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ARTICLE

Comparison of five commercial serological tests for the detection of anti-*Chlamydia trachomatis* antibodies

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Abstract Screening for *Chlamydia trachomatis*-specific antibodies is valuable in investigating recurrent miscarriage, tubal infertility and extrauterine pregnancy. We compared here the performance of immunofluorescence (IF) to four other commercial tests in detecting IgG antibodies directed against *C. trachomatis*: two enzyme-linked immunosorbent assays (ELISAs) using the major outer membrane protein (MOMP) as the antigen, commercialised respectively by Medac and R-Biopharm (RB), one ELISA using the chlamydial heat shock protein 60 (cHSP60) as the antigen (Medac), as well as a new automated epifluorescence immunoassay (InoDiag). A total of 405 patients with (n=251) and without (n=154) miscarriages were tested by all five tests. The prevalence of *C. trachomatis*-specific IgG antibodies as determined by the IF, cHSP60-Medac, MOMP-Medac, MOMP-RB and InoDiag was 14.3, 23.2, 14.3, 11.9 and 26.2%, respectively. InoDiag exhibited the highest sensitivity, whereas MOMP-RB showed the best specificity. Cross-reactivity was observed with *C. pneumoniae* using IF, MOMP-RB and InoDiag, and *Parachlamydia acanthamoebae* using the cHSP60 ELISA test. No cross-reactivity was observed between *C. trachomatis* and the other *Chlamydiales* (*Neochlamydia hartmannellae*, *Waddlia chondrophila* and *Simkania negevensis*). Given its high sensitivity, the new automated epifluorescence immunoassay from InoDiag represents an interesting alternative. The MOMP-based ELISA of R-Biopharm should be preferred for large serological studies, given the high throughput of ELISA and its excellent specificity.

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Introduction

Chlamydia trachomatis is the most common sexually transmitted bacterial infection in the world [1, 2]. In the majority of cases, *C. trachomatis* infection is asymptomatic [1]. Thus, few infected people seek medical care and treatment, resulting in continued transmission to sexual partners [1]. This explains the relatively high and increasing prevalence of *C. trachomatis* infection throughout the world, including countries with advanced medical care and public health programmes [3–7]. Chlamydial infection may cause urethritis, epididymitis, prostatitis, cervicitis, pelvic inflammatory disease, ectopic pregnancy and tubal infertility [2]. Chlamydial infection also increases the risk of acquisition of human immunodeficiency virus (HIV) [2, 8], has been associated with cervical cancer [9] and may induce severe adverse pregnancy outcomes [10, 11].

Serologic testing for *C. trachomatis* has been used to detect antichlamydial antibodies among women with tubal infertility [12, 13]. *C. trachomatis* serology is also useful to investigate women with ectopic pregnancies [14], recurrent miscarriages [10] and pelvic inflammatory disease (PID) [15]. The microimmunofluorescence assay (IF) is considered to be the 'gold standard' for the serological diagnosis of *C. trachomatis* infections [16]. However, IF is labour-intensive and reading of the assay is operator-dependant and subjective. To overcome these drawbacks, *C. tracho-*

matis-specific enzyme-linked immunosorbent assay (ELISA) tests have been developed, allowing large epidemiological studies to be performed. These ELISA generally use recombinant peptides of the major outer membrane protein (MOMP) or the 60-kDa chlamydial heat shock protein (cHSP60). A number of studies have demonstrated strong correlations between the presence of anti-MOMP antibodies [17–21] or cHSP60 [22–24] and the severity of genital *C. trachomatis* disease, PID, infertility and tubal pathology.

More recently, a new automated technique for the simultaneous testing and detection of *C. trachomatis*, as well as *C. pneumoniae* and *C. psittaci*, has been developed by InoDiag (Signes, France). This multiplex serology approach has already been used for the serodiagnosis of atypical pneumonia [25] and blood culture-negative endocarditis [26]. The InoDiag serological test uses calibrated antigens made of whole bacteria and is based on automated incubation, reading and results interpretation of this 'microarray' serology [27]. As several antigens may be tested simultaneously, this new test leads to decreased labour time and, thus, reduced costs [25, 26, 28].

The present study aimed at comparing the performance of five commercially available chlamydial IgG antibody methods, including IF, three different ELISA tests, as well as the new automated InoDiag serological test. Antibody cross-reactivity with other related pathogens was also assessed. Finally, the association between miscarriage and *Chlamydia* seropositivity was also investigated.

Materials and methods

Patient population

Sera were obtained from women attending the Recurrent Miscarriage Clinic at St. Mary's Hospital, London. These sera from 405 patients with (n=251) and without (n=154) miscarriages had already been investigated for *Coxiella burnetii*, *Brucella abortus*, *Waddlia chondrophila* and *Parachlamydia acanthamoebae* as part of two previous studies [29, 30]. Women without miscarriages were used as negative controls to assess the association between a positive *C. trachomatis*-positive serology and miscarriage.

Serological assays for *C. trachomatis*

The following five serological assays were compared: *C. trachomatis* IgG immunofluorescence (Micro IF Test, ANILabsystems, Vantaa, Finland), two *C. trachomatis* IgG ELISA both using the MOMP as the antigen (CT-IgG-pELISA; Medac, Wedel, Germany, and CT pELISA; R-Biopharm, Darmstadt, Germany), one ELISA using cHSP60 as the antigen (cHSP60-IgG-pELISA; Medac, Wedel, Germany) and a new automated epifluorescence immunoassay (MuST Chlamydiae; InoDiag, Signes, France). All five tests were performed according to the manufacturers' instructions. For clarity, these five tests were respectively abbreviated as follow: IF, MOMP-Medac, MOMP-RB, cHSP60-Medac and InoDiag.

InoDiag: a new automated epifluorescence immunoassay

The InoDiag test is an automated epifluorescence immunoassay that enable the simultaneous detection of *C. trachomatis*, *C. pneumoniae* and *C. psittaci* antibodies. Each multiplexed slide also contained four controls: *Staphylococcus aureus* ATCC 29213 to verify serum deposition, human IgG (Serotec, Oxford, UK) to verify the secondary antibody distribution and to detect the presence of rheumatoid factor in patient sera, human IgA (Sigma, St. Quentin Fallavier, France) to verify the secondary antibody distribution and double-stranded DNA (Diarect, Freiburg, Germany) to detect antinuclear antibodies in patient sera. The multiplexed slide included three nanolitre spots containing each of the following antigens: *C. trachomatis* (ATCC VR-348-B), *C. pneumoniae* (ATCC VR-1310) and *C. psittaci* (ATCC VR-601).

Four serum samples diluted at a ratio of 1:64 were tested at the same time. Further remaining InoDiag reaction steps (serum, secondary antibody addition, incubation, washing, drying) were fully automated as previously described [25, 26]. The slides were then analysed by an automatic InoDiag fluorescent camera analyser, allowing the simultaneous detection of IgG and IgA directed against all antigens tested. Finally, the data were automatically analysed for each spot using the data-processing software Analarray 4.4-1 (InoDiag).

Serological assays for other pathogens

Immunofluorescence slides from ANILabsystems used to detect *C. trachomatis* antibodies also contained *C. psittaci* and *C. pneumoniae* antigens, allowing the assessment of cross-reactivity. Each slide was read blindly by two independent observers.

In addition, an in-house IF assay was performed for *W. chondrophila* (ATCC 1470), *Simkania negevensis* (ATCC VR-1471), *Neochlamydia hartmannellae* (ATCC 50802) and *P. acanthamoebae* strain BN9 (ATCC VR-1476), as described previously [10, 29]. Briefly, sera were screened by immunofluorescence using as a secondary antibody FluolineH (bioMérieux, Marcy l'Etoile, France). Sera that exhibited a total Ig titre ≥1:64 were then tested for IgG reactivity using an anti-human IgG fluorescein (FluolineG,

bioMérieux). Each immunofluorescence was read blindly by two independent observers and congruent results were considered to be positive. The IgG positivity cut-off was ≥1:64 [10, 29, 31].

Sera were also tested for the presence of antibodies directed against *C. burnetii* using indirect immunofluorescence, as described previously [30]. *B. abortus* serological diagnosis was established by Wright's tube agglutination test (Brucella Antigen, Sanofi Diagnostics, Marnes-la-Coquette, France). Antibody reactivity against *Toxoplasma gondii* was assessed using a commercial latex agglutination kit, Toxo-Screen DA (bioMérieux).

Statistical analysis

To compare all five serological tests, the sensitivities, specificities, positive predictive values (PPVs), negative predictive values (NPVs), likelihood ratio for positive and negative tests (LR+ or −) were calculated using Stata (StataCorp, College Station, TX, USA) using different gold standards. First, we considered IF as the gold standard. Then, we used a combined gold standard based on the results from all five serological tests. Since MOMP-RB and MOMP-Medac are not completely independent tests, we also used two additional combined gold standards, respectively based on the results obtained with IF, cHSP60-Medac and InoDiag, as well as only one of both MOMP-based ELISA tests. Values in the grey zone (i.e. with optical densities between the values threshold for negativity and positivity, as defined by the manufacturer of cHSP60-Medac, MOMP-Medac and MOMP-RB) were excluded from these analyses. To assess cross-reactivity, we compared the correlation between serology directed against two different pathogens using the Chi^2 test. The Chi^2 test and logistic regression were used to assess the presence of a correlation between *C. trachomatis* seropositivity and miscarriage.

Results

Seroprevalence rates and concordance

Five different commercial serological tests were applied to 405 sera taken from 405 different patients. The *C. trachomatis* seroprevalence ranges from 11.8% with MOMP-RB to 26.2% with InoDiag (Table 1). The concordance of the *C. trachomatis* IgG results for all five assays is shown in Table 2. A total of 183 (45.2%) of the 405 patients were positive with at least one of the five serological tests, but only 20 (4.9%) were positive with all five tests. In 203 (50.1%) cases, all five tests were negative.

Table 1 Results of *Chlamydia trachomatis* serologies using the five serological tests

Serological tests	Positive		Negative		Grey zone	
	n	(%)	*n*	(%)	*n*	(%)
IF[a]	58	(14.3)	347	(85.7)	–	–
cHSP60-Medac[b]	94	(23.2)	292	(72.1)	19[f]	(4.7)
MOMP-Medac[c]	58	(14.3)	336	(83.0)	11	(2.7)
MOMP-RB[d]	48	(11.8)	339	(83.7)	18[f]	(4.4)
InoDiag[e]	106	(26.2)	299	(73.8)	–	–

[a] IF = Micro IF Test (ANILabsystems, Vantaa, Finland)

[b] cHSP60-Medac = cHSP60-IgG-pELISA (Medac, Wedel, Germany)

[c] MOMP-Medac = CT-IgG-pELISA (Medac, Wedel, Germany)

[d] MOMP-RB = CT pELISA (R-Biopharm, Darmstadt, Germany)

[e] InoDiag = MuST Chlamydiae (InoDiag, Signes, France)

[f] Two sera were in the grey zone with both cHSP60-Medac and MOMP-RB tests

Test performances

Using IF as the gold standard, the sensitivities ranged from 48.1 to 63.8%, whereas the specificities ranged from 80.9 to 93.1%. The InoDiag test exhibited the best sensitivity (63.8%), whereas MOMP-RB exhibited the best specificity (93.1%).

A second analysis was conducted using another gold standard based on the results from all five serological tests. True-positive was defined as 4/5 or 5/5 tests positive. True-negative was defined as 0/5 or 1/5 tests positive (i.e. 4/5 or 5/5 tests negative). Samples with indeterminate serology (i.e. 3/5 tests positive and 2/5 tests negative [n=9] or 2/5 tests positive and 3/5 tests negative [n=26]) and samples in the grey zone for at least one of the tests (n=46) were excluded from the analysis. Using this new gold standard, the sensitivities ranged from 83.3 to 100%, whereas the specificities ranged from 87.4 to 99.7% (Table 3). Again, the InoDiag test exhibited the best sensitivity (100%), whereas MOMP-RB exhibited the best specificity (99.7%).

Even when indeterminate serologies were also included (i.e. when true-positives were defined as three, four or five positive tests and true-negatives were defined as three, four or five negative tests), the InoDiag test exhibited the best sensitivity, whereas MOMP-RB exhibited the best specificity. Indeed, using this gold standard, sensitivities of 89.7, 92.3 and 97.4% and specificities of 95.9, 97.8 and 86.3% were observed for MOMP-Medac, MOMP-RB and InoDiag, respectively.

MOMP-Medac and MOMP-RB are using the same antigen (MOMP) to detect *C. trachomatis* IgG. Thus, these tests are not completely independent from each other and the expanded gold standard may be biased. Thus, we also

Table 2 Concordance of *C. trachomatis* IgG results with the five serological tests[a]

	Number of patients	
	n	%
Positive with all five tests		
IF/HSP60-Medac/MOMP-Medac/MOMP-RB/InoDiag	20	4.9
Positive with four tests	10	2.5
cHSP60-Medac/MOMP-Medac/MOMP-RB/InoDiag (not IF)	5	1.2
IF/MOMP-Medac/MOMP-RB/InoDiag (not cHSP60-Medac)	2	0.5
IF/cHSP60-Medac/MOMP-RB/InoDiag (not MOMP-Medac)	2	0.5
IF/cHSP60-Medac/MOMP-Medac/InoDiag (not MOMP-RB)	1	0.3
IF/cHSP60-Medac/MOMP-Medac/MOMP-RB (not InoDiag)	0	0.0
Positive with three tests	9	2.2
Positive with two tests	26	6.4
Positive with only one test	91	22.5
IF	13	3.2
cHSP60-Medac	37	9.1
MOMP-Medac	9	2.2
MOMP-RB	1	0.2
InoDiag	31	7.7
Negative with all five tests	203	50.1

[a] Samples in the grey zone for at least one of the tests (n=46) were excluded from the analysis

used two additional gold standards, respectively based on the results obtained with all three of the other tests (IF, cHSP60-Medac and InoDiag) and only one of the two MOMP-based ELISA tests. With the gold standard defined using MOMP-RB, IF, cHSP60-Medac and InoDiag, the test exhibiting the highest performance in terms of sensitivity and specificity was MOMP-RB, with a sensitivity of 97.1% and a specificity of 99.3%. Conversely, with the gold standard defined using MOMP-Medac, IF, cHSP60-Medac and InoDiag, the test exhibiting the highest performance in terms of sensitivity and specificity was MOMP-Medac, with a sensitivity of 94.9% and a specificity of 96.7%. Thus, the results obtained with these two additional gold standards confirmed that, overall, MOMP-RB exhibits the best performance.

Table 3 Performance of the five serological tests using all tests as the reference. True-positive was defined as 4/5 or 5/5 tests positive. True-negative was defined as 0/5 or 1/5 tests positive (i.e. 4/5 or 5/5 tests negative). Samples with indeterminable serology (i.e. 3/5 tests positive and 2/5 tests negative [n=9] or 2/5 tests positive and 3/5 tests negative [n=26]) and samples in the grey zone for at least one of the tests (n=46) were excluded from the analysis

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	LR +	LR −
IF	83.3	95.6	65.8	98.3	18.8	0.17
cHSP60-Medac	93.3	87.4	43.1	99.2	7.4	0.08
MOMP-Medac	93.3	96.9	75.7	99.3	30.5	0.07
MOMP-RB	96.7	99.7	96.7	99.7	284	0.03
InoDiag	100	89.5	49.2	100	9.5	0

PPV = positive predictive value

NPV = negative predictive value

LR+ = likelihood ratio for positive test

LR− = likelihood ratio for negative test

Cross-reactions

Table 4 shows the cross-reactivities observed between *C. trachomatis* and other related bacterial species according to the serological test used. All five tests cross-reacted with *C. psittaci* (p<0.001). IF, MOMP-RB and InoDiag also cross-reacted with *C. pneumoniae* (p<0.001), whereas both Medac tests did not cross-react with this pathogen. None of the serological tests exhibited cross-reactivity with *Chlamydia*-like organisms (*W. chondrophila*, *P. acanthamoebae*, *S. negevensis* and *N. hartmannellae*), except for cHSP60-Medac, which only cross-reacted with *P. acantha-*

Table 4 Cross-reactions with other related bacterial species

	C. pneumoniae	*C. psittaci*	*P. acanthamoebae*
IF	++	++	
cHSP60-Medac		++	+
MOMP-Medac		++	
MOMP-RB	++	++	
InoDiag	++	++	

+ = p<0.05

++ = p<0.001

moebae BN9 ($p<0.05$). There were no statistically significant cross-reactivities between *C. trachomatis* and *T. gondii*, *B. abortus* and *C. burnetii* ($p>0.05$).

Association between miscarriage and *C. trachomatis* seropositivity

A correlation between miscarriage and the presence of anti-*C. trachomatis* IgG antibodies was observed when using IF (9.6% versus 22.1%, $p<0.001$, odds ratio [OR] 2.68, 95% confidence interval [CI] 1.52–4.73), MOMP-Medac (8.5% versus 18.7%, $p=0.005$, OR 2.47, 95%CI 1.29–4.76) and MOMP-RB (6.8% versus 15.9%, $p=0.008$, OR 2.61, 95% CI 1.26–5.41). Although not reaching significance, a similar trend was observed when using InoDiag (22.7% versus 28.3%, $p=0.217$, OR 1.34, 95%CI 0.84–2.14) and cHSP60-Medac (19.1% versus 27.6%, $p=0.057$, OR 1.62, 95%CI 0.98–2.67).

Discussion

This study compared the performance of five commercially available tests for the detection of *C. trachomatis* IgG antibodies, including a new automated multiplex antigen microarray, developed by InoDiag for the detection of *C. trachomatis* antibodies. Considerable inter-assay variability was found in the number of patients with positive serology. Previous studies comparing *C. trachomatis* ELISA to IF have not reported such a large inter-assay variability [17, 20, 21, 32].

In the present study, seroprevalence rates were similar for IF and MOMP tests (11.8–14.3%), whereas InoDiag and cHSP60-Medac exhibited higher seroprevalences (23.2–26.2%), possibly due to a higher rate of false-positive results. ELISA MOMP-RB exhibited the best sensitivity/specificity ratio in our study. This is congruent with previous studies that showed the good sensitivities and specificities of ELISA tests based on peptides from the MOMP of *C. trachomatis* [20, 32]. Additional advantages of these ELISA assays are: (i) their high throughput due to their 96-well format, especially useful for large epidemiological studies, and (ii) their objective reading of the results.

The new automated epifluorescence immunoassay from InoDiag exhibited an apparently lower specificity than the ELISA tests. However, given its high sensitivity and high NPV in the present study, it may be used to screen patients suffering from tubal infertility or miscarriage, especially to rule out a possible role of *C. trachomatis* in the pathogenesis of these conditions. The flexibility of the machine allowing batches of two to four samples is particularly designed for a laboratory carrying a small number of tests per pathogen. The automated microarray could test several pathogens simultaneously, allowing pathology-driven testing instead of the common pathogen-driven testing [28]. Such antigen microarrays have already been used successfully for the simultaneous detection of specific aetiological pathogens of community-acquired pneumonia [25, 27] and culture-negative endocarditis [26]. Concerning pregnancy, this technique might be particularly useful to determine the serological status at the beginning of the pregnancy for various pathogens such as *T. gondii*, *Treponema pallidum*, rubella virus, cytomegalovirus, herpes, HIV, HBV and HCV. In the case of recurrent miscarriage and/or infertility, the simultaneous determination of anti-*C. trachomatis*, anti-*W. chondrophila* and anti-*Coxiella* antibodies, as well as rheumatoid factor and antiphospholipid antibodies, might greatly hasten diagnostic investigations. Additional benefits include rapid time to results, semi-quantitative antibody detection and low sera volume requirements.

The serological cross-reactivity between different *Chlamydia* species is well established [20, 33–35]. In one study of patients attending STD clinics, antibodies directed against two chlamydial antigens were found in 19 to 33% of patients, and 33 to 40% of sera reacted with antigens of these three species: *C. trachomatis*, *C. pneumoniae* and *C. psittaci* [35]. In the present study, all *C. trachomatis* tests cross-reacted with *C. psittaci*. IF and InoDiag tests also significantly cross-reacted with *C. pneumoniae*. Since both tests are using the whole elementary bodies of *C. trachomatis*, the observed cross-reactions are likely to be due to genus-reactive antigens exposed on the surface of the bacteria. MOMP-RB also exhibited cross-reactivity with *C. pneumoniae*, whereas MOMP-Medac failed to show such cross-reactivity, although both tests use the MOMP of *C. trachomatis* as the antigen. Similar observations were obtained by others [17, 20]. The information provided by the manufacturers about the antigenic epitopes used in their tests are very limited. Serotype-specific determinants differ between tests and may explain the differences found in the test performances between ELISA tests from different manufacturers. Furthermore, tests based on highly specific peptides may be so specific that they are not able to detect all relevant antigens [20, 36]. Finally, different mutations within MOMP and variants of serotypes have been identified in urogenital isolates [37].

Previous studies have suggested an association between *C. trachomatis*-positive serology and miscarriage [10, 11, 29]. In the present work, we confirmed this association using IF, MOMP-Medac and MOMP-RB. Using cHSP60-Medac and InoDiag, a trend towards a higher seroprevalence in the miscarriage group was observed that did not reach significance, likely due to the high seropositivity rate in the control group due to the lower specificity of these two tests.

In conclusion, all of the serological assays tested here performed as well as or even better than the IF assay for *C.*

trachomatis IgG detection. Since ELISA-based serological assays are well standardised, less time-consuming, less expensive and less labourious than IF, they might be good alternatives to IF for the detection of *C. trachomatis* antibodies, especially when a large number of samples are to be processed. The new automated epifluorescence immunoassay from InoDiag appears to be a promising assay, given its excellent sensitivity and its microarray format that enables the simultaneous testing of several pathogens.

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