Molecular Diagnosis of TORCH Infection of Pregnant Women in Iraq

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Abstract
Background: TORCH complex *(Toxoplasma gondii, others, Rubella virus, Cytomegalovirus,* and *Herpes simplex virus)* infections in pregnant women may attribute to bad obstetric outcomes.
Objective: To investigate the role of TORCH as an etiology of bad obstetric outcome using a molecular technique.
Materials and Methods: The samples were collected from women with Bad Obstetric History attending clinics in Mosul and Baghdad hospitals in Iraq over a period from (15/4/2013) to (1/6/2014) and from (1/5/2017) to (1/11/2017). The women included in the study were with mean age of (26±6.1) years and a range of 22 to 39 years. Blood samples, throat and cervical swabs were collected from 300 women ELISA positive seroprevalence of TORCH for PCR testing.
Results: DNA and RNA were extracted and Real-Time PCR indicates negative results for *T.gondii*, Rubella and HSV I&II, but were CMV positive in only four samples represented (1.3%) from total 300 positive samples in ELISA tests.
Conclusion: ELISA test is considered as a preliminary and screening test for TORCH infections. Real Time PCR is an essential tool in the research laboratory. It has engendered wider acceptance than the conventional PCR due to its improved rapidity, sensitivity, reproducibility and the reduced risk of carry-over contamination.

Keywords: TORCH, ELISA for TORCH, Real-Time PCR, Bad obstetric history.

Introduction

Pregnancy is considered a unique normal physiological episode in a woman's life. However, in some cases, many twists and turns occur which alter the good outcome of pregnancy into a fatality. For those women who have had a previous unsuccessful outcome, pregnancy may bring a lot of inevitable negative emotions [1].

Bad Obstetric History (BOH) implies previous unfavorable fetal outcome in terms of two or more consecutive spontaneous abortions, history of intrauterine fetal death, intrauterine growth retardation, stillbirth, early neonatal death and/or congenital anomalies. BOH etiology may be genetic, hormonal, abnormal maternal, immune response, and maternal infection. Primary infections caused by TORCH agents form the major cause of BOH [2, 3].Viral infections in pregnancy are major causes of maternal and fetal morbidity and mortality. Infections may affect the neonate transplacental, prenatal (from vaginal secretions or blood), or postnatal (from breast milk or other sources) [3, 4]. The clinical manifestations of neonatal infections vary depending on the viral agent and gestational age at exposure. The risk of infection is usually inversely related to gestational age at acquisition, some resulting in a congenital malformation syndrome [4].

Routine viral diagnostics includes techniques for both direct and indirect detection of viruses. Detection of viral nucleic acids is also referred to as Nucleic Acid Testing (NAT) is used. Indirect detection of viruses is performed by serological studies. Moreover, viral morphological structures can be investigated by Transmission Electron Microscopy (TEM) [5].

PCR techniques have advanced further with development of quantitative real – time PCR (qRT-PCR). This technique is more sensitive than conventional PCR and enables the number of viral RNA or DNA copies in sample to be measured. The most widely used variants of conventional amplification are real-time PCR (quantitative PCR) and reverse transcription-PCR (RT-PCR). Both are nowadays becoming benchmarks in assessing the viral load, and the first method that quantifies DNA throughout the reactions is real time [6]**.** In general real – time PCR assay for the detection and quantification of viral DNA or RNA are sensitive specific and reproducible and significantly reduce the time necessary to report results that may have impact on the care and management of patients [7].

Materials and Methods

The study included 300 samples which were previously proved positive for TORCH infection by ELISA test out of 1500 blood samples collected from women with BOH. The samples collected from Mosul and Baghdad hospitals within a period of two years. Three hospitals in Mosul City: Al-Salam Teaching Hospital, Al Khansaa Teaching Hospital for Maternity & Children and Al-Batool Hospital for Gynecology & Obstetrics were selected. In addition, three hospitals in Baghdad City: Al-Alwaiya Maternity Teaching Hospital, Al-Kademia Hospital for Children, Al -Yarmuk Teaching Hospital was selected. Information case reports are used for each case to get the information.

Venous blood is drawn from each woman with BOH who was positive with ELISA and was placed in EDTA blood tubes for Molecular tests. Throat and cervical swabs were taken and placed in Viral Transport Media (VTM) and used for molecular tests. QIAamp

® Viral RNA Min Kit and QIAamp® DNA Blood Min Kit from QIAGEN (Germany) were used for isolated of viral RNA and DNA according to the company instruction.

PCR procedure

Real-TM kits from Sacace (Italy) were used for *T.gondii*, *Rubella virus*, CMV and HSV I & II. Amplification program used was the real-time instrument according to the manual provided by the manufacturer and used the Applied Biosystem Fast Real time (7500) PCR system.

Results and Discussions

Overall seropositivity for IgM antibodies against *T. gondii*, Rubella, CMV, and HSV for either a single organism or in combination are shown in Table 1... Seropositivity for *T. gondii* is found to be 14.7% (n=44), CMV 48.7% (n=146), Rubella 12.3% (n=37) and 59% (n=177) are seropositive for HSV-II infections. The mean of *T. gondii* IgM seroprevalence that was extracted from 20 studies performed on women with bad obstetric history was 25.8% and a range of 0.97% to 58% [8]. Thus the present study IgM *T. gondii* seroprevalence was within the range of the previous studies performed in Iraqi community. While the CMV IgM seroprevalence was 20.44% of 14 studies reported previously in Iraqi community and a range of 3.8% - 60.2% [9].

Acute rubella infection (IgM positivity) was demonstrated in 22.3% of women with BOH (mean of 9 studies) [8], while the present study show lower prevalence, however, the present study finding confirmed the previous studies results that clarify a health care problem in our community.

HSV IgM seroprevalence as a mean of 6 studies performed in Iraq was 21.8%, which is lower than this study prevalence [9]. The range of HSV IgM seroprevalence in the above 6 studies was 3.1% to 73.9% and thus the present study prevalence was within the range of these studies. The high seroprevalence in some study cohort may not represent an acute infection in all cases with positive HSV IgM, but may most of them are positive due to latent infection reactivation. If acute high prevalence rate of the present study and one another Iraqi study that reported high prevalence rate, the overall rate of the other 5 studies was 11.6% which is more reasonable.

The present study indicated that the frequency of ***T.gondii***, CMV, rubella and HSV was more predominant at the age group of 20-29 years, which was consistent with previous studies reported for Iraq [10-17]. These findings suggest that acute herpetic infections were predominant in the sexually active age group.

The seropositivity for IgG antibodies against *T. gondii*, Rubella, CMV, and HSV for either a single organism or in combination are shown in Table 2. Seropositivity for *T. gondii* is 25% (n=75), CMV 63% (n=189), Rubella 20% (n=60) and 77.3% (n=232) are seropositive for HSV-II infections.

Globally, the Toxoplasma IgG seroprevalence was 0.48% - 55.2%, while IgM seroprevalence was 6.9% - 42.5%. In Iraq, the remote infection was with range of 18.5% to 81.5% (mean value of 23 studies was 37.3%) [8]. these figures variability indicated that the low detection rate of Toxoplasma by PCR in the present study may be influenced by the natural history and timing at which the samples were collected.

Previous studies in Iraq reported that CMV IgG seroprevalence in women with BOH was with a range of 8.02% - 96.6% and a mean value of 12 studies was 60.23% [9]. Thus the present study result was consistent with the previous studies performed in Iraq.

Table (1): Prevalence of mixed etiology of TORCH infections IgM according to age determined by ELISA.

Age in year	Number	IgM			
		T.gondii	CMV	Rubella	Herpes
Under 20	29	6	11	5	19
20 – 29	189	25	86	27	112
30 – 39	69	11	47	5	35
Above 39	13	2	2	0	11
Total number	300	44	146	37	177
Total %		14.7	48.7	12.3	59

Rubella non immune state as this study show is too high (80%) and this indicated that the majority of women in child bearing age was vulnerable to get rubella infection during their next pregnancy. This finding illustrates a public health problem that must be taken seriously and healthcare delivery must be aware of such problem. In a previous 9 studies that was performed in Iraqi community show that 51.3% of the Iraqi women were with non-immune state [8]. The present study finding and the previous studies in Iraqi community indicated a disruption in Iraqi National Vaccination Programs.

HSV IgG seroprevalence was 29.9% in Iraqi general population [13], with no significant differences between women with bad obstetric outcomes and those with normal pregnancy [13, 17]. Transmission of HSV-I is usually via direct contact with lesion while HSV-II is classically transmitted sexually [18], however, the trend of HSV-I epidemiology is different in primary episode from recurrent infection [19].

In 2013, a study conducted in Iraq, which included 2566 women with bad obstetric outcome shows that remote infection was 19.68%, 85.19%, and 88.58% for *T.gondii*, rubella and CMV respectively. While the acute infection was 1.05%, 9.28%, 12.9% and 3.3% for *T.gondii*, rubella, CMV and HSV-II respectively [20].

Overall Real time PCR for *T. gondii*, Rubella, CMV, and HSV found to be zero % for *T. gondii*, Rubella, and HSV, while CMV was positive in 1.3 % (n=4), Table 3 .

The samples of *T.gondii* are negative in RT-qPCR although they are positive in all serological methods. Al-Nasrawi *et al* [21] in 2014 elucidated that a positive PCR result was not always accompanied by positive serology indicating local synthesis of antibodies. Molecular detection of *T.gondii* by Real-Time PCR shows specific and highly sensitive assay. The study results are consistent with Bell and Ranford [22][8], who developed a rapid, sensitive, and quantitative real-time PCR for the detection of *T. gondii* from different clinical specimens. Therefore, this technique has the advantage of being

one of the rapid molecular tools for the diagnosis of Toxoplasmosis in a clinical laboratory, rather than a serological test [21, 22]. The disparity in the results of PCR from that of ELISA as this study shows may be explained on the basis that ELISA detected IgG antibodies which mainly indicated a remote rather than acute infection.

Table (2): Prevalence of Mixed Etiology of TORCH Infection IgG According to Age (Years) by ELISA.

AGE / Year	No.	ELISA IgG			
		T.gondii	CMV	Rubella	Herpes
Under 20	29	9	14	8	23
20 – 29	189	35	107	37	143
30 – 39	69	25	66	15	53
Above 39	13	6	2	Zero	13
Total number	300	75	189	60	232
Total %		25	63	20	77.3

Table 3: Real time PCR positivity of tested samples which are positive in ELISA.

AGE / Year	No.	Real time PCR positive			
		T.gondii	CMV	Rubella	Herpes
Under 20	29	Zero	zero	zero	Zero
20 - 29	189		4		
30 - 39	69		zero		
Above 39	13				
Total number	300	Zero	4	zero	Zero
Total %	%	Zero	1.3	zero	Zero

Gunel *et al* [23] show that *T. gondii* B1 gene is a target location with multi copies that allow reliable diagnosis of Toxoplasmosis. Compared to other methods, the real-time-PCR is a more rapid and specific diagnostic method and the specificity rate of real-time PCR for the diagnosis of Toxoplasmosis has been reported to be between (94%) and (100%). This method has a low false positive rate, decreasing the risk of contamination with a rapid diagnosis in a single tube analysis [23].

Only 4 samples (1.3%) from cervical swabs of CMV are positive in RT-PCR from 300 samples which are positive in all serological methods. This is confirmed by Tanaka

and others (2006) who found a relationship between CMV DNA in vaginal fluid during the first trimester and miscarriage risk. No other cause of miscarriage such as other virus infection or amniocentesis is detected. In addition, neither the frequency of premature delivery nor the gestational age at live birth is affected by the presence of CMV. Therefore, it is unlikely that the presence of CMV in the vagina is associated with the risks of chorioamnionitis or premature rupture of the fetal membranes [24]. Although, there was no significant differences in CMV IgG and IgM seroprevalence between women with BOH and those with normal pregnancy, the role of CMV in development of bad obstetric outcome can't be ruled out. Role of CMV in bad obstetric outcome may be that the virus induce immunosupression and this lead subsequently to Co-infections with other microbes and BOH induction [25, 26].

In the present study RT-PCR show a high CT value for both sample and internal control (IC) with a values < 33 and this finding is identical to that reported by Jamil *et al* [27]. These findings show that most of the ELISA results are confirmed by PCR which means that the seropositive results by ELISA are not specific or less significant due to the probability of false positive results as a result of other microbial infection. They also suggest that the best method to detect CMV and HSV is RT-PCR as Real time PCR is considered active, rapid and useful technique for diagnosis of active disease and monitoring response to therapy [27].

Dinc *et al* [28] (2010) finding agreed with the present study results as CMV secretion from cervix increases during pregnancy. A large spectrum of cells of the fetus is infected by CMV. The major target fetal organs for CMV infection are the lungs, pancreas, kidneys and the liver but comparing with these organs, CMV DNA level determined in uterine tissue and cervical smear is higher [28].

When pregnant women have primary CMV infection during the first trimester, approximately 25% of their fetuses will be infected [29]. Therefore, infants born to mothers with primary CMV infection during pregnancy are at high risk for the occurrence of congenital CMV infection. Maternal serum CMV immunoglobulin IgM antibody is often tested to identify primary infection. However, true primary CMV infection is determined in only 20–25% of pregnant women with positive results for CMV IgM. This is because CMV IgM may persist for (6–9) months following primary infection [30] or maybe detected during latent reactivation [29, 31]. In addition, fetal CMV infection occurs in (1–2.2%) of pregnant women with reactivation of a latent virus or re-infection with a new strain of CMV [32, 33]. Hassan et al (2014), Iraq, found that CMV IgM and IgG seroprevalence was more frequent in women with history of congenital anomalies than in those without [11].

All samples for Rubella virus are negative in RT-PCR although are positive in all serological methods. Van Nguyen *et al* (2013) explain that the pregnant women that not received rubella vaccination, their fetuses or new born has a congenital cataract [34]. Additionally, there was a significant difference in rubella IgG seroprevalence between women with BOH and control [16], while other study not shows such significant difference [12]. Mahmood *et al* confirm that the seropositive results by ELISA are not specific or less significant because of the probability of false positive that may be as a result of infection with other microorganism [35]. The high rate of false positive results in ELISA may be attributed to the kit sensitivity and specificity which it is an outcome of

bad quality control of the products. The reduction in financial support for the research leads to buy the cheapest kits from sources without quality control. The present study finding warranted performance of comparative study between ELISA and PCR for detection of TORCH agents using a high quality kits.

Rubella is now a vaccine-preventable disease. Live attenuated vaccines have been available since the late 1960s [36]. The vaccination programs have dramatically reduced the incidence of Rubella in developed countries. In 2009, they were in use in more than (67%) of countries worldwide, but vaccination coverage differed widely [37].

Viral infections such as Rubella virus have a significant risk among infants at birth and the risk of late manifestation is still unclear. Such data are fairly well known for Rubella [38]. The IgM appears during the first week, which might be inadequate to expect the presence of the pathogens by RT-PCR [35]. This gives the real incidence of infection of this microorganism and reflects the false positive results by routine ELISA investigation [39]. Additionally, *Toxoplasma gondii,* Rubella, CMV and HSV are characterized to be found as dormant infections in human being with continuous mini foci that may lead to immune response state without antigaenemia. Antigenic diversity in viruses may add another explanation to give false positive results in ELISA. However, this assumption may be clarified using monoclonal in ELISA.

Only a few Asian countries have introduced a Rubella-containing vaccine into their national immunization programs. So far, the control of Rubella with vaccination has been achieved only in Japan, Taiwan and Singapore. As a result, Rubella still remains poorly controlled in many countries in Asia. In particular, in the Southeast Asian continents, the vaccination coverage rate is only (4%) as for 2009 [37], while in Iraq, the vaccination coverage was 67.6% and 88% as demonstrated in retrospective and prospective study respectively [40]. Various inoculation strategies have been employed worldwide to prevent Rubella infections. Since active immunization is well tolerated, vaccination programs aim to protect all young people before puberty using a two-stage Rubella vaccination. In the case of a negative result, unknown status or the detection of specific IgM, passive immunization in the early stages of pregnancy is possible, although only within 7 days of exposure [41].

In the present study all the samples of HSV I/II Virus are negative in RT-PCR although being positive in all serological methods. Conde-Ferraez and others (2016), they dissented our study by showing that the presence of viral DNA without IgM detection could indicate a reactivation that may occur without IgM rises [42]. On the contrary, HSV I/II IgM positive test with a negative PCR could indicate one of the following possibilities: (1) infection of the oral mucosa, overestimating the rates of genital infection. (2) genital infection without detectable viral shedding at the moment of sampling. About the last, that shedding episodes are highly variable, and the detection depends on the applied methods, duration of the study and the anatomical site of sampling [43, 44]. Additionally, whether the case was with primary or recurrent herpetic infection since the viral shedding was more in primary than in recurrent episode [45].

Dinc and others [28] reported that not all positive samples of HSV-II in ELISA are positive in RT-PCR. HSV- II is rarely seen due to the monogamy in the sexual relations. Genital Herpes resulting from HSV-II infection of the urogenital tract is a worldwide public health problem [9]. European investigations of pregnant women have shown

overall prevalence of HSV-II to range between 9% and 33% [13, 46]. HSV-II DNA prevalence has been reported to be 2% in Turkish pregnant women [47].

In the infected women, there is an active and latent phase. After an incubation period of about a week, the active phase begins. During the active phase, the virus multiplies explosively between (50,000) and (200,000) new virions are produced from each infected cell. The primary infection and reactivation can occur without any symptoms and apparently healthy people can transmit HSV-II to their sexual partners or their newborns. The chance of acquiring infection increased with age [48, 49], however, in Iraqi population, the seroprevalence of HSV-II infection was more in the sexually active group (20-29 years) as compared to other age groups [13,17].

Kapranos and Kotronios (2009) reported a significant role of HSV in the first trimester pregnancy loss as it is detected by sensitive and accurate nested PCR which would permit prompt antiviral therapy for a successful future pregnancy. HSV is detected in 43.2% of early pregnancy loss and in 16.7% cases of elective pregnancy termination [50], which is contrasted to the present study findings. However, in Iraqi community, the mean HSV IgG was 36.6% in women with bad obstetric outcome, while the HSV IgM mean value was 21.8% [8].

In conclusion, ELISA test is considered as a preliminary and screening test for TORCH infections. However, the present study finding warranted a comparative large scale study. Real Time PCR is an essential tool in the research laboratory. It has engendered wider acceptance than the conventional PCR due to its improved rapidity, sensitivity, reproducibility and the reduced risk of carry-over contamination.

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