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RESEARCH ARTICLE

COMPARATIVE STUDY OF IMMUNOLOGICAL METHODS FOR THE DIAGNOSIS OF CHLAMYDIA TRACHOMATIS IN WOMEN WITH CERVICITIS AND INFERTILITY.

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Abstract

We have evaluated the diagnostic accuracy of three ELISA tests (IgM, IgG, IgA) and rapid test in detecting *C. trachomatis* by comparing with direct immunofluorescence (DIF), which is used as a gold standard. Blood samples and endocervical swabs of 100 suspectedfemales and 10 healthy women were collected. All sera were tested for chlamydial antibodies of the IgG, IgM and IgA classes by ELISA. Swabs were tested by Rapid test and DIF for detection of *C. trachomatis* antigen. Among 100 patients that were included in the study, 46 (46%) women with infertility and 4 (4%) with cervicitis were positive for chlamydia according to the standard method (DIF). None of the healthy women was positive. When compared to DIF results; the ELISA (IgM, IgG, IgA) and rapid test results showed noteworthy differences in the major performance characteristics. The sensitivity of IgM, IgA, IgG and Rapid test was (66, 62, 48, and 24%), specificity (96, 96, 92, and 94%), positive predictive values (94.3, 93.3, 58.7, and 80%), negative predictive values (73.8, 71.6, 63.9, and 55.3%) and Area Under a Curve (0.81, 0.81, 0.7, 0.59.3%) respectively. The results concluded that DIF, IgM, and IgA are valuable techniques to diagnose chlamydial infections. On the other hand, under the conditions of our study, the results suggested that IgG and rapid test are not reliable for diagnosis of chlamydial infections.

Introduction:-

Chlamydia trachomatis infection is the most common prevalent sexually transmitted infection worldwide (1). In 2012, WHO reported that there were about 130.9 million people that are infected yearly with *C. trachomatis* (1).The rate of infection is different worldwide (1). In Iraq, the rate of chlamydial infection reached 9.6% as reported in a study carried out in 1990 (2). AL-Husseineiet. al., 2009, used Enzyme linked fluorescent assay technique and found that 12.8 % of suspected women were infected with *C. trachomatis* (3). In 2009 Abdul-Karim et al, used ELIZA technique and stated that 20% of total 277 women tested were positive for IgG antibody against *C. trachomatis* (4).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 (5). Ahmed, 2012, used PCR technique and found that 26.5 % out of 147 endocervical women swabs were positive for Chlamydia infection (6).In Basra, the first study of *C. trachomatis* was carried by Tahir at 2016 and found that out of 200 infertile women 48% were PCR positive and

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11% and 5.5% were sero-positive for IgM and IgG antibodies (7). The variation of the positive results among many studies of diagnosis of *C. trachomatis*, in Iraq, might be due to the number 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 (8, 9). More than 40% offemales with untreated *C. trachomatis* infection may develop PID and about 20% of them become infertile, 18% results in chronic pelvic pain (8, 9). Additionally, women with chlamydial infection have 3-4 fold amplified risk of acquiring HIV and developing invasive cervical cancer (8, 9). Also, asymptomatically infected women may develop reproductive squeal. Therefore, screening and diagnosis of genital chlamydial infection is a high public health concern(10)Furthermore, thehigh level of chlamydial infection prevalence has produced considerable attention in development of sensitive, specific, cost effective, and rapid techniques for diagnosis of this disease (11). Bacterial isolation in tissue culture media and a nucleic acid amplification test (NAAT) are generally regarded as a gold standard for the diagnosis of Chlamydia trachomatis infection. However, culture method requires careful specimen collection and stringent transport condition and requires at least 48 to 72 h to perform (12, 13). On other hand, DNA amplification technique might not be cost effective for screening program in developing countries (14).

Many studies suggested that Chlamydial serology could be useful to detect current, chronic or persistent chlamydial infection, and for screening women with asymptomatic chlamydial infection (15 –19). The aim of this study was to evaluate the diagnostic accuracy of different immunological methods in the diagnosis of *Chlamydia trachomatis* in cervicitis and infertile women.

Method:-

Patient population:-

Between October 2017 and August 2018, a total of 100 women, aged 15 -42, with cervicitis and infertility and 10 apparently healthy women, who attended cervical cancer and infertility at Basra Hospital for Women and Children (Basrah – Iraq), 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.

Samples collection:-

Five ml of blood, from each participant, used for serological tests, and two endocervical swabs (cytologybrush swab & cotton polyester swab) from each contributor were collected, one of which was used for the direct immunofluorescence (DIF) and the other used for rapid test.

Dif Test:-

The swab specimen, used for DIF, was rolled using slight pressure, within the 6mm well area on the microscope slide. The specimen was allowed to air dry thoroughly at room temperature (15-30°C) and then fixed in fresh acetone for 10 minutes and allowed to air dry. The slides were subjected to the technique using a direct immunofluorescence test for the detection of Chlamydia trachomatis (IMAGEN Chlamydia, Oxoid, UK), according to the manufacturer's instructions and examined with a fluorescence microscope. The smears were investigated for the presence of extracellular elementary bodies (EBs) which appear as very small bright apple-green fluorescent smooth edged disc shapes approximately 300nm in diameter observed against background of a red counterstained cells and cellular debris (Fig. 1). The samples were considered positive if they presented at least ten EBs per slide, cut-off recommended by the manufacturer. The absence of Chlamydia bodies was considered negative.

Rapid Test:-

For rapid test the swabspecimen was immersed into the extraction tube and processed according to the manufacturer's recommendations (Chlamydia trachomatics antigen rapid test kit, PreCheck BIO), the results were interpreted against the positive and negative controls provided by the manufacture.

ElIZA Test:-

Sera were prepared from blood specimens and analysed by enzyme-linked immunosorbent assay for detection of immunoglobulin G and M and A antibodies to Chlamydia trachomatis. The technique used for the ELISA was according to the manufacturer's recommendations (Chlamydia trachomatis IgG ELIZA kit, AccuDiag; *Chlamydia*

trachomatis IgM ELIZA kit, AccuDiag, USA; *Chlamydia trachomatis* IgA, NOVA TEC, Germany). The reading of the results were interpreted by using a cutoff absorbance based on the absorbance of known negative and positive controls supplied by the manufactures.

Statistical analysis:-

The performances of ELIZA (IgG, IgM and IgA) and Rapid test were compared with that of DIF. Statistical analysis was carried out by 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 Undera Curve of each method tested were calculated and compared with the that ofthe gold standard (DIF). Chi-square (x2) was applied in the comparison of employed tests with the gold standard and the statistical significance was set at less than 5% level.

Results:-

A comparison of the five immunological tests showed noteworthy differences in the major performance characteristics (Table 1). Out of 100-suspected cases that were enrolled in this study, 15% (15) were positive for the rapid test, 35% (35) were positive for anti-chlamydial IgM Abs, 33% (33) were positive for anti-chlamydial IgA Abs, 28% (28) were positive for anti-chlamydial IgG Abs and 50% (50) were positive for DIF (Table 1).

The results of the present study showed that the positivity of rapid test in comparing with positive DIF was (24%), with significant difference ($P<0.05$). The sensitivity and specificity of this test were (24% and 94%) respectively. The PPV and NPV values were (80.0% and 55.29%). Additionally, the Area Under a Curve (AUC) was (0.59) as shown in table 1.

The positivity of anti-chlamydial IgM Abs in comparing with positive DIF was (66.0%) with significant difference ($P<0.05$). The sensitivity and specificity of anti-chlamydial IgM Abs were (66.0% and 96.0%) with PPV and NPV values of (94.28% and 73.84%) correspondingly. The AUC value was 0.81 as shown in table 1.

The results showed that the positivity of anti-chlamydial IgA Ab compared with positive DIF was (62.0%) with significant difference ($P<0.05$). The sensitivity and specificity of anti-chlamydial IgA Ab were (62% and 96%) with PPV and NPV values of (93.93% and 71.64%) respectively. The AUC value was (0.81) as shown in table 1.

The positivity of anti-chlamydial IgG Abs in comparing with DIF was 48% with significant difference ($P<0.05$). The sensitivity and specificity of anti-chlamydial IgG Abs were (48.0% and 92.0%) with PPV and NPV values of (68% and 88%) respectively. The AUC value of this test was (0.70) as shown in table 1.

The highest percentage of infection (75%) was observed in women aged 20 - 39. The illiterate and primary education and low socio-economic status showed high rate of infection. The comparison of obtained positive results with the histopathological finding showed that 35% and 10% of acute and chronic cervicitis respectively, 7.5% of CIN1 and 47.5% normal.The cervical appearance by inspection was about 15% bleeding on touch and 35% eroded with bleeding on touch and 50% normal. Patients with abortion showed 42.5% of the positive results and 57.5% without. Infertile women showed 87.5% of the positive results.

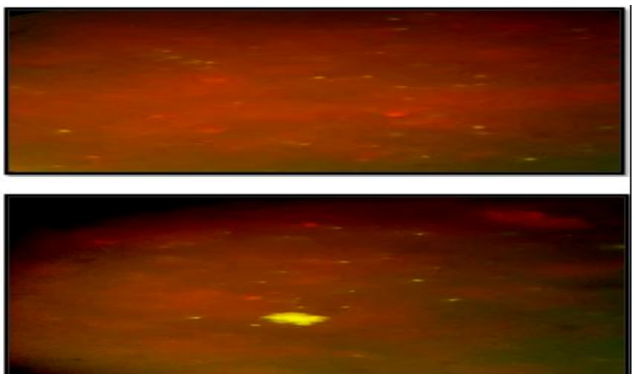

Figure 1:-Immunofluorescence microscopy (X1000 magnification) of *Chlamydia trachomatis* infected cells for endocervical swab slides showing extracellular elementary bodies (cut-off ≥10 EBs). EBs appear as a very small bright appel-green fluorescent smooth edged disc shapes approximately 300nm in diameter and can be observed against a background of red counterstained cells and cellular debris.

Table 1:-Comparison of immunological parameters within study subjects depending on DIF positivity of *C.trachomatis*.

Test type	DIF Positive	DIF Negative	Total	STATISTICS
Rapid test				X^2= 6.35, *P = 0.01
Positive	12	3	15	SP= 94.0%. Sn= 24.0%
Negative	38	47	85	PPV= 80.0%, NPV=55.29%
Total	50	50	100	AUC= 0.59
IgM				X^2= 42, *P = 0.001
Positive	33	2	35	SP= 96.0%, Sn= 66.0%
Negative	17	48	65	PPV= 94.28%, NPV= 73.84%
Total	50	50	100	AUC= 0.81
IgA				X^2= 38, * P = 0.001
Positive	31	2	33	SP= 96.0%, Sn= 62.0%
Negative	19	48	67	PPV= 93.93%, NPV= 71.64%
Total	50	50	100	AUC= 0.81
IgG				X^2= 20, *P = 0.001
Positive	24	4	28	SP= 92.0%, Sn=48.0 %
Negative	26	46	72	PPV= 85.71%, NPV= 63.88%
Total	50	50	100	AUC= 0.70

(*) Significant difference. (NS) non-Significant difference. (X2) Chi-square.. (P value) Probability. (Sn) Sensitivity. (Sp) specificity. (PPV) Predictive positive value and (PNV) predictive negative value, (AUC) Area Under a Curve

Discussion:-

The manifestation and consequences of *C. trachomatis* infections are more damaging to reproductive health in women than in men, so they are considered an important issue in women's healthcare (8, 9). Therefore, using specific and sensitive technique is essential to establish accurate diagnosis of chlamydial infections. Yet, there is no clinical or microbiological reference standard for diagnosis of *C. trachomatis* infection (20). Culture is no longer considered as a reference microbiological method because of the low sensitivity (75% to 85%) compared to PCR (21). In addition, the endocervical culture is an unreliable method to rule out the presence of endometrial or tubal pathogens, as the pathogen is not always present at the culture site (22). Therefore, the Immunological and PCR techniques are useful and reliable methods for the detection of *C. trachomatis* in endocervical swab and serum specimens (22). However, DNA Amplification technique might not be cost effective for screening programs in developing countries (14). Moreover, serological tests may be helpful in the diagnosis of chronic and invasive chlamydial infections such PID in which bacteria are undetectable in urogenital swabs or urine (23). In addition, persistent chlamydial infections and subsequent complications are usually associated with a positive antibody response; negative serology may exclude the chlamydial infection (23).

In this study, we used different immunological methods to diagnose chlamydial infections.The validity and the diagnostic accuracy of each method tested was quantified by measuring the sensitivity, specificity, positive and negative predictive values (PPV and NPV), the Area Under a Curve (AUC), of each test result and compared with that of DIF as a gold standard method. P value <0.05 was considered as a significant value (24).The selection of DIF as a gold standard was based on that, the validity of this technique was establishedby many studies (25, 26, 27).The comparison of DIF and culture showed that the range of DIF sensitivity between 80 to 90% and specificity was 98 to 99% (28).

Sensitivity defined as the probability of getting a positive test result in subjects with disease and specificity defined as the probability of a test to exclude a positive test result in subjects without the disease (24, 29). In the present study, the comparison of serological results with DIF showed highest sensitivity of IgM and IgA tests (66.0% and 62.0% respectively, table 1) comparing with low sensitivity of IgG and rapid test (48.0% and 24.0% respectively, table 1).These findings indicate that IgM and IgA have highest sensitivity among the serological tests used in this study. That may be due to the current infection or reinfection leading to high level of IgM and IgA in patient's

serum. On other hand, the rapid test showed the lowest sensitivity, which may be due to the low load of bacteria in the specimens, sample collection method or sample processing. These results agree with other studies, which demonstrated a low sensitivity of rapid tests (30, 31).

The specificity of IgM and IgA tests was 96.0% for both, with PPV 94.3% and 93.9%, respectively (table 1). The results revealed that the specificity of IgM and IgA tests approaches 100% and the PPV above 90% which approving that no false positive results will be expected according to the CDC guidelines for chlamydial diagnosis and other studies (31, 32).Therefore, the positive results of these tests do not need to be confirmed by other test. The NPV values of IgM and IgA were 73.8% and 71.6%, which indicate that the probability of having false negative results about 28%. This may be due to low sensitivity of the both test.

PPV defines the probability of having a disease of interest in a subject with a positive result and NPV probability of not having a disease in a subject with a negative result, unlike sensitivity and specificity, predictive values depend on the diseases prevalence in examined population (29, 30). We found that the specificity of IgG and rapid test was 92% and 94%with PPV 85.78% and 80% and NPV values 63.88% and 55.29% respectively. Based on the low sensitivity (48, 24% respectively) and PPV values, witha high probability of getting false positive results, the tests may needto be supplemented by another confirmatory.

Other measures of diagnostic accuracy used in this study is the ROC curve and the Area Under a Curve AUC, which helps to estimate the discriminative power of a given test. The diagnostic accuracy is determined by the value of AUC; the highest value of AUC (0.9-1.0) is the best and the lowest value (<0.5) means the test is useless. The present comparative study concluded that the AUC value in addition to other measurements proved that the IgM and IgA tests are more reliable serological tests, which could be used to diagnose acute and persistent chlamydial infections, preferably combined. On other hand, under the conditions of our study, the results indicated that IgG and rapid test are not trustworthy for diagnosis of chlamydial infections.

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