Diagnostic and prognostic aspects of tubal patency testing
Coppus, S.F.P.J.

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Chlamydia trachomatis IgG seropositivity is associated with lower natural conception rates in ovulatory women without visible tubal pathology.

Sjors Coppus
Jolande Land
Brent Opmeer
Pieternel Steures
Rene Eijkemans
Peter Hompes
Fulco van der Veen
Ben Willem Mol
Jan Willem van der Steeg

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Abstract

BACKGROUND: The relation between Chlamydia trachomatis infection and subsequent tubal damage is widely recognized. As such, C. trachomatis antibody (CAT) testing can be used to triage women for immediate tubal testing with hysterosalpingography (HSG) or laparoscopy. However, once invasive tubal testing has ruled out tubal pathology, CAT serology status is ignored, as its clinical significance is currently unknown. This study aimed to determine whether positive CAT serology is associated with lower spontaneous pregnancy rates in women in whom HSG and/or diagnostic laparoscopy showed no visible tubal pathology.

METHODS: We studied ovulatory women in whom HSG or laparoscopy showed patent tubes. Women were tested for C. trachomatis immunoglobulin G (IgG) antibodies with either micro-immunofluorescence (MIF) or an ELISA. CAT serology was positive if the MIF titre was ≥ 1:32 or if the ELISA index was > 1.1. The proportion of couples pregnant without treatment was estimated at 12 months of follow-up. Time to pregnancy was considered censored at the date of the last contact when the woman was not pregnant or at the start of treatment. The association between CAT positivity and an ongoing pregnancy was evaluated with Cox regression analyses.

RESULTS: Of the 1882 included women without visible tubal pathology, 338 (18%) had a treatment-independent pregnancy within 1 year [estimated cumulative pregnancy rate 31%; 95% confidence interval (CI): 27%-35%]. Because of differential censoring after 9 months of follow-up, regression analyses were limited to the first 9 months after tubal testing. Positive C. trachomatis IgG serology was associated with a statistically significant 33% lower probability of an ongoing pregnancy [adjusted fecundity rate ratio 0.66 (95% CI 0.49 to 0.89)].

CONCLUSIONS: Even after HSG or laparoscopy has shown no visible tubal pathology, subfertile women with a positive CAT have lower pregnancy chances than CAT negative women. After external validation, this finding could be incorporated into existing prognostic models.
Introduction

The devastating effect of *Chlamydia trachomatis* infections on the female reproductive tract is widely recognized (Paavonen and Eggert-Kruse, 1999). The diagnostic value of *C. trachomatis* immunoglobulin G (IgG) antibody titre (CAT) testing has been studied extensively, showing fairly good screening properties. In the Netherlands, this has resulted in a recommendation to include CAT testing as part of the routine fertility workup of subfertile couples (NVOG, 2004).

CAT is mainly used to identify women at high risk of tubal pathology and to triage them for further tubal testing (Coppus *et al.*, 2007a). CAT serology status is ignored, however, once invasive testing has ruled out tubal pathology, as its clinical significance is currently unknown. The causal relationship between Chlamydia infections and tubal factor subfertility has been well established, but the question whether positive CAT serology is associated with lower pregnancy rates has been studied less often. It has been postulated that Chlamydia infections have a detrimental effect on the endometrium resulting in impaired implantation and lower pregnancy rates (Witkin, 1999; Neuer *et al.*, 2000). Yet studies examining the association between elevated *C. trachomatis* IgG antibody levels and pregnancy rates in an IVF population were not uniformly conclusive. Whereas some studies suggested a lower implantation and a lower ongoing pregnancy rate (Rowland *et al.*, 1985; Lunenfeld *et al.*, 1989; Witkin *et al.*, 1994; Pacchiarotti *et al.*, 2009), other work suggested that IVF outcome was not associated with CAT status (Osser *et al.*, 1990; Tasdemir *et al.*, 1994; Claman *et al.*, 1996; Sharara *et al.*, 1997; Spandorfer *et al.*, 1999).

The prognostic value of *C. trachomatis* antibodies on spontaneous pregnancy rates in subfertile women is equally uncertain. To the best of our knowledge, four previous studies have examined this relation, with conflicting results (Idahl *et al.*, 2004; Kelz *et al.*, 2006; Perquin *et al.*, 2007; El Hakim *et al.*, 2009). As a consequence, it is still unclear whether positive CAT serology is associated with lower spontaneous pregnancy rates once invasive tubal testing has ruled out tubal pathology. The objective of this study was to assess in a prospective consecutive series of subfertile couples, whether evidence of a past Chlamydia infection affects the probability of spontaneous pregnancy in women without visible tubal pathology.
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Materials and Methods

Patients
Between January 2002 and February 2004, consecutive couples presenting at the fertility clinics of 38 hospitals in The Netherlands were invited to participate in a prospective cohort study. Institutional Review Board approval for this study was obtained from each participating hospital. For all couples, a thorough medical history was taken, including previous pelvic inflammatory disease (PID) and Chlamydia infection (Coppus et al., 2007b), and all couples underwent a basic fertility work-up according to the guidelines of the Dutch Society of Obstetrics and Gynaecology. The details of this workup have been described in detail elsewhere (van der Steeg et al., 2007).

Couples with a history of reversal of tubal ligation, tubal surgery, IVF, or previous tubal patency testing were excluded. The present study was limited to couples with a regular ovulatory cycle, defined as a cycle length between 23 and 35 days with a within cycle variation of less than 8 days. Ovulation was confirmed by a basal body temperature chart, mid-luteal serum progesterone, or by ultrasonographic monitoring of the cycle. Duration of subfertility was defined as the period between the date the couple had started unprotected intercourse and date of tubal testing. We calculated female age at the time of tubal testing. Subfertility was considered to be secondary if a woman had conceived in this or in a previous partnership, regardless of the pregnancy outcome.

Semen analysis was performed at least once, and a total motile sperm count (TMC) was calculated by multiplying semen volume, semen concentration and percentage of progressive motile spermatozoa. Couples in whom semen analysis showed a severe impairment of semen quality requiring IVF-ICSI (defined as a TMC < 1 x 10⁶) were excluded from the present analysis.

Chlamydia antibody testing was performed either with a micro-immuno-fluorescence (MIF) technique (BioMerieux, Paris, France) or with an ELISA (Medac GmbH, Wedel, Germany; Savyon Diagnostics LTD, Marne La Valle, France). CAT testing was performed according to the manufacturer’s instructions and the test result was considered positive at an MIF titre of ≥ 1:32 or an ELISA index of >1.1 (NVOG, 2004). Endocervical swabs were not routinely taken.
Tubal patency was evaluated by hysterosalpingography (HSG) and/or diagnostic laparoscopy, depending on the local protocol of the participating hospital. Tubal pathology was considered absent if the HSG showed passage of contrast medium through both tubes and normal intra-abdominal spread of contrast medium. When laparoscopy was used, tubal pathology was considered absent if there were no signs of tubal obstruction or severe adhesions interfering with ovum pickup. We did not take into account minimal stage endometriosis. In case a woman underwent both HSG and diagnostic laparoscopy, which was often done in case of an abnormal HSG, the results of the laparoscopy were decisive. No therapeutic interventions were performed during these procedures. Only women with bilateral patent tubes were included in the analyses; all women with intra-abdominal pockets of contrast after HSG or extensive adhesions at laparoscopy were excluded.

Follow-up started after tubal testing and ended 12 months thereafter. Primary end-point was spontaneous conception resulting in an ongoing pregnancy at 12 weeks of gestational age. The first day of the last menstrual cycle was considered to mark the end of time until spontaneous conception.

Statistical analysis
Time to spontaneous conception resulting in an ongoing pregnancy was considered censored at the moment treatment had started within 12 months after testing, or at the last date of contact during follow-up if the couple did not have an ongoing pregnancy at that date. If a woman became lost to follow-up within 12 months after evaluation of tubal status, her general practitioner was sent a questionnaire to document the most recent fertility status of the couple.

Kaplan-Meier curves for the chances of spontaneous ongoing pregnancy over time were constructed for CAT positive and CAT negative women separately. The difference in time to pregnancy between these groups was tested for significance with the log-rank test statistic. Subsequently, we evaluated the influence of CAT positivity using Cox regression modelling, expressing the effect as a fecundity rate ratio (FRR) with corresponding 95% confidence intervals (CIs). An FRR expresses the probability of an ongoing pregnancy per time unit for women with a particular feature relative to the probability in women without that feature. We estimated unconditional and conditional FRRs. For the conditional FRR, we built a multivariable Cox model by additionally including the following known prognostic factors:
duration of subfertility, female age at tubal testing, and secondary subfertility (Hunault et al., 2004).

The proportional hazard assumption of the Cox model was checked separately for each variable before performing the regression analysis. Such checking was done graphically by inspecting the graphs of logₐ[-logₑS(t)] versus logₑ(t) for each dichotomous covariate to assess whether the curves run parallel. Additionally, time-dependent covariates were included and we checked whether their coefficient differed significantly from zero. All data were analysed using SPSS 12.0.1 (SPSS Inc., Chicago, IL, USA).

Results

Data of 7860 couples were collected during the study period. After excluding couples with anovulation or severe male factor subfertility, and those that had undergone previous tubal testing, IVF or tubal surgery, data of 5522 ovulatory subfertile women were available. In 1569 women (28%) CAT was not measured; in 1602 other women (29%) CAT was measured but no tubal testing was performed. Tubal pathology was diagnosed in 469 (20%) at HSG or laparoscopy. The data of the remaining 1882 women with no visible tubal pathology and a known CAT result were included in this analysis (Fig. 1).

Baseline characteristics of the 1882 included couples are shown in Table 1. CAT positive women were slightly, but statistically significantly older and more often suffered from a secondary subfertility than did CAT negative women. In 1244 women (66%) tubal pathology was excluded by HSG, whereas in 638 of women (34%) this was done by laparoscopy. CAT testing was performed by MIF in 443 women (24%) and by ELISA in 1439 women (76%). Overall, 438 women (23%) had a positive CAT result.

Follow-up status is shown in Fig. 1. The median duration of follow-up, from time of tubal testing to pregnancy or start of treatment was 3.6 months (interquartile range 1.4 - 7.4 months). Of all included couples, 338 (18%) had an ongoing pregnancy without treatment within 1 year after evaluation of tubal function, including four multiple pregnancies. In 29 women pregnancy resulted in a miscarriage.
and one pregnancy was ectopic, resulting in an overall miscarriage and ectopic pregnancy rate of 8.6% and 0.3% respectively. A total of 1162 couples (62%) had started treatment within 12 months; 363 others (19%) neither started treatment nor became pregnant. The estimated overall fraction of untreated couples with a treatment independent pregnancy at 12 months of follow-up for all women was 31% (95% CI: 27%-35%).

FIGURE 1. Flow of patients
TABLE I. Baseline characteristics of 1882 couples

<table>
<thead>
<tr>
<th></th>
<th>CAT positive (n = 438)</th>
<th>CAT negative (n = 1444)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
<td>5(^{th})-95(^{th}) percentile</td>
<td>Median</td>
</tr>
<tr>
<td>Female age (year)</td>
<td>34.0</td>
<td>27.0-39.3</td>
<td>32.2</td>
</tr>
<tr>
<td>Male age (year)</td>
<td>36.2</td>
<td>29.1-46.8</td>
<td>34.1</td>
</tr>
<tr>
<td>Duration of subfertility (year)</td>
<td>2.0</td>
<td>1.1-5.2</td>
<td>1.9</td>
</tr>
<tr>
<td>Semen analysis – TMC (*10(^{6}))</td>
<td>52.2</td>
<td>4.8-284</td>
<td>50.9</td>
</tr>
<tr>
<td>Secondary subfertility</td>
<td>46%</td>
<td></td>
<td>36%</td>
</tr>
<tr>
<td>History of PID</td>
<td>5.3%</td>
<td>1.2%</td>
<td></td>
</tr>
<tr>
<td>History of Chlamydia</td>
<td>14.8%</td>
<td>2.4%</td>
<td></td>
</tr>
</tbody>
</table>

The Kaplan-Meier curves of women with a positive CAT status and those with a negative CAT status are shown in Fig. 2. There was a significant difference ($P = 0.02$). The proportional hazards assumption had to be rejected for the effect of CAT status on pregnancy chances over time ($P < 0.001$). Further analyses revealed that this deviance from proportionality was not constant over time, so we could not model the effect with a time-dependent covariate. Based on the $\log_e [-\log_e S(t)]$ versus $\log_e (t)$ plots, we came to the conclusion that there was differential censoring after 9 months. We therefore decided to limit the analysis to the first 9 months after tubal testing. In this time period, positive *C. trachomatis* IgG serology had a strong effect on time to natural conception, with 35% lower pregnancy chances (FRR 0.65, 95% CI 0.48-0.87) (Table II).

Multivariable Cox regression analysis showed that the conditional FRR was similar to the unconditional one, indicating that the lower pregnancy rates in CAT positive women were also present when known prognostic factors for spontaneous pregnancy were taken into account. This makes it unlikely that the observed association can be attributed to a confounding effect of duration of subfertility, female age and type of subfertility (Table II). As laparoscopy is considered the reference standard to exclude tubal pathology (NVOG, 2004), we performed a sub-analysis in which we compared women who had HSG only with those who underwent laparoscopy, which produced similar results.
CAT testing and natural conception

![Cumulative ongoing pregnancy rate (%) over time after spontaneous pregnancy (months)](image)

**FIGURE 2.** Spontaneous pregnancy rates for CAT IgG negative (upper line) and CAT positive (lower line) without visible tubal pathology.

**TABLE II.** The effect of C. trachomatis IgG serology on spontaneous pregnancy at 9 months follow-up*

<table>
<thead>
<tr>
<th></th>
<th>Unconditional</th>
<th>Conditional</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FRR 95% CI</td>
<td>P-value</td>
</tr>
<tr>
<td>Positive CAT</td>
<td>0.65 0.48-0.87</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Duration of subfertility (per year)</td>
<td>0.77 0.68-0.88</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Female age (per year)</td>
<td>0.97 0.94-0.99</td>
<td>0.03</td>
</tr>
<tr>
<td>Secondary subfertility</td>
<td>1.88 1.49-2.36</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Results obtained in Cox regression analyses; FRR, fecundity rate ratio; 95% CI, 95% confidence interval; *Analysis limited to first 9 months of follow-up due to differential censoring

**Discussion**

In this large prospective multicentre study, we observed that subfertile ovulatory women with a positive CAT result but without visible tubal pathology at HSG or laparoscopy, had significantly lower spontaneous pregnancy rates in the first 9 months after tubal testing than comparable women with a negative CAT test. This effect was also observed when other patient characteristics (secondary subfertility, female age and duration of subfertility) were taken into account. Pregnancy rates in CAT positive women were about a third lower.
Strength of our study is the multicentre design through which a large number of couples could be included. Although no uniform CAT assay was used in all clinics, the majority used ELISA assays, which are less laborious and reader-dependent than MIF. It has been shown that ELISAs in general show less cross-reactivity with other Chlamydia species than the MIF Biomerieux assay used in the present study (Gijssen et al., 2001; Land et al., 2003), although a recent study in general showed better diagnostic performance of MIF compared with ELISA (Broeze et al., 2011). However, as the CAT assays that were used in this study are all commercially available and routinely used in hospitals throughout the country, we feel that our study reflects routine daily practice, thereby improving the generalizability of the results of the study.

For tubal testing, both HSG and diagnostic laparoscopy were used in the participating hospitals. In the present study, hospitals were free to use their own protocol for evaluating tubal status. This generated the practice variation from which our analysis could benefit. The Dutch guideline on the diagnostic subfertility work-up (NVOG, 2004) recommends laparoscopy for CAT positive patients, and HSG or no tubal assessment in case of a negative CAT. Strict adherence to this guideline would have introduced a bias into our research, but in our study about half of CAT positive women underwent HSG, whereas 30% of CAT negative women underwent diagnostic laparoscopy. We acknowledge that, ideally, tubal status in all women would have been verified by laparoscopy, but feel that such a design would be hardly feasible for ethical reasons and due to restricted availability as a result of high costs and limited operating time. Around 10% of women with a normal HSG show tubal pathology on laparoscopy (Mol et al., 1999; den Hartog et al., 2008; Verhoeve et al., 2011). Taking into account that more CAT negative women underwent tubal testing with HSG than did CAT positive women, the effect of CAT found in the present study cannot be attributed to differential verification. Instead, it is because of this variability in the use of HSG and laparoscopy that we were able to evaluate the effect of CAT positivity on pregnancy chances. In addition to this differential verification, women who were tested with CAT were only partially verified. In the present study, 46% of women with an available CAT result did not undergo invasive tubal testing, of which 79% were CAT negative. The present study addressed women in whom pathology had been ruled out, but had different CAT status. We therefore believe that the main impact of the partial verification was a reduction in the precision, rather than a bias of fecundity rate estimates.
In the present study we tried to control for confounding effects of tubal pathology by limiting the analysis to those women without tubal abnormalities on HSG and/or laparoscopy. In addition, the confounding effect of the use of assisted reproductive technologies was eliminated by censoring time to pregnancy whenever treatment was started. Unfortunately, we were not able to analyse CAT titres as a continuous or categorical variable, taking into account the quantitative nature of this test, as the majority of clinics used an ELISA assay of which the results are reported as either positive or negative and no titres are available. Nevertheless, cut-off values advocated in the guideline of the Dutch Society for Obstetrics and Gynaecology were used, i.e. a titre of ≥1:32 in case of MIF and an index of >1.1 in case of ELISA. We feel this approach reflects daily clinical practice, where the CAT result is usually interpreted by the clinician as either being positive or negative.

Our results are in contrast with that of three other studies, which found no significant effect of *C. trachomatis* antibodies on pregnancy rates (Idahl et al., 2004; Perquin et al., 2007; El Hakim et al., 2009). The most recent study (El Hakim et al., 2009) explored the relation between CAT titres and probability of pregnancy in women with normal-looking Fallopian tubes at laparoscopy. However, with a total sample size of 174 women, the power of the study to detect an association was limited, due to the formation of multiple subgroups, including endometriosis.

Idahl and colleagues, studied 244 women followed for a mean of 37 months, but unfortunately their analysis did not take into account time to pregnancy (Idahl et al., 2004). Instead, pregnancy was evaluated as a dichotomous outcome, ignoring the fact that fecundity is a gradual continuum based on time to pregnancy. Furthermore, the analysis did not correct for the presence of tubal pathology and type of pregnancy (spontaneous or treatment-related). The third study, using data of 153 women who participated in a randomized controlled trial, evaluated the prognostic significance of CAT on cumulative pregnancy rates at 18 months follow-up (Perquin et al., 2007). No statistically significant difference in cumulative pregnancy rates was found (FRR 0.73, 95% CI 0.42-1.25). While this study used a time-to-event analysis, the interpretation of its results is hampered by heterogeneity in outcomes, as the authors did not solely evaluate treatment independent pregnancy rates, but also included pregnancies achieved after intrauterine insemination, IVF, tubal surgery and other therapeutic options.
The results of our study are in accordance with a previous study, which showed in 170 studied women that the number of treatment independent pregnancies was lower with increasing CAT MIF titres (Kelz et al., 2006). However, a drawback of that study is the fact that it did not correct for the effects of tubal pathology visible at HSG or laparoscopy related to a previous Chlamydia infection. As higher MIF titres are associated with a higher likelihood and severity of tubal disease (Akande et al., 2003), it is possible that an indirect association of titres on spontaneous pregnancy rates was measured; an association which in fact was due to the presence of tubal pathology.

We found a marked difference in effect of CAT positivity on pregnancy rates at 9 months of follow-up. This difference might be explained by the increasing duration of subfertility in those couples who did not conceive, which has been shown to be associated with a decrease in spontaneous pregnancy rates (van der Steeg et al., 2007). It might be that the remaining group of women at a later time in follow-up differs from the total cohort at the beginning of follow-up, as it can be hypothesized that when pregnancy has not occurred after 3 or 4 years, spontaneous pregnancy chances are low, due to a preponderance of severe -undefined- pathology that overrules other test results, such as the CAT that we investigated. Alternatively, the marked difference between pregnancy rates in CAT positive and CAT negative women at 9 months of follow-up could be explained by selection bias due to non-random censoring, i.e. higher proportions of CAT positive couples are starting treatment.

Two hypotheses could explain why CAT seropositivity influences pregnancy rates negatively even in case of patent tubes. First, it is known that C. trachomatis may persist in the upper female genital tract and this persistent exposure to the microorganism could result in a chronic inflammatory response that is responsible for damage to the Fallopian tubes (Patton et al., 1994; Den Hartog et al., 2005). It has been suggested that these persistent C. trachomatis infections also elicit an autoimmune response to human heat shock proteins (HSPs) due to their structural similarity with Chlamydia HSP (Neuer et al., 1997; Witkin, 1999). Human HSPs play an important role in early pregnancy (Witkin, 2002), and animal as well as human research indicates that autoimmunity to human HSP exerts a negative influence on embryo development and implantation (Witkin et al., 1994; Witkin 1999, Neuer et al., 2000). From this it has been hypothesized that women with Chlamydia
IgG antibodies, as marker of a previous infection, might be at risk for impaired implantation and lower pregnancy rates. Whether women who do not develop tubal pathology after Chlamydia infection are able to clear the micro-organism from their genital tracts before tubal pathology and an autoimmune response to human HSPs are elicited remains to be elucidated. Some research has focused on persistent endometrial infections that might either affect embryonic development or the implantation capacity of the endometrium, leading to implantation failure (Kamiyama et al., 2004; Romero et al., 2004; Den Hartog 2010). A second hypothesis to explain lower fecundity in CAT positive women without visible tubal pathology, is that intratubal microdamage may have resulted from a previous Chlamydia infection that cannot be detected with conventional patency tests such as HSG or laparoscopy. In addition, these tests are known to be imperfect, with some between-reader variability, but it is unlikely that this would explain the substantially lower pregnancy rates in CAT positive women.

We showed that in 9 months follow-up subfertile women with a positive CAT test without visible tubal pathology on HSG or laparoscopy have a 33% lower probability of pregnancy as compared to CAT negative women. This finding could be useful in counselling subfertile couples on their spontaneous fertility prospects. If our findings hold in external validation, CAT has to be incorporated in future prognostic models on spontaneous pregnancy. Our finding demonstrates that even in absence of tubal pathology, decreased fecundity is a late effect of lower genital tract Chlamydia infections. This might increase the late costs of Chlamydia infections, and as such alter the cost-effectiveness of Chlamydia screening (Land et al., 2010). CAT apparently does not only have diagnostic value, but also gives valuable additional prognostic information on the probability to conceive spontaneously, even after tubal testing has shown no visible tubal pathology.
Chapter 7

References


