Comparison of immunological and molecular methods for the detection of Chlamydia trachomatis among infertile women in Basra, Iraq

Abrar A. H. Sahi¹ and Khairallah A.S. Mohammed¹*

1. Department of Medical Lab Technology, Southern Technical University, Health & Medical Technology College, Basrah/Iraq.

*Corresponding Author:
Khairallah A.S. Mohammed
Southern Technical University
Health & Medical Technology College
Basrah, Iraq
Email: dr.kmohammed@stu.edu.iq

Abstract

Background: Chlamydial infection is one of the common causative agents of infertility. Hence, a rapid, sensitive, and specific diagnostic method is important in reducing the long-term squeal of the diseases. Aim: This study was performed in Basra/Iraq to detect the prevalence of Chlamydia trachomatis in infertile women and to compare ELISA, immunochromatography test (ICT) and direct immune fluorescence (DIF) with PCR for detection of C. trachomatis. Materials and Methods: The study included 100 women with cervical infection (81 infertile and 19 fertile women). Enocervical swabs and 5 ml blood samples were collected from all participants. ELISA, ICT, DIF and PCR were performed to detect C. trachomatis antibodies, antigens, and DNA respectively. PCR was used as the gold standard. Results: The results of PCR revealed that 35 (43.2%) of infertile women and 5 (26.3%) of fertile women were C. trachomatis positive. When compared to PCR results; the immunological tests showed varied differences in major
performance characteristics. The sensitivity of DIF, IgM, IgA, IgG, and ICT was (85, 67, 67.5, 47.5, and 30%), specificity (73.3, 86.6, 90, 85, and 95%), Positive predictive values (68, 77, 81.8, 67.8, and 89%), negative predictive values (88, 80, 80.5, 70.8, and 80%) and Area Under a Curve (0.79, 0.77, 0.78, 0.66, and 0.62%), respectively. **Conclusion:** The results concluded a higher frequency of chlamydial infection among women with infertility in comparison to fertile women. The results suggested that DIF, IgM, and IgA are trustworthy tests whereas IgG test and ICT are not reliable for the diagnosis of chlamydial infections.

**Key words:** *Chlamydia trachomatis*, female infertility, ELISA, PCR, endocervical swabs


**Introduction**

*Chlamydia trachomatis* (CT) infection is the most prevalent sexual transmitted bacterial infection (STI). In 2012, WHO reported that there were about 130.9 million people infected yearly with *C. trachomatis*. The rate of chlamydial infection is different worldwide. Various studies showed a significant variation in the prevalence of *C. trachomatis* infection among the community of the Middle Eastern region. In Egypt the chlamydial infection rate reached 30%, whereas, in Jordon the prevalence was 4.6%. In the United Arab Emirates (UAE), the frequency of *C. trachomatis* infection was low (2.6%) this may relate with the low risk of study population or insensitivity of rapid antigen detection test used by authors. The prevalence of chlamydial infection in Saudi Arabia in women with infertility is 8% and 1.1% in fertile women.

In Iraq, the rate of chlamydial infection reached 9.6% as reported in a study carried out in 1990. AL-Husseiniet al.2009, used Enzyme linked fluorescent assay technique and found that
12.8% of suspected women were infected with *C. trachomatis*.\textsuperscript{7} In 2009, Abdul-Karim et al. used the ELIZA technique and stated that 20% of total 277 women tested were positive for IgG antibody against *C. trachomatis*.\textsuperscript{8} Other studies carried in Baghdad city reported that 85.96% are positive for IgM, IgG and 59.64% for IgA chlamydial Abs in serum of suspected pregnant-women.\textsuperscript{9} Ahmed, 2012, used PCR technique and found that 39 out of 147 (26.5%) endocervical women swabs were positive for chlamydial infection.\textsuperscript{10} In Basra, the first study of *C. trachomatis* was carried by Tahir at 2016 and found that out of 200 infertile women 96 (48%) were PCR positive and 11% and 5.5% were sero-positive for IgM and IgG antibodies.\textsuperscript{11} The variation of the positive results among many studies of diagnosis of *C. trachomatis* might be due to the sample size and subject’s status, specimen collection techniques, transport time or methodology.

Urogenital infections with *C. trachomatis*, in women, can lead to pelvic inflammatory disease associated with late ectopic pregnancy and tubal infertility.\textsuperscript{12,13} More than 40% offemales with untreated *C. trachomatis* infection may develop pelvic inflammatory disease (PID) and about 20% of them become infertile, 18% results in chronic pelvic pain.\textsuperscript{12,13} Additionally, women with chlamydial infection have 3-4 fold amplified risk of acquiring HIV and developing invasive cervical cancer.\textsuperscript{12,13} Also, asymptotically infected women may develop reproductive squeal. Therefore, screening and diagnosis of genital chlamydial infection is a high public health concern.\textsuperscript{14} Furthermore, the high level of chlamydial infection prevalence has produced considerable attention in development of sensitive, specific, cost effective, and rapid techniques for diagnosis of this disease.\textsuperscript{15}

Different methods have been used to detect and identify *C. trachomatis*, such as direct smears to visualize inclusion bodies, culture of the organisms in cell lines, immunological methods to
detect the presence of chlamydial antigens /antibodies and molecular biological techniques, which based on the presence of *C. trachomati*s DNA.\textsuperscript{16-22} All these methods have advantages and disadvantages for their applicability in the diagnosis of *C. trachomatis*.

The aim of this study was to detect the prevalence of *C. trachomatis* infection among infertile women in Basrah province and to compare the diagnostic accuracy of direct immunofluorescence technique (DIF), immunochromotography test (ICT), and enzyme-linked immunosorbent assay (ELISA) with polymerase chain reaction (PCR) for diagnosis of chlamydial infection.

**Method**

**Patient population**

This study was conducted at the cervical cancer and the infertility Centre in Basra Hospital for Women and Children, Basra /Iraq. A total of 100 women with cervical infection (81 infertile and 19 fertile female), aged 16 - 60 were recruited in the present study. After examination by physicians, informed consent was obtained from all patients, and a questionnaire regarding, age, level of education, job, marital status, history of genital infections, history of aborting, contraceptive methods, history of genital surgery and symptoms was completed. Those with antibiotic therapy within 30 days before the assessment were excluded from the study.

**Specimen collection**

Three endocervical swabs (cytology brush swab & cotton polyester swab) from 100 participants were collected by specialized gynecologist. Before sampling, the endocervix was cleaned with a sterile cotton to remove excess mucus and exudate then three specimens were collected from each woman. Swabs were rubbed and rotated several times over the endocervical cells in the
cervical canal to collect samples. Swabs were withdrawn with careful attention to prevent touching the vaginal surface, and were placed into the transport medium. One swab was used for the direct immunofluorescence (DIF) and the other for Immunochromatography test (ICT). A third swab was kept in -20°C until PCR was performed. Five ml of blood were collected and centrifuged then have been refrigerated freezing at -20°C, until the serological tests (IgG, IgM and IgA) were performed.

**Polymerase Chain Reaction (PCR)**

DNA was extracted from endocervical swabs according to the manufacturer’s recommendations (Wizard Genomic DNA purification Kit, Promega, USA). Pair primers were designed to amplify 233 bp fragments of chlamydial plasmids, using Primer3 and BLAST software, with the following sequences:

F 5’TCACACCCAAAAGCTCTGG3’ and R 5’CCGCTTTCTAAACCAGCTAC’

The amplification reaction was performed in a volume of 25 µl containing 12.5 µl of Green master mix 2 X [Taq DNA polymerase, dNTPs, MgCl2 and reaction buffers] (Promega), 1µl (10 picomoles/µl) of each primer oligonucleotide, and 2 µl (50-100 ng) of DNA. The reaction mixture was made up to 25 µl with Nuclease – Free Water. The reaction mixture was amplified in a DNA thermal cycler (Applied Biosystem, UAS). The samples were subjected to an initial denaturation step at 95°C for four minutes, followed by 25 amplification cycles. Each amplification cycle consisted of denaturation at 95°C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 1 min.

Visualization of amplified product was carried out by agarose gel electrophoresis. A 7µl of PCR
product was loaded and separated by electrophoresis on 2% agarose gel in the presence of ethidium bromide, along with DNA ladder. PCR products were then visualized and photographed using a gel documentary machine (UV trans-illuminator).

**DIF, ELISA, and ICT**

Direct immunofluorescence technique (DIF), immunochromotography test (ICT), and ELISA for *C. trachomatis*-specific IgG, IgM and IgA were performed as described in our previous study.  

**Statistical analysis**

To determine the diagnostic accuracy of the evaluated methods, statistical analysis was carried out using the statistical package for social sciences (SPSS) version 22 and graphic pad prism version 6. The sensitivity, specificity, positive predictive value, negative predictive value, Area Under a Curve of each method tested were calculated and compared with the gold standard (swab based PCR). Chi-square ($\chi^2$) was applied in the comparison of employed tests with the gold standard and the statistical significance was set at 5% level.

**Results**

Of 100 specimens, 40 were positive by PCR using specific primers for cryptic plasmid, which was used as the gold standard (Fig.1). The comparison of results obtained by PCR in the present study with immunological results showed that of the 40 PCR positive 35 (85%) were positive by DIF, 27 (68%) were positive by IgM, 19 (48%) were positive by IgG, 27 (68%) were positive by IgA, and 12 (30%) were positive by ICT (Table 1, Fig. 1 - 2). Inten instances, DIF test was positive in the absence of a positive PCR. The frequency of *C. trachomatis* among suspected women according to age, level of education, infertility and clinical symptoms are shown in table 2. The highest frequency of *C. trachomatis* was in age group 20 -29 years and 1st age marriage
≤20, illiterate women, secondary infertility, vaginal discharge and irregular menstruation (table 2).

For comparison of the DIF, IgM, IgG, IgA, and ICT with the PCR technique, a two-by-two table was used to calculate sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and Area Under a Curve (AUC). The chi-square test was used to test the significance of the difference in frequency distribution. A P value of < 0.05 was considered significant (Table 3). The PCR technique was used as the gold standard.

**Figure 1.** Detection of *C. trachomatis* from cervical specimens by PCR (cryptic plasmid primers, PCR product 233pb). Lane M, DNA size markers; lane 1, 2, 3 positive patients for *C. trachomatis*; lane 4, 5, 6 negative patients for *C. trachomatis*; Lane C negative control.

**Figure 2.** Immunofluorescence microscopy of *C. trachomatis* infected cells for endocervical swab slides showing extracellular elementary bodies (cut-off ≥10 EBs). EBs appear as a very small bright apple-green fluorescent smooth edged disc shapes approximately 300nm in diameter and can be observed against a background of red counterstained cells and cellular debris.
As shown in table 3, the true positive results of DIF, IgM, IgG, IgA, and ICT compared with PCR were 85%, 68%, 68%, 48%, and 30% respectively, with P value <0.05. The range of the sensitivity was between 30 to 85%, the highest sensitivity given by DIF whereas the lowest given by ICT. The highest predictive negative value (88%) was given by DIF whereas ICT showed the lowest PNV (67%). In addition, DIF test showed the highest value of AUC, which ranged from 0.79 to 0.66. The specificity and the positive predictive values of the DIF (73 and 68%, respectively) were lower than those of other immunological tests, which ranged from 85-95% and 68 – 80% respectively (table 3). The values of the sensitivity, specificity, PNV, PPV and AUC of anti-chlamydial IgM and IgA antibodies were, significantly, higher than those of IgG (table 3).

Discussion

The manifestation and consequences of C. trachomatis infections in damaging the reproductive health in women is considered as an important issue in women’s healthcare. Therefore, using specific and sensitive technique is essential to establish accurate diagnosis of chlamydial infections.

In this study, we evaluated the diagnostic accuracy of different immunological and molecular methods to diagnose chlamydial infections amongst infertile women, using PCR technique as the standard gold. The selection of swab based PCRs as the standard gold because it is the most specific and sensitive non - culture test, for detection of C. trachomatis, now available.

In our study, we found that 35 (43.2%) of the tested infertile women with vaginal discharge were positive for C. trachomatis, 22 (62.8%) of them with secondary infertility and 13 (37.2%) with
primary infertility. Whereas 5 (26.3%) were positive among fertile group. The age group 20 -29 years and 1st age marriage ≤20 showed highest frequency of chlamydial infection. The high prevalence in infertile women was proved in previous by many studies. Also, there were some reports indicated greater prevalence for the infection in secondary than primary infertility. In Egypt, Naglaa et al. found through using PCR, DIF and ELISA technique prevalence rate of chlamydia infections in patients with infertility was 45%. In India, Malik et al. recorded chlamydial positivity in 20 of the 74 (27%) symptomatic women with primary infertility and in 11 of the 36 (30.6%) with secondary infertility by using cell culture and ELISA for detection of chlamydial antigen. Also, they found that the highest frequency of chlamydial (77.4%) in the 21-30 years age group which is higher than that we found. In Iran, Hossein Rashidi et al. reported prevalence of *C. trachomatis* in infertile women infection based on PCR and serologic detection of IgG and IgM respectively was 12.4%, 9.0% and 0.9%. Another study recorded that *C. trachomatis* was detected by direct immunofluorescence method in 15.3% and by PCR in 32% infertile women. In Jordan, the rate was reported to be 3.9% by PCR and in Turkey was 23.3% by direct immunofluorescence. In Basra/ Iraq, the first study of *C. trachomatis* was done by Tahir at 2016 and found that out of 200 infertile women 96 (48%) were PCR positive, which is slightly higher than our results. Our findings are compatible with the study done in Basra/Iraq by Tahir et al. (2016), which reported slightly higher prevalence (48%) for *C. trachomatis* infection in women with infertility.

The variations of positive results among many studies of diagnosis of *C. trachomatis* seem to be due to the socioeconomic status, sample size, specimen collection techniques, transport time and diagnostic methods.
In the present study, the comparison of the immunological tests results with PCR showed that DIF sensitivity and specificity 85% and 73.3% respectively. Similarly, several studies revealed that DIF sensitivity of 70-84%, thus far, more sensitive than other immunological techniques of diagnosing *C. Trachomatis* infection although less sensitive than culture and PCR.\(^{14}\) The lower specificity and PPV value of DIF recorded in our study versus the other studies may be due to the presence of ten DIF positive cases in the absence of a positive PCR. This may be due to inhibitory activity in PCR test, excessive debris in DIF test or may be due to presence of plasmid deficient strain of *C. trachomatis*. Overall, the sensitivity, specificity, PPV, PNV, and AUC values indicated that the DIF is a valuable method for the diagnosis of chlamydial infection.

In respect to ELISA for detection of *C. Trachomatis* IgG, IgM and IgA, we chose our cut off absorbance value based on the absorbance of known negative and positive controls as high significant limit to ensure good specificity and to prevent cross reactivity. Also, the outcome of our results were assessed in consistency with active infection as the serological test is not often supportive in diagnosis of chlamydial infection due to the high background of antichlamydial antibodies or seronegativity with persistent active infection. Seroprevalence with active infection indicated persistence or recurrent infection. Generally, the high sensitivity, specificity, PPV, PNV, and AUC values of IgM and IgA obtained in our study suggest that these tests are reliable for diagnosis of active and persistent chlamydial infections, preferably combined. The parameters measures of the both tests proved that unlikely producing false positive or negative results by these tests.\(^{34}\) On other hand, under the conditions of our study, the low sensitivity, NPV, and AUC values with high false negative results of IgG and ICT suggested that these tests are not trustworthy for diagnosis of chlamydial infections.
The results concluded the highest frequency of chlamydial infection were among women with infertility than fertile women. Women with secondary infertility showed high prevalence than primary infertility. Our findings suggested that screening of infertile women for *C. trachomatis* is highly recommended. The results in the present study suggested that, in the absence of requested equipment and skills for PCR, detection of antigen or antibodies (IgM and/or IgA) of *C. trachomatis* by direct immunofluorescent technique or ELISA assay, respectively, can play a substantial role in screening for *C. trachomatis* in infertile women. On the other hand, under the conditions of our study, the low sensitivity and AUC values with high false negative results of IgG and ICT suggested that these tests are not trustworthy for diagnosis of chlamydial infections.

The main limitation of our study was the limit number of specimens. Further studies with large size of samples will be required for comparison of different diagnostic test to determine the best method for detection of *C. trachomatis*.

**Conflicts of Interest**

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

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Table 1. Results of PCR compared to Direct Immunofluorescence, IgM, IgG, IgA, and Immunochromotography tests for Diagnosis of *C. trachomatis*.

*PCR was used as the gold standard*

Table 2. Frequency of *C. trachomatis* among cervicitis women according to age, level of education, infertility and clinical symptoms. The results based on PCR which was used as the gold standard.
### Table 3. Comparison of immunological method with swab based PCR for detection of *C. trachomatis*.

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P value: Probability, PPV: Predictive positive value, NPV: predictive negative value, AUC: Area Under a Curve