Serological markers of persistent C. trachomatis infections in women with tubal factor subfertility

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BACKGROUND: Persistent C. trachomatis infections are assumed to increase the risk of tubal pathology. We studied whether serological markers, assumed to be associated with persistent C. trachomatis infections, could identify subfertile women at risk of tubal pathology. METHODS: Sera of 313 subfertile women, who all underwent a laparoscopy with tubal testing to assess the grade of tubal pathology, were tested for the presence of immunoglobulin (Ig) G and IgA antibodies to C. trachomatis, IgG antibodies to chlamydia heat shock protein 60 (cHSP60) and C-reactive protein (CRP). RESULTS: C. trachomatis IgA, cHSP60 IgG and CRP, all serological markers of persistent infections, were significantly more prevalent in women with tubal pathology as compared to those without tubal pathology. The predictive value of the currently used screening test for tubal pathology (IgG to C. trachomatis) could be significantly improved by adding the CRP test. CONCLUSIONS: In subfertile women with tubal pathology, serological markers of persistent C. trachomatis infections are significantly more common as compared to women without tubal pathology. C. trachomatis IgG-positive subfertile women with slightly elevated (<10 mg/l) CRP levels are at highest risk of persistent C. trachomatis infections and tubal pathology.

Key words: Chlamydia trachomatis/chlamydia heat shock protein 60/C-reactive protein/immunoglobulin A/persistent infection

Introduction

C. trachomatis infections are a major cause of tubal factor subfertility. However, the precise pathogenesis of C. trachomatis infections remains to be elucidated. Not all women who have undergone a C. trachomatis infection will develop tubal pathology. Host factors, virulence of the micro-organism and environmental factors determine the course and morbidity of C. trachomatis infections. Depending on these factors and their interaction, C. trachomatis infections will either be cleared or persist. A clearance rate of 44.7% has been reported in asymptomatic and untreated women after 1 year follow-up (Morré et al., 2002). In women who clear a C. trachomatis infection adequately, the risk of tubal damage may be low, since the host has been exposed to the microorganism during a short period. Persistent exposure to the micro-organism may result in a chronic inflammatory response and may increase the risk of tubal damage, as has been suggested previously (Grayston et al., 1985; Patton et al., 1994a).

Previous studies, in which evidence of persistent C. trachomatis infections has been found in the upper genital tract of women with tubal damage, support this hypothesis. Gérard et al. (1998) have found viable C. trachomatis microorganisms in seven out of ten tubes of patients with ectopic pregnancies. Furthermore, C. trachomatis has been detected in 56–79% of the tubes of women with tubal factor subfertility, who underwent reconstructive surgery (Campbell et al., 1993; Patton et al., 1994b). Previously, we have demonstrated that genus-specific immunoglobulin (Ig) G antibodies to chlamydia lipopolysaccharide, which are supposed to be markers of persistent infections, are significantly more often detectable in sera of subfertile women with distal tubal pathology (62.7%) as compared to those without distal tubal pathology (33.9%) (Den Hartog et al., 2004).

Since the association between C. trachomatis-specific IgG antibodies and tubal pathology has been noted (Punnonen et al., 1979), measuring C. trachomatis IgG antibodies in serum is used as a screening method for tubal pathology. Although species-specific C. trachomatis IgG antibodies are markers of previous infections, their presence does not reflect the course of the infection. Therefore, measuring IgG antibodies to C. trachomatis is not useful in discriminating between clearance or persistence of the infection.

We hypothesize that persistent C. trachomatis infections play an important role in the development of tubal pathology, and have studied known serological markers of persistent infections in subfertile women with and without tubal pathology. Elevated levels of IgA antibodies and C-reactive protein (CRP) could be significantly improved by adding the CRP test.
protein (CRP), in the absence of an acute infection, have been suggested to be markers of chronic inflammation and infection, and have been evaluated previously in studies on the relationship between chronic C. pneumoniae infections and respiratory and cardiovascular disease (Rovinainen et al., 2000; Gattone et al., 2001; Johnston et al., 2001; Falck et al., 2002; Wong et al., 2002). IgG antibodies to chlamydia heat shock protein 60 (cHSP60) have also been associated with chronic inflammation (Morrison et al., 1989), and have been studied previously in sub fertile women with tubal pathology (Toye et al., 1993; Arno et al., 1995; Freidank et al., 1995; Claman et al., 1997).

Sera of sub fertile women were tested for the presence of IgG and IgA antibodies to C. trachomatis, IgG antibodies to cHSP60 and CRP. All women underwent a laparoscopy with tubal testing. We correlated the serological test results with the presence of tubal pathology at laparoscopy, and evaluated the role of single tests and test combinations in predicting the risk of persistent C. trachomatis infections and tubal pathology.

Materials and methods

The study was performed in sub fertile women who entered our clinic between December 1990 and November 2000. As part of their fertility work-up, in all female patients blood was drawn at their initial visit for a chlamydia IgG antibody test (CAT). All spare sera were cryopreserved and thawed for this study. Patients with a negative CAT and an otherwise normal fertility work-up underwent a hysterosalpingography (HSG) to evaluate the tubal status. If the HSG showed abnormalities, or if they did not conceive within 6 months after the HSG, a laparoscopy with tubal testing was performed. Patients with a positive CAT underwent a laparoscopy with tubal testing immediately after the fertility work-up. Only patients who had undergone a laparoscopy and tubal testing with Methylene Blue dye were included in the present study. Patients who had undergone previous pelvic surgery (except for an uneventful appendectomy or Caesarean section) were excluded.

Two independent investigators, who were unaware of the CAT results, scored 313 successive laparoscopy reports to assess the grade of tubal pathology. In cases of disagreement, consensus was reached by consultation. For the sake of the study, tubal pathology was defined as extensive peri-adnexal adhesions and/or distal occlusion of at least one tube (Land et al., 1998). Sub fertile women without distal tubal pathology served as controls. The controls had unexplained sub fertility, partners with mild male factor sub fertility, or proximal occlusion of at least one tube.

Serological methods

IgG antibodies to C. trachomatis were detected using the species-specific Chlamydia pneumoniae IgG micro-immunofluorescence (MIF) test (AniLabsystems, Finland), as described previously (Den Hartog et al., 2004). This species-specific test, which is the currently used screening test for C. trachomatis IgG antibodies in our clinic, has been found to be a good predictor of tubal pathology (Land et al., 2003). The threshold titre used for a positive test was 32.

IgA antibodies to C. trachomatis were detected using the Chlamydia trachomatis IgA enzyme immunoassay (EIA) (AniLabsystems, Finland). The test was used according to the manufacturer’s instructions. The threshold index for a positive test was 1.4.

IgG antibodies to cHSP60 were detected using the cHSP60 IgG enzyme-linked immunosorbent assay (ELISA; Medac, Germany), which is available for research use only. The test was used according to the manufacturer’s instructions. The threshold index for a positive test was 1.11.

CRP was determined using the CRP ELISA (DiaMed Eurogen, Belgium). The test was used according to the manufacturer’s instructions. In order to reliably detect low CRP concentrations, this high-sensitivity (hs) CRP test was used. CRP levels between 1.0 mg/l and 10.0 mg/l (slightly raised levels, but still within the normal range) are assumed to reflect a persistent infection, and were considered as positive. CRP levels >1.0 mg/l or CRP levels >10.0 mg/l (probably acute infection) were considered as negative (Pearson et al., 2003).

Statistical methods

Characteristics of women with and without distal tubal pathology were compared using the Mann–Whitney U-test. For comparison of the prevalence of IgG and IgA antibodies to C. trachomatis, IgG antibodies to cHSP60 and hs-CRP in women with and without distal tubal pathology, the $\chi^2$-test was used. The prognostic value of single tests as well as test combinations for distal tubal pathology was determined by calculating sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), odds ratio (OR) and 95% confidence interval (CI). A forward stepwise logistic regression analysis was used to select the best combination of tests. The bootstrap technique was used to test the difference between OR (Efron and Tibshirani, 1993), $P < 0.05$ was considered statistically significant.

Results

In 313 sub fertile women, serological test results and laparoscopy reports were available for analysis. Of those 313 women, 59 (11.8%) had patent tubes, 254 (81.2%) did not have patent tubal pathology and served as controls. Of those 254 women without distal tubal pathology, 94.9% had patent tubes and 5.1% had proximal occlusion of at least one tube. Since proximal tubal occlusion is considered not to be related to chlamydia disease, all 254 women without distal tubal pathology served as controls. In women with and without distal tubal pathology, median age (30.6 and 31.2 years respectively) and duration of sub fertility (2.4 and 2.3 years respectively) were comparable.

CRP levels between 1.0 and 10.0 mg/l (slightly raised levels, but still within the normal range) were assumed to reflect a persistent infection, and were considered as positive. CRP levels >10.0 mg/l were considered as negative, since these values were assumed to reflect acute infections. Twelve women (3.8%) had CRP levels >10.0 mg/l (median 16.1, range 11.2 > 130.3). Of these 12 patients, two had severe endometriosis, which is a known cause of elevated CRP levels (Abrâo et al., 1997). In the remaining ten patients, no clinical evidence of acute infections or other underlying inflammatory diseases could be found. Since seven out of these ten serum samples were obtained in autumn or winter, minor or subclinical infections (e.g. influenza-like infections) might have caused the elevated CRP levels.
First, we evaluated the prevalence of IgG and IgA antibodies to *C. trachomatis*. IgG antibodies to cHSP60 and a positive hs-CRP in subfertile women with and without distal tubal pathology. As shown in Table I, IgG and IgA antibodies to *C. trachomatis*, IgG antibodies to cHSP60 and a positive hs-CRP test were found significantly more often in women with distal tubal pathology as compared to women without distal tubal pathology.

Table II shows that, of all four single tests, the *C. trachomatis* IgG test was the best predictor of tubal pathology (OR 13.9). A forward stepwise logistic regression analysis and bootstrap analysis revealed that only adding the hs-CRP test significantly improved the diagnostic performance of the *C. trachomatis* IgG test (OR of test combination 39.7). Adding the *C. trachomatis* IgA test to the above-mentioned test combination led to an OR of 51.6, but the increase in OR was not statistically significant as compared to the combination *C. trachomatis* IgG/hs-CRP (39.7). Combining three or four tests led to 100% specificity, but sensitivity decreased to 15%. In order to limit the number of data in Table II, we have only shown data on test combinations which included a positive *C. trachomatis* IgG test (i.e. the best single test).

### Table I. The prevalence of positive tests in subfertile women with and without distal tubal pathology (DTP)

<table>
<thead>
<tr>
<th></th>
<th>DTP</th>
<th>No DTP</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 59)</td>
<td>(n = 254)</td>
<td></td>
</tr>
<tr>
<td>Ctr-IgG +</td>
<td>32 (54.2)</td>
<td>20 (7.9)</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Ctr-IgA +</td>
<td>21 (35.6)</td>
<td>21 (8.3)</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>cHSP60-IgG +</td>
<td>30 (50.8)</td>
<td>38 (15.0)</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>hs-CRP +</td>
<td>32 (54.2)</td>
<td>95 (37.4)</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>

Values in parentheses are percentages.

- aThreshold titre for a positive test: 32.
- bThreshold titre for a positive test: 1.4.
- cThreshold titre for a positive test: 1.11.
- dThreshold concentration for a positive test: 1.0–10.0 mg/l.

Ctr = *C. trachomatis*; cHSP60 = chlamydia heat shock protein 60; hs-CRP = high-sensitivity C-reactive protein.

### Table II. The predictive value of single tests as well as combinations of tests including a positive Ctr-IgG for distal tubal pathology (DTP)

<table>
<thead>
<tr>
<th>No. of tests performed</th>
<th>Ctr-IgG</th>
<th>Ctr-IgA</th>
<th>cHSP60-IgG</th>
<th>hs-CRP</th>
<th>No. of patients with positive test and DTP</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>OR</th>
<th>95% CI</th>
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</thead>
<tbody>
<tr>
<td>One test</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>52</td>
<td>32</td>
<td>54</td>
<td>92</td>
<td>62</td>
<td>90</td>
<td>13.9f 7.0–27.5</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>42</td>
<td>21</td>
<td>36</td>
<td>92</td>
<td>50</td>
<td>86</td>
<td>6.1f 3.1–12.3</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>68</td>
<td>30</td>
<td>51</td>
<td>85</td>
<td>44</td>
<td>88</td>
<td>5.9f 3.2–10.9</td>
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<tr>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>127</td>
<td>32</td>
<td>54</td>
<td>63</td>
<td>25</td>
<td>85</td>
<td>2.0f 1.1–3.5</td>
</tr>
<tr>
<td>Two tests</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>27</td>
<td>17</td>
<td>29</td>
<td>63</td>
<td>99</td>
<td>85</td>
<td>4.2–23.0</td>
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<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>41</td>
<td>28</td>
<td>47</td>
<td>95</td>
<td>68</td>
<td>89</td>
<td>16.7 7.9–35.7</td>
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<td></td>
<td>+</td>
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<td>+</td>
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<td>22</td>
<td>19</td>
<td>32</td>
<td>99</td>
<td>86</td>
<td>86</td>
<td>39.7f 11.2–140.5</td>
</tr>
<tr>
<td>Three tests</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>22</td>
<td>15</td>
<td>25</td>
<td>97</td>
<td>68</td>
<td>85</td>
<td>12.0 4.6–31.2</td>
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<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>11</td>
<td>10</td>
<td>17</td>
<td>100</td>
<td>91</td>
<td>84</td>
<td>51.6f 6.5–412.6</td>
</tr>
<tr>
<td>Four tests</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>16</td>
<td>16</td>
<td>27</td>
<td>100</td>
<td>100</td>
<td>86</td>
<td>∞</td>
</tr>
</tbody>
</table>

- aThreshold titre for a positive test: 32.
- bThreshold titre for a positive test: 1.4.
- cThreshold titre for a positive test: 1.11.
- dThreshold concentration for a positive test: 1.0–10.0 mg/l.

### Discussion

In this study, we hypothesized that the course of *C. trachomatis* infections is related to the risk of tubal damage. The natural course of *C. trachomatis* infections, duration of exposure to the micro-organism and re-infection rates are difficult to study, since *C. trachomatis* infections often remain asymptomatic, and consequently the onset of the infection is generally unknown. It is important, however, to identify subfertile women with persistent *C. trachomatis* infections, since they are supposed to have the highest risk of tubal pathology. In the present study, we have tested three serological markers of persistent infections in a cohort of 313 subfertile women. Our results indicate that persistent *C. trachomatis* infections play an important role in the development of tubal pathology.

IgA antibodies have been associated with chronic inflammation and infection. With respect to chlamydia, previous studies have demonstrated an association between *C. pneumoniae* IgA antibodies and its chronic sequelae, e.g. respiratory and cardiovascular morbidity. Falck *et al.* (2002) have found that the prevalence of symptoms of chronic respiratory tract disease increases parallel to the increase in *C. pneumoniae* IgA titre. In patients with coronary symptoms, a positive *C. pneumoniae* IgA titre significantly increases the risk of myocardial injury (OR 1.95) (Wong *et al.*, 2002). It has been suggested that serum IgA antibodies, as compared to IgG antibodies, may be more reliable markers of persistent *C. pneumoniae* infections (Saikku, 1999).

cHSP60 is a chlamydia genus-specific protein, serving as a strong antigenic target for the immune system (Morrison *et al.*, 1989; Kaufmann, 1990). It has been suggested that antibodies to cHSP60 are markers of chronic inflammation (Kaufmann, 1990). Studies have shown a strong association
between anti-cHSP60 antibodies and tubal factor subfertility. Anti-cHSP60 antibodies are significantly more prevalent in subfertile women with tubal disease (44–76%) as compared to those without tubal disease (8–19%) (Freidank et al., 1995; Claman et al., 1997). Among subfertile women with antibodies to C. trachomatis, anti-cHSP60 antibodies are significantly more prevalent in women with tubal pathology (76–81%) as compared to those without tubal pathology (0–43%) (Toye et al., 1993; Arno et al., 1995).

CRP is an acute phase protein. Slightly raised CRP concentrations, but still within the normal range, are known indicators of chronic inflammation. Research on the pathophysiology of coronary heart disease has shown that the association between C. pneumoniae infections and the risk of cardiovascular events is stronger if CRP is slightly raised, but within the normal range (Gattone et al., 2001). As compared to patients without C. pneumoniae antibodies and a low CRP, the risk of coronary events increased when C. pneumoniae antibodies were present (OR 1.22; 95% CI 0.74–2.01), but increased even more when both C. pneumoniae antibodies and a slightly elevated CRP were present (OR 5.40; 95% CI 2.35–12.43) (Roivainen et al., 2000). Serum CRP levels were significantly higher in patients with C. pneumoniae-infected atherosclerotic plaques (8 mg/l) as compared to patients with non-infected atherosclerotic plaques (undetectable CRP) (Johnston et al., 2001). The role of CRP in tubal factor subfertility has not yet been studied. In the present study, all evaluated serological markers of persistent infections were significantly more prevalent in women with tubal pathology as compared to women without tubal pathology. However, as single tests, the markers of persistent infections performed poorly as compared to the current screening test for tubal pathology (IgG to C. trachomatis). Odds ratios of IgA antibodies to C. trachomatis (6.1), IgG antibodies to cHSP60 (5.9) and hs-CRP (2.0) were significantly lower as compared to IgG antibodies to C. trachomatis (13.9).

The low OR of the C. trachomatis IgA and cHSP60 IgG tests might be explained by these antibodies being poorer markers of chronic inflammation than is currently presumed. The significantly lower OR of the cHSP60 IgG test, as compared to the C. trachomatis IgG test, might be explained by cross-reaction with the highly similar C. pneumoniae. Anti-C. pneumoniae antibodies are highly prevalent in subfertile women (detectable in 83.1% of women with distal tubal pathology and in 72.8% of women without distal tubal pathology), and are not associated with tubal disease (Den Hartog et al., 2004). The manufacturer of the cHSP60 IgG test mentions that cross-reaction with other chlamydia species may occur, due to the homology of >95% between cHSP60 of the different species. The low OR of the hs-CRP test as a single test might be explained by CRP being a general, and not a chlamydia-specific, marker of chronic inflammation.

A forward stepwise logistic regression analysis was performed in order to determine if the prognostic value of the best single test (i.e. C. trachomatis IgG) could be significantly improved by adding one or more tests. Only combining the C. trachomatis IgG test and the hs-CRP test resulted in a significantly higher OR (39.7) as compared to the C. trachomatis IgG test only (13.9). Measuring C. trachomatis IgG antibodies (markers of a previous infection), in combination with hs-CRP (a marker of the course of the infection), seems to identify a subset of subfertile women with the highest risk of persistent infections and distal tubal pathology. These results are comparable to previous studies, in which risk factors for cardiovascular disease were studied. In these studies, the association between C. pneumoniae and cardiovascular disease, which is commonly known, is stronger when elevated CRP levels, but within the normal range, are also detectable (Roivainen et al., 2000; Gattone et al., 2001; Johnston et al., 2001). Using the forward stepwise logistic regression model, no significant increase in test performance was noted when adding a third and fourth test to the combination C. trachomatis IgG/hs-CRP.

A limitation of this study is selection and referral bias. Only women who had undergone a laparoscopy with tubal testing, which is the reference standard in diagnosing tubal pathology, were included in the present study. This inclusion criterion will cause selection bias, as has been discussed previously (Den Hartog et al., 2004). This bias will be worsened by referral bias, since the C. trachomatis IgG test was used in the decision regarding who received a laparoscopy. However, it is hard to prevent selection and referral bias in a study like this, since a laparoscopy, which has costs and risks, is not a routine procedure in all subfertility patients.

The clinical purpose of serial testing is to find a combination of tests which can estimate the risk of tubal disease more accurately. The ultimate goal would be a test combination with a PPV and NPV of 100%. In these cases, invasive tubal testing may no longer be indicated. This goal has not yet been achieved in the present study. However, if our results could be confirmed in a larger study, the test combination C. trachomatis IgG/hs-CRP might be a better screening method for tubal pathology as compared to the current method (C. trachomatis IgG only). In daily practice, all C. trachomatis-positive samples could be retested with the hs-CRP test, in order to identify those women who are at highest risk of a persistent C. trachomatis infection and tubal disease.

In summary, we hypothesized that the risk of tubal pathology is increased in subfertile women with persistent C. trachomatis infections. We have studied serological markers of persistent infections in subfertile women. All evaluated serological markers of persistent C. trachomatis infections are significantly more common in subfertile women with tubal pathology as compared to women without tubal pathology. C. trachomatis IgG-positive subfertile women with raised CRP concentrations, but still within the normal range, are supposed to have persistent C. trachomatis infections and are at highest risk of tubal pathology.

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