**Chlamydia pneumoniae** and screening for tubal factor subfertility*

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Chlamydia antibody testing (CAT) by micro-immunofluorescence (MIF) tests has been introduced into the fertility work-up as a screening test for tubal factor subfertility. In this study the role of *C. pneumoniae* antibodies, as a cause for false positive CAT results due to cross-reactivity with *C. trachomatis* antibodies in the MIF test, has been evaluated. In 240 subfertile women serological data were compared to laparoscopy findings. The prevalence of *C. pneumoniae* antibodies using enzyme-linked immunosorbent assay (ELISA) was 75% and did not differ between patients with and without tubal pathology. *C. pneumoniae* antibodies were found in 87% of women with a positive MIF test (≥32), and in 66% with a negative MIF test (P < 0.0005). Using ELISA instead of MIF for the detection of *C. trachomatis* antibodies, *C. pneumoniae* antibodies were found in 87% of *C. trachomatis* positive women, and in 69% of *C. trachomatis* negative women (P < 0.0005). Patients without tubal factor subfertility but a positive MIF test showed *C. pneumoniae* antibodies more frequently than patients without tubal factor subfertility and a negative MIF test. Therefore it was suggested that *C. pneumoniae* antibodies may be the cause of false positive CAT results. Remarkably, tubal pathology was more common in patients who had antibodies to both *C. trachomatis* and *C. pneumoniae*.

Key words: Chlamydia antibody titre/Chlamydia trachomatis/Chlamydia pneumoniae/screening/tubal factor subfertility

**Introduction**

Nowadays, *C. trachomatis* is the most common sexually transmitted disease in industrialized countries. In the majority of female patients *Chlamydia* infections remain asymptomatic, but they may eventually compromise tubal function and increase the risk of tubal factor subfertility. Clinicians have searched for simple and inexpensive screening tests to estimate the risk for tubal factor subfertility in a particular patient, in order to subject a high risk patient to diagnostic testing (i.e. laparoscopy), and to postpone additional testing in a low risk patient. Since it was noted that the development of pelvic inflammatory disease and its chronic sequelae are associated with *C. trachomatis* immunoglobulin (Ig)G antibody formation, the relation between antibody titres and tubal factor subfertility has been confirmed in many studies (summarized by Mol et al., 1997).

*Chlamydia* antibody testing (CAT), e.g. by micro-immuno-

fluence microscopy (MIF) tests, has been introduced into the fertility work-up as a simple and inexpensive screening test for tubal factor subfertility. The clinical significance of CAT is limited by patients with chlamydial antibodies but without tubal pathology at laparoscopy (defined as false positive CAT results), and by patients without chlamydial antibodies but tubal pathology at laparoscopy (defined as false negative CAT results). False positive CAT results may be explained by non-chlamydial causes of tubal pathology. False positive CAT results increase health care costs by increasing the number of laparoscopies. Therefore, if CAT is applied for selecting patients for laparoscopy, the number of false positive CAT results should be minimized.

In discussing false positive CAT results (i.e. patients with positive antibody titres, without tubal pathology at laparoscopy), the possible cross-reactivity in MIF tests between *C. trachomatis* and *C. pneumoniae* antibodies is a major issue. Initially, in the MIF test, antigens from elementary bodies of each of the serotypes of *C. trachomatis* were included in the test, and provided serotype specific antibody testing (Wang and Grayston, 1970). The preparation of multiple immunotype antigens made the test technically difficult to perform and impractical, and therefore modifications were introduced.
Materials and methods

A prospective cohort study was performed in consecutive patients who sought treatment for subfertility in our clinic. Since 1992, CAT using MIF has been a routine procedure in our fertility work-up. In all female patients blood is drawn at their initial visit and an indirect micro-immunofluorescent antibody technique for C. trachomatis antibodies is used. All spare serum samples are cryopreserved. Between January 1992 and April 1999, 240 patients underwent laparoscopy and tubal testing with Methylene Blue dye as part of their fertility work-up and took part in the study. Patients who had undergone previous pelvic surgery (except for an uneventful appendectomy or Caesarean section) were excluded. For the sake of the study, tubal pathology at laparoscopy was defined as extensive periadnexal adhesions and/or distal occlusion of one or both tubes (Land et al., 1998).

A true positive CAT result was defined as a positive antibody titre using MIF, in a patient with tubal pathology at laparoscopy. A false positive CAT result was defined as a positive antibody titre using MIF, in a patient without tubal pathology at laparoscopy.

After thawing the spare sera of the participating patients, an ELISA for C. trachomatis antibodies (Labsystems, Helsinki, Finland) and an ELISA for C. pneumoniae antibodies (BioclonE Elegance, Marrickville, Australia) were performed. These ELISAs are considered specific for C. trachomatis antibodies (Närvänät et al., 1997) and for C. pneumoniae antibodies respectively.

Serological methods

For the indirect micro-immunofluorescent IgG antibody test, 20 µl of the serum was diluted eight times in phosphate buffered saline (PBS) and incubated on the C. trachomatis-spot immunofluorescence substitute slides (egg grown C. trachomatis biovar L2; BioMerieux, Hertogenbosch, The Netherlands) for 30 min at 37°C in a moist chamber. The slides were washed three times for 5 min in PBS and incubated with fluorescein-conjugated rabbit antihuman IgG (Dako, ITK Diagnostics BV, Uithoorn, The Netherlands) diluted in PBS for 30 min at 37°C. After three washings in PBS and one in ultra-pure water processed through a Milli-Q purification system (Millipore, Bedford, MA, USA), slides were embedded in Fluoprep mounting medium (BioMerieux). A positive reaction was characterized by specific fluorescence of the C. trachomatis elementary bodies. For quantitative determination serial dilutions in PBS were performed. All slides were evaluated independently by two readers. In case of disagreement, the judgement of a third reader was decisive. According to receiver operating characteristic curves constructed, an antibody titre of 32 appeared to be the optimum threshold value in predicting tubal pathology (Land et al., 1998). Therefore, a positive MIF result was defined as a titre ≥32.

For the C. trachomatis IgG ELISA (Labsystems, Helsinki, Finland), sera were diluted 1:10 with Tris-buffer and tested in microtitre plates coated with synthetic peptides derived from the major outer membrane proteins of C. trachomatis (L2, L3). The plates were incubated for 30 min at 37°C. The strips were washed five times in 300 µl washing solution (citrate buffered PBS) and dried. To each well 200 µl conjugate was added (sheep antihuman IgG/horseradish peroxidase). The plate was incubated again for 30 min at 37°C. The washing steps were repeated five times. Citrate-acetate buffered substrate in a volume of 200 µl was pipetted in each well, the plate was incubated at room temperature in the dark for 15 min. Finally, 50 µl stop reagent (2 mol/l H2SO4) was added. The absorbance of the plates was measured in a spectrophotometer at 405 nm. Threshold indexes were calculated according to the manufacturer’s instructions. The threshold index for a positive test was 1.1.

For the C. pneumoniae IgG ELISA (Elegance, Marrickville, Australia), human sera were diluted 1:210 with washing solution and tested in strips coated with highly purified C. pneumoniae-specific outer membrane complexes. The plate was covered and incubated for 30 min at 37°C. The strips were washed three times with 300 µl washing solution. To each well, 100 µl C. pneumoniae IgG antibody reagent (labelled anti-human IgG polyclonal antibodies) was added and the plate was incubated again for 60 min. The plate was washed as described above. To each well 100 µl substrate solution (diethanolamine) was added. The plate was incubated at room temperature for 10 min. Finally, 25 µl Chlamydia stop solution was added and the fluid on the plate gently mixed. The optical density of the plates was measured in a spectrophotometer at 405 nm. The threshold index for a positive test, as recommended by the manufacturer, was 1.1.

Statistical methods

Data obtained at laparoscopy were correlated to serological test results, using the χ² test. P values <0.05 were considered significant. Differences between proportions were calculated by using proportion tests for two independent proportions. 95% confidence intervals for proportion differences and P values were calculated. P values <0.05 were considered significant.

Results

In 240 patients serological data as well as laparoscopy results were available for analysis. The mean age of the patients was 30.3 years (range 19–40 years).

C. pneumoniae antibodies were detected by ELISA in 75% of patients. In 40% of patients a positive MIF test for C. trachomatis antibodies was found. C. pneumoniae antibodies were found in 83/95 (87%) of patients who had a positive MIF test for C. trachomatis, and in 96/145 (66%) of patients with a negative MIF test (P < 0.0005). C. pneumoniae antibodies were found in 66/76 (86%) of patients who had a positive ELISA for C. trachomatis antibodies, and in 51/164 (69%) of patients who had a negative ELISA for C. trachomatis antibodies (P < 0.0005). Table I summarizes positive serological test results in patients with and without tubal pathology at laparoscopy respectively.
C. pneumoniae and screening for tubal factor subfertility

### Table I. Positive antibody tests to different C. trachomatis and C. pneumoniae antigens in patients with and without tubal factor subfertility at laparoscopy

<table>
<thead>
<tr>
<th>Antibody test</th>
<th>Total group of patients (n = 240)</th>
<th>Patients with tubal pathology (n = 67)</th>
<th>Patients without tubal pathology (n = 173)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. trachomatis</td>
<td>95 (40%)</td>
<td>39 (58%)</td>
<td>56 (32%)</td>
</tr>
<tr>
<td>MIF (BioMerieux)</td>
<td>76 (32%)</td>
<td>35 (52%)</td>
<td>41 (24%)</td>
</tr>
<tr>
<td>C. pneumoniae ELISA</td>
<td>179 (75%)</td>
<td>51 (76%)</td>
<td>128 (74%)</td>
</tr>
<tr>
<td>ELISA (Bioncle Elegance)</td>
<td>81 (35%)</td>
<td>51 (76%)</td>
<td>128 (74%)</td>
</tr>
</tbody>
</table>

1Cut-off titre for positive test 32.
2Cut-off titre for positive test 1.1.
3Different superscripts denote significant differences; P < 0.01.

### Table II. Prevalence of C. pneumoniae antibodies in 173 patients without tubal pathology at laparoscopy and positive versus negative MIF test results

<table>
<thead>
<tr>
<th>Antibody test results1</th>
<th>Number of patients</th>
<th>Prevalence of C. pneumoniae antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. trachomatis MIF positive</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>C. trachomatis MIF positive and C. pneumoniae ELISA positive</td>
<td>47</td>
<td>84%2</td>
</tr>
<tr>
<td>C. trachomatis MIF negative</td>
<td>117</td>
<td></td>
</tr>
<tr>
<td>C. pneumoniae ELISA positive</td>
<td>81</td>
<td>69%2</td>
</tr>
</tbody>
</table>

1Threshold titre for positive MIF test 32. Threshold titre for positive C. pneumoniae ELISA 1.1.
295% confidence interval for the difference between proportions is 1–30; P < 0.05.

Cross-reactivity in the MIF test between C. pneumoniae and C. trachomatis antibodies is a possible cause for false positive CAT results. In order to study cross-reactivity, C. trachomatis and C. pneumoniae ELISA were performed. The prevalence of C. pneumoniae antibodies has been studied in two different subgroups of patients in whom no tubal pathology was found at laparoscopy (Table II). C. pneumoniae antibodies were found in 47/56 (84%) patients who had a positive MIF test and no tubal pathology at laparoscopy (false positive CAT). In a control group of patients who had a negative MIF test and no tubal pathology (true negative CAT), 81/117 (69%) had C. pneumoniae antibodies (P < 0.05). In Table III, in the same subgroups, ELISA for C. trachomatis was performed, which confirmed the results found by MIF. C. pneumoniae antibodies were found in 34/41 (83%) patients who had a positive ELISA for C. trachomatis and no tubal pathology at laparoscopy. In a control group of patients who had a negative ELISA for C. trachomatis and no tubal pathology, 94/132 (71%) had C. pneumoniae antibodies.

In Table IV the correlation between the presence of antibodies to C. trachomatis by MIF and/or to C. pneumoniae using ELISA, and tubal pathology found at laparoscopy is given. The presence of both antibodies to C. trachomatis and to C. pneumoniae was associated with a higher rate of tubal factor subfertility (43%), as compared to no antibodies (27%; P < 0.05), antibodies to C. trachomatis only (25%) or C. pneumoniae only (16%; P < 0.0001).

In Table V the correlation is given between the presence of antibodies to C. trachomatis using ELISA and/or to C. pneumoniae using ELISA, and tubal pathology found at laparoscopy. The presence of both antibodies to C. trachomatis using ELISA and to C. pneumoniae was associated with a

### Table III. Prevalence of C. pneumoniae antibodies in 173 patients without tubal pathology at laparoscopy and positive versus negative ELISA C. trachomatis results

<table>
<thead>
<tr>
<th>Antibody test results1</th>
<th>Number of patients</th>
<th>Prevalence of C. pneumoniae antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. trachomatis ELISA positive</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>C. trachomatis ELISA positive and C. pneumoniae ELISA positive</td>
<td>34</td>
<td>83%2</td>
</tr>
<tr>
<td>C. trachomatis ELISA negative</td>
<td>132</td>
<td></td>
</tr>
<tr>
<td>C. trachomatis ELISA negative and C. pneumoniae ELISA positive</td>
<td>94</td>
<td>71%2</td>
</tr>
</tbody>
</table>

1Threshold titre for positive C. trachomatis ELISA 1.1, threshold titre for positive C. pneumoniae ELISA 1.1.
295% confidence interval for the difference between proportions is –4 to 20; not statistically significant.

### Table IV. Relation between C. trachomatis antibodies (MIF), and C. pneumoniae antibodies (ELISA), and tubal pathology at laparoscopy in 240 subfertility patients

<table>
<thead>
<tr>
<th>C. trachomatis</th>
<th>C. pneumoniae</th>
<th>Number of patients</th>
<th>Patients with tubal pathology3</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIF1</td>
<td>ELISA2</td>
<td>n = 240</td>
<td>n = 67 (%)</td>
</tr>
<tr>
<td>Negative</td>
<td>Negative</td>
<td>49</td>
<td>13 (27)%</td>
</tr>
<tr>
<td>Negative</td>
<td>Positive</td>
<td>96</td>
<td>15 (16)%</td>
</tr>
<tr>
<td>Positive</td>
<td>Negative</td>
<td>12</td>
<td>3 (25)</td>
</tr>
<tr>
<td>Positive</td>
<td>Positive</td>
<td>83</td>
<td>36 (43)%</td>
</tr>
</tbody>
</table>

1Threshold titre for positive test 32.
2Threshold titre for positive test 1.1.
3Defined as extensive periadnexal adhesions and/or distal occlusion of both tubes.
4Reflects significant difference with P < 0.05.
5Reflects significant difference with P < 0.0001.

### Table V. Relation between C. trachomatis antibodies (ELISA) and C. pneumoniae antibodies (ELISA) and tubal pathology at laparoscopy in 240 subfertility patients

<table>
<thead>
<tr>
<th>C. trachomatis</th>
<th>C. pneumoniae</th>
<th>Number of patients</th>
<th>Patients with tubal pathology3</th>
</tr>
</thead>
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<tr>
<td>ELISA1</td>
<td>ELISA1</td>
<td>n = 240</td>
<td>n = 67 (%)</td>
</tr>
<tr>
<td>Negative</td>
<td>Negative</td>
<td>51</td>
<td>13 (26)%</td>
</tr>
<tr>
<td>Negative</td>
<td>Positive</td>
<td>113</td>
<td>19 (17)%</td>
</tr>
<tr>
<td>Positive</td>
<td>Negative</td>
<td>10</td>
<td>3 (30)</td>
</tr>
<tr>
<td>Positive</td>
<td>Positive</td>
<td>66</td>
<td>32 (49)%</td>
</tr>
</tbody>
</table>

1Threshold for positive test 1.1.
2Defined as extensive periadnexal adhesions and/or distal occlusion of both tubes.
3Reflects significant difference with P < 0.05.
4Reflects significant difference with P < 0.0001.

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higher rate of tubal factor subfertility (49%), as compared to no antibodies (26%; $P < 0.05$), antibodies to *C. trachomatis* only (30%) or *C. pneumoniae* only (17%; $P < 0.0001$).

**Discussion**

The aim of performing CAT as a standard procedure in subfertility patients is to identify patients with previous *C. trachomatis* infections. Using MIF, a positive test result is assumed to be associated with *C. trachomatis* antibodies, and the patient is considered to be at high risk for tubal pathology. However, the results reported in the literature on screening for tubal factor subfertility by CAT using MIF are heterogeneous. For the correlation of CAT results with laparoscopy results, both false positive and false negative results are found. In the present study the role of *C. pneumoniae* antibodies, as a cause of false positive CAT results, due to cross-reactivity of *C. pneumoniae* antibodies with *C. trachomatis* antibodies was evaluated. Furthermore, the prevalence of *C. pneumoniae* antibodies in subfertile women was calculated.

From Table I it can be concluded that the prevalence of *C. trachomatis* antibodies in the group of subfertile women studied here was comparable using MIF and ELISA, 40 and 32% respectively. Using ELISA the prevalence of *C. pneumoniae* IgG antibodies was 75%. In patients with tubal pathology at laparoscopy the prevalence was 76%. It was 74% in women without tubal factor subfertility (Table I). Several reports on the prevalence of *C. pneumoniae* antibodies in Western European countries have been published. In 21% of over 4500 women attending a genitourinary clinic *C. pneumoniae* antibodies were found using MIF (Moss et al., 1993). In adolescents the prevalence of *C. pneumoniae* antibodies using MIF was 69% in men, and 57% in women (Freidank and Brauer, 1992). Another study confirmed the prevalence of *C. pneumoniae* antibodies to be higher in men (72%) than in women (56%), and showed the prevalence to increase with increasing age (Karvonen et al., 1994). In female subfertility patients the prevalence of *C. pneumoniae* antibodies was significantly higher in patients with bilaterally occluded tubes at laparoscopy (75%), as compared to patients with normal tubes (56%) (Freidank et al., 1995). The prevalence of *C. pneumoniae* antibodies found in the current study (75%) is comparable with the prevalence reported in the literature in asymptomatic women and subfertility patients.

In discussing false positive test results for CAT, cross-reactivity in MIF tests with *C. pneumoniae* is a major issue. Since MIF tests have been modified and serotype specific antigens are no longer used, cross-reactivity with *Chlamydia* species other than *C. trachomatis* has been demonstrated (Mannion et al., 1991).

Comparison of subfertility patients with positive and negative MIF test results respectively, revealed a significant difference in the prevalence of *C. pneumoniae* antibodies (87 and 66% respectively, $P < 0.0005$). A possible explanation for the difference found is cross-reactivity between *C. pneumoniae* and *C. trachomatis* antibodies in the MIF test. To explore the likelihood of cross-reactivity further, the prevalence of *C. pneumoniae* antibodies was analysed in two subgroups of patients (Tables II and III). In patients without tubal factor subfertility but a positive MIF test result (Table II), *C. pneumoniae* antibodies were found more frequently than in patients without tubal factor subfertility and negative MIF test results. These data suggest that *C. pneumoniae* antibodies can be a cause of false positive CAT results. Analysis of the same groups using *C. trachomatis* ELISA instead of MIF (Table III) showed the same results.

Reports in the literature are rare and controversial concerning cross-reactivity between *C. pneumoniae* and *C. trachomatis* antibodies in MIF tests. In female patients with tubal factor subfertility and pregnant controls, the presence of *C. trachomatis* and *C. pneumoniae* antibodies has been studied (Persson et al., 1999). *C. trachomatis* antibodies were detected more often in patients (88%) than in controls (48%). In contrast, no difference was found for *C. pneumoniae* antibodies between patients (52%) and controls (58%). The authors concluded that cross-reactivity did not occur, as *C. trachomatis* antibodies were equally common in patients with and without *C. pneumoniae* antibodies (89 and 87% respectively). A study (Moss et al., 1993) in over 4500 serum samples of women attending a genitourinary clinic, using a modified MIF test to detect type-specific antibodies to *Chlamydia* species, has been performed. The IgG responses to *Chlamydia* species were detected in three distinct forms: species-specific (where IgG was directed against one species only), cross-reactive (where the IgG concentration against one species was 2-fold or higher than the IgG concentrations against other species), or group-specific (where IgG concentrations were similar against all species tested). In 67% of women *Chlamydia* IgG antibodies were found. Species-specific or cross-reactive IgG against *C. trachomatis*, *C. pneumoniae* and *C. psittaci* was present in 38, 21 and 0.1% of the samples respectively. In 8% of samples, the IgG concentration was similar against all three species (group-specific). The authors conclude that antibodies to *C. pneumoniae* may account for up to half of all *Chlamydia* positive test results using MIF. From the latter study (Moss et al., 1993) and the current study it can be concluded that, due to cross-reactivity, *C. pneumoniae* antibodies are probably major contributors to the low specificity of those MIF tests in which pooled antigens are used. Species-specific antibody tests for *C. trachomatis* are prerequisites for improving the predictive value of CAT.

In the current study, tubal pathology was more frequent in patients in whom both antibodies to *C. trachomatis* and to *C. pneumoniae* were found although the numbers of patients are small within the subgroups (Tables IV and V). The precise mechanism by which *Chlamydia* causes tissue damage remains to be elucidated, but immune-mediated inflammatory responses seem to be involved in the process (Brunham and Peeling, 1994). It has been suggested that primary chlamydial infections do not necessarily cause tubal tissue damage, but that tissue damage will develop only after reinfection or after reactivation of a primary infection (Patton and Kuo, 1989). Reinfection or reactivation of primary infections is considered to induce a booster immune response and subsequently tubal pathology. It has been postulated that this immune response may not be specific for *C. trachomatis*, and may be induced by *C. pneu-
niae antibodies as well (Freidank et al., 1995). In their study Freidank et al. (1995) found the presence of both antibodies to C. trachomatis and C. pneumoniae in the MIF test to be associated with a significantly higher rate of tubal occlusion. The findings prescribed here are in agreement with the findings of Freidank et al. (1995), i.e. more tubal pathology was found in patients with both C. trachomatis and C. pneumoniae antibodies. This supports the contention that C. trachomatis infection and (highly prevalent) C. pneumoniae infections might have a synergistic effect on the development of tubal pathology. However, the exact role of C. pneumoniae in this process needs further exploration.

In conclusion, in 240 subfertility patients a prevalence of C. pneumoniae antibodies of 75%, using a specific ELISA for C. pneumoniae was found. Comparison of laparoscopic and serological data suggests cross-reactivity between antibodies to C. trachomatis and antibodies to C. pneumoniae in the MIF test, as a cause for low specificity of CAT. The simultaneous presence of antibodies to C. trachomatis and to C. pneumoniae was associated with a significantly increased rate of tubal factor subfertility. C. pneumoniae antibodies, which are widespread, may be involved in chlamydial upper genital tract infections and/or subsequent tubal damage.

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References


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