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Detection of *Chlamydia trachomatis* inside spermatozoa using flow cytometry: Effects of antibiotic treatment (before and after) on sperm count parameters

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ABSTRACT

There is increasing evidence that *Chlamydia trachomatis* (CT) infection can directly affect male fertility. However, only few have investigated the effects of CT on semen parameters, and mostly with inconclusive results. The main aims of this study were to identify CT inside spermatozoa, and the possible pre and post antibiotic treatment effects on the overall semen parameters. We developed a flow cytometric method for the detection of CT inside spermatozoa (SPI™). Briefly, sperm cells were fixed, membrane permeabilized and DNA was loosened using DNAse. Sperm cells were incubated with a primary monoclonal antibody against CT and with a secondary fluorescent antibody (vs primary), and analysed using a flow cytometer. Of 2415 infertile individuals, 48.61% were found positive for CT. 170 CT+ samples were included in the CT antibiotic treatment study. 78.82% (134/170) of the CT+ showed a significant reduction in the percentage of the iCT infected spermatozoa after the antibiotic treatment; 59.70% (80/134) decreased to non–detectable levels. Spermcount data were also recorded. Spermatozoa morphology (normal and teratozoospermia index, TZI) and motility (fast progressive and non–progressive spermatozoa) were statistically significant altered in CT+ pre–treatment vs control group. CT antibiotic treatment showed statistically significant effects on normal spermatozoa morphology, mid–piece and tail defects, and TZI. The study demonstrated that semen flow cytometric analysis of semen could be a valuable tool for faster and accurate identification of individuals with asymptomatic CT infection. It also identified a positive effect of antibiotic therapy on semen parameters, that could help males with infertility.

1. Introduction

Chlamydia trachomatis (CT) infection is the leading bacterial cause of sexually transmitted diseases (STD) worldwide (Mackern-Oberti et al., 2013). CT belongs to one of the three species of the genus *Chlamydia* (*Chlamydiaceae* family). It is a Gram–negative aerobic, obligate, intracellular bacterium, with ~1.0 Mb genome size, encoding ~900 genes, encloses a 7.5 Kb double stranded (ds) DNA plasmid, and is reliant on the host's proliferating cells for its metabolic requirements (like viruses) (Thomson et al., 2008; Clarke, 2011; Abdelsamed et al., 2013; Elwell et al., 2016). CT has three distinct biovars, that are subdivided further into serovars depending on the bacteria's major outer membrane protein (MOMP) (Seth-Smith et al., 2013). Serovars A–C cause trachoma (non–congenital blindness), serovars D–K cause STDs, and serovars L1–L3 are

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the major cause of lymphogranuloma venereum (LGV) (Elwell et al., 2016; Taylor-Robinson, 2017). CT has a distinct biphasic developmental cycle. The first distinct form is the *elementary body* (EB), a small (0.2–0.3 μm), non–dividing extracellular infectious particle, with a spore–like cell wall, stabilized by crosslinked proteins with disulfide bonds. The second form is the *reticulate body* (RB) an intracellular, larger (0.6–1.5 μm) non–infectious, metabolically active form, specialized in replication and nutrient acquisition (Clarke, 2011; Elwell et al., 2016; Mpiga and Ravaoarinoro, 2006; Cossé et al., 2018). The EBs enter the mucosal cells, inside a membrane bound specialized compartment called the *inclusion*, and immediately differentiate into RBs. After a number of complete binary fissions, and towards the end of their cycle (48–72 h), RBs are re–differentiated back to EBs, and are released from the cell, either by lysis (and eventually necrosis of the cell) or by remaining inside the inclusion and extruded out of the cell, in a process similar to exocytosis (Clarke, 2011; Elwell et al., 2016; Cossé et al., 2018).

The impact of *Chlamydia* infection on a couple's infertility is internationally accepted (Zhou et al., 2022). However, most of the studies have focused on the female reproductive tract, with less emphasis given on the implications of CT infections on male fertility (Mackern-Oberti et al., 2013). In women, *Chlamydia* infections are usually clinically manifested as cervicitis, endometritis, salpingitis; both asymptomatic and symptomatic infections can cause complications, such as permanent scarring and functional impairment of the mucous membranes, chronic pelvic inflammatory disease and fallopian tube obstructions, leading to high risk of ectopic pregnancies and other important infertility related implications (Mackern-Oberti et al., 2013). In men, CT infections are usually thought of as a major cause of non–gonococcal urethritis, epididymitis, and prostatitis (Mackern-Oberti et al., 2013; Pacey and Eley, 2004; Motrich et al., 2018). Especially now, it is believed that CT can persist in the prostate, avoid the immune system and establish a chronic infection that eventually results in disrupting male fertility (Mackern-Oberti et al., 2013).

However, the correlation of male infertility and the pathophysiology of CT infections still remains controversial (Mackern-Oberti et al., 2013; Pajovic et al., 2013). Although there are studies that have reported that CT does not affect semen parameters, nor impairs male fertility (Puerta Suarez et al., 2017), there is an increasing research body that has provided clear evidence that CT genital infections contribute to male infertility, by affecting the quality of semen parameters (morphology and motility) (Veznik et al., 2004; Sellami et al., 2014), altering sperm DNA and increasing reactive oxygen species (ROS) (Gallegos et al., 2008; Agarwal et al., 2018; Moazenchi et al., 2018; AM et al., 2021; Pérez-Soto et al., 2021), inducing apoptosis (Sellami et al., 2014; Eley et al., 2005; Satta et al., 2006), increasing immunological factors (Pérez-Soto et al., 2021), changing the seminal microbiome (Farahani et al., 2021), increasing the number of leucocytes and spermatozoids (Pajovic et al., 2013). The major reason of the discrepancies in current research opinions is the different methodological approaches used by each one. The aims of the present study were to identify the presence of CT inside spermatozoa of infertile men, its possible negative effects, and the effects of CT antibiotic treatment on the spermcount parameters (concentration, motility, morphology, and vitality), respectively.

2. Materials and methods

2.1. Subject selection, sample size and location

Semen samples were provided from men attending the Locus Medicus Diagnostic and Research Center (Athens, Greece), over a 5–year period, as part of a work–up investigation for infertility, and subsequently failure to conceive with their partner, after regular intercourse with no contraception for more than one year. A total of 2,415 individuals were tested for CT from all age groups and educational backgrounds. This retrospective study was approved by the Bioethics and Ethics Committee, Faculty of Medicine, National and Kapodistrian University of Athens (EKPA, 19th of March 2014, protocol number 6805). All patients were asked to sign a written informed consent form.

2.2. Diagnostic and treatment protocols

Infertile men of unknown aetiology attending the Locus Medicus Diagnostic and Research Centre (Athens, Greece), were seen by the same team of doctors, comprised of a pathologist expert in infertility, a urologist, and an andrologist. The protocol started with the documentation of the subject's medical history, followed by the doctor's medical examination. Initial diagnostic tests included: ultrasound of the prostate–testis, doppler testis for exclusion of varicocele, semen examination according to WHO, peroxidase test for leucocyte exclusion, sperm cultures for aerobic, anaerobic (bacteria), mycoplasma and ureaplasma, flow cytometric examination using SPI™ for the detection of intracellular bacteria (CT) and viruses [Herpes Simplex Virus 1, 2 (HSV 1/2) and Cytomegalovirus (CMV)], DNA fragmentation index (data not in the current study), and oxidative stress by measuring 8–hydroxy–2′–deoxyguanosine (8–OHdG; data not in the current study). In case of CT positive individuals, they were included in the antibiotic therapy groups, if they fulfilled the inclusion criteria presented in Section 2.3.1.

The antibiotic protocol was based on the WHO Guidelines for the treatment of CT (World Health Organization, 2016). CT+ males were treated with the following double antibiotic per os treatment: One tablet of doxycycline, 100 mg twice a day; and one tablet of roxithromycin, 300 mg once daily, for a total of 16 days. Re–examination of CT by SPI™ was carried out at least 10 days after the end of the antibiotic treatment.

2.3. Antibiotic therapy groups

2.3.1. Inclusion criteria for the CT antibiotic therapy and Control groups

Inclusion criteria for the positive (CT+ group, $N = 170$) and the control groups were:

i) Initial semen analysis for CT infection was positive. Only applicable to CT+ group.
ii) No history of past infections and no antibiotic therapy taken prior (three months) to the first examination. Applicable to both groups.
iii) Re–examination for CT did not exceed two months. Applicable for both groups.
iv) No history of past infections and no antibiotic treatment taken prior to the first examination (three months). Applicable for both groups.
i) Low number of leucocytes was present in the semen samples. Applicable for both groups.

The control group (CT– group) was comprised from men found negative for CT, out of the total 2,415 individuals.

2.4. Semen analysis

Semen parameters were analysed according to the guidelines set by WHO (2021). Briefly, men were asked to abstain from sexual intercourse for 2–3 days prior to ejaculation. Samples were provided onsite. Following collection into standard containers, samples were left to liquefy at 37 °C in an incubator and were analysed immediately, no longer than one hour after liquefaction. Additionally, for each sample, semen smears were taken and stained according to the Papanicolaou staining procedure, and the spermatozoa morphology was analysed according to Tygerberg criteria (World Health Organization, 2021). For counting white blood (WBC) cells, the LeucoScreen Plus (FertiPro nv, Belgium), a semi–quantitative histochemical kit for determining peroxidase–positive WBCs in semen samples, from other round cells was used, according to the manufacturer instructions (El Feky et al., 2009;

FertiPro NV, 2022).

2.5. Experimental materials and methods

2.5.1. Materials used in flow cytometry method

The following materials were used:

- Anti–*Chlamydia trachomatis* MOMP, monoclonal antibody, clone 10B2228, IgG2a, 1 mg/ml (US Biological, USA). Final concentration: 1/ 5,000. Used for CT staining.
- Purified IgG2a, k isotype control, clone MG2a–53, 500 μg (Biolegend, USA). Final concentration: 1/ 2,500. Used as control of the staining.
- Anti–mouse PE, Goat polyclonal antibody, F(ab')2 (DAKO, USA). Final concentration: 1/ 500. Used as a secondary antibody to the primary for staining.
- PE anti–human CD45, clone HI30 (Biolegend, USA). Final concentration: 1/ 40. Used to stain leucocytes.
- 7–Amino–Actinomycin D, 7AAD (BD Pharmingen™, USA). Final concentration: 1 μl/ 100 μl.

Ready to use nucleic acid dye solution. For exclusion of non–viable cells in flow cytometric analysis. In our method, because the WB contained 0.1% saponin, 7–AAD was used to dye all nucleic acid positive cells.

2.5.2. Detection of CT in semen samples by flow cytometry

The Sperm Pathogen Immunophenotyping (SPI™) test, a new patented (EU patent: EP 13721796.4/24.10.2014, entitled *"Method of intracellular infectious agent detection in sperm cells"*. International Patent: PCT/GR2013/000016/29.03.2013) flow cytometric (FC) diagnostic method developed in our laboratories was used to detect CT inside spermatozoa.

2.5.3. Sperm pathogen immunophenotyping (SPI™) flow cytometry method

a. Sample preparation:

After semen liquefaction, 200 μl were taken and placed in a 5 ml Falcon round bottom tube for FC (Corning, USA). Sperm cells were washed twice using 2 ml phosphate buffer saline (PBS, Sigma–Aldrich, USA), mixed gently and centrifuged at 300*g* for 5 min.

b. Fixation:

Supernatant was decanted and sperm cells were fixed in 4% paraformaldehyde (PFA) (Sigma–Aldrich, USA) solution in 1× PBS and stored at 4 °C for minimum of 30 min. Following fixation, cells were centrifuged at 300*g* for 5 min.

c. Permeabilization:

Supernatant was decanted and pellet was incubated with 200 μl of a 4% PFA/ 1× PBS solution with 0.1% saponin (solution A) (Sigma–Aldrich, USA), for 30 min at 4 °C. After incubation, cells were washed with 2 ml washing buffer containing PBS, 2% FBS, and 0.1% saponin (WB) and centrifuged at 300*g* for 5 min. Supernatant was decanted and pellet was incubated with 200 μl PBS solution containing 0.1% saponin and 10% dimethylsulfoxide (DMSO, AppliChem, USA) (solution B), for 10 min at 4 °C. Then, cells were washed with 2 ml of WB, centrifuged at 300*g* for 5 min, decanted and pellet was incubated with 200 μl of solution A, for 10 min at 4 °C.

d. DNAse incubation:

After incubation, cells were washed in WB and centrifuged at 300*g* for 5 min. At this step, supernatant was decanted and pellet was incubated with 10 μl DNAse buffer, 3 μl recombinant DNAse I (1000 U, Takara, Japan) and 90 μl of PBS for 60 min at 37 °C with 5% CO_2 (Fig. 1).

e. Staining steps:

After incubation with DNAse, cells were washed with 2 ml of WB, centrifuged at 300*g* for 5 min, decanted and 200 μl WB were added at the cell pellet. At this point, the cell pellet was aliquoted in 4 Falcon tubes at 30 μl each. Tube 1 was left blank, tube 2 and 4 were incubated with purified IgG2a, k isotype control (clone MG2a–53, 500 μg, Biolegend, USA) and anti–*Chlamydia trachomatis* MOMP, primary mouse mAb (clone 10B2228, IgG2a, 1 mg/ml, US Biological, USA), respectively, for 30 min at 4 °C.

Following incubation, sperm cells were washed twice with WB, centrifuged at 300*g* for 5 min, decanted and pellets in tube 2 and 4 were incubated with polyclonal goat anti–mouse phycoerythrin (PE) secondary antibody (DAKO, USA) and tube 3 was incubated with CD45 PE mouse anti–human antibody, for 30 min at 4 °C.

After incubation, sperm cells were washed twice with WB, centrifuged at 300*g* for 5 min, decanted and pellets in all tubes were incubated with 7–Amino–Actinomycin D (7AAD, BD Pharmingen™, USA), for 8 min at room temperature (R.T.), prior to acquisition.

f. Acquisition:

Finally, ~20.000 events were acquired using a FACSCalibur (Becton Dickinson, USA) equipped with a 488–nm argon laser [band–pass (bp) filters at 530 nm (FL1), 585 nm (FL2) and 670 nm (FL3)] and a 635 nm red diode laser [bp at 661 nm (FL4)], and were analysed using the CELL Quest Pro™ software (Becton Dickinson, US).

2.5.4. Gating strategy

The gating strategy was based on the gating strategy described by Perticarari et al., 2007 (Perticarari et al., 2007b). The entire sperm population was determined based on scattering measurements, i.e., forward scatter (FSC) vs side scatter (SSC) parameters. A region R1 was set on the entire semen cell population (Fig. 2A). Because the R1 also included debris or cells with similar sized and granularities as sperm, the analysis was also based on 7–AAD staining, to allow for a more precise identification of the spermatozoa. Since, the cell pellet was always under WB containing 0.1% saponin (permeabilized), using 7–AAD allowed to stain all cells with nucleic acid, and therefore, excluding debris and other cell bodies (Fig. 2B). A new R2 region was set on the 7–AAD$^+$ cell population. Next, a new dot plot was created using CD45–PE vs SSC–Height, gated from R1 and R2 (Fig. 2C). A new region R3 was created around the sperm cell population, i.e., excluding the CD45$^+$ cells, and therefore making it possible to more precisely identify spermatozoa population. Then, the CT analysis was carried out on two new dot plots (Fig. 2D and E). The new dot plots were gated from R1 and R2 and R3 cell populations, and based on FL4 vs isotype antibody/ anti–mouse PE (Fig. 2D) and FL4 vs CT monoclonal antibody/ anti–mouse PE (Fig. 2E) parameters, respectively. The FL4 was used to minimize spill over or overlapping emission spectra from fluorescence interference created by the dyes. A quadrant cross was made on both plots to measure the percentage of intracellular CT$^+$ spermatozoa.

2.6. Statistical analysis

Data were analysed using the SPSS, version 28.0 (SPSS, USA). For data that were not normally distributed, their non–parametric equivalent statistical tests were used. To investigate the effect of treatment on spermcount parameters, the Wilcoxon test for paired samples was used. Comparison of semen parameters, between the CT+ and control groups (between subjects) was carried out using the nonparametric Mann–Whitney *U*–test. The results in the text and figures are expresses as

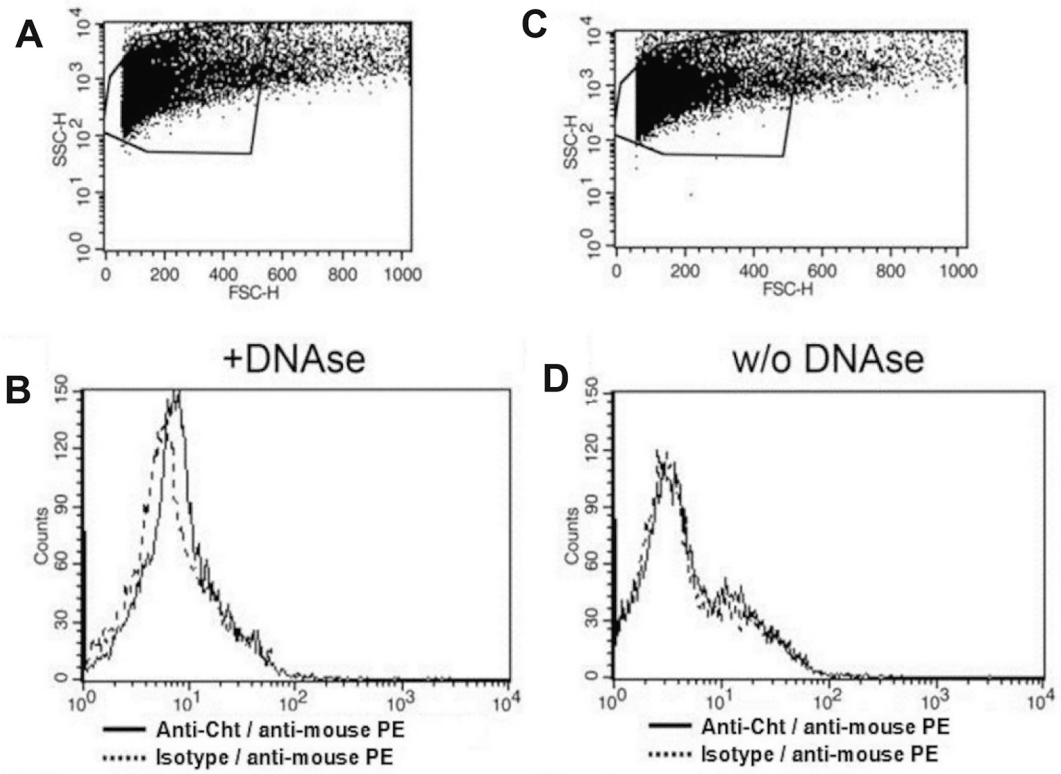

Fig. 1. Effect of DNAse I. Cellular distribution of spermatozoa according to scatter (size and cell complexity) parameters FSC/ SSC. Gate R1 was set in the region of spermatozoa (A & C). The left panels indicate a semen sample that was treated with DNAse (A, B). The right panels indicate the same sample without the step of DNAse treatment (C, D). Note the differences between the isotype controls/ anti–mouse PE and anti–Cht/ anti–mouse PE. SSC–H: Side Scatter; FSC–H: Forward Scatter; PE: Phycoerythrin; Anti–Cht: Anti–*Chlamydia trachomatis* antibody.

mean ± S.E.M. The p value was set at <0.05.

3. Results

3.1. Detection of CT in semen samples by SPI™ flow cytometry

2415 men were tested for CT using the SPI™ FC method. 1174 individuals were identified positive (CT+ group; 48.61%), whereas the rest were tested negative (CT– group; 51.39%). From the CT+ group, 170 cases satisfied the criteria (Section 2.3.1) to be included to the CT antibiotic therapy.

3.2. Demographic, clinical and semen characteristics for the CT antibiotic therapy groups

Table 1. shows the demographic, clinical and basic semen characteristics of the antibiotic therapy groups and controls. Major conditions for the symptomatic individuals included urethritis, epididymitis, chronic prostatitis, frequent urination, and balanitis, in both groups.

3.3. CT antibiotic therapy: overall treatment response

170 CT+ men were included in the antibiotic treatment study. 134 (78.82%) showed a decrease in the percentage (%) of the intracellular CT (iCT) infected spermatozoa, in semen re–examination; 7.06% remained unaltered, whereas 14.12% showed an increased. Further analysis of the 134 CT+ men responding to the CT antibiotic treatment, showed that 40.30% (54/134) had a decrease of the iCT infected spermatozoa, whereas the rest 59.70% (80/134) showed a decrease to non–detectable levels, after the first re–examination.

3.4. iCT presence after antibiotic treatment

Statistical analysis of the 134 individuals revealed that after antibiotic treatment there was a statistically significant decrease in the % of the iCT, from a mean value of 3.36% pre–treatment to a 0.63% post–treatment (Wilcoxon, $N = 134$, $z = -10.044$, 2–tailed, $p = 0.001$).

3.5. CT antibiotic treatment and semen parameters

For the 134 men that responded with reduction in the % of iCT, the effects of antibiotic treatment on the overall semen parameters (concentration, motility, morphology and the teratozoospermia index (TZI)) were also examined. Only 22 CT+ cases (22/134) had both before and after spermcount data. The control group (CT– group) comprised of 67 individuals (67/1241) with spermograms.

3.5.1. CT antibiotic treatment and spermatozoa morphology

The analysis revealed a statistically significant increase in the % of spermatozoa with normal/ typical morphology (Wilcoxon, $N = 22$, $z = -2.717$, 2–tailed, $p = 0.007$); and a statistically significant reduction in the % of spermatozoa with mid–piece (neck) (Wilcoxon, $N = 22$, $z = -3.231$, 2–tailed, $p = 0.001$) and tail defects (Wilcoxon, $N = 22$, $z = -1.966$, 2–tailed, $p = 0.049$), respectively. When compared to the control group, it was found that the CT+ pre–treatment group showed a statistically significant lower % of spermatozoa with normal/ typical morphology ($U = 383.0$, $N_1 = 67$, $N_2 = 22$, 2–tailed, $p < 0.001$). Furthermore, there was a significant decrease in the tail defects between the control group and the CT+ post–treatment group ($U = 532.0$, $N_1 = 67$, $N_2 = 22$, 2–tailed, $p = 0.050$) (Fig. 3). Additionally, the TZI index showed a statistically significant decrease after antibiotic treatment, when compared to both the control ($U = 455.0$, $N_1 = 67$, $N_2 = 22$, 2–tailed, $p = 0.007$) and the CT+ pre–treatment group (Wilcoxon, $N =$

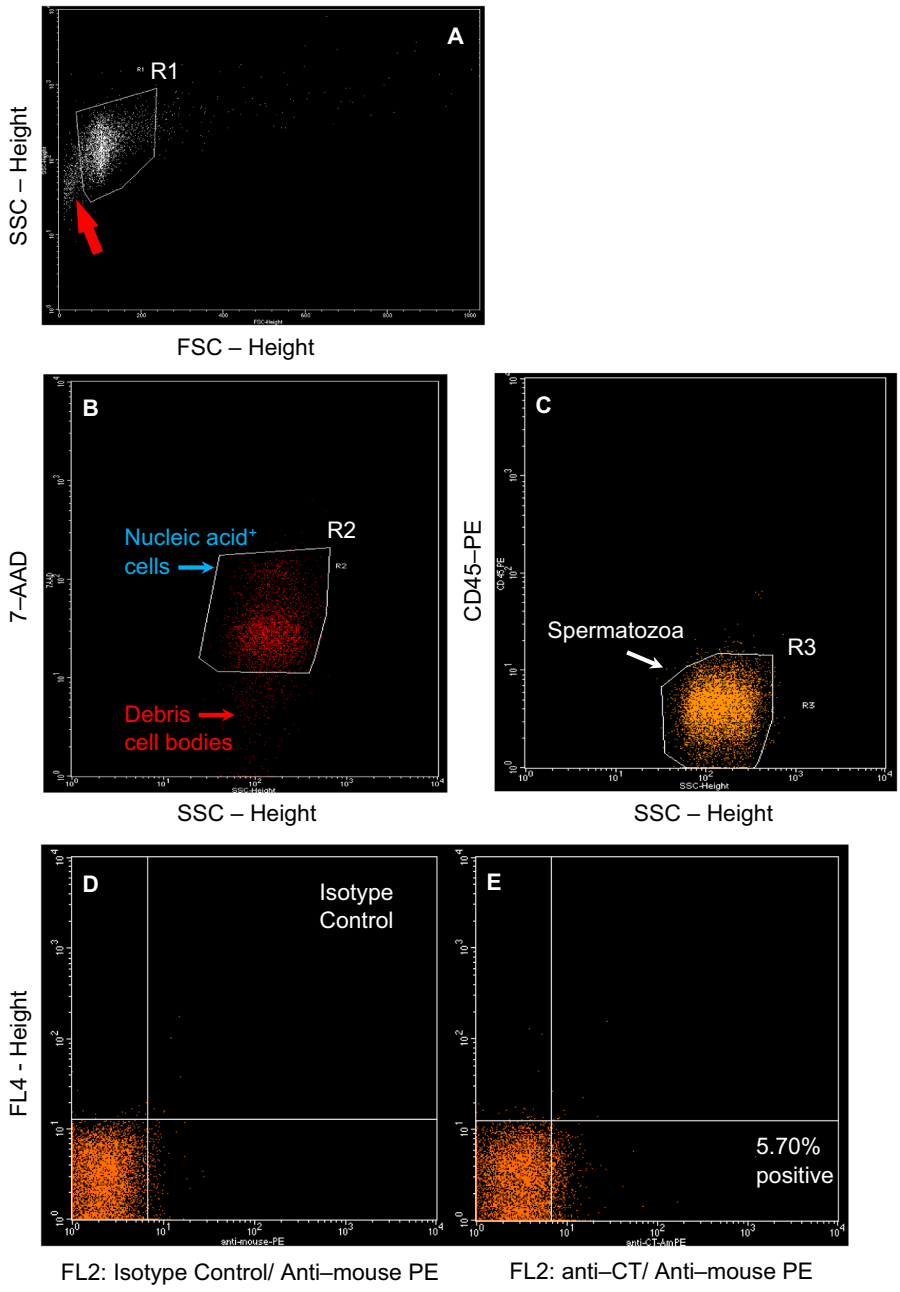

Fig. 2. Flow cytometer data acquisition dot plots of intracellular immunostaining of CT in semen samples. Dot plot A indicates semen population gated according to the SSC vs. FSC scatter parameters. A region R1 was made to identify spermatozoa and exclude debris (red arrow). **Dot plot B** shows all nucleic acid positive cells (blue arrow), identified using 7–AAD. Debris and cell bodies are also shown. **Dot plot C** represent spermatozoa gated from FSC vs SSC (R1) and 7–AAD fluorescence vs SSC (R2) regions, using CD45–PE vs SSC parameters. A new region R3 was set on the $CD45^{-}$ cell population, representing spermatozoa (white arrow). **Dot plots D & E**. Gated from R1 and R2 and R3, dot plot **D** shows the isotype control (FL4 vs Isotype control antibody/ anti–mouse PE) and dot plot **E** shows the test sample (FL4 vs anti–CT/ anti–mouse PE). Quadrant analysis was used to measure the % of intracellular CT. Using this strategy, it was possible to more accurately define the spermatozoa and calculate the % of infected cells. SSC: Side Scatter; FSC: Forward Scatter. PE: Phycoerythrin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

22, $z = -2.972$, 2–tailed, $p = 0.003$), respectively (Fig. 4).

For sperm motility parameters, there was a statistically significant lower % of the sperm motility of the fast progressive spermatozoa (PR) ($U = 512.0$, $N_1 = 67$, $N_2 = 22$, 2–tailed, $p = 0.032$), and a statistically significant higher % of non–progressive motile spermatozoa (NP) of the CT pre–treatment group, when compared to the control group ($U = 424.5$, $N_1 = 67$, $N_2 = 22$, 2–tailed, $p = 0.003$). In addition, NP spermatozoa were statistically significant increased after antibiotic treatment ($U = 360.0$, $N_1 = 67$, $N_2 = 22$, 2–tailed, $p < 0.001$). Interestingly, there was an almost significant decrease of antibiotic treatment on immotile spermatozoa (IM) (Wilcoxon, $N = 22$, $z = -1.901$, 2–tailed, $p = 0.057$) (Fig. 5). No significant differences were found on other motility parameters, semen volume, pH and concentration.

4. Discussion

This research study utilized a novel immunophenotypic FC method (SPI™) for the detection of CT inside spermatozoa. Using this technique, we identified that 48.61% of men, who visited our clinic following fertility issues, tested positive for CT. A study carried out by El Feky et al., (El Feky et al., 2009) using FC analysis, found 46.60% CT+ semen samples, very similar to our result. This finding is significant, since infertility in males has been linked to CT infections (Zhou et al., 2022). Studies have already implicated semen as an important vector of several different pathogens. For instance, viruses, such as HSV, have been found in semen and in seminal cells, and if they are transferred to the embryo, they could lead to miscarriages (Kapranos et al., 2003; Klimova et al., 2010; Michou et al., 2012). Therefore, it is vital to screen males for seminal intracellular pathogens, before natural conception or assisted reproductive techniques (ART), in order to achieve successful

Table 1
Demographic, clinical and basic semen characteristics of the CT antibiotic treatment groups and controls (Mean ± SEM: Standard Error of Mean).

CLINICAL	*CT+ group (n = 170)*	*Control Group (n = 67)*	*P*
Age (years)	*37.5 ± 0.46 (21–52)*	*39.55 ± 0.71 (25–53)*	*NS*
Infertility Duration (Years)	*>1 year*		–
Asymptomatic	*80.68%*	*84.91%*	–
Symptomatic	*19.32%*	*15.09%*	–
Primary Infertility	*56.82%*	*50.94%*	–
Secondary Infertility	*43.18%*	*49.06%*	–
Miscarriages	*0 = 2.63%, 1 = 34.21%,2 = 41.11%, 3 = 18.42%, 4 = 2.63%*	*1 = 19.23%,2 = 34.62%, 3 = 38.46%, 5 = 7.69%*	–
Round cells/ HPF *	*4.34 ± 0.41*	*3.77 ± 0.41*	*NS*
WBC / HPF †	*4.59 ± 0.38*	*4.69 ± 0.37*	*NS*
WBC 1 × 10^6/ ml	*1,147,500 ± 95,000*	*1,172,500 ± 92,500*	*NS*

SEMEN	*Pre–treatment (n = 22)*	*Post–Treatment (n = 22)*		
Volume (ml)	*3.90 ± 0.41*	*3.75 ± 0.35*	*3.67 ± 0.22*	*NS*
pH	*8.37 ± 0.09*	*8.22 ± 0.07*	*8.23 ± 0.05*	*NS*
Concentration / ml	*16.53 ± 4.62*	*20.96 ± 5.28*	*28.00 ± 4.32*	*NS*
Total Concentration	*66.31 ± 19.30*	*84.23 ± 26.21*	*95.66 ± 14.17*	*NS*
Live Spermatozoa ‡	*62.45% ± 3.11%*	*64.28% ± 2.81%*	*63.26% ± 2.18%*	*NS*
Dead Spermatozoa ‡	*37.55% ± 3.11%*	*35.32% ± 2.81%*	*36.73% ± 2.18%*	*NS*

* *HPF: High Power Field. Round cells except WBC.* † *LeucoScreen Plus staining kit.* ‡ *Negrosin – Eosin staining. p = 0.01.*

fecondations and pregnancies. As CT infections could alter semen parameters that subsequently have important implications on male infertility (Zhou et al., 2022), screening for CT is to be considered vital.

Our results clearly showed that CT infection had negative effects on semen parameters, such as motility and morphology. Despite the fact that our results are in parallel with reports from other groups that have associated CT infections with decreased sperm concentration, motility, altered semen pH, reduced volume, and altered morphology (Pajovic et al., 2013; Veznik et al., 2004; AM et al., 2021), others have revealed no correlations between the two (Puerta Suarez et al., 2017; Farahani et al., 2021). The above discrepancies could be largely due to the different experimental approaches of each study.

In the present study, an antibiotic treatment consisting of doxycycline and erythromycin for 16 days was followed. However, not all individuals responded positively to the antibiotic therapy. Under certain conditions, antibiotics could favor persistence, instead of resolving the infection (Mpiga and Ravaoarinoro, 2006). Persistence is the state where CT has entered a metabolically inactive, non–infectious, aberrant form, after "sensing" alterations in the microenvironment (deprivation of essential amino acids, like tryptophan and cysteine, decreased inflammation response due to low cytokine secretion, etc) (Mpiga and Ravaoarinoro, 2006). Therefore, it can be extrapolated that the proportion of individuals that did not respond to the treatment could have an ongoing persistent CT infection, contributing to the development of chronic disease, and therefore will present positive on subsequent re–examination.

On the other hand, administration of the CT antibiotic therapy showed significant improvements on spermcount parameters. The increased NP spermatozoa, could be the result of the large decrease observed in the IM spermatozoa (Fig. 5; 44.77% to 38.00%). This decrease, which continues under the level of the control group was very interesting and could be attributed to the effects of antibiotic therapy against other potential pathogens that were not identified. Regarding viable spermatozoa, our analysis showed no significant differences, indicating that CT infection or antibiotic treatment did not affect viability. It would be interesting for future investigation to see whether apoptotic markers are increased during the CT infection, as others have suggested (Sellami et al., 2014; Satta et al., 2006), and whether apoptotic markers are decreased after antibiotic treatment. Therefore, the positive effects of CT antibiotic therapy on spermcount parameters could have significant implications on male fertility and future pregnancies.

Spermatozoa are not considered to be the cell of choice, when it comes to CT's targeting repertoire. The first cell that CT will invade (urogenital infections) are polarized epithelial cells of mucosal surfaces (Cossé et al., 2018). In males, the epithelium of penile urethra is the primary site of infection (epididymis and orchitis), and resulting in impaired sperm function, and subsequently infertility (Cunningham and Beagley, 2000) (Fig. 6). Studies in mice have detected *Chlamydia* infected Sertoli cells, resulting in decreased sperm concentration, motility, and morphology (Sobinoff et al., 2015). EBs are more likely to infect Sertoli cells, since the latter have a full composition of organelles [Golgi apparatus, mitochondria, smooth endoplasmic reticulum (SEM), rough endoplasmic reticulum (RER), microtubules], which are vital for CT's developmental cycle, but most of them are not present in sperm cells (Elwell et al., 2016). Furthermore, the receptors found on the mature spermatozoa surface (hormonal, cytokine, growth, neurotransmitter and other) (Naz and Sellamuthu, 2006) are different from the

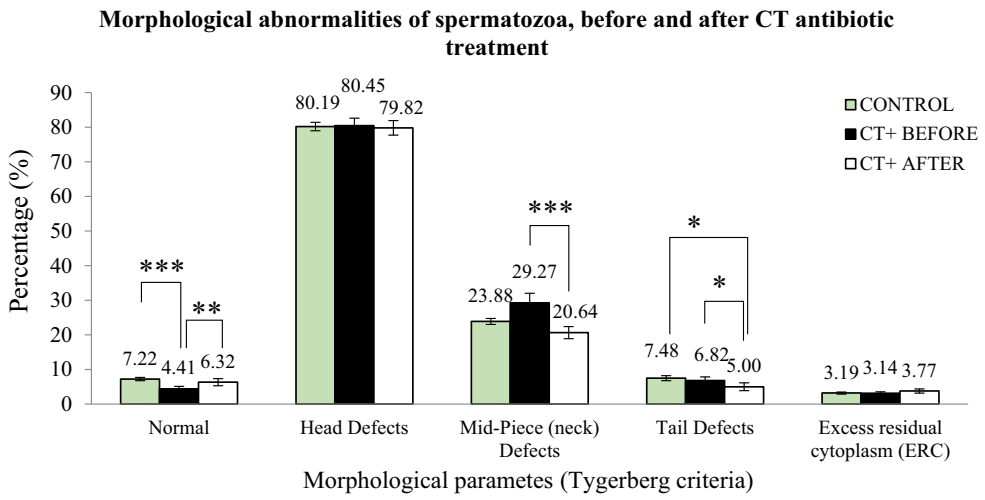

Fig. 3. Morphological parameters according to Tygerberg criteria. The effect of CT is clearly evident when comparing the control and CT+ pre–treatment group. The CT antibiotic therapy had a statistically significant positive effect on the % of normal/ typical spermatozoa, and the % of spermatozoa with mid–piece (neck) and tail defects. The results indicate a beneficial role of the CT antibiotic treatment, on spermatozoa morphology. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$. Control group ($N = 67$; green), CT+ pre–treatment group ($N = 22$; black) and CT+ post–treatment (N = 22; white). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

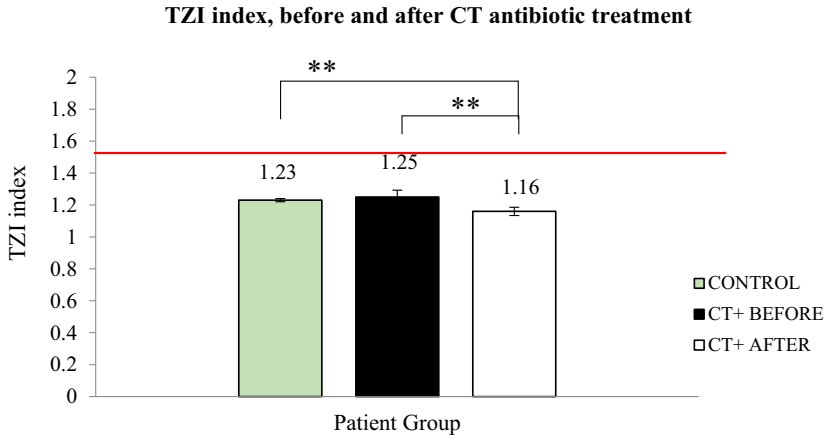

Fig. 4. Pre– and post–treatment results for the teratozoospermia index (TZI). Although all groups were below the normal index (red line), there was a statistically significant decrease of the TZI index of the CT+ post–treatment group, when compared to both the control and CT+ pre–treatment group, respectively. $^{**}p \leq 0.01$. Control group (N = 67; green), CT+ pre–treatment group (N = 22; black) and CT+ post–treatment (N = 22; white). Note: The red line indicates the normal TZI index (from fertile couples) value at ≤1.51, according to the new edition of the WHO laboratory manual for the examination and processing of human semen (6th edition. Geneva: World Health Organization; 2021. Licence: CC BY–NC–SA 3.0 IGO). However, according to a study by Menkveld et al., (Menkveld et al., 2001), when taking into account the adjusted cut–off points (based on 50% prevalence for a possible subfertile population), the index value can be increased to ≤2.09. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

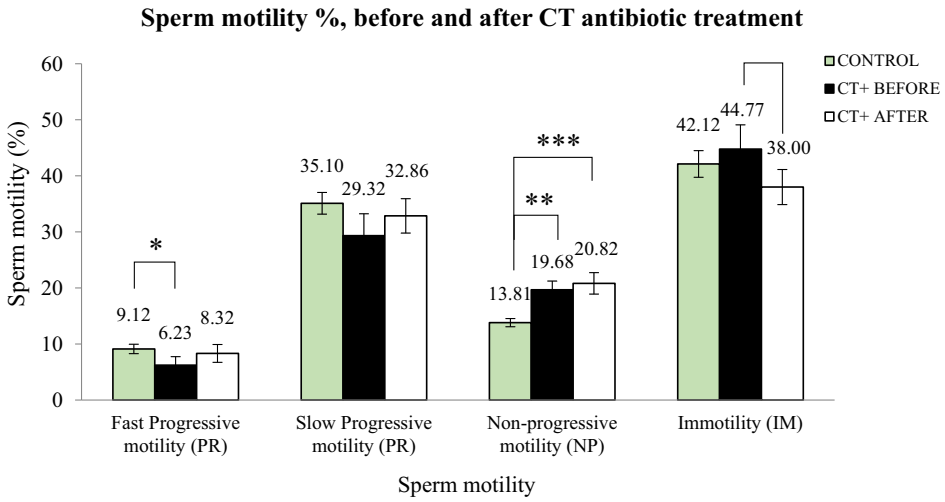

Fig. 5. Pre– and post–treatment sperm motility results. The effect of the CT infection is evident when comparing the control and CT+ pre–treatment group. CT antibiotic treatment showed a positive effect on all parameters (note PR and IM), but was only statistically significant for the non progressive motility (NP) spermatozoa. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. Control group ($N = 67$; green), CT+ pre–treatment group (N = 22; black) and CT+ post–treatment (N = 22; white). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

molecules and ligands usually deployed by CT, in order to bind and gain access to the cell. Up to now, the data suggests that CT adhesion is dependent on multiple superfluous adhesion molecules (Cossé et al., 2018), first by a low affinity interaction of CT's outer membrane protein, OmcB (CT442), to the host's cell heparan sulfate proteoglycans (HSPGs) and then by a high affinity binding, to other host cell receptors (e.g., b1 integrin, the epidermal growth factor receptor (EGFR), e.t.c.]. Apparently, apart from EGFR, there seems to be no other receptors that CT can use to gain access inside spermatozoa. As a result, presence of EBs (after release from Sertoli cells) to the microvicinity of the testis, could lead to their entry into adjacent cells, such as, spermatogonia, spermatocyte, round spermatids and eventually mature spermatozoa, as these progress through the spermatogenesis steps (Varuzhanyan and Chan, 2020). The fact that we have identified increased defects in the mid–piece (neck), an area abundant in mitochondria, could further justify that these are due to the presence of EBs. In addition, it provides clues about the level of infection, and supports the theory that the male gametes could act as a "*vehicle*" for the pathogen. Subsequently, vertical transmission of pathogens to the female gamete (Pacey and Eley, 2004), could have detrimental effects on the normal embryo development, leading to miscarriages and couple's fertility problems.

FC has already been used to evaluate several semen parameters, such as leukocytospermia (Ricci et al., 2009), measurements of activated caspases and mitochondria function (Brugnon et al., 2009; Zou et al., 2010), apoptosis (Perticarari et al., 2007a), DNA fragmentation (Agarwal et al., 2020; Omran et al., 2021), reactive oxygen species (Agarwal et al., 2020; Gosalvez et al., 2017; Riley et al., 2021), and antisperm antibodies (Nikolaeva et al., 2000). In addition, FC has been used to evaluate the presence of CT in different samples types (Tjagur et al., 2020), even monitor CT developmental cycle (Vromman et al., 2014). Consequently, we have used a novel patented FC method to directly investigate the presence of iCT in spermatozoa of asymptomatic men. Certainly, most of the studies detecting seminal pathogens (viruses), have used PCR, which is the most effective and reliable approach available today (Kapranos et al., 2003; Michou et al., 2012; Eley, 2011). However, PCR techniques cannot distinguish between extracellular or intracellular pathogens, and cannot indicate the specific infected cell types from the promiscuous seminal cell population (Eley, 2011). The same applies for other techniques (e.g., enzyme–linked immunosorbent assay (ELISA), fluorescent or chromogenic in situ hybridization techniques, electron microscopy (Wølner-Hanssen and Mårdh, 1984), e.t.c.), but these have lower sensitivity, can be very expensive, are time consuming, and could require special equipment. Therefore, FC is cheaper, faster, quantitative by giving immediate information of the percentage and type of the infected cells, and can be used to analyze a large number of cells simultaneously and repeatedly to monitor, e.g., the infectious status of the individual or the progress of the antibiotic therapy (Gosalvez et al., 2017).

Our study has several strengths including the large number of semen samples and CT+ cases. In addition, the SPI™ method showed better performance characteristics when compared to PCR or ELISA techniques, similar to those reported by others (El Feky et al., 2009; Álvarez-

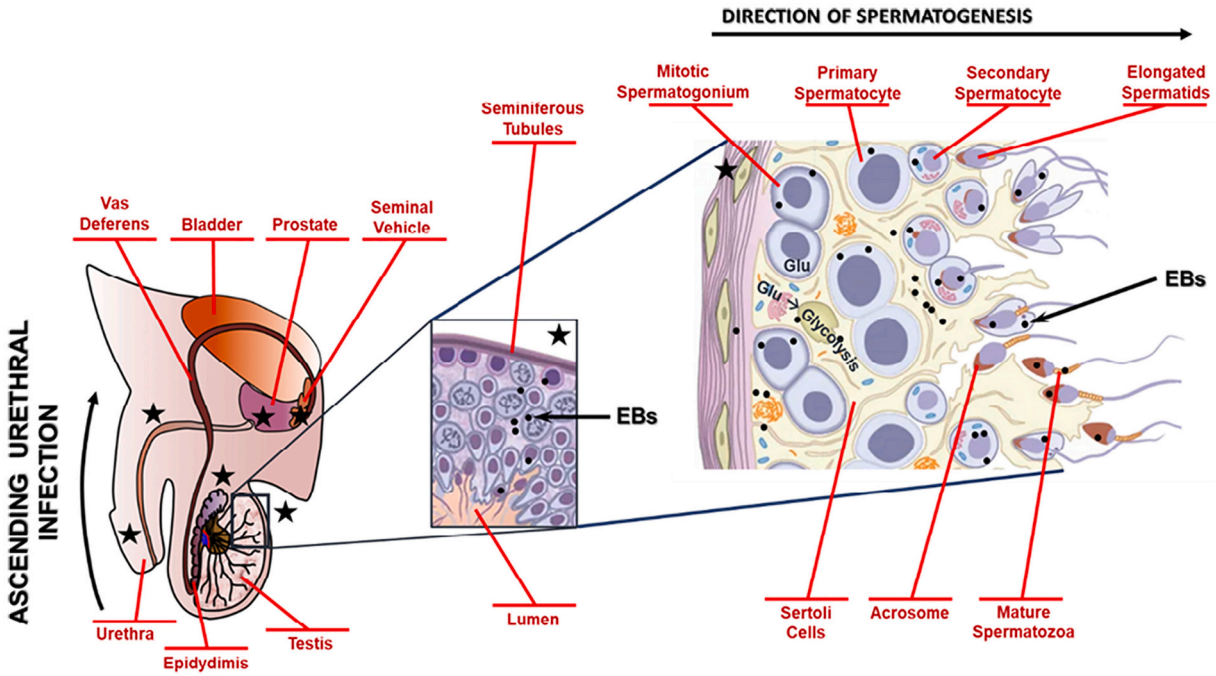

Fig. 6. Ascending CT urethral infection. The stars (*) indicate the possible sites of CT infection in the urogenital system. In the testis, and more specifically, in the seminiferous tubules, the elementary bodies (EBs) can infect Sertoli cells, that provide physical support and nutrition to the developing sperm cells. The EBs could gain access to the spermatogonia, which are then "trapped" and carried throughout the development of spermatogenesis. Moreover, glucose (Glu) is present in Sertoli cells and spermatogonia (energy through glycolysis) (Varuzhanyan and Chan, 2020), making the microenvironment of testis favorable to EBs. Also, since, the mid–piece area of the mature spermatozoa is rich in mitochondria, it is expected that EBs will be present there, and as a result, more possible to induce defects. Image adapted and modified from Encyclopedia Britannica, Inc., 2013.

Barrientos et al., 2000). Moreover, we have attributed the inability of previous studies using FC, to detect any microorganism inside the head of spermatozoa, to the special condensation of the DNA. It was therefore necessary to loosen the DNA through enzymatic processing (digestion) to create pathways for the antibody molecules to find their targets (microorganisms), and therefore, to be able analyze the content of spermatozoa. Moreover, the use of monoclonal, instead of polyclonal antibodies, vastly improved the sensitivity and resolution of the positive samples. Lastly, with this technique a large number of spermatozoa (~20.000 cells) was analysed, per sample, minimizing the possibility of not detecting the pathogenic agent. In contrast, this study has limitations, like the small number of pre and post CT antibiotic treatment spermcount data. In addition, being a retrospective study limited the level of evidence compared to prospective studies. Also, the control group comprised of aged–matched individuals that were negative for CT. Instead, an aged–matched control group from healthy, fertile men should be used.

Future considerations, in view of a large cohort prospective study, should account for extended semen analysis (e.g., DFI, ROS, apoptosis) including relevant sperm functions tests [e.g., acrosin activity test, mixed antiglobulin reaction (MAR) test, etc.]. Lastly, with the use of special fluorescent beads, a curve that corresponds to the intensity of the fluorochrome and the concentration of microorganisms per infected spermatozoon could be created in FC analysis, providing valuable quantitative information (Haidl and Allam, 2022).

5. Conclusions

In conclusion, this study was able to identify spermatozoa with iCT using a new immunophenotypic FC method. In addition, it was able to identify negative effects on the semen parameters, due to the CT infection, and that these could be subsequently and effectively reversed, after CT antibiotic therapy. Monitoring the impact and the level of improvements of CT antibiotic therapy on semen parameters of infertile men is of outmost importance, and could be used as a prognostic factor of male infertility. While acknowledging the limitations of the study, nevertheless, we believe that CT antibiotic therapy provides a positive outcome, both in acute, as well as, in chronic CT infections, that may be of particular importance in natural conception or ART techniques. These results indicate that the use of empirical therapeutic protocols for infertility and IVF should be avoided, before determining the intracellular pathogenic niche of the seminal microenvironment.

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CRediT authorship contribution statement

Konstantinos Makarounis: Data curation, Formal analysis, Investigation, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Michail Leventopoulos:** Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing. **Georgios Georgoulias:** Investigation, Supervision, Writing – review & editing. **Dimitris Nikolopoulos:** Investigation, Methodology, Writing – review & editing. **Maria Xountasi:** Investigation, Methodology, Writing – review & editing. **Panagiotis Kotrotsos:** Investigation, Writing – review & editing. **Effrosyni Nosi:** Investigation, Methodology, Writing – review & editing. **Vasiliki Gennimata:** Project administration, Supervision, Visualization, Writing – review & editing. **Dionysios Venieratos:** Project administration, Supervision, Visualization, Writing – review & editing. **Vassilis Protogerou:** Project administration, Supervision, Visualization, Writing – review & editing. **Vassilis Tsilivakos:** Conceptualization, Investigation, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors report no conflicts of interest relevant to this article.

Data availability

The data that has been used is confidential, but could be made available upon request.

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