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The relationship between *C.trachomatis* and *M. genitalium* infection and pregnancy rate and outcome in Iranian infertile couples.

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Running title:

STDs infection in infertile couples and pregnancy rate and outcome

Summary

To study the prevalence of *C. trachomatis* and *M. genitalium* in a population of infertile couples from Iran and how this relates to tubal factor infertility, pregnancy rate and outcome of pregnancy. Blood, semen and first void urine samples were obtained from 250 infertile couples and 250 fertile women as a control. Infertile couples were followed up after 24 months to determine diagnosis, referral for assisted conception, any pregnancy and pregnancy outcome. Data were analyzed with regard to the results of(i) serological analysis for specific antibodies to *C. trachomatis* in serum; (ii) the presence of *C. trachomatis* and *M. genitalium* DNA in first void urine ; and (iii) in a semen sample of the male partner. Prevalence of *C. trachomatis* in our study population was comparable to other studies using similar methods and test specimens. No evidence of *M. genitalium* infection was found. Detection of *C. trachomatis* in one partner rarely correlated with infection in the other. The risk of tubal factor infertility and the probability of pregnancy and pregnancy outcome were unrelated to the results of serological tests for *C. trachomatis* antibodies or the presence of *C. trachomatis* DNA in first void urine of both partners and in a semen sample provided by the male.

Keywords:*C. trachomatis*, *M. genitalium*, Infertility, PCR, pregnancy outcome.

INTRODUCTION

Chlamydia trachomatis and *Mycoplasma genitalium* are bacterial infections of the male and female reproductive epithelium and are common causes of non-gonococcal urethritis (Taylor and Haggerty, 2011; McGowin *et al.,* 2012).However, their prevalence depends on sex, age, sexual activity, study population, the test specimen taken and the diagnostic methods used (Dorey *et al*., 2012) leading to a confusing picture of their role in infertility.

Of the two organisms, *C. trachomatis* that has been more extensively studied. There is controversy between results from a new systematic review in non-pregnant women and pregnant women. The studies showed results available for non-pregnant women are indicating test and treat of *C. trachomatis* infection in antenatal care to prevent adverse pregnancy and neonatal outcomes (de Cortina *et al*., 2016), another systematic review in pregnant women showed wide variation of Sexually Transmitted Infection (STI) burden in pregnancy (Joseph Davey *et al*., 2106) and therefore further studies would be needed.

C. trachomatis incidence in different studies has ranged from 52.8% when tested by PCR of endocervical samples from sub-fertile women in Brazil(de Lima Freitas *et al.,* 2011) to as low as 1.0% in a population of asymptomatic subfertile women in Germany by PCR of urine samples(Eggert-Kruse *et al*., 2003).In male partners,*C. trachomatis* infection has ranged from 39.4% in Tunisia when detected by PCR in semen and first void urine samples(Gdoura *et al*., 2008) to 0.304% in a Canadian cohort study where both urine and semen samples were tested(Domes *et al*., 2012). Also a prospective cross-sectional study in 2013 showed the presence of chlamydial antibodies was quantitatively related to the likelihood of hysterosalpingography diagnosed tubal disease(Olaleye & Olamijulo, 2016).

In contrast to *C. trachomatis*, the incidence of *M. genitalium* in infertile couples is not as well studied and moreover its prevalence as a STI is also highly variable. Although most

investigations have considered men with urethritis, in a single study the prevalence among male partners of infertile couples in Tunisia was found to be 18.3 %(Gdoura *et al*., 2008). By contrast, the incidence among infertile women with tubal factor infertility (TFI) was 22.0% compared to 6.3% in women with no tubal abnormality detected(Clausen *et al.,* 2001).

However, in the setting of a genitourinary medicine (GUM) clinic its incidence in women who were considered low risk was found to be as low as 5%and in high risk populations was 7.3%(McGowin and Anderson-Smits, 2011).

Bacterial infections are of concern in men and women of reproductive age because of potential direct effects on conception. In women, for example, genital tract infection can give rise to Pelvic Inflammatory Disease (PID) and TFI (Haggerty *et al*., 2010). Whereas in men it has been shown that semen quality (Hosseinzadeh *et al*., 2000; Idahl *et al*., 2007) and sperm function (Hosseinzadeh *et al*., 2000; 2001; 2003; Eley *et al.,* 2005) can be affected by past or current infection. Therefore, it might be hypothesized that the risk of TFI as well as the pregnancy rate and/or pregnancy outcome in couples with an active bacterial infection might be poorer than in those with no evidence of infection.

To investigate this, we have examined the prevalence of *C. trachomatis* and *M. genitalium* infection in a population of couples from Iran seeking their first medical consultation for infertility. In addition, we also examine the pregnancy rate and outcome of pregnancy in relation to the diagnosis of *C. trachomatis* and *M. genitalium* in either partner.

MATERIALS AND METHODS

Study population and samples obtained

Sequential couples (n=324) attending the Research and Clinical Centre for Infertility (Yazd, Iran)presenting with primary and secondary infertility were screened for inclusion in the study between September 2009 and October 2010. All were approached with informed consent and were asked to participate unless one or both of them had: (i) abnormal karyotype; (ii) history of chemotherapy or radiotherapy treatment; (iii) previous sterilisation; (iv) low semen volume (<1.0 ml) or retrograde ejaculation in the male partner; (v) hypogonadotropic hypogonadism; (vi) a genital tract anomaly; or (vii) where the female age was >35 years old. Using these criteria, seventy-four couples were excluded and the remainder (n=250) were enrolled, with each partner giving informed consent.

Two hundred and fifty pregnant women attending the antenatal clinic in the Akbary Public Health centre (Yazd, Iran) were recruited as a control group between May 2010 and September 2010. Only women with naturally conceived pregnancies, as recorded in medical records, were recruited and gave written informed consent to take part. Extensive attempts were also made to recruit fertile men but this was not successful.

The Ministry of Health Research Ethics Committee, Iran and the University of Sheffield School Of Medicine Research Ethics Committee approved all recruitment procedures and the collection and processing of biological samples.

Collection, processing and transport of samples

All enrolled participants (each individual) provided a 2-ml blood sample (1.5-ml serum) and 20-40 ml urine and the male partners provided a semen sample. Blood was collected into a tube without any anticoagulant and within 6 hours was centrifuged (blood was clotted) at 1500 g for 10 minutes then the serum removed and stored at -20ºC.First void urine samples for both partners of infertile couples as well as the fertile controls were stored in a

refrigerator immediately after collection and DNA extraction (see below)was performed within 2 days. Ejaculates were produced after at least 48 hours sexual abstinence and semen samples were stored -80ºC prior to DNA extraction.DNA was extracted from all urine and semen samples using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instruction. DNA was stored at -20ºC prior to transfer of specimens to the UK.Frozen sera and extracted DNA from urine and semen were transferred on dry ice to Sheffield at the end of the recruitment phase. Upon arrival in Sheffield the samples were stored at -20ºC prior to further analysis as outlined below.

***Chlamydia trachomatis* serology**

To detect specific IgA, IgMand IgG antibodies to *C. trachomatis* an immunofluorescence assay (SeroFIA^{TM}*C. trachomatis*) kit was used (Savyon, Ashdod, Israel). Two people examined each slide and a positive resultdeclared when both were in agreement. Positive and negative controls on each slide were included from the kit.

***Chlamydia trachomatis* PCR**

Nested plasmid PCR for *C. trachomatis* was conducted according to a previously published method on all extracted DNA from urine and semen and two pairs of primers (directed against the cryptic plasmid) used to detect *C. trachomatis* as previously described (Hosseinzadeh *et al*., 2004). Products were analyzed by gel electrophoresis in 1.0% (w/v) agarose with ethidium bromide staining.Positive results were compared with *C. trachomatis* plasmid ($pCTT_1$) sequence, accession: M19487 (J03304).

***Mycoplasma genitalium* PCR**

PCR was carried out on all urine and semen DNA samples to identify the *M. genitalium* 16SrRNA gene (Jensen *et al*., 1991; 2003).The DNA template for positive control was supplied by the Health Protection Agency (London, UK), giving a band size of 427 bp,and distilled water used as a negative control.Products were analyzed by gel electrophoresis in 0.8% (w/v) agarose with ethidium bromide staining.

Clinical information

Clinical information was collected from questionnaires and medical records. Primary infertility was defined as the lack of conception after a year of unprotected coitus, whereas secondary infertility was defined as the inability of a couple to conceive after a year of unprotected and appropriately timed intercourse when one or both partners had previously conceived children (Shaw, 2003) . TFI was defined as the occlusion of one or both tubes as diagnosed either by laparoscopy and/or HSG (Patil, 2009). Endometriosis was confirmed by laparoscopy using European Society of Human Reproduction and Embryology(ESHRE) guidelines for diagnosis and treatment of endometriosis (Kennedy *et al*., 2005). PCOS was diagnosed by vaginal sonography and/or laparoscopy and considering hirsutism (hyperandrogenism) and oligo-amenorrhea in a general examination as described in the Rotterdam 2003 guidelines (Fauser *et al*., 2004). A regular menstrual cycle was defined as being 25-34 days. Miscarriage indicates loss of an embryo or fetus before the 20th week of pregnancy (Shaw, 2003).

Follow up

During follow up period patients were followed up for 24 months after their enrolment into the study. Data collected from their medical records including the treatment and diagnostic procedure performed.

Follow up details included the outcome of any pregnancy (spontaneous or assisted) including live birth, still birth, miscarriage and ongoing pregnancy. Also the sex and weight of baby was born during follow up.

The Statistical Package for the Social Sciences (SPSS) 18.0 software (SPSS Inc., Chicago, IL, USA) was used for data analysis. Chi-square test was employed to compare positivity of *C. trachomatis* by different tests between couples to show the concordance. Relative Risk (RR) and 95% Confidence Interval (95% CI) were used to find the relationship between past medical and reproductive history and infection. Logistic regression was used to examine confounding factors among clinical data obtained in the study population.

RESULTS

The age of all participants ranged from 15 to 52 years of age, with a median age for infertile males of 32 (range 21-52), for infertile women 28(range 15-35) and for fertile women (control group) 28 (range 16-39).The duration of infertility in each couple was ≥1 year but in individual cases ranging up to 18 years. Primary infertility was seen in 72.8% of couples and secondary infertility was seen in 27.2%. The duration of infertility was 5.8 ± 3.5 years among couples with primary infertility and 6.3 ± 3.6 (mean ± SD) years among couples with secondary infertility. None of the patients complained of any symptoms of on-going sexually transmitted infections. Table 1a details the principle diagnoses encountered, with Table 1b showing the type of assisted conception undertaken and Table 1c the proportion of couples achieving a pregnancy. After 24 months, 25 couples were still undergoing treatment. However, four couples (1.6%) had been divorced and 14 couples (5.6%) were

lost to follow-up, of whom 5 couples had emigrated and the rest did not respond or were unreachable. Therefore, pregnancy (end-point) data were only available for232/250 (92.8%) of couples enrolled at the start of the study.

In the infertile couples, the prevalence of *C. trachomatis* defined by serology (IgG positive) was 18% (45/250) and 15.6% (39/250) in male and female partners respectively.This compared to 12.8% observed in the fertile women. In only 9 couples were both partners IgG positive. No IgA positive samples were found in infertile couples or fertile women and only 1.2% (3/250) and 4% (10/250) of samples were IgM positive in infertile males and females.All serum samples from fertile controls were also tested for IgM and IgA but no positive samples were found.

PCR of urine from infertile men and women were positive for *C. trachomatis* DNA in 4.4%(11/250) and 4.8% (12/250) of cases. However, although the incidence seemed very similar between males and females in only one couple did the urine samples from both partners test positive. None of the semen samples from the male partners tested positive for *C. trachomatis* DNA. Similarly, PCR of the urine DNA from fertile women (n=250) did not find any evidence of *C. trachomatis*.

In addition to PCR for evidence of *C. trachomatis* DNA, urine from each group and semen samples from male partners of infertile couples were also examined for evidence of *M. genitalium* DNA, but no samples were found to be positive.

In total, 41 out of the 250 women in infertile partnerships (16.4%) were found to have one or both tubes blocked as diagnosed either by laparoscopy and/or HSG. Therefore, Table 2 shows the risk of TFI according to the *C. trachomatis* status in either the female (Table 2a)

or male (Table 2b) partner. These data show that the risk of TFI was not associated with the *C. trachomatis* status in either partner, regardless of whether this was defined by PCR or serology (IgM&IgG).

Table 3 shows the risk of women in infertile partnerships achieving a pregnancy either naturally (n=56) or following assisted conception (n=59) as a function of her (Table 3a) or her partner's (Table 3b) *C. trachomatis* status. Briefly, this shows that there was no relationship between pregnancy and *C. trachomatis* status in either partner.

Table 4 shows data for pregnancy outcome (live birth or pregnancy loss) in the 115 couples for which outcome data was available. There was no relationship between pregnancy outcome and *C. trachomatis* status in either partner as assessed by serology (IgM&IgG) and PCR of first void urine.

DISCUSSION

We aimed to determine the prevalence of *C. trachomatis* and/or *M. genitalium* among a population of infertile couples in provincial Iran and relate this information to their probability of pregnancy (natural or through assisted conception)and the outcome of pregnancy (live birth or pregnancy loss). To our knowledge, this is the first study of prevalence undertaken on infertile couples in Iran using PCR and serology. Moreover, this is only the second study we are aware of at any location to examine the relationship between prevalence and outcome data in such a cohort.

In contrast with other studies this study did not support the relationship between C. trachomatis infection and TFI. Our other main findings are low prevalence of *C. trachomatis* (comparable to other studies), a zero incidence of *M. genitalium*.

C. trachomatis infection by IgA antibodies in serum detect signs of early infection, (Hamdad-Daudi *et al.,* 2004) and serum IgM and PCR of first void urine establish current infection (Hamdad-Daudi *et al.,* 2004; Eggert-Kruse *et al.,* 2011) and serum IgG provides evidence of past infection (Hamdad-Daudi *et al.,* 2004). As anticipated, a higher prevalence of *C. trachomatis* IgG antibodies was observed in both male and female partners of infertile couples, compared to women of proven fertility (control group).The prevalence of serum IgM and DNA positive samples was about three times lower than seen for IgG. However, overall these data were similar to rates found in non-Islamic countries of Europe and North America when like-for-like comparisons for test and test-specimen are made.

In Sweden the prevalence of IgG antibodies was 24.2% for women and 20.1% for men presenting as couples for infertility and 15.6% for pregnant women acting as controls (Idahl *et al.,* 2004).Similarly, the prevalence of *C. trachomatis* DNA in first void urine of infertile couples was lower, at 6.8% and 7.1% for the female and male partner respectively (there was no first void urine available for the pregnant women acting as controls). In contrast, only 4.5% of French males from infertile couples had detectable levels of IgG antibodies in serum and *C. trachomatis* DNA was detected in 5.4% of first void urine samples (Hamdad-Daoudi *et al*., 2004).

Interestingly, in our population there was no evidence of concordance within couples with regard to *C. trachomatis* infection. This was unexpected and is in contrast with previous studies although that may represent differences in the populations studied and the testing strategies used to detect current or past infection. In a study of infertile couples a significant relationship between IgG positivity in the male and female partner was reported,

and as well as a significant correlation between their serum IgG titre levels (Idahl *et al.,* 2004).

In addition, we failed to find evidence of *C. trachomatis* or DNA in semen samples provided by the male partner or any evidence of *M. genitalium*in any urine or semen samples tested. Whilst this may reflect problems with our PCR, we think this unlikely since the positive and negative controls worked as expected. The prevalence of *M. genatalium* in infertile couples has to our knowledge not been studied. In infertile women, antibodies to *M. genitalium* were found in 22% of their patients with TFI (Clausen *et al.,* 2001).This is similar to an investigation on women in Kenya with endometritis (16%) (Cohen, 2002) and women in the United Kingdom with clinically suspected PID (13%) (Simms, 2003). It remains possible therefore, that *M. genitalium* is a rare infection in infertile couples in this part of Iran. However, a recent study in Tehran found a prevalence of 12% and 2% in symptomatic and asymptomatic men respectively using PCR of first void urine (Yeganeh *et al.,* 2013). Clearly, this is an area which requires further investigation.

Given the prevalence of *C. trachomatis* seen in the infertile couples recruited to this study, we were surprised that there were no negative relationships between past or current *C. trachomatis* infection and TFI or the probability of pregnancy (either natural or with assisted conception) and/or pregnancy outcome (live birth or pregnancy loss) in those women who did get pregnant. A study similar in design to ours found that IgG antibodies in women was related to TFI, but that decreased pregnancy rates were only seen in couples where the man was IgG positive (Idahl *et al.,* 2004). The difference between the two studies is hard to explain given they recruited a similar number of couples (n=250 vs. n=244), had similar levels of serum IgG antibodies to *C. trachomatis* (24.2% vs. 15.6% in infertile women and 20.1% vs. 18.0% in infertile men) and a similar incidence of TFI (16.4% in this study vs. 19%(Idahl *et al.,* 2004). However, both studies have found that

among couples that did achieve a pregnancy, pregnancy outcome was unrelated to past *C. trachomatis* infection in either partner (i.e. IgG positive) although we can also conclude from our PCR results that pregnancy outcome was also unrelated to current *C. trachomatis* infection. This is strengthened by the fact that, unlike the study by Idahl and colleagues (Idahl *et al.,* 2004). where presumably the results of serological tests were available quickly – our couples were not given antibiotic therapy, since the nature of recruitment (in Iran) and subsequent analysis in Sheffield (up to two years later) meant that most /all patients had concluded the follow-up period before the results of screening tests were known. Therefore, if current infection were an important determinant in the probability of pregnancy or pregnancy outcome, we would argue that it would be more obvious in the current study than the one previously conducted (Idahl *et al.,* 2004).

Although previous studies suggesting a relationship between *C. trachomatis* antibodiesand TFI (Taylor and Haggerty, 2011; Clausen *et al.,* 2001;Idahl *et al.,* 2004).,[1, 8, 25]most were carried out on women based on a positive result for *C. trachomatis* and/ora medical history of TFI. In our study, women were not symptomatic and the serology results were obtained after patient recruitment and the completion of all diagnostic procedures. Therefore recruitment was carried out blind to diagnosis and without reference to their diagnosis or reason for infertility. Among 41 women with a TFI diagnosis, only 6 female and 9 male partners were IgG positive, with only one couple where both partners were IgG positive. The rest (26 women) were negative for IgG antibody to chlamydia. Therefore, we feel confident that this is a genuine result and worthy of reporting.

Although clinical guidelines suggest *C. trachomatis* screening is vital, authors have questioned the strength of the evidence base to suggest that genital chlamydial infection

leads to infertility. A systematic review of 3,349 studies published in this journal concluded there was an 'absence of valid evidence on the attributable risk of post-infective tubal factor infertility after genital chlamydial infection' (Wallace *et al.,* 2008).This has been given subsequent credence by modelling studies (Kavanagh *et al.,* 2013), which have suggested that 'at the population level, the likelihood of all-cause TFI in those with past or current chlamydial infection is low'. Clearly this remains a controversial area where well-conducted population based studies are still required.

In conclusion, our findings suggest that in a population of infertile couples in Iran, current or past*C. trachomatis* infection had little bearing on TFI and moreover had no influence on the chance of pregnancy or pregnancy outcome in those who conceived. With regard to *M. genitalium,* we can find no evidence of a relationship with infertility and pregnancy outcome by virtue of the fact that no evidence of infection could be found.

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Competing Interests

None declared.

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Table 1: Diagnoses, treatment and outcome summary of the infertile women (n=250) after 24 months follow up.

	No of couples	Percent (%)
(a) Principal diagnoses:		
Male Factor	100	40.0
PCOS	56	22.4
Tubal damage	41	16.4
Unexplained	31	12.4
Oligomenorrhea	30	12.0
Endometriosis	22	8.8
(b) Treatments:		
None	64	25.6
Ovulation Induction	63	25.2
IUI	18	7.2
IVF	39	15.6
ICSI	66	26.4
(c) Pregnancy outcomes:		
Spontaneous	56	48.7
Assisted Conception	59	51.3

Table 2: The probability of tubal factor infertility (TFI) in 41 women according to *C. trachomatis* antibodies (IgM / IgG) in serum and detection of *C. trachomatis* DNA in urine in both the (a) female and (b) male partner.

(a) Female *C. trachomatis* status				(b) Male *C. trachomatis* status			
Diagnosis		**TFI Status**	**RR (95% CI)**	**Diagnosis**		**Partners TFI**	**RR (95% CI)**
IgM	Positive	2/10	1.23 (0.34-4.39)	IgM	Positive	0/3	1.45 (0.26-8.11)
	Negative	39/240			Negative	41/247	
DNA	Positive	0/12	0.47 (0.07-3.16)	DNA	Positive	2/11	1.11 (0.31-4.03)
	Negative	41/238			Negative	39/239	
IgG	Positive	6/39	0.93 (0.41-2.05)	IgG	Positive	9/45	1.28 (0.66-2.49)
	Negative	35/211			Negative	32/205	

Table 3: Chances of achieving a pregnancy either naturally (n=56) or by Assisted Conception (n=59) in 232 sub-fertile couples according to the presence of IgM&IgG antibodies to *C. trachomatis*or the presence of *C. trachomatis* DNA detected by PCR of first void urine in either the (a) female or (b) male partner.

		No of pregnancies		RR (95%CI)	
		Natural conception	Assisted conception	Natural conception	Assisted conception
(a) Female partner:					
IgM	Positive	1/9	3/9	0.45 (0.07-2.90)	1.33 (0.51-3.44)
	Negative	55/223	56/223		
DNA	Positive	4/11	1/11	1.54 (0.68-3.49)	0.35 (0.05-2.27)
	Negative	52/221	58/221		
IgG	Positive	11/39	13/39	1.21 (0.69-2.12)	1.39 (0.84-2.33)
	Negative	45/193	46/193		
(b) Male partner:					
IgM	Positive	0/2	1/2	1.35 (0.27-6.79)	1.98 (0.49-8.07)
	Negative	56/230	58/230		
DNA	Positive	4/10	2/10	1.71 (0.77-3.78)	0.78 (0.22-2.75)
	Negative	52/222	57/222		
IgG	Positive	14/44	12/44	1.42 (0.86-2.37)	1.09 (0.63-1.88)
	Negative	42/188	47/188		

Table 4: *C. trachomatis* antibodies (IgM&IgG) and the presence of *C. trachomatis* DNA detected by PCR of first void urinePCR in the (a) female and (b) male partner showing the relationship between live birth and pregnancy loss in naturalor assisted conception pregnancies.

(a)

		Pregnancy Outcome		RR (95% CI)	
		Natural conception or Assisted Conception		Natural conception or Assisted Conception	
		Live birth	Pregnancy loss	Live birth	Pregnancy loss
IgM	Positive	3/9	1/9	0.59 (0.23-1.53)	0.69 (0.10-4.63)
	Negative	59/106	17/106		
DNA	Positive	4/11	0/11	0.65 (0.29-1.45)	0.46 (0.07-3.14)
	Negative	58/104	18/104		
IgG	Positive	13/39	4/39	0.52 (0.32-0.83)	0.56 (0.19-1.58)
	Negative	49/76	14/76		

(b)

		Pregnancy Outcome		RR (95% CI)	
		Natural conception or Assisted Conception		Natural conception or Assisted Conception	
		Live birth	Pregnancy loss	Live birth	Pregnancy loss
IgM	Positive	1/2	0/2	0.93 (0.23-3.74)	1.03 (0.08-13.34)
	Negative	61/113	18/113		
DNA	Positive	3/10	0/10	0.53 (0.20-1.39)	0.51 (0.07-3.43)
	Negative	59/105	18/105		
IgG	Positive	17/44	1/44	0.61 (0.40-0.92)	0.09 (0.01-0.69)
	Negative	45/71	17/71		