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RESEARCH ARTICLE

Assessment of ELISA and PCR in Detection of Cytomegalovirus Viremia in Pregnant Women

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Abstract

Background: Maternal infection with Cytomegalovirus (CMV) during pregnancy is frequently associated with transplacental transmission to the fetus. Early diagnosis of CMV in pregnant women is necessary to get effective treatment and prevent fetal complications.
Objective: The present study aimed to assess the use of PCR and compare it with enzyme-linked immunosorbent assay (ELISA) for detection of CMV infections in pregnant women.
Methodology: Forty six pregnant women were included in this study ranging in age from 20-35 years selected from patients attending outpatient clinic of the Obstetrics & Gynecology Department at Sohag University Hospital, over a six month period.
They were tested for the presence of CMV DNA in their blood by PCR and specific antibodies to CMV by ELISA. The results obtained were recorded to evaluate the best technique to detect CMV infection.
Results: Out of 46 subjects in this study, 37(80. 4%) cases had positive results for ELISA anti CMV (IgG) and 15(32.6%) cases had positive results for ELISA anti CMV (IgM). Two cases (4.3%) had positive PCR results, and 44 cases (95.7 %) were negative. Sensitivity & Specificity of ELISA IgG were 100 % & 20.6% respectively and of IgM was 100% & 70.5% respectively when compared with PCR**.**
Conclusion: The results suggest that screening pregnant women for CMV antibodies using ELISA is a specific and sensitive tool for the early identification of patients exposed to CMV. This information can be used as an alarm for babies at high risk of developing serious disabilities.

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INTRODUCTION

Cytomegalovirus (CMV) is the most common source of congenital malformations resulting from viral intrauterine infection in developed countries **(Jahromi et al., 2010)**. Congenital CMV infection is transmitted from mother to fetus as a consequence of primary or recurrent infection of the mother during pregnancy **(Adler, 2011)**. Rapid and correct diagnosis of CMV infection during pregnancy is very important to advocate proper management and adequate therapy of the case. Virologic and molecular detection of CMV and serological demonstration of a specific immune response are used for diagnosis. ELISA is the commonest method employed for detection of CMV specific IgM antibodies to establish CMV infection. Low specificity and sensitivity of the ELISA systems used have been reported in some evaluation studies **(Priya and Madhavan , 2002)**. Many studies have been conducted to identify CMV DNA during pregnancy, and relate that to the outcome of congenital infection of fetuses **(Fabbri et al.,**

2011). PCR is the method of choice for CMV DNA identification which has been used in various clinical specimens as amniotic fluid, urine, blood and cerebrospinal fluid **(Ross et al., 2006).** PCR assay is more versatile and can be used either qualitatively (diagnostic PCR) or quantitatively to measure the viral load, which is proportional to the level of CMV DNA **(Jahan, 2010)**. This study was undertaken for the assessment of ELISA and PCR in detection of exposure to cytomegalovirus in pregnant women at risk for transmission of the virus to the fetus.

PATIENTS AND METHODS

Study design and patients

This study was carried out during over a six month period from August 2013 to January 2014. Forty six pregnant women ranging in age from 20- 38 years were selected from patients attending the Outpatient Clinic of the Obstetrics & Gynecology Department at Sohag University Hospital. These 46 women with complicated previous pregnancy states had to fulfill one of the following criteria:-Women with repeated abortion (> two times), premature delivery, congenial anomaly, Intrauterine fetal death (IUFD), Still birth (S.B). These data were obtained from the pregnant women who were interviewed using a structured questionnaire. For the ELISA and PCR techniques, 10 ml of venous blood were collected by venipuncture into vacutainer tubes from each participant in the study. Each blood sample was divided into two tubes, one heparinized tube was used for PCR (whole blood) and the second a blank tube to obtain serum for ELISA. For serum preparation, the blood was allowed to clot for 30 minutes and serum was obtained by centrifugation at 1500 rpm for 10-15 minutes. Serum samples were stored at -20°C until analyzed.

- **Molecular detection of CMV gene by PCR:**

a) Sample treatment & DNA extraction:

Genomic DNA of the study isolates was extracted for molecular analysis from the buffy coat of blood samples according to the manufacturer's instructions by the use of QIAamp DNA Kits **(QIAGEN GmbH)**. Buffy coat is a leukocyte-enriched fraction of whole blood. Preparing a buffy-coat fraction from whole blood is simple and yields approximately 5–10 times more DNA than an equivalent volume of whole blood. Buffy coat was prepared by centrifuging whole blood at 2500 x g for 10 minutes at room temperature. After centrifugation, 3 different fractions are distinguishable: the upper clear layer is plasma; the intermediate layer is buffy coat, containing concentrated leukocytes; and the bottom layer contains concentrated erythrocytes.

Primers (Kaneko et al., 2006):

Oligonucleotide primer sequences used **(synthesized by metabion international AG, Germany)** were as follows: The 2 oligonucleotide primers A_1 and A_2 resulting in the amplification of 426 -bp PCR fragments for detection of CMV major immediate-early gene; Primer A_1: 5′- TATACCCAGACGGAAGAGAAATTCA -3′. Primer A_2: 5′- ATAAGCCATAATCTCATCAGGGGAG -3′.

b) PCR (Kaneko et al., 2006):

In a sterile thermal cycler 0.5ml tube, 25 µl PCR reaction mix containing 12.5 µl PCR mastermix **(Jena Bioscience GmbH, Lobstedter, Germany)**, 4.5 µl sterile water, 2 µl of each primer and 4 µl of the DNA sample was added. In each set of experiments, a negative control was included. The negative control was prepared by replacing the DNA template with PCR grade water. Amplification of the sample was performed using a Biometra thermal cycler **(T-Gradient PCR system version 4 - Biometra Whatman, Goettingen, Germany).** The PCR amplification cycling profile of CMV major immediate-early gene was 5 min of denaturation at 94°C (1 cycle), followed by 35 cycles of amplification; each of heat denaturation at 94 °C for 60 s, primer annealing at 60 °C for 60 s, and DNA extension at 72 °C for 60 s then one cycle for final extension at 72°C for 5 minutes. The amplified DNA was electrophoresed using 2% agarose gel electrophoresis **(Electrophoresis power supply-Biometra Whatman company, Goettingen, Germany)**, stained with ethidium bromide, and visualized under UV trans illumination and photographed. The specific DNA product for CMV of each sample was determined by identifying the 426 bp amplified DNA bands in comparison with the 100-bp DNA ladder **(Fermentas Germany)**, used as DNA size marker (Figure 3).

II. Enzyme-linked Immunosorbent Assay (ELISA) for the detection of human Cytomegalovirus IgM and IgG:

Principle of the assay:

Purified CMV antigen is coated on the surface of microwells. Diluted patient serum is added to the wells, and the CMV Ig specific-antibody, if present, binds to the antigen. All unbound materials are washed away. HRP-conjugate is added, which binds to the antibody-antigen complex. Excess HRP-conjugate is washed off and a solution of TMB Reagent is added. The enzyme conjugate catalytic reaction is stopped at a specific time. The intensity of the color generated is proportional to the amount of CMV Ig-specific antibody in the sample. The results are read by a microwell reader compared in a parallel manner with calibrators and controls. All tests were done according to the manufacturer's instructions using Stat Fax 2600 ELISA work station **(Awareness Technology Inc., Palm City, USA).**

Reagent preparation:

The Solutions and reagent provided in ELISA kit **(BioCheck, USA with Lot number 42385)**. All reagents should be allowed to reach room temperature (18-25 °C) before use. Dilute 1 volume of Wash Buffer (20x) with 19 volumes of distilled water.

Assay procedure: According to the manufacturer's instructions:-

- The desired number of coated wells was placed into the holder .
- 1:40 dilution of test samples, Negative Control, Positive Control, and Calibrator were Prepared by adding 5 µl of the sample to 200 µl of Sample Diluent, followed by proper mixing .
- 100 µl of diluted sera, Calibrator, and Controls were dispensed into the appropriate wells. For the reagent blank, 100 µl of Sample Diluent in 1A well position were dispensed.
- The holder was incubated at 37°C for 30 minutes .
- At the end of incubation period, liquid was removed from all wells. The microtiter wells were rinsed and flicked 4 times with diluted Wash Buffer (1x) and then one time with distilled water .
- 100 µl of Enzyme Conjugate was dispensed into each well. And was mixed well for 10 seconds.
- Then was incubated at 37°C for 30 minutes .
- Enzyme Conjugate was removed from all wells. The microtiter wells were rinsed and flicked 4 times with diluted Wash Buffer (1x) and then one time with distilled water .
- 100µl of TMB Reagent was dispensed into each well. And was mixed well for 10 seconds .
- Then was incubated 37°C for 15 minutes .
- 100µl of Stop Solution (1N HCl) was added to stop the reaction.
- The O.D. was detected at 450 nm within 15 minutes using a Stat Fax 2600 Microplate Reader (**Awareness Technology Inc., Palm City, USA**).

Interpretation

- Negative: CMV IgG/IgM Index less than 0.90 is seronegative for IgG/IgM antibody to CMV .
- Equivocal: CMV IgG/IgM Index between 0.91-0.99 is equivocal. Sample should be retested .
- Positive: CMV IgG/IgM Index of 1.00 or greater is seropositive.

Statistical Analysis

Statistical differences were determined by using analysis of variance (ANOVA) and Student´s t-test. Results were expressed as mean ± standard deviation of the mean (SD).

RESULTS

All blood samples from 46 patients in this study were tested using ELISA and PCR.

I. Serological results:

According to this study, the results obtained from the serological test were 37 patients positive for IgG and 9 negative for IgG, while the 15 patients were positive for IgM and the other 31 patients were negative for IgM. **(Table 1) (figures 1 &2)**

Table 1: Showing ELISA results of anti CMV IgG and IgM antibodies.

Elisa	Positive	Negative
IgG	37(80. 4%)	9(19. 9%)
IgM	15(32. 6%)	31(67. 4%)

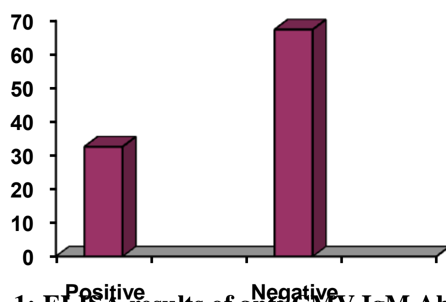

Fig. 1: ELISA results of anti CMV IgM Ab.

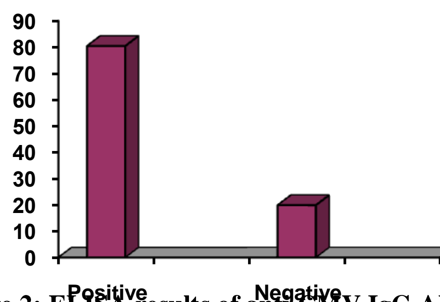

Figure 2: ELISA results of anti CMV IgG Ab

II. Cytomegalovirus polymerase chain reaction (PCR) Results:

The results for the PCR showed that; 2 out of 46 (4.3%) patients were found to be CMV positive while 44 out of 46 (95.7%) patients were found CMV negative. Both results for the serological test and PCR showed in (Table 2).

Table (2): Serological and PCR Results for all patients tested in this study.

No. of Specimens	ELISA		PCR	
	IgG	IgM	Positive	Negative
22	Positive	Negative	-	22
9	Negative	Negative	-	9
15	Positive	Positive	2	13
Total:46			2	44

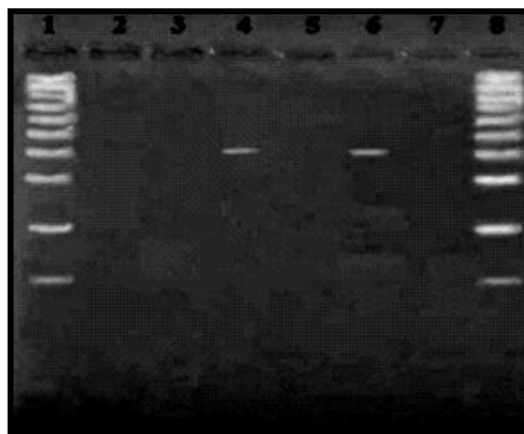

Figure 3: Gel picture of amplified DNA product of CMV. Lanes 1 & 8: 100 bp ladder marker. Lanes 4 & 6 CMV positive (426 bp); lane 7: negative control; lanes 2, 3 & 5 Negative.

Sensitivity & Specificity of ELISA in relation to PCR:

Sensitivity & Specificity of ELISA IgG were 100 % & 20.6% respectively and of IgM was 100% & 70.5% respectively when compared with PCR. The negative predictive values of ELISA IgG and ELISA IgM were 100 %. While the positive of ELISA IgG and ELISA IgM were 5.4 % and 13.3% respectively when compared with PCR (Tables 4&5).

Table (4) Accuracy of ELISA IgG compared to PCR

PCR IgG	+ve (n=2)	-ve (n=44)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV(%)	Accuracy (%)
Positive (n=37)	**2 TP**	**35 FP**	**100**	**20.6**	**5.4**	**100**	**24**
Negative (n=9)	**- FN**	**9 TN**					

Table (5) Accuracy of ELISA IgM compared to PCR

PCR IgM	+ve (n=2)	-ve (n=44)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
Positive (n=15)	**2 TP**	**13 FP**	**100**	**70.5**	**13.3**	**100**	**71.7**
Negative (n=31)	**- FN**	**31 TN**					

DISCUSSION

The most common known congenital viral infection is CMV, where in different parts of the world, its incidence has been estimated to be 0.2 to 2.2% of all live births **(Fisher et al., 2000).** On the basis of population sampling, CMV has been estimated to be the leading infectious cause of damage to the developing fetus in utero in the United States, as well as in Europe and other developed areas of the world where improved hygiene has delayed acquisition of CMV to the childbearing years. The disease burden for congenital CMV infection is similar to that for congenital rubella before vaccination was introduced to control this disease **(NVAC, 2004).** In this study all blood samples from 46 patients who had complicated previous pregnancy states were monitored by using serological test (ELISA) and PCR. According to this study, the results obtained from the serological test were 37(80.4%) patients positive for IgG due to prior CMV infection, and 9 (19 .9%) negative for IgG, while 15 (32.6%) patients were positive for IgM (acute or recent infection) and the other 31(67.4%) patients were negative. Similar results were acquired in Egypt (96%) **(El Nawawy and Solliman , 1996)**, Israel (84.3%) **(Stein et al., 1997)** and Iran (98%) **(Moddares and Moddares , 1994)**.The results for the PCR showed that; 2 out of 46 (4.3%) patients were found to be CMV positive while 44 out of 46 (95.7%) patients were found to be CMV negative. Sensitivity & Specificity of ELISA IgG were 100 % & 20.6% respectively and of IgM was 100% & 70.5% respectively when compared with PCR. According to the results of serologic assay we can divide participants in to 4 groups; **Group 1:** Women with negative serology for IgG and IgM antibodies, they were not contaminated with CMV and were susceptible to infection (primary infection). In this situation there is a risk for transmission of the virus to the fetus during the pregnancy **(Enders et al., 2001).** These women were sensitive to CMV infection, and it was related to some extent to socioeconomic status and geographic location. **Group 2:** Women with IgM seropositivity without positive serology for IgG antibody. These women were considered acutely contaminated with CMV. They were also asymptomatic and indicated the substantial prevalence of infection in the local population **(Wreghitt et al., 2003).** This group was not found in our study population. **Group 3:** Women with positive serology for IgG and negative for IgM antibodies, these women were considered immune and their primary infection with CMV was assumed to have been taken place before the current pregnancy. CMV IgG antibody was detected in (80.4%). **Group 4:** Women with positive serology for IgG and IgM antibodies, at the same time. These women were considered to be possibly infected with CMV during the current pregnancy or a

chronic infection which can be confirmed by IgG avidity test because antibody binds to the antigen with less avidity during acute infection than chronic infection **(Wreghitt et al., 2003).** The results for the PCR showed that; 2 out of 46 (4.3%) patients were found to be CMV positive who were with positive serology for IgG and IgM antibodies, at the same time**. Remington and Klein, (1995)** concluded that IgG ELISA may give false positive result. The explanation of this is that IgG antibodies usually appear within 1-2 weeks of infection, peak within 1-2 months, declined at various rates and usually persist for life. IgM antibodies may appear earlier and decline more rapidly than IgG antibodies. In a high seroprevalence population, a pregnant woman has a higher likelihood of exposure to CMV-infected people. Thus, in a high risk population, seropositive women have a higher risk of reactivation and seronegative women have a higher risk of primary infection **(Kenneson and Cannon, 2007)**. Previous studies have shown that detection of CMV by PCR can be applied to blood, all nucleated cells, plasma, and serum, but we do not know which is the best source for the diagnosis of CMV disease by PCR **(Machida et al.,2000)**. In this study, we found non significant correlation between the results of the antigenemia assay and blood PCR. The main limitation of this study was that it lacks a follow-up of newborns to correlate the studied markers with the development of congenital CMV infection and the appearance of long-term sequelae. The molecular assay should be used side by side with traditional laboratory methods for a rapid and an accurate detection of CMV DNA in pregnant women with complicated previous pregnancy states, with serious consideration to performing PCR on amniotic fluid samples rather than blood samples. The extremely low positive results for PCR in our study could be attributable to many factors, possibly technical but most probably biological; in a population with high incidence of CMV such as ours, more extensive study has to be performed to determine the kinetics of CMV viremia as compared to the production of antibodies, and if in the face of frequently recurring infections, there is a tendency for IgM antibodies to persist for much longer durations, in which case lack of viraemia could be an expected finding.

CONCLUSION

Severe life-threatening complications of CMV infection in pregnant women may not be as rare as previously thought. The screening of women before pregnancy for CMV antibodies should reduce abortion rate and also the number of congenitally infected infants. PCR for CMV although a more sensitive and effective test than ELISA, yielded very low positivity in blood specimens submitted to the virology laboratories. For extremely high risk patients, amniotic fluid sampling seems to be a logical approach.

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