### Table 4. 7-AAD, $\Delta \Psi_m$, caspase 3 activation and sperm DNA fragmentation of semen of *C. trachomatis* positive patients compared to uninfected men.

| Parameters                      | Uninfected Semen | *C. trachomatis* positive semen | $p$ value $^a$ |
|--------------------------------|------------------|---------------------------------|----------------|
| Negative 7-AAD (%)             | 63.2 ± 13.9      | 51.3 ± 21.1                     | 0.014           |
| Low $\Delta \Psi_m$ (%)        | 24.5 ± 9.7       | 33.7 ± 13.3                    | 0.006           |
| CP 3 activation (%)            | 20.8 ± 14        | 54.5 ± 18.1                    | <0.001          |
| DNA fragmentation (%)          | 25.1 ± 14.3      | 29.2 ± 17.2                    | 0.62            |

Values are means (# Standard Error).

$^a$Unless indicated, variables were tested T-Test.

7-AAD: 7-amino-actinomycin-D.

$\Delta \Psi_m$: Mitochondrial membrane potential.

CP3: Caspase 3.

doi:10.1371/journal.pone.0098903.t004
dysfunction in spermatozoa and caspase 3 activation. However, sperm DNA damage was not significantly associated to C. trachomatis infection. This leads us to suggest that caspase 3 could be implicated during C. trachomatis infection but does not cause directly DNA damage.

Author Contributions
Conceived and designed the experiments: HS AZ RG AS. Performed the experiments: HS SB AZ HM NL. Analyzed the data: HS AZ RG AS HM. Contributed reagents/materials/analysis tools: HM TR AH LK. Wrote the paper: HS AZ RG AS. Contributed to specimens collection: LK.

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Figure 5. Distributions of percentages of different apoptotic markers among patients positive for C. trachomatis qPCR compared to uninfected patients. (A) Mean percentage of Sperm Vitality, evaluated with 7-amino-actinomycin-D Dye (7-AAD). (B) Mean percentage of Sperm mitochondrial membrane potential ($\Psi_m$), evaluated with JC-1. (C) Mean percentage of Caspase 3 activation, evaluated with fluorescein-labeled inhibitor of caspases (FLICA). (D) Mean percentage of Sperm DNA fragmentation, evaluated with (TUNEL). ** Indicates significant differences compared with uninfected semen (P<0.05). * Indicates significant differences compared with uninfected semen (P<0.001).

doi:10.1371/journal.pone.0098903.g005

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Figure 5. Distributions of percentages of different apoptotic markers among patients positive for C. trachomatis qPCR compared to uninfected patients. (A) Mean percentage of Sperm Vitality, evaluated with 7-amino-actinomycin-D Dye (7-AAD). (B) Mean percentage of Sperm mitochondrial membrane potential ($\Psi_m$), evaluated with JC-1. (C) Mean percentage of Caspase 3 activation, evaluated with fluorescein-labeled inhibitor of caspases (FLICA). (D) Mean percentage of Sperm DNA fragmentation, evaluated with (TUNEL). ** Indicates significant differences compared with uninfected semen (P<0.05). * Indicates significant differences compared with uninfected semen (P<0.001).

doi:10.1371/journal.pone.0098903.g005

Author Contributions
Conceived and designed the experiments: HS AZ RG AS. Performed the experiments: HS SB AZ HM NL. Analyzed the data: HS AZ RG AS HM. Contributed reagents/materials/analysis tools: HM TR AH LK. Wrote the paper: HS AZ RG AS. Contributed to specimens collection: LK.
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