Human Reproduction, Vol.34, No.10, pp. 1891–1898, 2019
Advance Access Publication on October 4, 2019 doi:10.1093/humrep/dez169

human reproduction

ORIGINAL ARTICLE *Andrology*

Detection of chlamydia infection within human testicular biopsies

Emily R. Bryan[1], Robert I. McLachlan[2,3,4], Luk Rombauts[2,3], Darren J. Katz[5,6], Anusch Yazdani[7], Kristofor Bogoevski[8], Crystal Chang[8], Michelle L. Giles[9], Alison J. Carey[1], Charles W. Armitage[1], Logan K. Trim[1], Eileen A. McLaughlin[10,11,12], and Kenneth W. Beagley[1,*]

[1]School of Biomedical Sciences and Institute of Health & Biomedical Innovation, Queensland University of Technology, 300 Herston Rd, Herston, QLD 4006, Australia [2]Monash IVF Group, 89 Bridge Road, Richmond, VIC 3121, Australia [3]Department of Obstetrics and Gynecology, Monash Medical Centre, Monash University, 246 Clayton Road, Clayton, VIC 3168, Australia [4]Hudson Institute of Medical Research, Monash Medical Centre, Monash University, 246 Clayton Road, Clayton, VIC 3168, Australia [5]Men's Health Melbourne, 233 Collins Street, Melbourne, VIC 3000, Australia [6]Department of Surgery, Western Health, Melbourne, VIC 3000, Australia [7]Queensland Fertility Group, 55 Little Edward Street, Spring Hill, QLD 4000, Australia [8]Histology Services, QIMR Berghofer Medical Research Institute, 300 Herston Road, Herston, QLD 4006, Australia [9]Ritchie Centre, Department of Obstetrics and Gynecology, Monash University, Melbourne, VIC 3000, Australia [10]School of Environmental and Life Sciences, Faculty of Science, The University of Newcastle, University Drive, Callaghan, NSW 2308, Australia [11]School of Science Faculty of Science and Technology, University of Canberra, Kirinari Street, Bruce, ACT 2617, Australia [12]School of Biological Sciences, University of Auckland, Private Bag 92019, Auckland 1142, New Zealand

*Correspondence address. School of Biomedical Sciences and Institute of Health & Biomedical Innovation, Queensland University of Technology, 300 Herston Rd, Herston, QLD 4006, Australia. E-mail: k2.beagley@qut.edu.au

Submitted on January 20, 2019; resubmitted on July 12, 2019; editorial decision on July 23, 2019

STUDY QUESTION: Can *Chlamydia* be found in the testes of infertile men?

SUMMARY ANSWER: Chlamydia can be found in 16.7% of fresh testicular biopsies and 45.3% of fixed testicular biopsies taken from a selection of infertile men.

WHAT IS KNOWN ALREADY: Male chlamydial infection has been understudied despite male and female infections occurring at similar rates. This is particularly true of asymptomatic infections, which occur in 50% of cases. Chlamydial infection has also been associated with increased sperm DNA damage and reduced male fertility.

STUDY DESIGN, SIZE, DURATION: We collected diagnostic (fixed, $n = 100$) and therapeutic (fresh, $n = 18$) human testicular biopsies during sperm recovery procedures from moderately to severely infertile men in a cross-sectional approach to sampling.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The diagnostic and therapeutic biopsies were tested for *Chlamydia*-specific DNA and protein, using real-time PCR and immunohistochemical approaches, respectively. Serum samples matched to the fresh biopsies were also assayed for the presence of *Chlamydia*-specific antibodies using immunoblotting techniques.

MAIN RESULTS AND THE ROLE OF CHANCE: Chlamydial major outer membrane protein was detected in fixed biopsies at a rate of 45.3%. This was confirmed by detection of chlamydial DNA and TC0500 protein (replication marker). *C. trachomatis* DNA was detected in fresh biopsies at a rate of 16.7%, and the sera from each of these three positive patients contained *C. trachomatis*-specific antibodies. Overall, *C. trachomatis*-specific antibodies were detected in 72.2% of the serum samples from the patients providing fresh biopsies, although none of the patients were symptomatic nor had they reported a previous sexually transmitted infection diagnosis including *Chlamydia*.

LIMITATIONS, REASONS FOR CAUTION: No reproductively healthy male testicular biopsies were tested for the presence of *Chlamydia* DNA or proteins or *Chlamydia*-specific antibodies due to the unavailability of these samples.

WIDER IMPLICATIONS FOR THE FINDINGS: Application of *Chlamydia*-specific PCR and immunohistochemistry in this human male infertility context of testicular biopsies reveals evidence of a high prevalence of previously unrecognised infection, which may potentially have a pathogenic role in spermatogenic failure.

STUDY FUNDING/COMPETING INTEREST(S): Funding for this project was provided by the Australian NHMRC under project grant number APP1062198. We also acknowledge assistance from the Monash IVF Group and Queensland Fertility Group in the collection of fresh

biopsies, and the Monash Health and co-author McLachlan (declared equity interest) in retrieval and sectioning of fixed biopsies. E.M. declares an equity interest in the study due to financing of fixed biopsy sectioning. All other authors declare no conflicts of interest.

TRIAL REGISTRATION NUMBER: N/A

Key words: *Chlamydia trachomatis* / STI / testicular infection / male infertility / *Chlamydia*-specific antibodies

Introduction

The current number of *Chlamydia trachomatis* (*C. trachomatis*) infections diagnosed globally (~127 million people/year; WHO, 2019) is likely to be grossly underestimated as ~50% of male and 75% of female infections are asymptomatic (Cunningham and Beagley, 2008). These asymptomatic infections form an unrecognised chlamydial reservoir contributing to ongoing transmission in the community. The effect of asymptomatic infection on male fertility remains unclear (Cunningham and Beagley, 2008). Chlamydial infection has been associated with infertility in women, but much less is known about the impact of chlamydial infection on male fertility (Cunningham and Beagley, 2008).

Male factors account for ~40% of human infertility; most are described as idiopathic but with genetic and environmental factors also proposed (Kumar and Singh, 2015). In the absence of a known pathological process, a descriptive approach is applied clinically, based on abnormalities of sperm number, motility and/or morphology (a surrogate marker of function) identified by semen analysis (WHO, 2010). Excluding obstructive azoospermia, changes in sperm number, motility and/or morphology are thought to reflect dysfunctional spermatogenesis which occurs in the seminiferous tubules, within which germ cell replication and development proceeds in close association with the supporting Sertoli cells. Testicular histological patterns range from Sertoli cell only appearance, arrested germ cell development or a reduction in all germ cell populations, termed hypospermatogenesis (McLachlan *et al.*, 2007). Each pattern can arise from a range of insults including genetic, infectious or toxic exposure.

Infectious diseases, such as mumps virus infection of the human testis, are known to play a role in spermatogenic dysfunction and have been associated with abnormal sperm parameters (Masarani *et al.*, 2006). Bacteria including *E. coli*, mycoplasmas and *C. trachomatis* in human semen are also associated with abnormal sperm parameters (Gallegos *et al.*, 2008; Moretti *et al.*, 2009). Yet there are no consistent histological indicators or markers observed during semen analysis for male infertility associated with chlamydial infection in humans. However, a mouse model demonstrated that chlamydial infection impairs testicular health and reduces sperm motility and normal morphology (Sobinoff *et al.*, 2014).

Challenges in attributing male infertility to chlamydial infection include the wide diversity in diagnostic approaches (Samplaski *et al.*, 2014). Options for the diagnosis of *C. trachomatis* infection include detection of *Chlamydia*-specific antibodies (which may not be *C. trachomatis*-specific or discriminate between current versus previous infection), culture (which is rarely used clinically), immunofluorescent detection of *Chlamydia* within urine, semen or urethral swab and PCR amplification of chlamydial DNA from urine, semen or urethral swab (Samplaski *et al.*, 2014). Conventionally, first-void urine testing by PCR is used, yet this may not be sensitive enough to detect chronic or asymptomatic infections in the testis (Samplaski *et al.*, 2014). Testing of semen may be more sensitive, with detection being reported when urine testing is negative and in semen donated for artificial insemination (Gregoriou *et al.*, 1989; van den Brule *et al.*, 1993; Witkin *et al.*, 1993; Pannekoek *et al.*, 2000; Eley and Pacey, 2011). This suggests that the upper reproductive tract may be a source of sexually transmissible *Chlamydia* that is undetected by the current nucleic acid amplification test (NAAT), and this requires further investigation.

We sought to establish whether testicular infection is associated with impaired spermatogenesis and male infertility by examining human testicular biopsies of men presenting with idiopathic nonobstructive azoospermia (NOA). Tissue was obtained from men presenting for testicular sperm extraction for use in intracytoplasmic sperm injection treatment and from men undergoing testicular biopsy for diagnostic evaluation of fertility.

Materials and Methods

Ethics

Bouin's fixed biopsies were collected under ethical approval given by Monash University (UHREC: RES-16-0000-559L) and Queensland University of Technology (QUT, UHREC: 1700000362). Fresh biopsies were collected under ethical approval given by Epworth (HREC: 666-15), Monash Health (HREC: 15489M), Queensland Fertility Group (QFG) (HREC: QFG12.15) and Queensland University of Technology (QUT) (UHREC: 1500000394) after patients provided signed patient informed consent forms.

Tissue collection

Bouin's fixed, paraffin-embedded testicular biopsies (n = 100) were obtained from Monash Health Anatomical Pathology Department. Subjects were selected by the clinical note of 'azoospermia' and absence of defined causes for spermatogenic failure (e.g. Klinefelter syndrome, prior chemoradiotherapy, testicular infarction or trauma) and thus were presumed idiopathic NOA. Samples were deidentified (now MB001 to MB100).

Additionally, deidentified fresh testicular biopsies (n = 18) were obtained by microdissection testicular sperm extraction (micro-TESE) and testicular sperm aspiration biopsy (TESA), from patients attending Monash IVF Group (Melbourne) and QFG for assisted reproductive technology (ART) procedures. In contrast with the fixed biopsies, this cohort contained samples from patients with both known and unknown causes of infertility (e.g. cystic fibrosis, Klinefelter syndrome, chemotherapy and azoospermia). These were sent to QUT for analysis. These patients also answered a survey on previous diagnosis of sexually transmitted infections (STIs).

Fixed biopsy immunohistochemistry

Two chlamydial markers were detected: the general marker major outer membrane protein (MOMP) and the active replication marker TC0500 (O'Meara *et al.*, 2016). Sections (4 µm) were dewaxed and rehydrated (Leica ST5010-CV5030 Integrated Workstation). Endogenous peroxidase activity was quenched (2% H_2O_2). Antigen retrieval was performed (Diva Decloaker, Biocare Medical, CA, USA) as per the manufacturer's instructions. Nonspecific binding was blocked (2% BSA in Background Sniper, Biocare Medical). The primary anti-MOMP IgG (raised in sheep) or primary anti-TC0500 IgG (mouse derived hybridoma: generated by the Monoclonal Antibody Technologies Facility, Monash University, Australia) was applied at room temperature. The primary antibody was then washed off and followed with anti-sheep or anti-mouse IgG-HRP. The secondary antibody was washed off and followed with DAB chromogen. Finally, sections were counterstained using haematoxylin (Leica XL Autostainer). Staining was validated using primary antibody only, secondary antibody only and DAB only controls. Stained slides were scanned (Aperio AT Turbo, Leica Biosystems). Positive staining was determined by four individuals, by the presence of chlamydial inclusions, which stained brown-black. Staining was conducted in collaboration with QIMR Berghofer Histology Services. A chi-squared test statistic was applied to MOMP staining results.

DNA extraction from biopsies

QIAamp DNA FFPE Tissue Kit (56404, Qiagen, VIC, Australia) was used to extract DNA from fixed biopsies. DNA was extracted from fresh biopsies using DNeasy Blood and Tissue kit (69506, Qiagen). DNA was extracted from *C. trachomatis* serovar D (ATCC® VR-855™) as a positive control. Kits were used as per the manufacturer's instructions.

Chlamydial real-time PCR

C. trachomatis 16S rRNA DNA was detected using real-time PCR (RG6000, Qiagen). Primers were 5′-GCGAAGGCGCTTTTCTAATT TAT-3′ (forward) and 5′-CCAGGGTATCTAATCCTGTTTGCT-3′ (reverse). Amplification conditions included 95°C for 10 min, 40 cycles of 95°C for 30 s, 52°C for 30 s, 74°C for 30 s, then 74°C for 2 min before standard melt analysis. Amplicons were confirmed as chlamydial in origin when they melted within 4°C of the positive control. Amplicons were electrophoresed on 2% agarose gel for size comparison.

C. trachomatis-specific antibodies

Serum samples matched to fresh biopsies were assayed for *C. trachomatis*-specific antibodies. *C. trachomatis* serovars D and E (20 µg/drop) were spotted onto nitrocellulose membranes. Membranes were probed with the serum samples (diluted 1:100), then with anti-human IgG-HRP (Southern Biotech), then developed and viewed with enhanced chemiluminescence (GE Healthcare). Additionally, HeLa cell monolayers were infected with *C. trachomatis* serovar D for 48 h, fixed (100% methanol), then probed with serum to detect *C. trachomatis*-specific antibodies, followed by anti-human IgG-AF594 (diluted 1:1000, Thermo Fisher Scientific). Stained cells were viewed using epifluorescent microscopy (Zeiss, Axio Vert.A1).

Table I Chlamydial MOMP positivity rate in fixed human testicular biopsies.

Histological pattern	Number with MOMP positivity (%)
Sertoli cell only appearance	21/51 (41.2%)
Germ cell arrest	6/11 (54.5%)
Combined hypospermatogenesis groups	15/33 (45.5%)
• Mild hypospermatogenesis	7/16 (43.7%)
• Moderate hypospermatogenesis	2/5 (40.0%)
• Marked/severe hypospermatogenesis	6/12 (50.0%)
Combined total	43/95 (45.3%)

n = 95; 5 of the 100 samples were eliminated due to insufficient tissue. MOMP, major outer membrane protein.

Results

Actively replicating *Chlamydia* detected in testicular biopsies

Of the 100 fixed biopsies, five were eliminated due to insufficient tissue. Sections were probed for MOMP (Fig. 1a–c) and TC0500 (Fig. 1d–f). Staining controls (primary or secondary antibody only, and DAB only) each showed little or no positive staining, thereby validating the localisation patterns shown (Supplementary Fig. S1).

Chlamydial MOMP was detected in 43 of 95 samples, equating to a 45.3% rate of infection overall. When categorised by histological pattern, the rate of infection ranged from 40 to 54% (Table I). No significant difference ($P = 0.1$) was found in infection rates between histological categorisations.

A range of MOMP positive ($n = 11$) and negative ($n = 9$) sections were selected where sufficient tissue was available. These were stained to detect the active replication marker TC0500. Positive TC0500 staining was observed in all MOMP-positive sections, indicating that *Chlamydia* was actively replicating within the testes at the time of biopsy. There was a 100% concordance between MOMP and TC0500 staining (Table II).

Confirmation of positive staining was achieved *via C. trachomatis* 16S rRNA-specific PCR on DNA extracted from the fixed biopsies (Fig. 2). A range of MOMP-positive ($n = 8$) and negative ($n = 8$) samples were selected where sufficient tissue was available. *C. trachomatis* DNA was detected in 100% of MOMP-positive samples (Table II). There was a 100% concordance between immunohistochemistry and PCR techniques for MOMP-positive samples. For MOMP-negative samples, there was a 50% concordance between immunohistochemistry and PCR techniques as four of eight samples returned *C. trachomatis*-positive PCR results. A representative gel electrophoresis image of PCR-positive and -negative samples is shown (Fig. 2).

Detection of *C. trachomatis* DNA in fresh testicular biopsies

Testicular biopsies ($n = 18$) were obtained from severely infertile men undergoing testicular sperm recovery. None of these patients

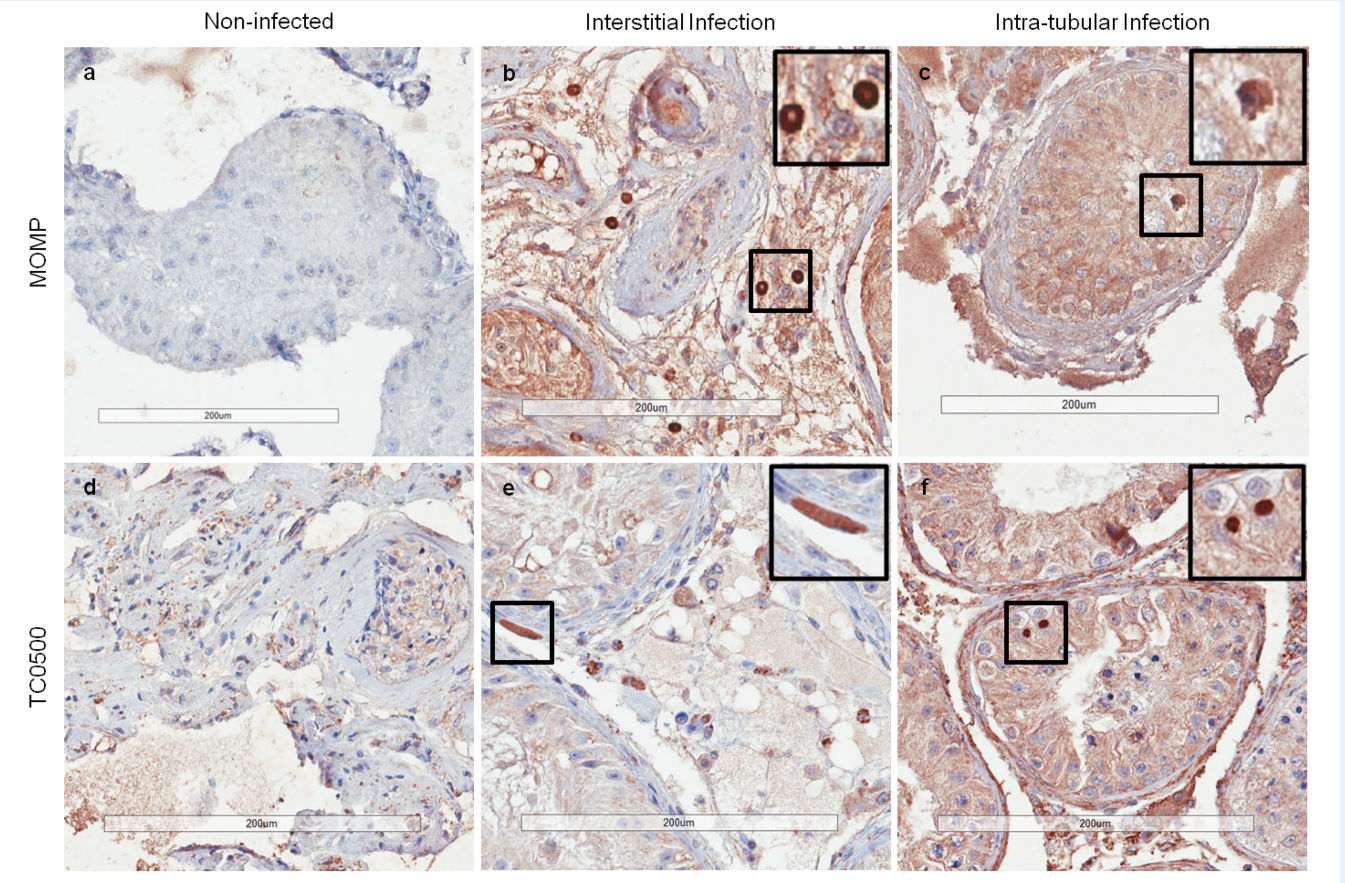

Figure 1 Histological detection of *Chlamydia* in human testicular biopsies. Tissues were provided by Monash Health Anatomical Pathology Department and stained using immunohistochemistry techniques for chlamydial major outer membrane protein (MOMP, **a**–**c**) and the active replication marker TC0500 (DAB chromogen, dark brown, **d**–**f**) then counterstained to show tissue structure (haematoxylin, blue). Panels (a) and (d) noninfected testicular tissue (patient code MB020), (b) and (e) *Chlamydia* identified in interstitial human testicular tissue (patient code MB058) and (c) and (f) *Chlamydia* identified in seminiferous tubules of human testicular tissue (patient code MB036). Scale bars represent 200 μm; images are representative of $n = 5$ noninfected and $n = 5$ infected samples; images were captured on ×20 magnification.

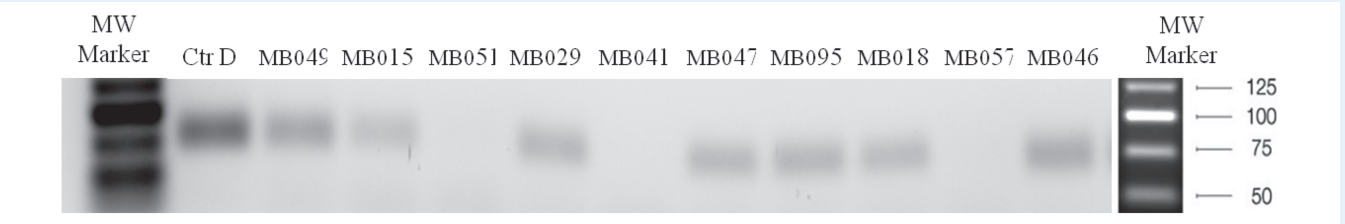

Figure 2 Detection of *C. trachomatis* DNA in fixed testicular biopsies from male-factor infertility patients. Tissues were provided by Monash Health Anatomical Pathology Department, DNA was extracted from tissue sections (Qiagen FFPE DNA kit) and real-time PCR specific to *C. trachomatis* 16S rRNA DNA was performed. Amplicons were electrophoresed on agarose gel to obtain the representative image pictured, which includes *C. trachomatis* serovar D (Ctr D) positive control and several positive (MB049, MB015, MB029, MB047, MB095, MB018, MB046) and negative (MB051, MB041, MB057) samples. The molecular weight (MW) marker shows the amplicon size to be <100 bp.

reported previous diagnosis with an STI, including *C. trachomatis*. Thirteen men had a diagnosis of idiopathic infertility with severe oligo-azoospermia, and five azoospermic men had an identified cause including obstruction (cystic fibrosis, $n = 2$), Klinefelter syndrome ($n = 1$), Yq chromosome microdeletion ($n = 1$) and post-chemotherapy ($n = 1$) (Table III). *C. trachomatis* DNA was found in 3 of the 18 specimens (16.7%): Patient 1, Patient 4 and Patient 5 (Table III). Amplicons of all three specimens melted within 4°C of the positive control (Table III). The gel electrophoresis shows all three specimens produced amplicons of similar size to the positive control (Fig. 3). These tests assisted in confirming the detection of *C. trachomatis* DNA within the biopsies.

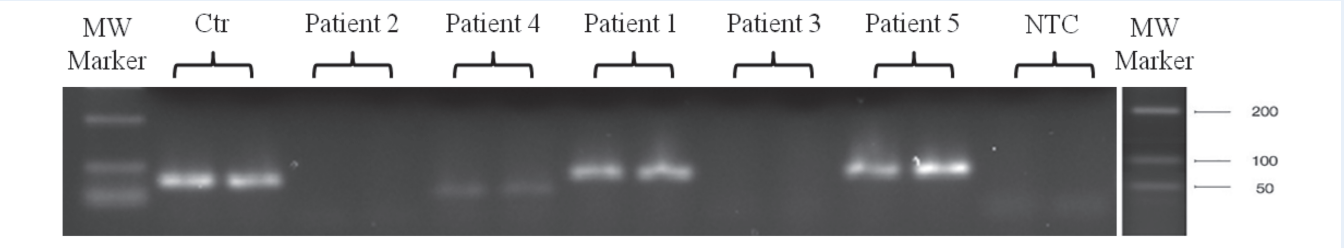

Figure 3 Detection of *C. trachomatis* DNA in fresh testicular biopsies from male-factor infertility patients. Testicular biopsies were provided by Monash IVF Group and Queensland Fertility Group, DNA was extracted from tissue (QIAmp Blood and Tissue kit) and real-time PCR specific to *C. trachomatis* 16S rRNA DNA was performed. Amplicons were electrophoresed agarose gel to obtain the representative image pictured, which includes positive (Patient 1, 4 and 5) and negative (Patient 2 and 3) samples. The positive control used was *C. trachomatis* serovar D DNA. The molecular weight (MW) marker shows the amplicon size to be <100 bp.

Table II Concordance between chlamydial immunohistochemistry markers and PCR in selected fixed samples.

Sample identifier	MOMP	TC0500	PCR
MB049	+	+	+
MB015	+	+	+
MB029	+	+	+
MB095	+	+	+
MB018	+	+	+
MB014	+	+	+
MB060	+	+	+
MB045	+	N/A	+
MB028	+	+	N/A
MB096	+	+	N/A
MB058	+	+	N/A
MB036	+	+	N/A
MB039	–	–	+
MB047	–	–	+
MB046	–	–	+
MB052	–	N/A	+
MB085	–	N/A	–
MB051	–	–	–
MB041	–	–	–
MB057	–	–	–
MB075	–	–	N/A

Detection of *C. trachomatis*-specific antibodies in human serum

Serum samples from the 18 patients were assessed for *C. trachomatis* serovar D- and E-specific IgG by dot blotting. Serum samples from three patients whose biopsies were PCR-positive showed seropositivity to one or both of the serovar: D and/or E (Table III). Serum samples from eight other patients also showed seropositivity to one serovar ('+'), while two additional patients were serovar D and E seropositive ('++'). This gives a total of 13 positive samples: a *C. trachomatis*-specific antibody seropositivity rate of 72.2%. Examples of negative, single and double seropositive dot blots are shown in Fig. 4a–f, along with immunocytochemistry of infected cell monolayers (Fig. 4g and h). As the fresh biopsy cohort was small in this pilot study, it was not possible to show a correlation between PCR and serum positivity of patients.

Discussion

Our findings demonstrate for the first time that replicating *C. trachomatis* is present in human testicular tissue and may be associated with moderate to severe spermatogenic impairment. We utilised highly specific DNA and protein markers to diagnose an unexpectedly high prevalence of chlamydial infection in two distinct populations of infertile men, who underwent either diagnostic (fixed) or therapeutic (fresh) biopsy. Furthermore, similarly high rates of detection (40–54%) were present in all of the major histological categories of spermatogenic failure represented in the fixed biopsies. These findings have profound implications for the study of the etiology of idiopathic male infertility and the pathogenesis of testicular chlamydial infection. There are also broader implications for clinical practice in terms of detection of infection in the testis and role for therapy.

These samples may represent a population that is enriched for chlamydial positivity, and the rate is most likely lower in the general population that includes reproductively healthy men, at least when conventional detection systems are used. Unsurprisingly, there was no difference in infection prevalence rates amongst histology types as many testicular insults lead to each of these histological patterns over the evolution of spermatogenic damage, culminating, for example, in the Sertoli cell only phenotype. While a causative role for chlamydial infection and the time course of damage cannot be assessed in the cross-sectional sampling approach, the data could potentially implicate chlamydial infection in damage to the seminiferous tubules.

The presence of *Chlamydia* within fixed biopsies was assessed by two distinct methods. The immunohistochemistry-positive samples were also PCR-positive, and there were no false-positive samples. In particular, the use of the active replication marker TC0500 indicates that at the time of biopsy, these patients had live *C. trachomatis* replicating within the testicles.

However, 50% of immunohistochemistry-negative fixed biopsy samples were PCR-positive. This may highlight the increased sensitivity of

Table III Characteristics of patients providing fresh testicular biopsies

Participant	Clinical presentation	*C. trachomatis* DNA in biopsies	PCR amplicon melt temperature	*C. trachomatis* antibody seropositivity in serum
C. trachomatis	N/A	Positive Control	83.7	N/A
Patient 1	Azoospermia	+	83.7	+
Patient 2	Oligozoospermia	_	N/A	+
Patient 3	Cystic fibrosis	_	N/A	+
Patient 4	Azoospermia	+	83.5	+
Patient 5	Severe oligozoospermia, Y deletion	+	83.0	++
Patient 6	Oligozoospermia	_	N/A	_
Patient 7	Klinefelter syndrome, undescended testis	_	N/A	++
Patient 8	Azoospermia	_	N/A	+
Patient 9	Azoospermia	_	N/A	_
Patient 10	Azoospermia	_	N/A	_
Patient 11	Azoospermia	_	N/A	+
Patient 12	Azoospermia	_	N/A	+
Patient 13	Azoospermia	_	N/A	_
Patient 14	Cystic fibrosis	_	N/A	_
Patient 15	Azoospermia	_	N/A	+
Patient 16	Severe oligozoospermia	_	N/A	+
Patient 17	Post-chemotherapy	_	N/A	++
Patient 18	Azoospermia	_	N/A	+

Bold text: Chlamydia positive patients.

PCR compared to immunohistochemistry, as some samples may have a bacterial burden below the limit of immunohistochemistry detection. Alternatively, PCR will also identify DNA from dead *Chlamydia* (Lleo *et al.*, 2014), whereas immunohistochemistry may only identify live *Chlamydia* within the intracellular inclusion structures produced during replication (AbdelRahman and Belland, 2005). In these cases, infection may have been cleared by the host immune response or antibiotic therapy. The past *C. trachomatis* infections seem likely to have damaged the seminiferous tubules. Unfortunately, patient information for these archived samples was not available to investigate this further.

The fresh biopsies were *C. trachomatis* PCR-positive in 3 of 18 cases, and of these 18 samples, 5 patients had conditions known to cause azoospermia. Normally, diagnosis of *C. trachomatis* is based on urine PCR. Both Patient 4 and Patient 5 had tested urine-negative; unfortunately, no result could be obtained for Patient 1. Taken together, the results indicate that upper reproductive tract infection may not shed into the urinary system or may do so only intermittently. Higher rates of detection are possible with semen analysis, including in an ART setting, highlighting the need for more robust chlamydial detection methods (van den Brule *et al.*, 1993; Witkin *et al.*, 1993; Eley and Pacey, 2011). A more sensitive approach may include testing of testicular needle aspiration or open biopsy samples at the time of collection for sperm retrieval procedures. Given that PCR is utilised as a clinical tool for diagnosis of *Chlamydia* in other body sites, a testicular PCR assay may be a powerful instrument in future fertility evaluation protocols. Chlamydial screening of diagnostic and therapeutic testicular biopsies in male infertility patients could inform clinicians about the course of action required for patients and improve reproductive outcomes. Antibiotic therapy that eradicates testicular infection may improve sperm quality and improve reproductive outcomes (Wong *et al.*, 1986; Pajovic *et al.*, 2013). However, this also requires further investigation into the mechanisms of chlamydial pathophysiology, which has begun in a mouse model (Bryan *et al.*, 2019; Sobinoff *et al.*, 2014).

Additionally, as is already the case in public health practice for many STIs, once a diagnosis is made, an effort should be undertaken to screen and treat all sexual contacts. Transmission between partners may occur unknowingly, as the testicular infection may be asymptomatic and pose a risk to future fertility.

Serum samples matched to the fresh biopsies were assayed for the presence of *C. trachomatis*-specific antibodies. The long serum half-life of IgG confounds exact determination of when a chlamydial infection occurred and whether it is active (Correia, 2010). The rate of *C. trachomatis*-specific antibody detection in serum in our study was at the high end of the range identified in literature, which varies widely between 0 and 90.3% depending on geographic and demographic factors, antibody type and testing material (Idahl *et al.*, 2004; Samplaski *et al.*, 2014). The incidence is also likely higher in infertility cohorts than in the general population. However, it could be inferred that the seropositivity confirms the finding of *C. trachomatis* DNA within the fresh testicular biopsies, while seropositivity in the *C. trachomatis* DNA-negative patients is suggestive of a past infection.

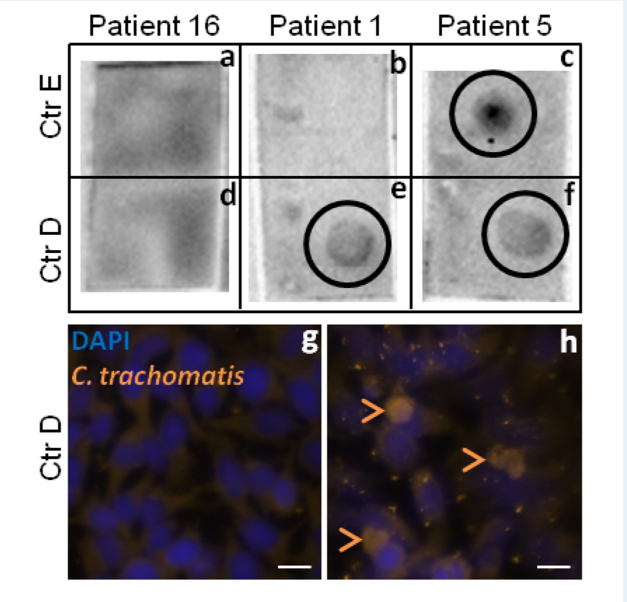

Figure 4 Detection of *C. trachomatis*-specific antibodies in serum from male-factor infertility patients. *C. trachomatis* serovar E (Ctr E)- and serovar D (Ctr D)-loaded nitrocellulose membranes were probed with patient serum. Ctr D-infected HeLa cells were also probed with human serum as a negative control. Examples of these tests from patients with no Ctr-specific antibodies (Patient 16: **a**, **d**), Ctr D specific antibodies (Patient 1: **b**, **e**) and Ctr D- and Ctr E-specific antibodies (Patient 5: **c**, **f**) are shown along with immunocytochemistry of infected cell monolayers (Patient 16: **g** and Patient 5: **h**). Fluorescent images were captured using epifluorescent microscopy on ×40 magnification.

Conclusion

No evidence of human testicular chlamydial infection has been reported in the literature; therefore, this study describes the novel utilisation of PCR and immunohistochemistry to detect a previously unrecognised infection in nonobstructive azoospermic men. *Chlamydia* was detected in fixed diagnostic (40–54%) and fresh therapeutic (16.7%) testicular biopsies, and *Chlamydia*-specific antibodies were detected in serum samples from 72.2% of patients including all three patients who returned PCR-positive fresh biopsies. Although causality cannot be determined at this point, the detection of *Chlamydia* in otherwise asymptomatic infertile men is highly significant and will direct future studies on the mechanisms of damage by male chlamydial infection and approaches to the detection, prevention and treatment of a potentially preventable cause of human infertility.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Acknowledgements

We acknowledge the support from the Monash IVF Group and Queensland Fertility Group in the collection of fresh biopsies, and Monash Health in the retrieval and sectioning of fixed biopsies. We acknowledge the Ian Potter Foundation for equipment used during this study and the QIMR Berghofer Histology Facility for staining expertise.

Authors' roles

E.B.: data acquisition, data and statistical analysis and interpretation, manuscript drafting and critical revision and approval of the final manuscript. R.M.: sample acquisition, data analysis and interpretation, critical revision of the manuscript, funding, student supervision and approval of the final manuscript. L.R.: sample acquisition, critical revision of the manuscript and approval of the final manuscript. D.K.: sample acquisition, critical revision of manuscript and approval of the final manuscript. A.Y.: sample acquisition, critical revision of the manuscript and approval of the final manuscript. K.B.: data acquisition, critical revision of the manuscript, technical/material support and approval of the final manuscript. C.C.: data acquisition, critical revision of the manuscript, technical/material support and approval of the final manuscript. M.G.: analysis and interpretation of data, critical revision of the manuscript and approval of the final manuscript. A.C.: data and statistical analysis and interpretation, drafting and critical revision of the manuscript, administrative/technical/material support, student supervision and approval of the final manuscript. C.A.: data acquisition, critical revision and approval of the final manuscript. L.T.: data acquisition, critical revision and approval of the final manuscript. E.M.: funding, student supervision, critical revision and approval of the final manuscript. K.B.: analysis and interpretation of data, drafting, critical revision and approval of final manuscript, funding, administrative support, technical/material support and student supervision.

Funding

Australian National Health and Medical Research Council (NHMRC) (APP1062198).

Conflict of interest

R.M. declares an equity interest in the study due to financing of fixed biopsy sectioning. All other authors declare no conflicts of interest.

References

AbdelRahman YM, Belland RJ. The chlamydial developmental cycle. *FEMS Microbiol Rev* 2005;**29**:949–959.

Bryan ER, Kollipara A, Trim LK, Armitage CW, Carey AJ, Mihalas B, Redgrove KA, McLaughlin EA, Beagley KW. Hematogenous dissemination of Chlamydia muridarum from the urethra in macrophages causes testicular infection and sperm DNA damage. *Biol Reprod* 2019. ioz146.

Correia IR. Stability of IgG isotypes in serum. *MAbs* 2010;**2**:221–232.

Cunningham KA, Beagley KW. Male genital tract chlamydial infection: implications for pathology and infertility. *Biol Reprod* 2008;**79**:180–189.

Eley A, Pacey AA. The value of testing semen for Chlamydia trachomatis in men of infertile couples. *Int J Androl* 2011;**34**:391–401.

Gallegos G, Ramos B, Santiso R, Goyanes V, Gosálvez J, Fernández JL. Sperm DNA fragmentation in infertile men with genitourinary infection by Chlamydia trachomatis and Mycoplasma. *Fertil Steril* 2008;**90**:328–334

Gregoriou O, Botsis D, Papadias K, Kassanos D, Liapis A, Zourlas PA. Culture of seminal fluid in infertile men and relationship to semen evaluation. *Int J Gynecol Obstet* 1989;**28**:149–153.

Idahl A, Boman J, Kumlin U, Olofsson JI. Demonstration of Chlamydia trachomatis IgG antibodies in the male partner of the infertile couple is correlated with a reduced likelihood of achieving pregnancy. *Hum Reprod* 2004;**19**:1121–1126.

Kumar N, Singh AK. Trends of male factor infertility, an important cause of infertility: a review of literature. *J Hum Reprod Sci* 2015;**8**:191–196.

Lleo MM, Ghidini V, Tafi MC, Castellani F, Trento I, Boaretti M. Detecting the presence of bacterial DNA by PCR can be useful in diagnosing culture-negative cases of infection, especially in patients with suspected infection and antibiotic therapy. *FEMS Microbiol Lett* 2014;**354**:153–160.

Masarani M, Wazait H, Dinneen M. Mumps orchitis. *J R Soc Med* 2006;**99**:573–575.

McLachlan RI, Rajpert-De Meyts E, Hoei-Hansen CE , de Kretser DM, Skakkebaek NE. Histological evaluation of the human testis–approaches to optimizing the clinical value of the assessment: mini review. *Hum Reprod* 2007;**22**:2–16.

Moretti E, Capitani S, Figura N, Pammolli A, Federico MG, Giannerini V, Collodel G. The presence of bacteria species in semen and sperm quality. *J Assist Reprod Genet* 2009;**26**:47–56.

O'Meara CP, Armitage CW, Kollipara A, Andrew DW, Trim L, Plenderleith MB, Beagley KW. Induction of partial immunity in both males and females is sufficient to protect females against sexual transmission of Chlamydia. *Mucosal Immunol* 2016;**9**:1076–1088.

Pajovic B, Radojevic N, Vukovic, M, Stjepcevic A. Semen analysis before and after antibiotic treatment of asymptomatic Chlamydia- and Ureaplasma-related pyospermia. *Andrologia* 2013;**45**:266–271.

Pannekoek Y, Westenberg SM, de Vries J, Repping S, Spanjaard L, Eijk PP, van der Ende A, Dankert J. PCR assessment of Chlamydia trachomatis infection of semen specimens processed for artificial insemination. *J Clin Microbiol* 2000;**38**:3763–3767.

Samplaski MK, Domes T, Jarvi KA. Chlamydial infection and its role in male infertility. *Adv Androl* 2014;**2014**:11.

Sobinoff, AP, Dando SJ, Redgrove KA, Sutherland JM, Stanger SJ, Armitage CW, Timms P, McLaughlin EA, Beagley KW. Chlamydia muridarum infection-induced destruction of male germ cells and Sertoli cells is partially prevented by Chlamydia major outer membrane protein-specific immune CD4 cells 1. *Biol Reprod* 2014;**92**(1): Article 27, 21–13.

van den Brule, AJC, Hemrika DJ, Walboomers JMM, Raaphorst P, van Amstel N, Bleker OP, Meijer CJLM. Detection of Chlamydia trachomatis in semen of artificial insemination donors by the polymerase chain reaction. *Fertil Steril* 1993;**59**:1098–1104.

WHO. WHO laboratory manual for the examination and processing of human semen. In: *ISBN 978 92 4 154778 9*. World Health: Organization. Fifth Edition, 2010

WHO. (2019, 14 June 2019). Sexually transmitted infections (STIs). *Fact Sheets*, from http://www.who.int/mediacentre/factsheets/fs110/en/.

Witkin, SS, Jeremias J, Grifo JA, Ledger WJ. Detection of Chlamydia trachomatis in semen by the polymerase chain reaction in male members of infertile couples. *Am J Obstet Gynecol* 1993;**168**:1457–1462.

Wong, PC, Balmaceda JP, Blanco JD, Gibbs RS, Asch RH. Sperm washing and swim-up technique using antibiotics removes microbes from human semen. *Fertil Steril* 1986;**45**:97–100.