Serological and Molecular Diagnosis of Rubella virus and Cytomegalovirus in aborted Patients in Al-Najaf province

التشخيص المصلي والجزيئي المقارن بعض أنواع العدوى الفيروسية (فيروس الحصبة الألمانية والفيروس المضخم للخلايا) في النساء المجهضات في محافظة النجف

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الخلاصة :
خلفية البحث: العدوى الفيروسية مثل الحصبة الألمانية والفيروس المضخم للخلايا يخشى من مخاطرها أثناء الحمل. الحصبة الألمانية والفيروس المضخم للخلايا هي إلى حد ما من القضايا الرئيسية التي يتم تشخيصها مختبريا أثناء الحمل ، وترشيح استخدامها مع المعلومات السريرية، أيضا للوقاية، والعلاج، والتشخيص قبل الولادة فضلا عن إمكانية التقليل من مشاكل الإصابة بها أثناء الحمل .
أهداف الدراسة: هدفت الدراسة إلى تقييم ELISA ينتج عن الحصبة الألمانية والفيروس المضخم للخلايا (IgM (CMV و مفتش ومقارنة نتائج ELISA مع RT-PCR للتحكيم النتائج المتنافرة.
المنهجية : أجريت هذه الدراسة في محافظه النجف الاشرف / العراق ، وتضمنت جمع عينات من دم النساء الحوامل اللواتي يعانين من الإجهاض أول مرة أو متكررة وكان عدد الحالات ٥٧ حالة. أعمارهن يتراوح بين ١٥سنة إلى٤٠ سنة واللواتي يراجعن مستشفى الزهراء التعليمي والمختبر المركزي وبعض العيادات الخاصة للمدة من أيار 2012 إلى آيار2013 آذ تم أخذ عينات الدم من النساء المشمولات بالدراسة وأجراء الفحص المصلي الأليزا (فحص الامتصاص المناعي المرتبط بالأنزيم) للأضداد المناعية (IgM,IgG) وبعدها الفحص الجزئي (PCR).
النتائج : أظهرت نتائج 12 مريضا (21.1٪) كانت إيجابية المصل للفيروس الحصبة الألمانية الكلوبولين المناعي، و 46 (80.7٪) كانت إيجابية المصل للفيروس الحصبة الألمانية مفتش، في حين كانت 24 فقط (42.1٪) مصليا لكل مفتش و الكلوبولين المناعي في نفس الوقت. في هذه الدراسة كانت 2 المرضى (7.69٪) مصليا لكل من الحصبة الألمانية فحص الأمتصاص المناعي المرتبط بالأنزيم والفيروس المضخم للخلايا معا و 12 (30٪) كانت إيجابية المصل للكل من الحصبة الألمانية والفيروس المضخم للخلايا فحص الأمتصاص المناعي المرتبط بالأنزيم وفيما يتعلق للعدوى الحصبة الألمانية، أظهرت النتائج أن 12 (21.1٪) كانت إيجابية المصل للحصبة الألمانية IgM و 26 (45.6٪) كانت إيجابية فحص الأمتصاص المناعي المرتبط بالأنزيم الحصبة الألمانية، في حين أن 6 فقط (17.5٪) كانت إيجابية المصل للكل من الغلوبولين المناعي ومفتش. أظهرت النتائج في الوقت الحقيقي PCR 6 فقط (10.5٪) كانت إيجابية لفيروس الحصبة الألمانية و 3 (5.26٪) كانت إيجابية للفيروس المضخم للخلايا .
الاستنتاج : وينبغي أن يتم التشخيص الدقيق للعدوى الحصبة الألمانية والفيروس المضخم للخلايا بواسطة طريقة الجزيئية وليس على أساس اختبار ELISAالتي ينبغي أن تستخدم اختبار الفحص.
التوصيات: ننصح الطبيبة النسائية بأن توصي المرأة الحامل بأجراء الفحوصات الجزيئي إضافة إلى الفحوصات المصلية أثناء الشك في الإصابة المشتركة بعدة مسببات مرضية .

ABSTRACT
Background: Viral infections such as Rubella and Cytomegalovirus are feared risk during pregnancy. Rubella and Cytomegalovirus virus are fairly the respective major diagnostic issues in pregnancy, the laboratory diagnosis, and its rational use in combination with clinical information are presented , also the value of passive prophylaxis, therapy, and prenatal diagnosis as well as the possible management for diminishing the infection problems in pregnancy .
Aims of study : The study was aimed to evaluate the ELISA result for rubella and cytomegalovirus (CMV) IgM and IgG and compare the results of the ELISA with RT-PCR to arbitrate discordant results**.**
Methodology :The present study was carried out on 57 pregnant women, all of them had bad obstetric history (BOH) . Patients were between 15-40 years old , and they were attending Al- Zahra Teaching Hospital during the period from May 2012 till May 2013. Serological evaluation for Rubella Cytomegalovirus infections were carried out by using IgM and IgG Enzyme Linked Immunosorbent Assay (ELISA) method and molecular technique (Real Time - PCR) .
Results : The results showed 12 patients (21.1%) were Seropositive for Rubella virus IgM , and 46 (80.7%) were Seropositive for Rubella virus IgG , while only 24 (42.1%) were Seropositive for both IgG and IgM at

same time . In this study 2 patients (7.69 %) were Seropositive for both Rubella IgM and Cytomegalovirus together and 12 (30 %) were Seropositive for both Rubella IgG and Cytomegalovirus IgG .
Regarding to Rubella infection ,the results showed that 12 (21.1%) were seropositive for Rubella IgM and 26 (45.6 %) were seropositive for Rubella IgG , while only 6(17.5%) were seropositive for both IgM and IgG . The Real Time PCR results showed only 6(10.5%) were positive for Rubella virus and 3(5.26 %) were positive for Cytomegalovirus .
Conclusion : The accurate diagnosis of Rubella and Cytomegalovirus infection should be done by molecular method and not based on ELISA test which should be used as screening test .
Recommendations :Advice the gynecologist to take care before give management for pregnant women that present with more than one infection at same time unless confirm the diagnosis by more than one technique like RT-PCR .

INTRODUCTION

Maternal infections play a critical role in pregnancy wastage and their occurrence in patients with bad obstetric history (BOH) is a significant factor [(1)] . The rate of spontaneous abortion from fetal infection by infectious agents like (, Rubella virus and Cytomegalovirus) and others is 10 -15 % [(2)] . Rubella is a common childhood infection usually with minimal systemic upset although transient arthropathy may occur in adults. Serious . Acquired (not congenital) Rubella is transmitted via airborne droplet emission from the upper respiratory tract of active cases (can be passed along by the breath of people sick from Rubella [(3)] . Rubella virus specific IgM antibodies are present in people recently infected by Rubella virus but these antibodies can persist for over a year and a positive test result needs to be interpreted with caution [(4)] . Maternal Cytomegalovirus is commonest viral infection in prenatal period and is the leading cause of congenital CMV infection [(5)]. The incidence of congenital CMV ranges from 0.5-3.0% in all live births [(6)] .

This review focuses on the application of real-time PCR in the clinical microbiology laboratory [(7)]**.** Conventional methods for the detection of antibodies to Rubella and a include immunofluorescence assay (IFA) , enzyme immunoassay (EIA), and enzyme-linked fluorescent assay (ELFA) , these techniques have been used for years in both diagnostic and screening protocols for (Toxoplasma gondii ,Rubella CMV and Herpes virus) infection and have demonstrated reliable performance [(8)] **.**

Recently, Real Time- PCR technology emerged as a novel approach to assess the molecular response to various infectious diseases [(9)] **.** This technology is similar to traditional PCR but allows for the simultaneous detection and identification of multiple samples [(10)] **.**

MATERIALS AND METHODS:

Study design and Patients : Case control study is a 57 blood samples from pregnant women with previous unfavorable fetal outcome in terms of two or more consecutive spontaneous abortion and other bad obstetrical history (intrauterine fetal death , retardation ,still births , early neonatal death and congenital anomalies) ,their ages were between 14-45 years. Timing of abortion were between first trimester and second trimester .Patients were attending Al-Najaf province hospital from May 2012 to May 2013 .
Collection of Samples : Five milliliters (ml) of venous blood were drawn from each patient by venipuncture 5ml disposable syringe. Blood was divided into 2 groups .
1. 3ml of blood were collected in sterile serum tube and left for serum collection, which was stored at-20 C˚ till used for ELISA test (Cytomegalovirus IgG and IgM , Rubella IgG and IgM).

2. 2 ml of blood were collected in EDTA tube and extraction of DNA and RNA from blood was done in each sample in the Eppendorf tubes(1-2) ml and were done immediately for RT- PCR test .

SEROLOGICAL DIAGNOSIS

A-Enzyme Immunoassay for Qualitative and Quantities Determination of Serum Antibody IgM and IgG . Complete ELISA Kits were used . This kit was supplied by (Human company / German) which included :

1. Enzyme Immunoassay for Qualitative and quantities detection of Cytomegalovirus in human was measured by ELISA technique using ELISA Kit IgM .
2. Enzyme Immunoassay for Qualitative and quantities detection of Cytomegalovirus in human was measured by ELISA technique using ELISA Kit IgG.
3. Enzyme Immunoassay for Qualitative detection of Rubella Virus in human Serum was measured by ELISA technique using ELISA Kit IgM:
4. Enzyme Immunoassay for Qualitative detection of Rubella Virus in human Serum was measured by ELISA technique using ELISA Kit IgG.

MOLECULAR DIAGNOSIS

Extraction and Estimation .

1. DNA Extraction : DNA extraction kit was supplied by Geneaid Company (USA), DNA extraction was performed according to the manufacturer's instructions.

2. RNA Extraction: RNA extraction kit was supplied by Geneaid Company(USA) .

Real Time PCR

1-Real – Time PCR test for qualitative detection of Cytomegalovirus in human was measured by RT-PCR Kit (Sacace Biotechnologies –company –Italy) .Programming the Real-Time PCR Thermo cycler conditions (Amplification): Real-Time PCR Thermo cycler conditions were set according to kit instructions .

2-Real – Time PCR test for qualitative detection of Rubella Virus in human was measured by RT-PCR Kit (Sacace Biotechnologies –company –Italy) . Real-Time PCR Thermo cycler conditions (Amplification) : Real-Time PCR Thermo cycler conditions were set according to kit instructions .

RESULTS:

1. Incidence of Rubella Virus

Table (1): Frequency of women with positive Rubella antibodies detected by ELISA in the study sample (n=57) .

Rubella antibodies	Sample N=57	%
IgM	12	21.1
IgG	28	49.1
IgM and IgG	10	17.5

The results showed that 12/57 patients (21.1**%**) were Seropositive for Rubella virus IgM and 28/57(49.1%) were Seropositive for Rubella virus IgG , while only 10 (17.5 %) were Seropositive for both IgM and IgG Rubella virus **(Table 1)** .

2.Incidence of CMV

Table(2): Frequency of women with positive Cytomegalovirus (CMV) antibodies detected by ELISA in the study sample (n=57).

CMV antibodies	Sample N=57	%
IgM	26	45.6
IgG	40	70.2
IgM and IgG	23	40.4

The results showed that 26 patients (45.6**%**) were Seropositive for CMV IgM and 40 ; 57(70.2%) were Seropositive for CMV IgG , while only 23 (40.4 %) were Seropositive for both IgM and IgG **(Table 2) .**

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Table (3): Proportion of Patients (N=57) with positive IgM, IgG and IgM+IgG antibodies in comparison with RT-PCR positivity .

Pathogen type N= 57	IgM No.(%)	IgG No.(%)	IgM + IgG No.(%)	PCR No.(%)
Cytomegalovirus	26 (45.61)	40 (70.18)	24 (42.10)	3 (5.26)
Rubella	12 (21.05)	28 (49.12)	10 (17.54)	6 (10.53)

$X^2 = 5.375$ df= 6 P= 0.496

The Real Time PCR result was only 10/57 (17,45%) . It was positive for Cytomegalovirus and 6/57 (10.53%) for Rubella virus **(Table 3)** .

FREQUENCY OF MIXED INFECTION OF CMV AND RUBELLA BY ELISA

Table (4) : Frequency of Mix infection of CMV and Rubella by ELISA.

Parameters	Positive Cytomegalovirus Patients N= 57	CMV + Rubella positive No.(%)	Rubella positive No.(%)
IgM	26	2(7.69 %)	12(46.15 %)
IgG	40	12(30%)	28(60.86 %)
IgM and IgG	23	13(56.52) %	10 (41.66 %)

X^2 = 10.23 df=2 P= 0.0167

The results of a comparative investigation of commercial tests for the measurement of IgM and IgG antibody on a series of 57 Seropositive for both *CMV and Rubella virus* IgM 2(7.69 %), 12(30%) ,respectively **)** , IgG 12(30%) , 28(60.86 %)respectively **,** and IgM with IgG 13(56.52) % , 10 (41.66 %), respectively **(Table 4).**

CROSS TABULATION

CMV IgG and Rubella virus IgG Cross tabulation.

Table (5) : Seropositive IgG for both Cytomegalovirus and Rubella virus.

Test		CMV IgG		
		Negative	Positive	Total
Rubella IgG	Negative	10	18	28
	Positive	21	8	29
Total		31	26	57

X^2 = 0.704 df=1 P= 0.293

While in table(5) , the coexistence of CMV and Rubella virus IgG showed increase result (21/57) because of previous infection may play role in the cross reaction **(Table 5**) .

CMV PCR and Rubella virus PCR cross tabulation.

Table(6) : PCR Result Cytomegalovirus for Rubella virus

Test		CMVPCR		Total
		Negative	Positive	
Rub.PCR	Negative	48	3	51
	Positive	6	0	6
Total		54	3	57

X^2 = 0.373 df=1 P= 0.712.

The results were very interesting which interpretation the real condition of patients whose suspected table infected by TORCH infection because the PCR result of appearance only in one disease and no role of cross reaction that was why all the result was positive Rubella virus PCR which consists of about 6/57 and for Cytomegalovirus 3/57 **(Table 6**) .

DISCUSSION

The present study was conducted on patients suffers from abortion and bad obstetric history. Women were selected to detect serum IgM and IgG specific for Rubella Virus and Cytomegalovirus. In this study , the seropositive of the aborted women cases for Rubella IgG (49.12%) which appear to be a low in compare with other studies [11], that indicate either a previous vaccination with Rubella vaccine during teen age group and before pregnancy or due to a previous infection with Rubella [(12)] . In present study the percentage of CMV IgM among aborted women and BOH was 26 (45.6%) **.** Primary CMV infection in pregnancy has a higher incidence of symptomatic congentital infection and fetal loss. This infection, being asympotomatic in adults , is difficult to diagnosis clinically [(13)]**.** Several studies have been reported between 84.5 – 95 % prevalence of CMV IgG among aborted women in Turkey [(14)] , and this result was higher than our result which was 70.2 % so CMV infection in pregnancy has a higher incidence of fetal loss [(15)] **.**

About 1/3 of seropositive cases by ELISA were confirmed by PCR , this means either the seropositive results by ELISA were not specific or less significant because the probability of false positive that may be as a result of infection with other microorganism [(16)] **.** The results indicates that the percentage of Rubella IgG in Al-Najaf was 49.12%which was less than the result recorded by **Lenochove** in Turkey [(17)] **.**

Viral infections such as Rubella virus and Cytomegalovirus have a significant risk among infants at birth and the risk of late manifestation is still unclear, whereas such data are fairly well known for Rubella [10)] **.** The IgM appears during the first week , which may be inadequate to expect the presence of the pathogens by RT-PCR [(18)] **.** This study revealed that out of 57 patients only 12 patients were seropositive IgM and IgG for Rubella and only (50%) of there seropositive results were positive by PCR techineque . This result nearly agreed with the result of **Nolan** [19)] whose showed that out of 17 positive cases of Rubella by ELISA only 6 were positive by PCR. This study reveals that the percentage of seropositive IgG for Rubella and Cytomegalovirus were 49.12 % and 70.2% respectively. PCR technique will find the percentage was decline to 10.53 % in Rubella virus , which give the real incidence of infection of this microorganism and reflect the false of tests by routine ELISA investigation [(20)] .

CONCLUSIONS:

1- The accurate diagnosis of Rubella virus and Cytomegalovirus infections should be done by molecular method and not based on ELISA test which should be used as screening test.
2- Low prevalence of Rubella IgG in Iraq is mainly due to default of vaccination (MMR) program that concentration on secondary school girls only .
3- There is no mixed RT-PCR results of Rubella virus and Cytomegalovirus in contrary to ELISA test which reflect the specifity and sensitivity of RT- PCR and in same time reflect the cross reaction and false result of ELISA technique , so that there is no role of treatment for mixed Rubella virus and Cytomegalovirus infections in pregnant women.

RECOMMENDATIONS :

1- Advice the gynecologist to take care before give management for pregnant women that present with more than one infection at same time unless confirm the diagnosis by more than one technique like RT-PCR .
2- Rubella virus IgG should be included in the listed tests for pre married girls in order to give vaccine to negative one .
3- Pre pregnant screening for antibodies to Rubella virus and Cytomegalovirus are recommended to be done as a routine practice that prevent fetus from infection by Rubella virus and Cytomegalovirus in uterus.
4- The type of sample detection should be according to shedding of microorganisms that reflect to serological test .

REFERENCES

1. **Alsamarai M, A; Aljumaili ,M and Zainab Khalil (2013).** Seroepidemiology of Toxoplasma, Rubella, Cytomegalovirus and Herpes simplex virus -2 in women with bad obstetric history. part i: toxoplasma and rubella infections. Nasza Dermatologia Online ., 14 (Issue 4) : 522-535.
2. **Kumari N ;Morris N,and Dutta R(2011)** . Is screening of TORCH worthwhile in women with bad obstetric history ?An observation from eastern Nepal . Journal of Health , Population ,and Nutrition ; 29(1):77-80.
3. **Roussos, N. and Falagas, M. E. (2009)** . Toxoplasmosis snapshots: Global status of *Toxoplasma gondii* seroprevalence and implications for pregnancy and congenital toxoplasmosis. Int. J. Parasitol. Ed. 39, p. 1385-1394
4. **Best, J.M., Cooray, S., and Banatvala J.E (2005)** . Rubella in Topley and Wilson's Microbiology and Microbial Infections. Virology , Chapter 45(2) : 960- 992 .
5. **Hamdan ZH; Ismail EA ; Nasser MN and Ishag (2011).** Seroprevalence of cytomegalovirus and rubella among pregnant women in western Sudan .Virology Journal; 8:217-220.
6. **Mifune K., Desmyter J., and Williams W. E (2010)** . Effect of exogenous interferon in rubella virus production in carrier cultures of cells defective in interferon production. MMWR Morb Mortal Wkly Rep. 59 (40) : 1307-1310 .
7. **Holland GN; Crespi CM, ten Dam-van Loon N, (2008) .** Analysis of recurrence patterns associated with toxoplasmic retinochoroiditis . 6 : 145 .
8. **Berth, M., and E. Bosmans(2010)** . Comparison of three automated immunoassay methods for the determination of Cytomegalovirus specific immunoglobulin M. Clin. Vaccine Immunol., 17:559-563

9. **Binnicker MJ , Jespersen DJ and Harring JA (2010)**.Multiplex detection of IgM and IgG class antibodies to *Toxoplasma gondii* ,Rubella virus ,and cytomegalovirus using a novel multiplex flow immunoassay .Clinical and Vaccine Immunology.17(11):1734-38.
10. **Yasodhara P, Ramalakshmi BA, Lakshmi V, Krishna TP (2004).**Socioecomic status and prevalence of toxoplasmosis during pregnancy**.** Indian J. Med. Microbiol. , 22(4):241-243.
11. **Vijayalakshmi P ; Anuradha R; Prakash K; Narendran K;Ravindran M and Prajna L,(2004)** . Rubella serosurveys at three Aravind eye hospitals in Tamil Nadu ,India . Bull World Health Organ ; 82: 259-264.
12. **Balsam J; Ossandon M, and Bruck HA (2013)** . Low-cost technologies for medical diagnostics in low-resource settings. Expert Opin Med. Diagn., 7:243–255 .
13. **Mimi, O.(2009).** Is there any role of Toxoplasma gondii in the etiology of obsessive-compulsive disorder? Psychiatry Res. ,177 : 263-265.
14. **Surpam RB ,Kamlakar UP ,Khadse RK ,Qazi MS and Jalgaonkar SV (2009).**Serological study for TORCH infection in women with bad obstetric history .J Obstet Gynecol India ; 56:41-43.
15. **Gulden ST ; Devrim D, and Eray C (2009)** . Seroprevalence of *Toxoplasma gondii* ,Rubella and Cytomegalovirus among pregnant women in western region of Turky ,Clin Invest Med ; 32 (1) :43-47.
16. **Lenochova, P., Hodny, Z. and Vondrova, M. (2011) .** Fatal attraction phenomenon in humans: cat odour attractiveness increased for toxoplasma-infected men while decreased for infected women. , 5 : 1389 .
17. **Balsam J,Ossandon M and Bruck HA(2013).** Low –cost technologies for medical diagnostics in low-resource setting .Expert Opin Med .Diagn.,7:243-255.
18. **Aaiz ,N.N.(2010) .** Genotyping Analysis to determine the main lineages types of *Toxoplasma gondii* with study of autoantibodies production by Toxoplasma , Ph.D thesis.College of Medicine Kufa university .
19. **Nolan T; Hands RE, and Bustin SA(2006) .** "Quantification of mRNA using real-time RT-PCR" ., 1 : 236 .
20. **Gulden ST ; Devrim D, and Eray C (2009)** . Seroprevalence of *Toxoplasma gondii* ,Rubella and Cytomegalovirus among pregnant women in western region of Turky ,Clin Invest Med ; 32 (1) :43-47.