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# *Waddlia chondrophila*, a *Chlamydia*-related bacterium, has a negative impact on human spermatozoa

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**STUDY QUESTION:** What is the impact of *Waddlia chondrophila*, an emerging *Chlamydia*-related bacterium associated with miscarriage, on human spermatozoa?

**SUMMARY ANSWER:** *W. chondrophila* had a negative impact on human spermatozoa (decrease in viability and mitochondrial membrane potential) and was not entirely removed from infected samples by density gradient centrifugation.

**WHAT IS KNOWN ALREADY:** Bacterial infection or colonization might have a deleterious effect on male fertility. *Waddlia chondrophila* was previously associated with miscarriage, but its impact on male reproductive function has never been studied.

**STUDY DESIGN SIZE, DURATION:** An *in vitro* model of human spermatozoa infection was used to assess the effects of *W. chondrophila* infection. Controls included *Chlamydia trachomatis* serovar D and latex beads with similar size to bacteria.

**PARTICIPANTS/MATERIALS, SETTING, METHODS:** Purified motile spermatozoa were infected with *W. chondrophila* (multiplicity of infection of 1). Immunohistochemistry combined with confocal microscopy was used to evaluate how bacteria interact with spermatozoa. The impact on physiology was assessed by monitoring cell viability, mitochondrial membrane potential and DNA fragmentation.

**MAIN RESULTS AND THE ROLE OF CHANCE:** Using super-resolution confocal microscopy, bacteria were localized on spermatozoa surface, as well as inside the cytoplasm. Compared to controls, *W. chondrophila* caused a 20% increase in mortality over 72 h of incubation ($P < 0.05$). Moreover, higher bacterial loads significantly reduced mitochondrial membrane potential. Bacteria present on spermatozoa surface were able to further infect a cell-monolayer, indicating that sperm might vector bacteria during sexual intercourse.

**LIMITATIONS REASONS FOR CAUTION:** The main limitation of the study is the use of an *in vitro* model of infection, which might be too simplistic compared to an actual infection. An animal model of infection should be developed to better evaluate the *in vivo* impact of *W. chondrophila*.

**WIDER IMPLICATIONS OF THE FINDINGS:** Intracellular bacteria, including *C. trachomatis*, *Mycoplasma* spp. and *Ureaplasma* spp., are associated with male infertility. *Waddlia chondrophila* might represent yet another member of this group, highlighting the need for more rigorous microbiological analysis during investigations for male infertility.

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**Key words:** *Waddlia chondrophila* / *Chlamydia*-related / infection / male infertility / spermatozoa

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# Introduction

Infertility, which affects one in seven couples, is a significant health issue. The cause of infertility can involve the female, the male or both partners. A male infertility factor is present in up to 50% of couples seeking infertility treatment (Hirsh, 2003; Brugh and Lipshultz, 2004). Classic causes of male infertility are genetic anomalies, hypogonadism, cryptorchidism or tumours, but unfortunately male infertility is still considered 'idiopathic' in a large proportion of cases (50%).

There is substantial evidence that bacterial infections of the male genital tract are associated with infertility (Weidner *et al.*, 2013; Gimenes *et al.*, 2014; La Vignera *et al.*, 2014; Shang *et al.*, 2014). Pathogenic bacteria may impair fertility at several levels including, epididymitis, orchitis, obstruction of reproductive tracts and spermatozoal damage (Gimenes *et al.*, 2014). Presence of bacteria in semen (bacteriospermia) and leukocytospermia may be direct causes of subfertility or be additional negative factors worsening the prognosis of fertility in natural and assisted reproduction (Dahlberg, 1976; Fraczek *et al.*, 2015).

Intracellular bacteria, which grow poorly on routine culture media, may be agents of male infertility of unknown aetiology. Among them, *Chlamydia trachomatis* is the most common sexually transmitted bacterial infection with more than 100 million new cases reported annually, of which 90% remain asymptomatic (Vasilevsky *et al.*, 2014). *C. trachomatis* can directly or indirectly affect sperm function at different levels (Cengiz *et al.*, 1997; Joki-Korpela *et al.*, 2009; Mackern-Oberti *et al.*, 2013). In a study of 627 men, the *Chlamydia*-positive group showed a deterioration of spermatozoa morphology (−14.4%), concentration (−8.3%), motility (−7.8%) and velocity (−9.3%), as well as ejaculation volume (−6.4%), compared to *Chlamydia*-negative samples (Veznik *et al.*, 2004). Among 1161 men, Mazzoli *et al.* (2010) reported significantly lower sperm concentration, percentage of motile sperm and normal morphologic forms in *Chlamydia*-positive patients.

Other *Chlamydia*-related bacteria have recently been described as emerging pathogens (Baud *et al.*, 2008; Greub, 2009; Baud and Greub, 2011). Among them, *Waddlia chondrophila* has been strongly associated with miscarriage in humans based on serology (Baud *et al.*, 2007, 2014; Hornung *et al.*, 2015). Moreover, the presence of *W. chondrophila* was observed in human samples (placenta, vaginal swab, urine) following miscarriage (Baud *et al.*, 2011, 2014). Similarly to other members of the *Chlamydiales* order, *W. chondrophila* has a biphasic developmental cycle. Initially, infectious elementary bodies (EBs) adhere to cell surface and enter into the host cell. Once internalized, bacteria escape the endocytic pathway and prevent fusion with lysosomes, thus their degradation. After a latent phase of ~8 h, EBs convert to metabolically active reticulate bodies (RBs), which start replicating inside the vacuole by binary fission. Once the replication cycle is finished, RBs dedifferentiate into EBs, which are released into the extracellular space after cell lysis. A new infectious cycle can then start with infection of new host cells (de Barsy and Greub, 2013).

Initially isolated from aborted bovine foetuses (Dilbeck *et al.*, 1990; Rurangirwa *et al.*, 1999), *W. chondrophila* has the potential to grow in several human cell lines including McCoy cells, human fibroblasts, peripheral blood mononuclear cells, Vero cells, A549 pneumocytes, macrophages and Ishikawa endometrial cells (Goy *et al.*, 2008; Kebbi-Beghdadi *et al.*, 2011; de Barsy and Greub, 2013). Using a mouse model of infection, we have shown that *W. chondrophila* was able to induce systemic infection, organ pathology and elicited Th1-associated humoral immunity after genital inoculation (Vasilevsky *et al.*, 2015).

We have recently observed a high *W. chondrophila* seroprevalence in male patients from infertile couples with suspected male infertility (unpublished data). As it is taxonomically related to *C. trachomatis*, which has a negative effect on male infertility, we have decided to assess the impact of *W. chondrophila* on human spermatozoa using an *in vitro* model of infection. We have investigated the ability of bacteria to attach to and penetrate into spermatozoa. We have assessed the impact on the viability. Lastly, we have evaluated the possibility that spermatozoa could act as vectors for bacterial dissemination.

# Materials and Methods

## *Waddlia chondrophila* and *C. trachomatis* culturing

*Waddlia chondrophila* strain WSU 86–1044 (ATCC® VR-1470™) was replicated in *Acanthamoeba castellanii* cultures grown in 75 $cm^2$ flasks (Corning, NY, USA) at 32°C in Peptone–Yeast–Glucose (PYG) broth as previously described (Greub and Raoult, 2002). To obtain bacterial preparations, 7-day amoeba-*Waddlia* co-cultures were harvested and filtered through a 5-µm filter (Merck Millipore, Darmstadt, Germany) to eliminate amoebal debris.

*Chlamydia trachomatis* strain D/UW-3/Cx (ATCC® VR-885™) was cultured as previously described (Ripa and Mårdh, 1977). Briefly, *C. trachomatis* was grown on monolayers of McCoy cells in 75 $cm^2$ flasks (Corning) using Dulbecco's Modified Eagles Medium (DMEM, GE Healthcare, Glattbrugg, Zurich, Switzerland) supplemented with 10% foetal bovine serum (Connectorate AG, Dietikon, Switzerland) and 2 µg/ml of cycloheximide (Life technologies, Carlsbad, CA). After inoculation, flasks were incubated for 72 h at 37°C in presence of 5% $CO_2$. To obtain bacterial preparations, cells were detached by trypsinization and homogenized with glass beads.

## Quantification of *W. chondrophila* and *C. trachomatis*

Prior to infection, bacterial preparations were quantified in order to prepare inocula. Quantification of *W. chondrophila* was performed using a previously described real-time PCR method (Goy *et al.*, 2009). Briefly, we used forward primer WadF4 (5′-GGCCCTTGGGTCGTAAAGTTCT-3′), reverse primer WadR4 (5′-CGGAGTTAGCCGGTGCTTCT-3′) and WadS2 probe (5′-FAM-CATGGGAACAAGAGAAGGATG-BHQ-3′, underlined based are locked nucleotides) to amplify the 16 S rRNA gene of *W. chondrophila*.

*C. trachomatis* was quantified as previously described (Lienard *et al.*, 2011). Briefly, we used forward primer panCh16F2 (5′-CCGCCAACAC TGGGACT-3′), reverse primer panCh16R2 (5′-GGAGTTAGCCGGTGC TTCTTTAC-3′), and the probe panCh16S (5′-FAM [6-carboxyfluorescein]-C TACGGGAGGCTGCAGTCGAGAATC-BHQ1 [Black Hole Quencher 1] −3′), targeting the 16 S rRNA gene of bacteria belonging to *Chlamydiales* order.

Quantitative PCR reaction was performed with the iTaq supermix (BioRad, California, USA), according to manufacturer's instructions using 200 nM of primers (forward and reverse), 100 nM of probe and iTaq supermix (BioRad, California, USA) in a StepOne Plus (Applied Biosystems, California, USA).

Quantification of human DNA was done with the Investigator Quantiplex HYresTM assay (Qiagen AG, Basel, Switzerland) on an AB

7500 Real-time PCR system (Applied Biosystems, Zug, Switzerland) following standard procedure.

The equivalent of one well (1 ml) of infected or mock-treated spermatozoa was transferred to microcentrifuge tubes and stored at −20°C prior to processing. DNA was extracted using the QIAamp DNA mini kit (Qiagen AG, Basel, Switzerland) following a slightly modified manufacturer's protocol, in which 43 mM DTT was added to the lysis buffer and elution was performed in 100 µl.

## Ethical approval

The study was approved by the local ethics committee (protocol 265-14).

## Semen samples

Semen specimens were collected from healthy volunteers by masturbation after 2–4 days of sexual abstinence.

Spermatozoa were washed at the Reproductive Medicine Unit (CHUV) using the density gradient centrifugation method (ISolate kit, Irvine Scientific, Irvine, CA, USA), according to manufacturer's specifications. Recovered spermatozoa were subsequently washed twice (300 *g*, 10 min) with the G-MOPS™ PLUS medium (Vitrolife Sweden AB, Sweden) and resuspended in 1 ml of the same medium.

## Human spermatozoa infection procedure

Experimental infection of human spermatozoa was carried out in 24-well low-binding plates (Costar Ultra-Low attachment multiwell plates, Corning) in HEPES buffered media and Earle's balanced salt solution with osmolality adjusted at 285 ± 10 mOsm/kg. Spermatozoa concentration was adjusted to $10^6$ per well and 5 µl of bacterial suspension (final multiplicity of infection [MOI] of 1) or mock solution (filtered supernatant of naïve cells used to replicate bacteria) was added, respectively. After an incubation step of 2 h at 37°C with 5% $CO_2$, gentamycin (Bioconcept, Allschwil, Switzerland) and ampicillin (Sigma-Aldrich Chemie, Buchs, Switzerland) were added at 50 and 30 µg/µl, respectively, and this represented the initial point of the analysis. Throughout the experiment, plates were kept at 37°C with 5% $CO_2$. The same infection procedure was used with latex beads (polystyrene, 0.6 µm mean particle size, Sigma-Aldrich Chemie), which served as a control.

Infection of McCoy cells by *W. chondrophila*-infected spermatozoa was performed similarly as previously described by Al-Mously *et al.* (2009). Spermatozoa were incubated with *W. chondrophila* at an MOI of 1 for 48 h. Samples were centrifuged at 500 *g* for 10 min and resuspended in 1 ml DMEM supplemented with 10% foetal bovine serum and used to infect a monolayer of McCoy cells, previously grown to confluence on 13 mm diameter glass coverslips in 24-well plates containing DMEM supplemented with 10% foetal bovine serum. Presence of *W. chondrophila* growth was assessed by confocal microscopy using a specific antibody (see below).

## Confocal microscopy

Samples (300 µl) were placed on microscopy glass slides using a Cytospin 4 cytocentrifuge (Thermo Scientific, Zurich, Switzerland). Slides were then fixed in Phosphate buffered saline (PBS) supplemented with 4% paraformaldehyde (Thermo Scientific). Rabbit anti-*W. chondrophila* polyclonal antibody (diluted 1/1000) was added to the slides in a wet chamber and incubated for 1 h at room temperature. After three washing steps with PBS for 5 min each, the secondary antibody was added and incubated for 1 h at room temperature (goat anti-rabbit Alexa Fluor 488-conjugated, diluted at 1/1000 in blocking solution, Molecular Probes/Life Technologies). After a PBS washing step, slides were stained with DAPI (4′, 6-diamidino-2-phenylindole, AppliChem GmbH, Darmstadt, Germany) and rhodamine-labelled Concanavalin A (Vector Laboratories Ltd, Cambridgeshire, UK) for 1 h. An anti-fading agent, Mowiol (Sigma-Aldrich Chemie) was added before mounting a cover slip and kept at 4°C overnight. Image capture was performed using a Zeiss LSM 780 confocal microscope (Carl Zeiss Vision Swiss AG, Feldbach, Switzerland) and processed by LSM software ZEN 2012 (Carl Zeiss Vision Swiss AG). For the detection of internalized bacteria, z-stacks of pseudo-super resolution images were acquired using the airyscan module (Carl Zeiss Vision Swiss AG).

The abovementioned confocal microscopy protocol was also used to quantify the inclusion forming units (IFU). For this, different dilution of pre- and post-wash sperm samples, experimentally infected with *W. chondrophila*, were incubated on monolayers of McCoy cells, previously seeded on glass coverslips in 24-well plates.

## Viability staining

Viability of spermatozoa was determined using the LIVE/DEAD Sperm Viability Kit (Thermo Scientific). Samples (500 µl) were centrifuged at 500 *g* for 10 min and resuspended gently in 500 µl PBS. Staining was performed by adding 1 µl LIVE/DEAD dye and incubating the samples in the dark at room temperature for 30 min. After 15 min of incubation, samples were mixed by gently inverting the tubes. Samples were then centrifuged at 500 *g* for 10 min, resuspended in the fixation solution (PBS supplemented with 4% paraformaldehyde) and incubated for 10 min in the dark. After two washing steps with 500 µl PBS, samples were stored at 4°C until flow cytometry analysis.

## Mitochondrial membrane potential assay

The mitochondrial membrane potential was determined as previously described (Slabbert *et al.*, 2015), using the MitoTracker Red CMXRos kit (Life technologies). Briefly, MitoTracker dye was used at a final concentration of 250 nM in PBS to stain $10^6$ spermatozoa infected with different MOI of *W. chondrophila* or with mock control. After 30 min of incubation in the dark, samples were washed with PBS and consequently fixed with 500 µl fixation solution (PBS supplemented with 4% paraformaldehyde). After two washing steps with 500 µl PBS, samples were stored at 4°C until flow cytometry analysis. As a positive control, we used the antineoplastic agent betulinic acid (BA), which was previously shown to disrupt spermatozoa mitochondrial membrane potential (Espinoza *et al.*, 2009).

## DNA fragmentation assay

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay was used to assess the fragmentation of spermatozoa DNA, as previously described (Villani *et al.*, 2010). TUNEL Label Mix and TUNEL Enzyme kits (Roche, Switzerland) were used as indicated by the manufacturer. A total of $10^6$ *W. chondrophila*- or mock-infected spermatozoa were washed and fixed (PBS supplemented with 2% paraformaldehyde) for 1 h. After two PBS washing steps, spermatozoa were permeabilized (0.1% sodium citrate, 0.1% Triton X-100 solution) and washed again. TUNEL kit was then used to detect DNA fragmentation. Treatment with DNase I (New England Biolabs, Ipswich, MA, USA) served as the positive control.

## Flow cytometry analysis

Flow cytometry measurements were performed using a BD Accuri C6 Flow Cytometer (BD Biosciences, Erembodegem, Belgium). Green fluorescence was recorded with the FL-1 channel while red fluorescence was recorded with the FL-3 channel. For each sample, 10 000 events were recorded. Results were analysed with the BD Accuri™ C6 Software (BD Biosciences).

## Statistical analyses

Statistical analyses were performed using GraphPad Prism (version 7.00 for Windows, GraphPad Software, La Jolla California USA). A $P$-value $< 0.05$ was considered to be statistically significant. Comparisons for sperm viability, mitochondrial membrane potential and DNA fragmentation

assays were performed using ANCOVA. Post-hoc analysis was performed with the test for homogeneity of regressions.

# Results

## *Waddlia chondrophila* adheres to the cell membrane, penetrates inside spermatozoa and is not removed completely by density gradient centrifugation

Density gradient centrifugation, routinely used for the washing of sperm, was applied to raw sperm samples experimentally infected with *W. chondrophila*. In each of the purified samples, bacteria were detected by qPCR with an average of 3.49 ± 1.33% of the initial load (Table IA). Similarly, when pre- and post-wash samples were incubated on a monolayer of McCoy cells, the latter still contained viable bacteria, as IFU were detected at the level of 1.43 ± 0.43% of the initial load (Table IB).

**Table I Effect of density gradient washing on the bacterial load of sperm samples experimentally infected with *W. chondrophila* (MOI of 0.01).**

| A: Replicate | Pre-wash | Post-wash | % |
|---|---|---|---|
| 1 | $1.31 \times 10^6$ | $6.74 \times 10^4$ | 5.14 |
| 2 | $1.98 \times 10^6$ | $7.74 \times 10^4$ | 3.91 |
| 3 | $1.44 \times 10^6$ | $3.82 \times 10^4$ | 2.65 |
| 4 | $1.67 \times 10^6$ | $5.88 \times 10^4$ | 3.52 |
| 5 | $1.97 \times 10^6$ | $8.54 \times 10^4$ | 4.33 |
| 6 | $1.26 \times 10^6$ | $1.71 \times 10^4$ | 1.36 |
| Mean | $1.61 \times 10^6$ | $5.74 \times 10^4$ | 3.49 |
| **B: Replicate** | **Pre-wash** | **Post-wash** | **%** |
| 1 | 7.63 × 105 | 1.10 × 104 | 1.44 |
| 2 | $5.72 \times 10^5$ | $4.99 \times 10^3$ | 0.87 |
| 3 | $5.13 \times 10^5$ | $8.80 \times 10^3$ | 1.71 |
| Mean | $6.16 \times 10^5$ | $8.26 \times 10^3$ | 1.34 |

A: Bacterial loads pre- and post-wash determined by qPCR. The difference in bacterial load between the pre- and post-wash samples is statistically significant ($P < 0.0001$). B: Bacterial loads (IFU) determined by inoculation of pre- and post-wash samples on McCoy cell monolayers. Again, the difference in bacterial load between the pre- and post-wash samples is statistically significant ($P = 0.0013$).

To further characterize their interaction, spermatozoa incubated with *W. chondrophila* (MOI of 1) were analysed by confocal microscopy. The infection led to the attachment of bacteria to the spermatozoa membrane already at early time points (2 h) (Fig. 1A). Attachment was observed to the spermatozoal head, midpiece and tail, with the majority of bacteria adhering to the head. Numbers of attached bacteria per spermatozoon was variable and increased by using an MOI of 10. In addition to attachment, this intracellular bacterium was able to penetrate inside spermatozoa and localize in the head (Fig. 1B).

Concentration of bacterial DNA in samples remained stable over time, suggesting that replication of *W. chondrophila* did not occur or was below the level of detection (Supplementary Information Fig. S1).

## Exposure to *W. chondrophila* impairs spermatozoa physiology

In order to evaluate the impact of *W. chondrophila* internalization and adherence to spermatozoa, a fluorescence-based flow cytometric assay was used to analyse viability. The *C. trachomatis* strain D/UW-3/Cx was included in the analysis, as well as a negative control consisting of latex beads similar in size to the bacteria (0.6 μm). Viability was monitored at 0 and 72 h of incubation and values of the mock controls were used to remove the effect of the physiological mortality of spermatozoa. Exposure to *W. chondrophila* resulted in an increase in mortality of 20.66% ($P = 0.04$) after 72 h of incubation (Fig. 2). In comparison, both *C. trachomatis* serovar D and latex beads did not negatively influence spermatozoa viability over time despite adherence to the membrane (Supplementary Information Fig. S2). Interestingly, incubation of spermatozoa with latex beads had a positive effect on viability.

We further evaluated the impact of the bacterium on spermatozoa physiology by analysing the mitochondrial membrane potential and detecting DNA fragmentation. As seen in Fig. 2B, a significant reduction of 42.67% ($P = 0.03$) of mitochondrial membrane potential was

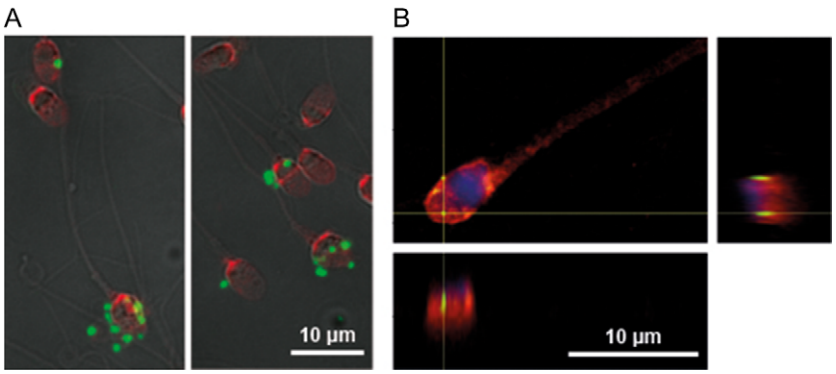


**Figure 1** Attachment and internalization of *W. chondrophila* in human spermatozoa. (**A**) Attachment of *W. chondrophila* on the spermatozoa surface. (**B**) Internalized *W. chondrophila*. The orthogonal view of the complete z-stack allows a clear vision of the position of the bacterium inside the spermatozoon head. Samples were stained using a specific anti-*W. chondrophila* antibody (green), Concanavalin A-rhodamine (red) and DAPI (blue).

observed for the MOI of 10 after 72 h of incubation. On the other hand, infection by *W. chondrophila* did not have any significant impact on the DNA fragmentation (Fig. 2C).

### Ability of spermatozoa to vector *W. chondrophila*

The observation that *W. chondrophila* is able to attach to the membrane raises the question of whether spermatozoa could act as a vector for bacterial dissemination. In a first set of experiments, spermatozoa were incubated with *W. chondrophila* (MOI of 10) for 48 h. After two washes with PBS, they were spotted on a layer of confluent McCoy cells. Negative control samples consisted of bacteria alone that were processed in the same way.

Interestingly, spermatozoa were able to spread bacteria over the cell layer (Fig. 3A–C) as several paths of infected McCoy cells were observed. The spread appeared linear, potentially following the movement of spermatozoa across the plate. In contrast, negative controls showed only a few isolated foci of infection (Fig. 3C and D).

## Discussion

*Waddlia chondrophila*, an emerging intracellular bacterium, was first isolated from aborted bovine foetuses (Dilbeck *et al.*, 1990; Rurangirwa *et al.*, 1999), which raised concern for a new abortigenic agent (Henning *et al.*, 2002; Dilbeck-Robertson *et al.*, 2003; Barkallah *et al.*, 2014), as well as whether *W. chondrophila* could infect other mammals such as humans. Three large prospective studies indicated that

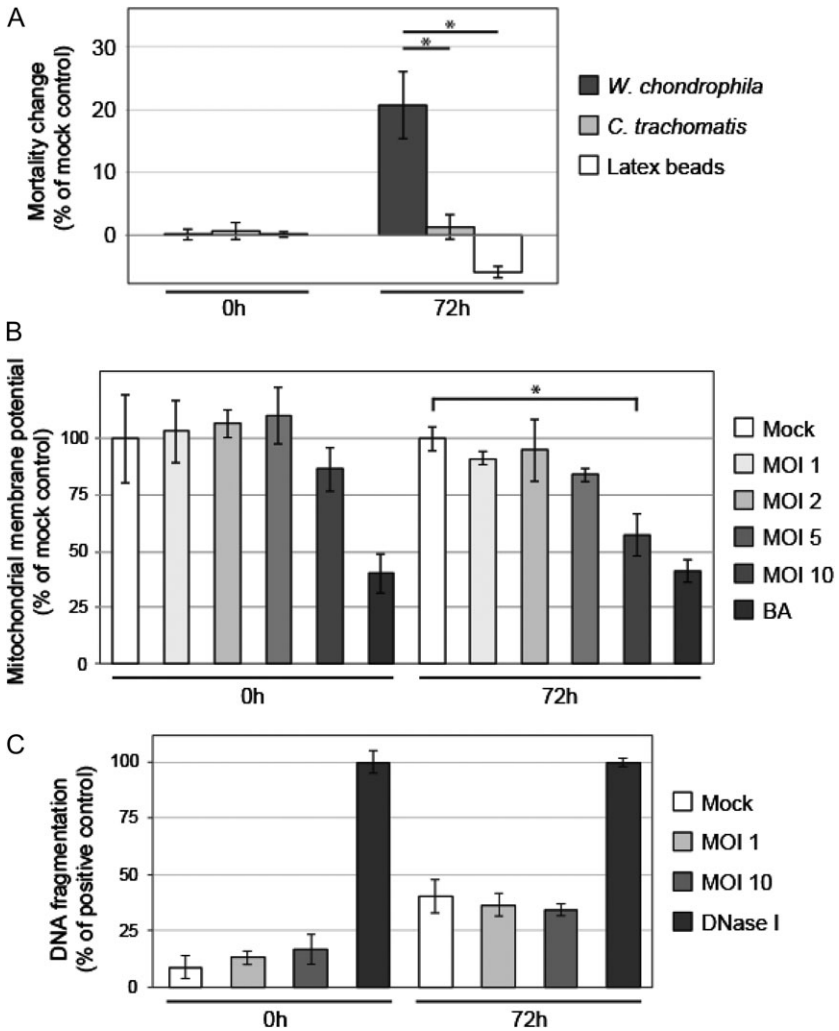


**Figure 2** Impact of *W. chondrophila* to spermatozoa physiology. (**A**) Fluorescence-based viability assay was used to monitor spermatozoa mortality in the presence of *W. chondrophila*, *C. trachomatis* or latex beads. Data were determined by subtracting the effect of the physiological mortality of spermatozoa (mock control). Results are representative of three independent experiments using different semen samples. (**B**) Evaluation of the mitochondrial membrane potential of mock- and *W. chondrophila*-infected spermatozoa. Betulinic acid (BA) was used as positive control. (**C**) TUNEL assay performed to assess DNA fragmentation of mock- and *W. chondrophila*-infected spermatozoa. DNase I treatment served as the positive control. MOI, multiplicity of infection. Error bars represent mean ± SD. Asterisks indicate $P < 0.05$ (one-way ANCOVA).

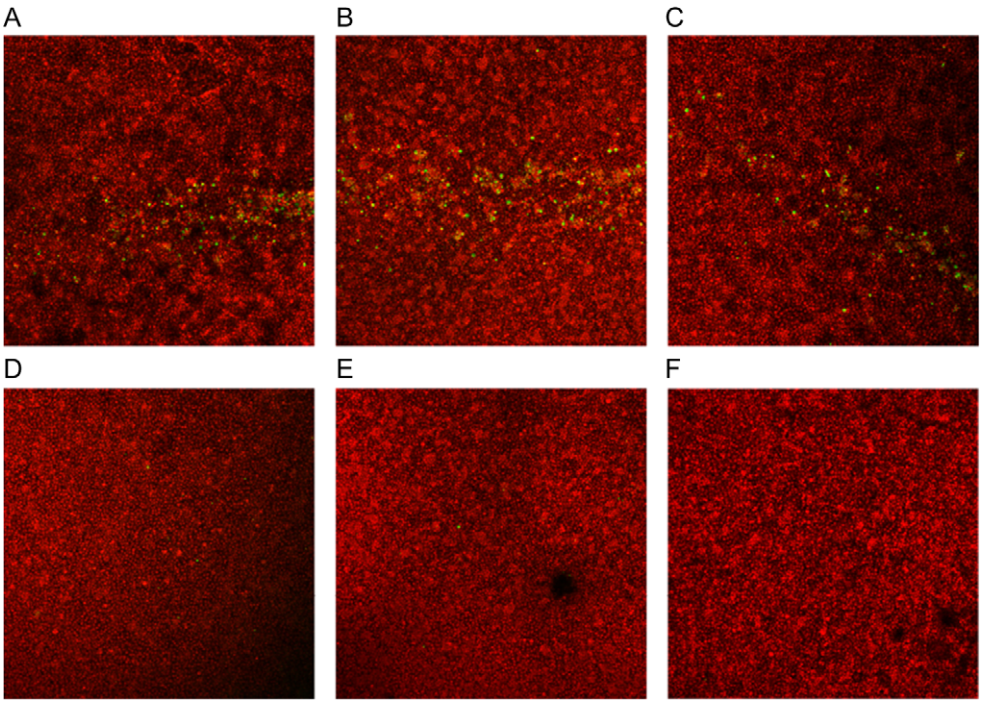


**Figure 3** Ability of spermatozoa to vector bacteria over a monolayer of McCoy cells. Samples consisting of spermatozoa incubated with *W. chondrophila* (**A**–**C**) and bacteria alone (**D** and **E**) were used to infect a monolayer of McCoy cells. (**F**) Mock control. Bacteria were stained using a specific anti-*W. chondrophila* antibody (green) and cells with concanavalin A-rhodamine (red).

*W. chondrophila* potentially induced miscarriage in pregnant women (Baud *et al.*, 2007, 2014; Hornung *et al.*, 2015). With almost all research efforts focusing on women, little is known about the impact of this bacterium on male reproduction. Of note, the seroprevalence in healthy men (8%) is similar to the seroprevalence in healthy female controls (7.1%) (Baud *et al.*, 2007, 2009).

In this study, we assessed the effects of *W. chondrophila* infection of human spermatozoa. We found that bacteria could not be entirely removed from spermatozoa preparation by the standard density gradient centrifugation, as similarly observed for *C. trachomatis* (Al-Mously *et al.*, 2009). In agreement with this observation, *W. chondrophila* was able to attach to and penetrate inside spermatozoa. Known sexually transmitted pathogens, such as *C. trachomatis*, *Mycoplasma* spp. or *Ureaplasma* spp., which are known to negatively impact male fertility, have also previously been detected on the surface of and inside spermatozoa (Erbengi, 1993; Reichart *et al.*, 2000; Svenstrup, 2003; Gallegos-Avila *et al.*, 2009; Buzinhani *et al.*, 2011).

This interaction had a negative impact on spermatozoa, as exposure to *W. chondrophila* caused an increase in mortality over time. We did not observe any significant change in viability compared to mock controls when spermatozoa were incubated with the *C. trachomatis* strain D/UW-3/Cx. In a previous study, *C. trachomatis* serovar E reduced both spermatozoa viability and motility, while *C. trachomatis* serovar LGV had only a minor effect (Hosseinzadeh *et al.*, 2001). Interestingly, although both are associated with urogenital infections, serovars D and E show a clear difference in the impact on spermatozoa. A control, consisting of latex beads that are a comparable size to the bacteria, did not negatively impact the viability.

Mitochondrial membrane potential was also altered upon the infection with W. chondrophila. However, a significant reduction was only observed for MOI of 10, while this was not the case for MOIs of 1, 2 and 5. This might be explained by the fact that with a lower MOI, only a minority of spermatozoa are actually in contact with bacteria. Indeed, this was observed by confocal microscopy analyses. By increasing the MOI to 10, we also increase the chance for spermatozoa to encounter *W. chondrophila* and observe a negative effect on their physiology. Interestingly, the infection did not impair DNA fragmentation compared to the mock control.

Very little is known about the transmission of *W. chondrophila*, although, by analogy to *C. trachomatis*, a sexual route may be a plausible mechanism of transmission. In this study we have experimentally shown that infected spermatozoa were able to spread *W. chondrophila* onto a cellular monolayer, indicating that sperm might vector this bacterium.

As with *C. trachomatis*, a large number of patients probably remain asymptomatic after *W. chondrophila* infection. In a mouse model of infection (Vasilevsky *et al.*, 2015), *W. chondrophila* infection of the genital tract did not translate into an overt illness, despite evidence of systemic infection.

Our results indicate that *W. chondrophila* might represent a novel sexually transmitted pathogen and that semen might represent a vector of transmission of this intracellular bacterium. The ability of *W. chondrophila* to penetrate and attach to human spermatozoa might impair fertility of infected individuals and/or induce miscarriage in their partner upon exposure. Future research should assess the impact of *W. chondrophila* on fertility in a male animal model of infection, as well

as the prevalence of this emerging pathogen among men and its potential impact on male fertility.

## Supplementary data

Supplementary data are available at *Human Reproduction* online.

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## Authors' roles

Study concept and design: M.S. and D.B. Acquisition of data: M.S., J.G, N.V. and V.C. Statistical analysis: M.S. and D.B. Interpretation and synthesis of data: M.S., N.V. and D.B. Writing of the article: M.S. and D.B. Critical revision of the article: G.G. All authors agree with the article's results and conclusions. All authors have read, and confirm that they meet, the authorship criteria.

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## Conflict of interest

None to declare.

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