Original Article

Comparison of four serological assays for the diagnosis of *Chlamydia trachomatis* in subfertile women

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Abstract

Introduction: Chlamydia antibody testing (CAT) in serum has been introduced as a screening method in the infertility workup. We evaluated the test characteristics of two ELISA tests compared to micro-immunofluorescence tests (MIFs). MIFs are considered the gold standard in the *C. trachomatis* IgG antibodies detection. We also compared the accuracy of all CAT tests in predicting tubal subfertility, using laparoscopy as a reference.
Methodology: Four commercial serological methods were used to analyse 101 serum samples for the presence of *C. trachomatis* IgG antibodies from patients at the Infertility Clinic of Ghent University Hospital. The diagnostic utility for prediction of tubal infertility of serological methods was evaluated based on patients' medical records.
Results: A comparison of the serological assays showed little difference in the major performance characteristics: the sensitivities of all MIFs and ELISAs were 100% for all assays (except the ELISA Vircell, with a sensitivity of 90%), and the specificities ranged from 92% for MIF Ani Labsystems to 98% for the MIF Focus and ELISA Vircell. As compared to laparoscopy data, CAT positivity in subfertile women with tubal damage (n=40) did not significantly differ from that of subfertile women without tubal damage (n=61): Positive predictive values (PPV) of CAT ranged from 53% to 60% and negative predictive values (NPV) ranged from 62% to 64%.
Conclusion: evaluated ELISAs are comparable to MIFs in the detection of *C. trachomatis* IgG antibodies and should be preferred for large serological studies, especially in resource poor settings.

Key words: antibody; *Chlamydia trachomatis*; serology; subfertility

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Introduction

Chlamydia trachomatis is the commonest sexually transmitted bacterial infection in the world [1,2]. In women, up to 80% of *C. trachomatis* infection is asymptomatic; thus few infected women seek medical care, resulting in continued transmission to sexual partners [1]. These untreated women are at risk of developing chronic sequelae such as periadnexal adhesions and tubal occlusion. Tubal pathology is one of the causes of subfertility being responsible for 10% to 30% of the cases in developed countries and up to 85% in developing countries [3-5]. The reference method of assessment for tubal damage is laparoscopy and, when laparoscopy is not available such as in low-resource diagnostic settings, hysterosalpingography can be used [6]. However, both methods are costly and invasive, and therefore unsuitable for screening on a large scale. Since tubal pathology and infertility have been associated with asymptomatic *Chlamydia trachomatis* infections in the past, chlamydia antibody testing (CAT) in serum has been introduced as a screening method for tubal factor subfertility [7-10]. Hence the development of a simple and reliable assay for the detection of *C. trachomatis* antibodies is essential.

The micro-immunofluorescence test (MIF) is generally regarded as a gold standard in the serological diagnosis of *C. trachomatis* infection [11,12]. However, MIF is not ideal for routine

serodiagnostics because it is labour intensive, highly observer dependent, and interlaboratory variation is significant[13]. Enzyme immunoassays (EIA) based on synthetic peptides characterized by high throughput, objective endpoints, and technical accessibility are commercially available [14], but are generally considered to be inferior to MIF in predicting tubal factor subfertility [15].

The aim of this study was to evaluate the possibility of the use of ELISA tests for fast throughput of large numbers of sera, to be used particularly in a study foreseen to investigate the association of tubal pathology and past chlamydia infection in Rwanda, a developing country. Their performances were evaluated against those of two microimmunofluorescence assays for the detection of *Chlamydia trachomatis* IgG antibodies in sera of subfertile women. Secondly, we compared all CAT in their accuracy to predict tubal subfertility using laparoscopy.

Methodology

Sera of patients, who underwent a laparoscopy as a part of the infertility workup at the Infertility Clinic at Ghent University Hospital between September 2005 and May 2007, were included in the study. The sera were stored at -20°C prior to analyses. A total of 101 sera were analysed, comprising 40 (39.6%) sera from patients with tubal damage and 60 (60.4%) sera from patients without tubal damage as assessed by laparoscopy. Tubal damage at laparoscopy was defined as extensive periadnexal adhesions and/or distal or proximal occlusion of one or both fallopian tubes. After thawing the cryopreserved sera, four different CAT tests were performed and the results were correlated to the laparoscopy data. The study was approved by the Ghent University Hospital Ethics Committee under Belgian Registration Number B67020072676.

Serological methods

Chlamydia pneumoniae Ig G/Ig M Micro-IF test: The *Chlamydia pneumoniae* Ig G/Ig M Micro-IF test (Ani Labsystems Ltd., Vantaa, Finland) is a species-specific test based on indirect detection of *C. pneumoniae, C. trachomatis* and *C. psittaci* antibodies. In short, patient's serum was diluted 1:8 in phosphate-buffered saline (PBS) and incubated on the microscope slides dotted with three *Chlamydia* antigens for 30 minutes at 37°C in a moist chamber. The slides were washed twice with PBS and twice with distilled water and incubated with goat anti-human IgG-fluorescein isothiocyanate conjugate for 30 minutes at 37°C. The slides were washed twice again with PBS and twice with distilled water. Mounting fluid was added on the slides, and a cover slip was placed. The slides were read using a Leitz Laborlux 12 fluorescent microscope (WILD Leitz, Heerbrugg, Germany) with 25x objective by two independent readers. In case of disagreement, the judgement of a third reader was decisive. For a quantitative determination of endpoint titres, serial dilutions in PBS were performed. For *C. trachomatis*, a titre of 1:32 was considered the cut-off for positivity according to the manufacturer's instructions.

Chlamydia Micro-IF IgG test (Focus Diagnostic, Cypress, USA): This micro-immunofluorescence antibody assay is a two-stage “sandwich” procedure. In the first stage, the patient sera were diluted in PBS. The diluted sera were added to appropriate slide wells in contact with the substrate, and incubated for 30 minutes. Following incubation, the slide was washed twice with PBS and twice with distilled water. In the second stage, each well was overlaid with fluorescein-labelled antibody to IgG and incubated for 30 minutes. After the slide was washed, dried, and mounted, it was examined using a Leitz Laborlux 12 fluorescent microscope (WILD Leitz, Heerbrugg, Germany) with 25x objective by two independent readers. In case of disagreement, the judgement of a third reader was decisive. For a quantitative determination, serial dilutions in PBS were performed. According to the manufacturer's instructions, a serum was considered to be MIF IgG positive if it was reactive at a dilution of 1:16.

Chlamydia trachomatis IgG EIA (Ani Labsystems Ltd., Vantaa, Finland): This test was developed for the detection of species-specific IgG antibodies to surface-expressed peptides of *C. trachomatis*. Sera were tested manually according to the manufacturer's instruction. Briefly, sera diluted 1:100 in PBS were incubated with the *C. trachomatis* antigens coated onto a 96-well plate. After washing, horseradish peroxidase-conjugated anti-immunoglobulin (IgG) was added to the wells and incubated for 30 minutes at 37°C. After further washing, the chromogen containing tetramethylbenzindine was added. The reaction was stopped with sulphuric acid after 15 minutes and optical density was read immediately at 450 nm using a BEP III Behring ELISA Processor (Siemens AG, Erlangen, Germany) spectrophotometer. The signal to cut-off indices was categorized per the

manufacturer's instructions as negative, equivocal and positive.

Chlamydia trachomatis ELISA IgG/IgM (Vircell, Santa Fe, Spain): Synthetic peptides derived from complexes of outer membrane proteins (COMP) of *C. trachomatis* free from lipopolysaccharide (LPS) were used in this indirect enzyme immunoassay. Sera were tested manually according to the manufacturer's instructions. In short, sera were diluted 0.5:100 in PBS and tested in microplates coated with *C. trachomatis* antigens. The plates were incubated for 45 minutes at 37°C in a chamber. The plates were washed five times with PBS. To each well, conjugate (goat anti-human IgG, horseradish peroxidase-conjugated) was added and the plates were incubated for 30 minutes at 37°C. The plates were washed again five times with PBS. To each well, tetramethylbenzidine substrate was added and the plates were incubated for 20 minutes at room temperature. Finally, sulphuric acid was added to stop the colouring reaction. The optical density of the plates was measured at 450 nm using a BEP III Behring ELISA Processor (Siemens AG, Erlangen, Germany) spectrophotometer. Threshold indexes were calculated according to the manufacturer's instructions as negative, equivocal and positive.

Statistical analysis

For statistical analysis SPSS 11.5 for windows programme (SPSS Inc., Chicago, USA) was utilized. For the comparison of the ELISA tests to the MIF assays and the test ability to detect tubal pathology, two-by-two tables were used to calculate sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and odds ratio (OR). The chi-square test was used to test significance of the difference in frequency distribution. A p-value of < 0.05 was considered as significant.

Results

Four different commercial serological assays were applied to 101 sera taken from subfertile women. The *C. trachomatis* seropositivity ranged from 9.9% with Vircell ELISA to 16.9% with Ani Labsystems MIF (Table 1). The concordances of the IgG results for all of the different assays are shown in Table 2. Diagnostically significant reactions were found in 19 patients. In nine patients all four CAT tests were positive, whereas only one patient had three positive tests, three patients had two positive tests and six patients had one positive test.

Further comparison of the assays was done on the basis of the current gold standard, MIF. Since the results of the two different MIFs were not homogeneous, we defined a positive sample as one that was positive by the two MIFs [16]. Based on this internal standard, we found that 10 (10%) samples were positive and 91 (90%) samples were negative. In addition, by using our internal gold standard, we determined the specificities, sensitivities, PPVs and NPVs of the different tests (Table 3). The sensitivities of all MIFs and ELISAs for the detection of–*C. trachomatis* antibodies were 100 % for all assays except the Vircell ELISA, for which the sensitivity was 90%. The specificities were 98% for the MIF from Focus and ELISA from Vircell, 96 % for the Ani Labsystems ELISA, and 92% for Ani Labsystems MIF. The PPVs were 90% for MIF from Focus and ELISA from Vircell, 76 % for the Ani Labsystems ELISA, and 58 % for the Ani Labsystems MIF. All assays had relatively high NPV between 98% and 100%.

The results of four different CAT tests and laparoscopy in 101 subfertile women are presented in Table 4. As compared to laparoscopy data, CAT positivity in subfertile women with tubal damage (n = 40) did not significantly differ from that of subfertile women without tubal damage (n = 61). None of the evaluated serological assays had acceptable PPV (from 53% for ELISA Ani Labsystems to 60% for ELISA Vircell) and NPV (from 62% for MIF Focus, ELISA Ani Labsystems and ELISA Vircell to 64% for MIF Ani Labsystems).

Discussion

This study was designed to compare the performance of four different commercially available tests for the detection of *C. trachomatis* specific IgG antibodies in sera of 101 subfertile women. The second objective of the study was to evaluate diagnostic utility of serology in the prediction of tubal infertility as compared to laparoscopic findings.

Two different versions of MIF (including the recently developed MIF Focus), the current gold standard for the serodiagnosis of *Chlamydia* infection, were included in the study, as were two ELISAs, which differed in the particular antigen preparations used. In general, these peptide-based assays performed as well as the MIF assay.

In the present study, seroprevalence rates of the two ELISA assays were similar to those of the Vircell MIF assay (9.9-12.9%), whereas the Ani Labsystems MIF showed higher seroprevalence

Table 1. *C. trachomatis* antibody testing by four different serological assays and laparoscopical data on tubal damage (TD) in 101 subfertile women

Test	Number of women with positive CAT test	Number of women with positive test and TD	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	OR(95%CI)	p-value
MIF Ani Labsystems	17	10	25	88	58	64	2.5 (0.9-7.4)	NS
MIF Focus	11	6	15	91	54	62	1.9 (0.5-6.9)	NS
ELISA Ani Labsystems	13	7	17	90	53	62	1.9 (0.6-6.2)	NS
ELISA Vircell	10	6	15	93	60	62	2.5 (0.6-9.5)	NS

NS: Not significant

Table 2. Diagnostically significant reactions of different serological tests

Number of Positive tests	Test with positive result	Number of positive women	Accumulated positive cases
4	MIF Anilabsystem, MIFFocus, ELISA Anilabsystem, ELISA Vircell	9	9
3	MIF Anilabsystem, MIFFocus, ELISA Anilabsystem	1	1
2	MIFFocus, ELISA Anilabsystem	2	
	MIF Anilabsystem, ELISA Anilabsystem	1	
	MIF Anilabsystem, ELISA Vircell	1	
	Total	4	4
1	MIF Anilabsystem	4	
	ELISA Anilabsystem	1	
	Total	5	5
0		82	19

(16.8%), possibly due to a higher rate of false-positive results.

Based on our internal gold standard, the test characteristics of both MIFs, including the recently developed MIF Focus and two ELISAs for the detection of serological evidence of *C. trachomatis* infection, were similar. All tests had reasonably high sensitivity, specificity, and NPV and would therefore match the criteria of a screening test (Table 3). Thus far, few studies have compared these new serological assays with the MIF assay. The findings of our study are consistent with previous studies that revealed good sensitivities and specificities of ELISA assays based on peptides from the major outer membrane protein (MOMP) [15,17]. Paukku *et al.* [18] compared the MIF assay to the ELISA Ani Labsystems for 78 patients with tubal factor infertility and showed a good correlation between the results of these assays. Morré *et al*. [19], in a study involving 43 women with PCR positive cervical swabs for *C. trachomatis*, showed that the results of the ELISA tests correlated well with the antibody results for *C. trachomatis* obtained by the MIF tests.

The association of serum IgG antibodies to *C. trachomatis* and tubal pathology is commonly known [20,21]. In the present work, we observed that *C. trachomatis* antibody positivity in subfertile women with tubal damage did not differ significantly from that of subfertile women without tubal damage (Table 1). Although not reaching significant levels, a trend

Table 3. Sensitivities, specificities, PPVs, and NPVs of different assays in relation to the study's internal gold standard (i.e., positive by both MIFs)

Test	Frequency of each outcome*				Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
	True positive	False positive	True negative	False negative				
MIF Ani Labsystems	10	7	84	0	100	92	58	100
MIF Focus	10	1	90	0	100	98	90	100
ELISA Ani Labsystems	10	3	88	0	100	96	76	100
ELISA Vircell	9	1	90	1	90	98	90	98

Table 4. *C. trachomatis* antibody testing by four different serological assays and laparoscopical data on tubal damage (TD) in 101 subfertile women

Assays	CAT Result	Tubal damage n= 40	Normal tube n= 61	Sensitivity (%)	Specifity (%)	PPV	NPV	OR (95% CI)
MIF Ani Labsystems	Positive	10	7	25	88	58	64	2.5 (0.9-7.4)
	Negative	30	54					
MIF Focus	Positive	6	5	15	91	54	62	1.9 (0.5-6.9)
	Negative	34	56					
ELISA Ani Labsystems	Positive	7	6	17	90	53	62	1.9 (0.6-6.2)
	Negative	33	55					
ELISA Vircell	Positive	6	4	15	93	60	62	2.5 (0.6-9.5)
	Negative	34	57					

*n = 101

toward a higher seroprevalence in the group of women with tubal damage was observed for all CAT tests. Our findings are consistent with those of an earlier published critical reappraisal [21] of the literature on screening for tubal factor subfertility by CAT tests. CAT screening as a strategy reveals heterogeneous results and the meta-analysis indicates that the predictive value of CAT for tubal pathology is limited: the sensitivity of CAT varies between 30% and 88%, and the specificity varies between 45% and 100%. In a recent study, there was also no significant difference found in *C. trachomatis* antibody positivity when 104 infertile women were compared with 80 fertile women [22]. In contrast, there are earlier published reports on the high predictive value of *C. trachomatis* antibody positivity on tubal damage [23,24]. Another study has shown that combined use of the CAT test and medical history taking has superior diagnostic accuracy over one of these approaches alone [25].

The absence of a significant association of *C. trachomatis*-positive serology and tubal damage observed in our study and some of the others mentioned above [21,22] is likely due to a relatively small sample size. Secondly, our findings are based on the use of retrospective laparoscopic data and are therefore lacking systematic information regarding the type of tubal abnormalities with the more stringent selection of the *Chlamydia*-associated pathology.

Of particular note, *Chlamydia* antibodies are associated with *Chlamydia*-induced tubal pathology only, and, as a consequence, the predictive value of CAT will be poor for disease not associated with *Chlamydia* infection. It has been shown that CAT is more accurate in predicting distal tubal pathology, instead of unspecified tuboperitoneal abnormalities or proximal tubal occlusion [11]. Moreover, in studying the implications of different cutoff titres for a positive test, it has been noted that increasing the cutoff titre will improve the specificity, at the expense of sensitivity [7,12,15]. In patients with laparoscopically detected tubal pathology but negative antibody titres, diminished antibody titres related to time has been considered by some authors but not by others [26,27]. Finally, immunity status in *C. trachomatis* infection has not yet been fully understood but there is growing evidence that persistent *C. trachomatis* infections present an important risk group for tubal pathology [8].

In conclusion, our data show that the two ELISAs performed equally as well as or slightly better than MIF assays for the detection of antibodies to *C. trachomatis*. Since these ELISA tests are easier to perform, less expensive, and able to be read more objectively than the MIF assay, they might be good alternatives to the MIF assay for the detection of *C. trachomatis* antibodies, especially when a large number of samples are to be processed. However, with all CAT tests evaluated, a trend toward a similar higher seroprevalence in the group of women with tubal damage was observed that did not reach significance, likely due to the relatively small sample size in this study.

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