ISSN: 0067-2904

Molecular diagnosis of *Chlamydia trachomatis* in infertile Iraqi women using Real time-PCR and comparison with other methods

Iqbal Harbi Mohammed*[1], Salwa J. Al-Awadei[2], Shurook.M.K.Saadedin[3]

[1] Ministry of Agriculture office –Horticulture, Baghdad, Iraq.
[2] College of Applied Biotechnology, University of Al-Nahraan, Baghdad, Iraq
[3]Genetic Engineering and Biotechnology, Institute for Postgraduate Studies, Baghdad, Iraq.

Abstract

Chlamydia trachomatis is the most common of negative gram bacteria that cause sexually transmitted diseases. It affects the reproductive system in women, not the symptoms of the disease, but the most serious is the long-term effects of the reproductive system.. out of 100 women were attending different hospitals in Baghdad included the Gynaecology Departments of Women Health Center at Al-Elwyia Obstetrics Hospital . Ibn Al balady Maternity and Children's Hospital , Kamal al-Samarrai hospital Fertility Center infertility treatment and In Vitro Fertilization (IVF) (20 control and 80 women with infertility) DNA was extracted from the Endocervical Swabs of all infertili women, to investigate the bacteria by using Real time -PCR technique and another swab were subjected to direct Rapid Test . The venous blood sample was obtained, serum were diagnosis by Anti-*Chlamydia trachomitis* Chlamydia trachomitis IgG ELISA.

The result of the Real time-PCR revealed was significant associated with infertility women (OR= 0.733; 13.75%, P= 0.041), among 80 infertili women, while was no significant diagnosis by ELISA (OR= 0.063; 2.50%, p= 0.095). and no significant associated with Rapid Test .

In summary Real time-PCR has the possibility to contribute in the primary screening for infertility in women.

Keywords: *Chlamydia trachomatis*, female infertility, Real time -PCR, ELISA, Rapid Test, endocervical swabs,Blood.

التشخيص الجزيئي لـ *Chlamydia trachomatis* في النساء العراقيات العقيمات باستخدام Real time-PCR والمقارنة مع طرق اخرى

أقبال حربي محمد*[1]، سلوى جابر عبد الله العوادي[2]، شروق محمد كاظم سعد الدين[3]

[1]وزارة الزراعة ، دائرة البستنة، بغداد، العراق.
[2]كلية التقنيات الحيوية التطبيقية، جامعة النهرين، بغداد، العراق.
[3]معهد الهندسة الوراثية والتقنيات الأحيائية ، جامعة بغداد، بغداد، العراق.

*Email: iqbalharbbi743@yahoo.com

الخلاصة

تعد *Chlamydia trachomatis* واحدة من اكثر انواع البكترية السالبة لصبغة كرام انتشارا والمسببة للامراض المنتقلة جنسيا والتي تصيب الجهاز التناسلي لدى النساء ، ليست في أعراض المرض ولكن الأمر الأكثر خطورة يكمن في الآثار طويلة الأمد التي يحدثها في الجهاز التناسلي.

تم فحص 100 امرأة من المراجعات لمستشفيات مختلفة في بغداد تضمنت قسم الامراض النسائية لمركز صحة المرأة في مستشفى العلوية للولادة ومستشفى كمال السامرائي مركز الخصوبة وعلاج العقم والتخصيب الخارجي ومستشفى ابن بلدي للاطفال والنسائية وشملت (80 امراة عقيمة و20 سيطرة) تم استخلاص الحامض النووي من مسحات عنق الرحم وذلك للتحري عن لبكتريا *C.trachomatis* باستخدام تقنية Real time PCR اما المسحة الثانية فقط شخصت مباشرة باستخدام الاختبار السريع وشخص مصل الدم مناعيا للكشف عن البكتريا باستخدام Anti-*Chlamydia trachomitis* IgG ELIS . اثبتت نتائج هذه الدراسة بأن تقنية Real time-PCR هي طريقة كفوءة للكشف عن بكتريا *C. trachomatis* من المسحات المأخوذة من عنق الرحم للنساء العقيمات .

اظهرت النتائج بان هنالك احد عشر عينة موجبة من ضمن 80 عينة التي تم اختبارها باستخدام تقنية Real –time pcrوالتي اعطت فروقات معنوية مرتبطة مع النساء العقيمات ,13.75% ;0.733 =OR) ,P= 0.041) في حين لم يظهر اي فرق معنوي وارتباط بتقنيتي المناعية والاختبار السريع ;0.063 =OR) . 2.50%, p= 0.095).

Introduction

Sexually transmitted infections(STIs) which are mainly transferred from one person to another through sentimental contact [1,2] .

The infection can be spread through vaginal, anal sex, or though contact with blood during sexual activity In developing countries 70 % of all pelvic inflammatory diseases (PIDs), which can lead drive to infertility[3].

The most common STIs are the bacterial infections: chlamydia, syphilis, gonorrhoea [4]of the "top ten" sexually transmitted infections, *Chlamydia trachomatis* (*CT*) and *Neisseria gonorrhoeae* ranked second and fifth, respectively, worldwide [5] .

Screening for these infections is important not only to identify infected symptomatic individuals for the diagnosis and management directive of their infections but also to identify asymptomatic individuals who serve as reservoirs for*C. trachomatis* infections [6].

One of the main reasons for the rise prevalence rates of *C. trachomatis* is that in most cases (80% of women and 50% of men) the infection remains asymptomatic. Untreated, the infection increases the risk of ectopic pregnancy and thus leading to causes of female infertility worldwide.[7,8]

rise prevalence rates of *C. trachomatis* and the asymptomatic course of infection have drive several countries to implement screening checking programmes to prevent the development of reproductive sequelae [9,10].

Diagnosis of genital chlamydial infection has been by culture, Rapid antigen and antibody testing. classiccell culture methods for *C. trachomatis* can differ in sensitivity between 60-80 % and are complicated to standardize, technically imperious and expensive [11,12]. Antigen detection tests requires skill and proficiency and is only proper for laboratories that test a limited number of sample [11, 13]. In addition, the sensitivity of antigen detection tests as low as 60 % [14]. Serological tests are of limited value in diagnosing this bacterial infection in the individual, because antibodies may not exist in every case of uncomplicated infection and tests do not distinguish current from past infection. is no longer used as diagnostic tool for acute infections [15]. Recently nucleic acid amplification techniques (NAATs), have become available with the possibility to offer improved sensitivity for diagnosing *CT* infections [16, 17].

Checking for CT by polymerase chain reaction (PCR) assays performed on endocervical swab provides the highest sensitivity, by early diagnosis which reducing the risk of disease sequel and persistent transmission of infections[18,19]. qPCR has increasingly been used in recent years for detecting genital chlamydia , since it is easier to do ,furnish faster results and being performed in a closed-tube is less prone to contamination[20].

The objectives of this study were to appraise the diagnostic utility of a qPCR assay for detection and prevalence of CT from endocervical swab samples in infertile Iraqi women . In addition, comparison the efficiency of the Real time-PCR with IgG Anti-Chlamydia trachomitis IgG ELISA and Chlamydia Rapid Test .

Material & Methods

Study population: Endocervical swab samples were obtained from 100 women, out them 40 women were attending the Gynaecology Departments of Women Health Center at Al-Elwyia Obstetrics Hospital in Baghdad, Ibn Al balady Maternity & Children's Hospital and the rest 40 women from Kamal Al-Samarrai hospital Fertility Center infertility treatment and In Vitro Fertilization with an age range of 16-40 years (In addition 20 healthy women).

Specimens collection

Endocervical swab samples were collected from 100 samples women (80 samples Infertile women and20 samples control) . For each patients three endocervical swabbing by speculum examination were obtained from all patients by the gynecologist. The first swab was used to clean off the mucus, while the second swab was rotated against the wall of the endocervical canal several times for 10-30 seconds, and withdrawn without touching surfaces and placed in the 300µl phosphate buffered saline (PH 2-4) and extraction direact by DNA extraction kit(AmpliSens® \Russia) according to the manufacturer's directive DNA was stored at -20°C until used and the third swab for Chlamydia Rapid Test directly using chlamydia cassette test (Healgen /USA).[11]

Collecting Blood Samples

From each participating women The serum was obtained by placing the blood sample (3ml) in a clean dry plain plastic tube and allowed to clot at room temperature for 15 minutes before centrifugation. The tubes were centrifuged at 4000 rpm for 6 minutes. Then, serum was collected and kept in freezer (-20 C̊) until used using Anti-Chlamydia trachomitis IgG ELISA kit **(**Demeditec /Germany) Depending on the manufacturer's directive. and Elisa plate reader (Human – Germany) were used. The results were compared with standard amount and density of antibodies and at last Optical Dencity (OD) was calculated by sample OD division OD cut off 10.Negative <9, 9-11 Equivocal positive ≥11.

Diagnosis of *Chlamydia trachomatis* by Real time- PCR

Chlamydia trachomatis detection by the Real-time PCR, the amplified product is detected with the use of fluorescent dyes. These dyes are linked to oligonucleotide probes, which bind specifically to the amplified product during thermocycling. The Real-time monitoring of fluorescence intensities during the real-time PCR allows the detection of accumulating product without re-opening the reaction tubes after the PCR run.

Chlamydia trachomatis-FRT PCR kit (AmpliSens® \Russia) is a qualitative test that contains the Internal Control (Internal Control-FL (IC)). It must be used in the extraction procedure in order to control the extraction process of each individual sample and to identify possible reaction inhibition**.**

PCR Reaction setups: PCR Reaction was performed in a 25 µl total volume for sample also performed for Positive Control (C+), Negative Control (C–) and Internal Control (IC) according to the manufacturer's directive , as described in Table-1.

Table1-PCR reaction setup.

Component	*Reaction volume (µl)*
PCR-mix-1-FL *Chlamydia trachomatis*	5 (µl)
PCR-mix-2-FL	10 µl
Template DNA	10 µl
Total reaction volume	25 (µl)

PCR Amplification profiles

Reaction profiles were optimized Depending on the manufacturer's directive and the program as described in Table-2

Table 2- PCR Amplification setup.

Step	Temperature,°c	Time	Cycles
1	95	15 min	1
2	95	5s	5
	60	20s	
	72	15s	
3	95	5s	40
	60	20s *Fluorescent signal	
	72	15s	

*Fluorescent signal is detected in the channels for the FAM and JOE fluorophores

Data analysis

Analysis of results is performed by the Rotor-Gene Q Software 2.3.1.49 of the real-time PCR instrument used by measuring fluorescence signal accumulation in two channels:

The signal of the Chlamydia trachomatis DNA amplification product is detected in the channel for the FAM fluorophore. The signal of the IC DNA amplification product is detected in the channel for the JOE fluorophore.

Results are interpreted by the crossing (or not-crossing) the fluorescence curve with the threshold line set at the specific level that corresponds to the presence (or absence) of a Ct value of the DNA sample in the corresponding column of the results grid.

Results

During study period, 150 infertile women, in whom male factor infertility, hormonal and ovulatory disorders, uterine fibroid, through initial screening, 80 women were found eligible. The mean age of patients women was 24.82 ± 1.99yr. with 20 women control mean age was 27.52 ± 1.81 yr.DNA was extracted from all by collected endocervical swab for diagnosis by q *PCR* amplification.

Controls for the q *PCR*: The kit containing the target gene was established and used as positive control in each run. A negative control in the form of template-free master mix solution was included in each run of amplification. Internal Control during the DNA extraction procedure directly to the sample.Amplification was deemed efficient for a sample if the conforming Internalcontrol demonstrated the same threshold cycle (Ct) (±1) of positive control. A sample was considered negative if the signal did not increase within 35 cycles. An amplification plot shown in Figure-1.

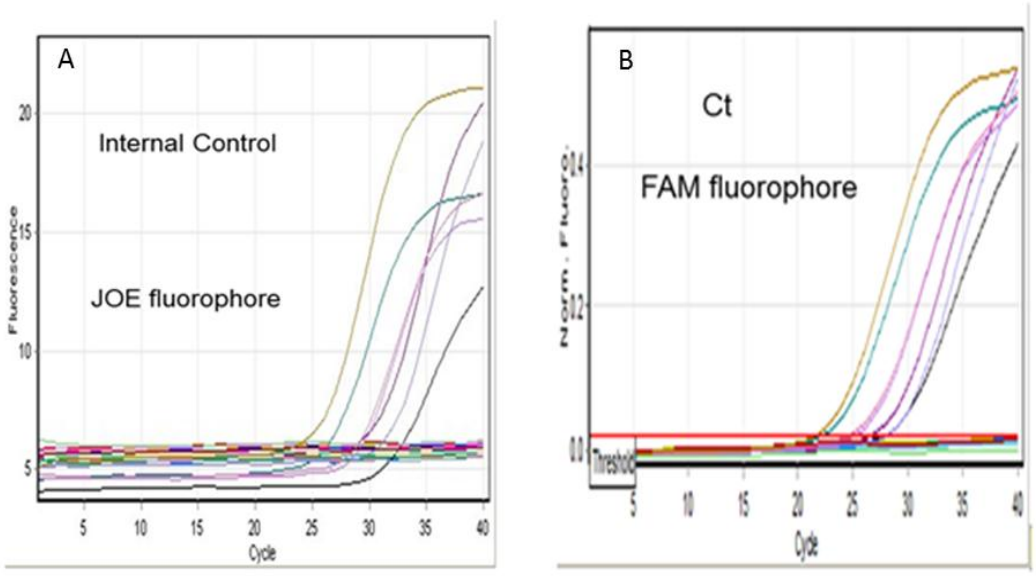

Figure 1- q-PCR amplification plot (fluorence vs Cycle) A: The signal of the Internal Control (IC) is detected in the JOE fluorophore. B: The signal of the Chlamydia trachomatis DNA amplification product is detected the FAM fluorophore.

The result of the qPCR revealed was significant associated with infertility women (OR= 0.733; 13.75%, P= 0.041), among 80 infertili women

Table 3-Diagnosis of *Chlamydia trachomatis in* women infertility using qPCR

Sample	IC	NCA	C+	C–	Ct	%
Patients /80	+	-	+	-	11	13.75
Controls /20	+	-	+	-	0	0.00
P-value O.R.	--	--	--	--	--	0.041 * 0.733

IC= Internal Control, NCA= Negative Control of Amplification, C+= Positive Control of Amplification, C– = Negative Control of Extraction, Ct= *Chlamydia trachomatisin*.
*(P<0.05).

In this study The most frequent cause of infertility was blocked fallopian in 6 from 11 Patients (54.5%) of women, followed by the presence of blocked fallopian tubes and removed another in 5 (45.5%) patients. Which Diagnosis infection of *Chlamydia trachomatis*. However, we found association between detection of *C. trachomatis* in infertile women and the causes of infertility. signs and symptoms could be identified as significantly associated with *C. trachomatis* infection in infertile women. (Table-4) (Figure- 2)

Table 4- signs and symptoms could be identified as significantly associated with *C. trachomatis* infection in infertile women

*No. of patients sample	Symptoms and signs	%
6	blocked fallopian	54.5
5	blocked fallopian tubes and removed another	45.5
Total \ 11		100%

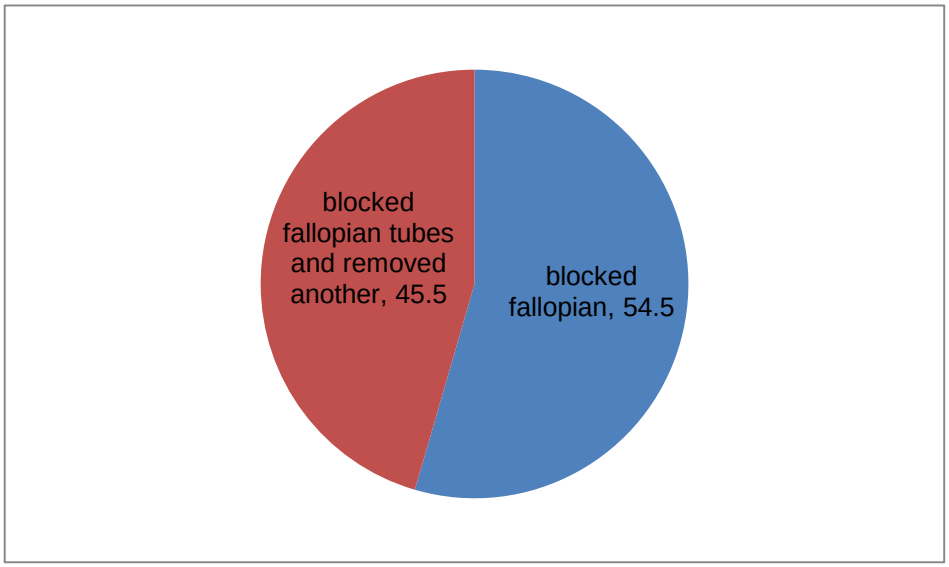

Figure 2- percentage of frequent cause of infertility was blocked fallopian tubes and remove another in 11 Patients.

In this study to test the thoroughness the qPCR assay, all the 80 endocervical swab samples previously tested by qPCR, were also tested by Chlamydia Rapid Test. These 11 samples were found to be positive by qPCR,but no ditected by Chlamydia Rapid Test. While two samples were detected to be positive by ELISA test, Table-5, Figure-3 The result of the Real team-PCR revealed was significant associated with infertility women (OR= 0.733; 13.75%, P= 0.041), among 80 infertili women, while was no significant diagnosis by ELISA (OR= 0.063; 2.50%, p= 0.095) and no significant associated with Rapid Test .

Table 5 -Comparison between different methods for the diagnosis of *Chlamydia trachomatis in* Female infertility

Sample	Real time\ PCR	%	Chlamydia trachomatis	%	Chlamydia Rapid	%	Total
Patients /80	11	13.75	2	2.50	0	0	13
Controls /20	-	0.00	-	0.00	0	0	0
P-value O.R.	---	0.041 * 0.733	---	0.095 NS 0.063	0		

* (P<0.05), NS: Non-significant

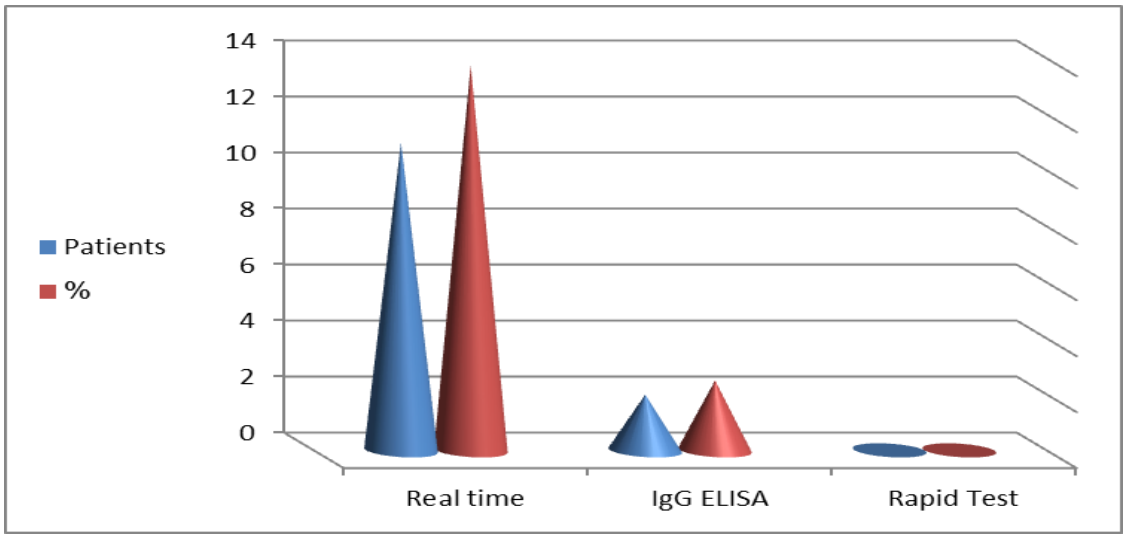

Figure 3- Comparison of different diagnostic methods and percentage of infection.

In the Table-6 compare between difference methods in percentage of results The result of the Real time-PCR revealed was significant associated with infertility women (OR= 0.784; 13.75%, P= 0.0395), among 80 infertili women, while was no significant diagnosis by ELISA and Rapid Test . Molecular genetic techniques are useful for the identification of microorganisms that are difficult diagnostic such as *C. trachomatis* .. RT-q PCR is more sensitive test than cell culture; it has a high sensitivity and specificity when compared to other tests used for *C. trchomatis* diagnosis, such ELISA, Which give some false-positive results. Since CT serology does not yield certain results we think it is useless to perform it on a routine basis CT antibody testing has no predictive value to consider tubal damage. For this reason it should not be used as a screening test.

Table 6- Compare between difference methods in percentage of results

Methods	Total No	+ve	
1	80	11	13.57 A
2	80	2	2.50 B
3	80	0	0.00 B
P-value	---	---	0.0395 *
O.R.	---	---	0.784
* (P<0.05). A. significant B .NO- significant			

Discussion

Chlamydial infection is one of most frequently reported STI WHO [21] are an ultimate public health and socio-economic concern due to the possibility for severe long-term effects in women including infertility.

Tubal tissue damage happen when acute PID progresses into a chronic form of the disease resulting in scarifications and adhesions in the fallopian tubes [22, 23].

so,repeated chlamydial infections are a risk factor for PID. and more importantly is linked to inflammation of fallopian tubes, which can result in scarring and thus chronic pelvic pain, infertility and ectopic pregnancy [24].

This warrants obligatory checking of women attending infertility dispensary [25, 26]. Early and rigorous diagnosis of ct infection requires the use of highly specific laboratory techniques.

The present results showed the highly sensitive as determined by q PCR assay for presence of C. trachomatis among infertile women. Similar detection rates of *C. trachomatis* in infertile women by PCR have been reported in previous studies from developing countries [27, 28]. Ahmed by a specific PCR for the *Chlamydia* plasmid (KL1 and KL2) genes detection revealed that *Chlamydia trachomatis* in 39(26.5%) of endocervical swabs from women Iraqi [29]. El Qouqa *et al.* [27] detected C. trachomatis in 20.2 % of from infertile Palestine women infertility by plasmid-based PCR. Also C. trachomatis infection detection in 200 Indian infertile women found in 13.5 % ,11.5 % .6.5 % by real time-PCR,cryptic plasmid , EIA_Respectively [30] . Similarly in infertile Iranian women was found 13.7 %frequency of C. trachomatis infection by PCR [28]

diversity of Chlamydia spread between countries and studies could be due to several factors such as, study population, rate of infection in the study area, hygiene level and socioeconomic status of the study area, culture of the society whether it is conservative or open , and the DNA target of PCR used. [31].

In this study Low of anti-chlamydia antibodies 2.5 %among infertile women had been observed. Sönmez *et al.* [32]found out that tubo-peritoneal adhesions could not be predicted by the presence of CT in serum. In a study from India by Malik *et al* [33] Chlamydia IgG antibodies were find out in 55 %of infertile womenin. a previous study for the same group of researchers [34] showed a comparatively lower prevalence of C. trachomatis infection in infertile women by culture and antigen detection assays (30 % in secondary infertility patients). Similar were observed disagreement in a study from Rwanda[35] in which the overall positivity of C. trachomatis in infertile women as determined by PCR was 3.6 % compared to an IgG ranging between 17.4 - 18.8 %. A higher frequency of this bacterial infection by serological testing was observed in infertile women compared to the detection rates by culture, antigen detection and PCR assays. This can be imputed to the persistence of antibodies for Chlamydia in serum long after treatment and resolution of C. trachomatis infection. Thus, presence of antibodies cannot differentiate between an acute, chronic or a resolved C. trachomatis infection which is the major limitation of serological testing. Also, cross-reactive antibodies can be stimulated in response to lipopolysaccharides of organisms of the genus Chlamydia [36].

(NAATs) are the most sensitive assays with a specificity and are considered the method of choice for CT detection because to The intracellular life style in this bacterial and the capability to persistent infections Makes it difficult to detect it

Thus. The q PCR had specificity used in the present study was able to detect a very low amount of DNA per reaction, and this maybe explains the higher specificity compared to that of our conventional gene PCR.

Larger study population with multicentric patient recruitment over a lengthy time period can be beneficial for a thorough evaluation of the diagnostic interest of qPCR assays for chlamydia in Iraqi with infertile there are limited data for checking Ct in the infertile Iraqi women. Hence, is not a routine ingredient of infertility practice.

Conclusions

The qPCR has the possibility to support to the enhanced rates of *C. trachomatis* detection in the primary checking for infertility in Iraqi laboratories. comparison with the Rapid Test and ELISA .

References

1. Centers for Disease Control and Prevention (CDC). **2010**. Sexually transmitted diseases treatment guidelines. *Morbidity and Mortality Weekly Report*. Vol. 59, No. Recommendations and Reports - 12
2. WHO. **2011**. Sexually transmitted infections. WorldHealth Organization, Geneva
3. Paavonen J, Eggert-Kruse W. **1999**.*Chlamydia trachomatis*: impact on human reproduction. *Hum Reprod Update*. **5**(5): 433-447.
4. Centers for Disease Control and Prevention (CDC). **2009**. Sexually transmitted disease surveillance,. Atlanta,
5. Alzahrani, A.J., Obeid, O.E., Hassan M.I., and Almulhim, A.A. **2010**. Screening of pregnant women attending the antenatal care clinic of a tertiary hospital in eastern Saudi Arabia for Chlamydia trachomatis and Neisseria gonorrhoeae infections. *Indian J. Sex. Transm. Dis.*, **31**: 81-86.

6. White, R. and Perry, K. **2004**. Chlamydia trachomatis Nucleic Acid Amplification Tests *(NAATs): Review of evaluation literature. MicrobiologicalDiagnosticsAssessment Service*(MIDAS)

7. Baud, D., Regan, L. and Greub .G. **2008**. Emerging role of Chlamydia and Chlamydia-like organisms in adverse pregnancy outcomes. *Curr Opin Infect Dis*, **21**: 70-76.

8. Shaw, J.L., Wills, G.S., Lee, K.F., Horner, P.J., McClure, M.O., Abrahams, V.M., Wheelhouse, N. Jabbour, H.N., Critchley, H.O., Entrican, G. and Horne, A.W. **2011**. Chlamydia trachomatis infection increases fallopian tube PROKR2 via TLR2 and NFkB activation resulting in a microenvironment predisposed to ectopic pregnancy. *Am J Pathol*,**178**: 253-260

9. Low, N., Bender, N., Nartey, L., Shang, A. and Stephenson, J. M. **2009**. Effectiveness of chlamydia screening: systematic review. *Int J Epidemiol*, **38**: 435–448.

10. Low, N. and Hocking, J. **2010**.The POPI trial: what does it mean for chlamydia control now? *Sex Transm Infect*, **86**: 158–159.

11. Black, C.M. **1997**.Current methods of laboratory diagnosis of *Chlamydia trachomatis* infections. *Clin Microbiol Rev*., **10**: 160–184.

12. Watson, E.J.Templeton A, Russell I, Paavonen J, Mardh PA, Stary A, et al. **2002**. The accuracy and efficacy of screening tests for *Chlamydia trachomatis*: a systematic review. *J Med Microbiol*., **51**: 1021–1031

13. Schachter, J. Stamm, W.E. and Quinn, T.C. **1996**. Discrepant analysis and screening for *Chlamydia trachomatis*. *Lancet*., **348**: 1308–1309.

14. Mylonas , I . **2012**. Female genital *Chlamydia trachomatis* infection: where are we heading? *Arch Gynecol Obstet*., **285**: 1271–1285.

15. Akande, V. **2002.** Tubal pelvic damage: prediction and prognosis. *Human Fertil*.5, S15-S20.

16. Mushanski ,L.M. Brandt, K. Coffin, N. Levett, P.N. Horsman, G.B. Rank, E.L. **2012**. Comparison of the BD Viper System with XTR Technology to the Gen-Probe APTIMA COMBO 2 assay using the TIGRIS DTS system for the detection of *Chlamydia trachomatis and Neisseria gonorrhoeae in urine specimens*. *Sex Transm Dis*. **39**: 514–517.

17. Van Der Pol, B. Liesenfeld, O. Williams, J.A. Taylor ,S.N. Lillis, R.A. and Body, B.A. **2012**. Performance of the Cobas CT/NG test compared to the Aptima AC2 and Viper CTQ/GCQ assays for detection of *Chlamydia trachomatis and Neisseria gonorrhoeae*. *J Clin Microbiol.* **50**: 2244–2249.

18. Michel, C.E. Solomon, A.W. Magbanua, J.P. Massae, P.A., Huang, L. and Mosha, J. **2006**. Field evaluation of a rapid point-of-care assay for targeting antibiotic treatment for trachoma control: a comparative study. *Lancet.* **367**: 1585–1590.

19. Semeniuk, H. Zentner, A. Read, R. and Church, D. **2002**. Evaluation of sequential testing strategies using non-amplified and amplified methods for detection of *Chlamydia trachomatis* in endocervical and urine specimens from women. *Diagn Microbiol Infect Dis*. **42**: 43–51.

20. Sachdeva, P. Patel, A.L. Sachdev, D. Ali, M. Mittal, A. and Saluja, M. **2009**. Comparison of an in-house PCR assay, direct fluorescence assay and the Roche Amplicor *Chlamydia trachomatis* kit for detection of C. *trachomatis*. *J Med Microbiol*. **58**: 867–873.

21. WHO .**2012**. Global Incidence and Prevalence of Selected Curable Sexually Transmitted Infections. WorldHealth Organization, Geneva.

22. Mardh, P.A. **2004**. Tubal Factor Infertility, with Special Regard to Chlamydial Salpingitis. *Current Opinion in InfectiousDiseases*, **17**: 49-52

23. Schindlbeck, C., Dziura, D. and Mylonas, I. **2014**. Diagnosis of Pelvic Inflammatory Disease (PID): Intra-Operative

24. Soper, D. E. **2010**. Pelvic inflammatory disease, *Obstet Gynecol*, **116**: 419-428.

25. Oakeshott, P. Kerry, S. Atherton, H. Aghaizu , A. Hay, S. and Taylor-Robinson, D. **2008**. Community-based trial of screening for *Chlamydia trachomatis* to prevent pelvic inflammatory disease: the POPI (prevention of pelvic infection) trial. Trials, **9**: 73.

26. Oakeshott, P. Kerry, S. Aghaizu, A. Atherton, H. Hay, S. and Taylor-Robinson, D. **2010**.Randomised controlled trial of screening for *Chlamydia trachomatis* to prevent pelvic inflammatory disease: the POPI (prevention of pelvic infection) trial. BMJ., **340**:c1642

27. El Qouqa, I.A. Shubair, M.E. Al Jarousha, A.M., and Sharif, F.A. **2009**. Prevalence of *Chlamydia trachomatis* among women attending gynecology and infertility clinics in Gaza, Palestine. *Int J Infect Dis*. **13**: 334–41.

28. Hossein Rashidi, B. Chamani Tabriz, L. Haghollahi, F. Ramezangadeh, F. Shariat, M. Rahimi and Foroushani, A. **2009**. Prevalence of *Chlamydia trachomatis* infection in fertile and infertile women: a molecular and serological study. *J Reprod Infertil*., **10**: 32–41

29. Ahmed, S.T. **2012**. Detection of *Chlamydia trachomatis* Using polymerase chain reaction (PCR). *Al- Mustansiriyah J. Sci*. **23**(6).

30. Dhawan,B.Rawre,J.Arnab,G.Malhotra,N.Ahmed,M.M.Sreenivas,V. and Chaudhry, R. **2014** . Diagnostic efficacy of a real time-PCR assay for *Chlamydia trachomatis* infection in infertile women in north India. *Indian J Med Res*. **140**(2): 252–261

31. Nassar, F.A. Abu-Elamreen , F.H. Shubair,M.E. and Sharif, F.A. **2008**. Detection of *Chlamydia trachomatis* and *Mycoplasma hominis*, *genitalium* and *Ureaplasma urealyticum* by Polymerase Chain Reaction in patients with sterile pyuria *Advances in Medical Sciences*, **53**(1): 80-86 ·

32. Sönmez, S. Sönmez, E. Yasar, L. Aydin, F. Coskun, A. and Süt,N. **2008** . Can screening *Chlamydia trachomatis* by serological tests predict tubal damagein infertile patients. New microbiologics, **31:** 75-79

33. Malik, A. Jain, S. Rizvi, M. Shukla ,I. and Hakim, S. **2009**.*Chlamydia trachomatis* infection in women with secondary infertility. *Fertil Steril*. **91**: 91–95.

34. Malik, A. Jain, S. Hakim, S. Shukla, I. and Rizvi , M. **2006**. *Chlamydia trachomatis* infection & female infertility. *Indian J Med Res.* **123**: 730-734

35. Muvunyi, C.M. Dhont, N. Verhelst, R. Temmerman, M. Claeys, G. and Padalko, E. **2011**. *Chlamydia trachomatis* infection in fertile and subfertile women in Rwanda: prevalence and diagnostic significance of IgG and IgA antibodies testing. *Hum Reprod*. **26**: 3319–3326.

36. Mårdh, P.A. **2004**. Tubal factor infertility, with special regard to chlamydial salpingitis. *Curr Opin Infect Dis*. **17**: 49–52.