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Page: 01-10

Serological Markers as Indicators for Congenital Cytomegalovirus Infection

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To assess the problem of CMV infection in pregnant women in Sohag community and to determine the risk of congenital CMV infection. The study was performed on 900 pregnant women residing in Sohag, during the period from June, 2006 till January 2010. Detection of CMV antibodies (IgM and IgG) was carried out using microparticle enzyme immunoassay. Women proved positive (+ve) for CMV IgM or IgG antibodies were considered the seropositive group (group1), while women proved negative (-ve) for both antibodies were the seronegative group (group II). Serum samples of IgM +ve females and with rising IgG titers were further subjected to PCR to detect CMV DNA. Of the 900 pregnant women, 850 (94.4%) were seropositive for CMV antibodies. Of these, 828 females (97.4%) had +ve IgG and -ve IgM antibodies, and 22 (2.6%) women had positive IgM. Viral DNA was detected in 12 of the 22 IgM +ve and 8 out of 10 women with rising IgG titer. CMV antibodies and serum PCR were done for 15 live births whose mothers had positive CMV PCR. Of 7 infants whose mothers had CMV IgM and CMV PCR positive, 4 infants had positive PCR results. Eight live births, whose mothers had rising IgG titer and positive PCR test, were negative for viral DNA. In conclusions, CMV specific IgM or rising IgG antibodies in maternal serum could predict congenital CMV infection.

Cytomegalovirus (CMV) is a member of the Herpesviridae family, which includes Epstein-Barr virus (EBV), Herpes Simplex virus tipes1 and 2 (HSV-1,2), Varicella- Zoster virus, and Human Herpes virus types 6 and 7 (HHV-6,7). Similar to infection with other viruses in the family (Vareria *et al.,* 2006). The human cytomegalovirus (CMV) or human herpes virus 5 is one of the major causes of congenital infections. Its clinical manifestations range from asymptomatic forms (90% of cases) in healthy adults, but some pregnant women may have mild fever, swollen glands, and flu-like symptoms to severe foetal damage and, in rare cases, death due to abortion (C.D.C Accessed 2009). Furthermore, 10 % –15% of the children who are asymptomatic at birth may develop sequelae, especially hearing defects, after a period of months or even years (Colugnati *et al.,* 2007), (Massimo *et al.,* 2009) and some neuro-developmental disabilities in children (Malm & Engman 2007). However, most of them are born without any clinical findings (Oroy & Diav-Ctrin 2006). The prevalence of congenital CMV in fertile women offspring varies in different countries between 0.15 - 2.0% (Fowler & Boppana 2006). Latency following a primary infection (first contact with the virus) may be punctuated by periodic reactivations that give rise to recurrent infections (Michael *et al*., 2004), latent infection, due to the ability of the virus to remain integrated in the DNA of host cells (Vareria *et al*., 2006). In utero transmission may occur during either primary or recurrent infections (Massimo *et al*., 2009). Primary CMV infections are reported in 1-4% of seronegative women during pregnancy, the risk of congenital infection is much higher during primary infection, when the rate of transmission from mother to fetus is 30%–40%, reactivation of a CMV infection during

pregnancy is reported in 10 - 30% of seropositive women and the risk of transmitting the virus to the fetus is about 1-3% against 0.15%–2.2% during re-infections (Malm & Engman, 2007). In re-infection there is protection by preexisting CMV antibodies (Inoue *et al*., 2001). Furthermore, most of the newborns are asymptomatic. Symptomatic cases are due more to re-infection than reactivation of these, 5% to 18% will be symptomatic at birth. The mortality rate in these children is almost 30%; approximately 80% of the survivors will have severe neurological morbidities (Pass *et al*., 2006).

In Europe, 45% of pregnant women are seropositive for CMV at the beginning of pregnancy. Of the seronegative patients, only 1% to 4% will undergo seroconversion during pregnancy, as detected by the appearance of immunoglobulin G and immunoglobulin M antibodies (Collinet *et al*., 2004). Seropositivity was correlated with lower socioeconomic status, multi-gravidity, older age, a first pregnancy at 15 years of age, and multiple sexual partners (Morris, 1990). In Egypt, El-Sayed Zaki & Goda (2007) studied the frequency of parvovirus B 19, herpes simplex 2, and cytomegalovirus infections in sera from Egyptian pregnant women with history of recurrent spontaneous abortion. They reported parvovirus B19 IgM in 84% of studied women, herpes simplex IgM in 40% and CMV IgM in only 12%. Their observation, of low CMV IgM rates, could be attributed to the possibility that primary CMV infection could probably be acquired during childhood so that the risk of primary infection during pregnancy would be lower than that of other viruses. CMV transmission routes include transplacental, cervical and vaginal secretions, breast milk, saliva, urine, stools, blood, semen, and tissue transplant (Sergio, 2008).

Diagnosis of cytomegalovirus infection can be made by detection of CMV specific IgM or IgG or both in recent serologic assays using enzyme-linked immunosorbent assay. Detection of viral DNA in clinical samples (e.g maternal blood, umbilical cord blood, amniotic fluid, or neonatal blood) involves DNA extraction and analysis. Plasma-based polymerase chain reaction (PCR) assay that is significantly more sensitive than pp65 antigenemia and blood cultures for detection of CMV in blood specimens (Michael *et al*., 2004), (Babbal *et al*., 2009). PCR has become the preferred method for rapid and accurate viral diagnosis (Revello *et al*., 1998), (Lipitz *et al*, 2002).

Foetal ultrasound findings can predict symptomatic CMV infection of the newborn. Foetal findings include one or more of the following: placental enlargement, ventriculomegaly, microcephaly, intrauterine growth restriction (IUGR), foetal ascites, organomegaly, megaloureter, or hepatic and intestinal echodensities (Collinet *et al*., 2004). About 10% of infected babies that do have symptoms at birth (jaundice, enlarged spleen, seizures, liver symptoms, and/or a characteristic rash) have a more negative prognosis. Up to 20% of these babies may die due to complications from the infection, and survivors may have up to a 90% risk of developing mental retardation, cerebral palsy, or other serious disabilities (Krissi, 2009).

The aim of the current study was to estimate the frequency of CMV infection among pregnant women with poor obstetric history in Sohag community using CMV IgM and IgG seriological tests. The risk of CMV intra uterine transmission was also evaluated.

Patients and Methods

Study design and Patients

The current study included 900 pregnant women attending the outpatient clinic of Obstetrics and Gynecology Department, Sohag Faculty of Medicine

and fulfilling the inclusion criteria of the study. Their age ranged from 18- 35 years, they were recruited in the study during the period from June 2006 to January 2010. Inclusion criteria of the study include multigravidae (in their 1st, 2nd or 3rd trimester of pregnancy), females with poor obstetric history such as habitual abortion, recurrent late pregnancy loss, congenital foetal CNS anomalies, neonatal neurological sequels, females not assumed to have a probable cause of their poor obstetric history such as poorly controlled diabetes, sensitized Rh-negative patients, females negative for TORCH (Toxoplasma, Rubella, Cytomegalovirus, and Herpes) organisms other than cytomegalovirus. The assigned women were counseled about their participation in the study. Written informed consents were obtained prior to recruitment, they had the right to refuse the participation without being denied their regular clinical care. Personal information and medical data collected were confidential and were not made available to a third party. After delivery, newborns, whose mothers were positive for viral DNA were followed up for two years. The study was approved by the local institutional ethics committee.

Blood Sampling and Laboratory Techniques

Seven ml of blood were withdrawn from each female and divided into: 3ml was added to K–EDTA tube for routine investigation, the other 4 ml were delivered into plain tube, after centrifugation the serum of each sample was divided into 3 aliquots, kept at -20°C till the procedures were done. The newborns and the infants samples were 3 ml blood which were delivered into special pediatric vacutainers. The vacutainers were supplied by (B.D). The routine investigations, including blood grouping and Rh typing, the kits were supplied by Biotec, complete blood count was done by H-Max Coulter system, after centrifugation the plasma was subjected for liver and renal function tests and random blood glucose which were done on chemical autoanalyser Synchron CX-9 Beckman system.

Serology for IgM and IgG for CMV

CMV IgM and IgG antibodies detection were performed at the department of clinical pathology, faculty of medicine, Sohag university in one of the serum aliquots using (AxSYM fully automated system), which is based on microparticle enzyme immunoassay (MEIA) which is Enzyme Linked Immunosorbent Assay(ELISA) technology. The samples and all AxSYM CMV antibodies reagents were placed into the reaction vessels in the sample center, recombinant Ag coated microparticles were added then the reaction vessels were transferred into the processing center, the CMV Abs in the samples bind to Ag coated microparticles forming Ag/Ab complex, the mixture was transferred to matrix cell where the microparticles bind irreversibly to the glass fiber matrix. Goat antihuman immunoglobulin alkaline phosphatase conjugate dispensed onto the matrix cell, where it bind to the Ag/Ab complex, after washing the substrate (4-methylumbelliferyl phosphate) was added, the conjugate catalyzes the removal of phosphate group from the substrate yielding fluorescent product (4-methylumbelliferone),which measured by optical assay, the sample was considered positive if the rate of formation of fluorescent product was equal to or greater than cutoff rate. CMV IgM index value based on the ratio of the sample rate to the index calibrator mean rate, patients who proved positive for IgM, had a titer more than (0.5 index). IgG positive titer was more than (10 AU /ml). Patients with only IgG +ve antibodies were re-tested again after 4 weeks to detect elevated antibody titers (reactivated disease).

Detection of CMV DNA by PCR

CMV PCR was performed on serum samples for all women who were positive for CMV IgM antibodies, and rising CMV IgG antibody titer. PCR for virus DNA detection was also carried on serum samples obtained from newborns whose mothers were positive for IgM and /or with rising IgG antibody titer, and positive for PCR. CMV PCR was performed at the Medical Microbiology & Immunology department, Faculty of Medicine, Assiut University.

DNA Extraction from Serum

DNA was extracted from serum using the Qiamp Blood Kit (Qiagen), 25 µl Qiagen proteinase K (19.33 mg/ml) and 200 µl of buffer AL (Qiagen) were mixed with 200 µl serum before incubation at 70°C for 10 minutes and the addition of 210 µl of isoprepranol. This mixture was applied to a silicone resin (Qiamp spin column, Qiagen) by centrifugation and washed three times with 500 µl buffer AW (Qiagen). DNA was eluted into 200 µl preheated (70°C) water after incubation at 70°C for 10 minutes. This elute was used for PCR amplification.

Primers and Amplification

PCR for the detection of CMV DNA was performed using oligonucleotide primers complementary to CMV conserved region of the fourth exon of the immediate early (IE) gene, located in the *Hind* III-X fragment region that amplifies a 406 bp fragment as described by Drouet *et al*., (1993) and Mendez *et al*., (1998). Primer sequences were forward primer, P1:5'-**GGA TCC GCA TGG CAT TCA CGT ATG T**-3', and reverse primer, P2:5'- **GAAT TC AGT GGA TAA CCT GCG GCG A**-3'. The reaction mixtures consisted of 5 µl of

template DNA, 100 pmol of each of the oligonucleotide primers, 1.25 U of the enzyme Taq polymerase, 200 µM deoxynucleotide triphosphate , 5 µl of 10X reaction buffer (500 mM KCl, 100 mM Tris-HCl [pH 8.3], 15 mM $MgCl_2$, 0.01% gelatin), 10 µl of a 50% glycerol solution, and high-grade distilled water to a total volume of 50 µl in a microcentrifuge tube. The tubes were overlaid with 2 drops of mineral oil and were subjected to PCR protocol that consisted of 95° C 10 min, followed by 35 cycles of amplification (94°C for 1 min, 55°C for 2 min, and 72°C for 3 min) with a final extension at 72° C 5 min. using OmniGene cycler, USA. The amplified PCR products were electrophoresed on an agarose gel and were visualized with UV light as a single band by staining with ethidium bromide (10µg/ml). The positive amplicon appeared as a single band with a length of 406bp. To determine the size of the amplified product, a molecular weight PCR marker with a ladder bands 100 – 1000 bp (Promega Company) was run in a separate lane on each gel. Measures were taken to prevent contamination as DNA extraction, reaction mix preparation (in a positive pressure laminar flow hood) and product electrophoresis were conducted in separate rooms with separate pipettes, aerosol- resistant tips and reagents. Negative (water) control was included in each.

Main Outcome Measures

Live births were examined for birth weight, apparent jaundice, any abnormalities as neurological defect, abdominal ultrasound was done for detection of organomegally, X-ray for diagnosis of intracranial calcification at birth and then followed up by a pediatrician for two years. Newborns, whose mothers were positive for IgM and /or rising IgG antibody titer and positive for PCR, were subjected for complete blood count, liver function tests, renal function tests, CMV antibodies detection and PCR for virus DNA detection. Bone marrow aspiration was done for the infants with thrombocytopenia, the marrow aspiration was done by Rt.tabial aspiration for the less than one year old infants, Rt.ant. Sup.iliac spine was done for 2 years old one, the marrow was subjected to staining by the ordinary stain and examination.

Statistical Analysis

Statistical analysis was performed using SPSS (Statistical Package for the Social Sciences), software, and version 15.0 for windows. Numeric data were presented as mean ± standard deviation (SD). A p-value less than 0.05 were considered statistically significant.

Results

A total of 900 women were enrolled in the study. Group I consisted of 850 women who were +ve for CMV antibodies (seropositive), while group II consisted of 50 women, who were seronegative for CMV antibodies. Of the 850 women (group I), 828 females (97.4 %) were positive for CMV IgG and negative for IgM antibodies and only ten patients had rising IgG antibody titer. The other 22 women of group I were positive for CMV IgM antibodies.

There were no significant differences between the group I and the group II regarding the age and gravidity. However, Most of the patients in the studied groups were in their first trimester as shown in table 1.

Table 1. Sociodemographic Characteristics of The Pregnant Study Groups.

Variables	Group I (n=850)	Group II (n=50)
Age (years)	25±6.2	24±5.3
Gravidity (Mean ±SD)	3±1.5	4±1.8
Gestational age (n (%):		
1st trimester	350 (41.17%)	20 (40%)
2nd trimester	250 (29.41%)	15 (30%)
3rd trimester	250 (29.41%)	15 (30%)

Figure (1) shows cytomegalovirus (CMV) testing in all patients. Eight hundred and fifty (94.44 %) out of 900 women were seropositive for CMV antibodies (group I), while 828 (92%) patients had +ve IgG and –ve IgM antibodies, and only 22 (2.4%) patients had +ve IgM with or without IgG antibodies. Fifty females (6.66%) were seronegative for both CMV antibodies (group II) Qualitative Polymerase Chain Reaction (PCR) was done on the serum for CMV DNA, the test was performed to IgM +ve females (22 patients) and females with rising IgG titers (10 patients). Of the 22 IgM +ve patients, 12 ladies (54.54%), and 8 out of 10 patients with rising IgG titers (80%) proved +ve for viral DNA (PCR +ve). Serum PCR testing was also carried out to 15 live births whose mothers had +ve PCR, (the other 5 cases of PCR +ve mothers had no live births). Four out of seven infants of IgM/PCR +ve mothers were PCR +ve for CMV DNA (57.14%), and all the 8 live births (100%) of IgG rising titer /PCR+ve mothers were negative for CMV DNA.

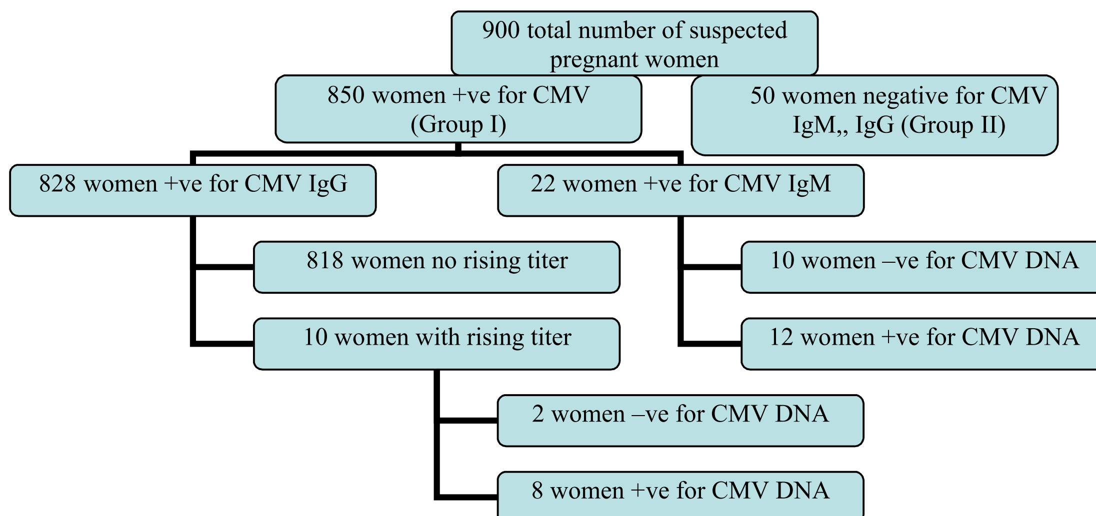

Figure 1. CMV Testing in pregnant females.

As regards to laboratory results, all PCR +ve mothers (12 in IgM +ve subgroup and 8 in IgG +ve subgroup with rising titer) were subjected to laboratory tests, where some abnormalities in the form of elevated liver enzymes, anemia, and thrombocytopenia were found. Also, 15 infants (7 in IgM +ve subgroup, and 8 in IgG +ve subgroup) were subjected to laboratory tests, where 8 out of 15 (53.3%) had some abnormalities in the form of elevated liver enzymes with clinical hepatosplenomegaly and jaundice, high serum creatinine level (one infant), anemia, thrombocytopenia and hypoblastic bone marrow as shown in table 2, 3 and figure 2.

Table 2. Hematological findings in the mothers and the infants +ve for IgM & IgG

Variables	IgG+ve mothers (n= 828)	IgM +ve mothers (n= 22)	IgG+ve infants (n= 8)	IgM +ve infants (n= 7)
Hb g/dl	10.4±1	9.7± 0.5	11.4 ± 1.3*	*8.5±1.3
Leukocytes X10^9/L	8.4± 1.4	7.5± 2.2	7.4± 2.5 *	* 3.8 ± 1.4
Neutrophils X10^9/L	5.3± 1.6*	* 2.5± 1.4	4.8±1.3 *	* 1.7±0.8
Lymphocytes X10^9/L	2.6±0.7	4.5± 1.2	2.4± 1.1	2.2± 0.4
Platelets X10^9/L	265 ± 32.8	154±10.4	150± 20 *	* 55± 11
Bone marrow finding	Not done	Not done	Not done	Hypoplastic marrow with little erythroid dysplasia

*Significant *(P* < 0.05).

Table 3. Liver Function Tests in IgM& IgG +ve Mothers and Infants

Variables	IgG+ve mothers (n= 828)	IgM+ve mothers (n= 22)	IgG+ve infants (n=8)	IgM +ve infants (n= 7)
ALT (IU/L)	35±3.5*	* 55.4± 6.3	47.4± 6.3 *	60±5.8 *
AST (IU/L)	27±3.4*	* 58.3± 4.5	48.4±4.7 *	76±8.4 *
Bilirubin (mg/dl)	0.84±0.2	1.4± 0.7	1.2±0.6	1.7±0.5
T.P (g/l)	7.5± 1.5	8±1.2	7.8±2.3	7.6±1.3
Albumin (g/l)	4.38±0.4	3.4±0..8	3.4±0.7	3.23± 0.3

*Significant (*P* < 0.05)

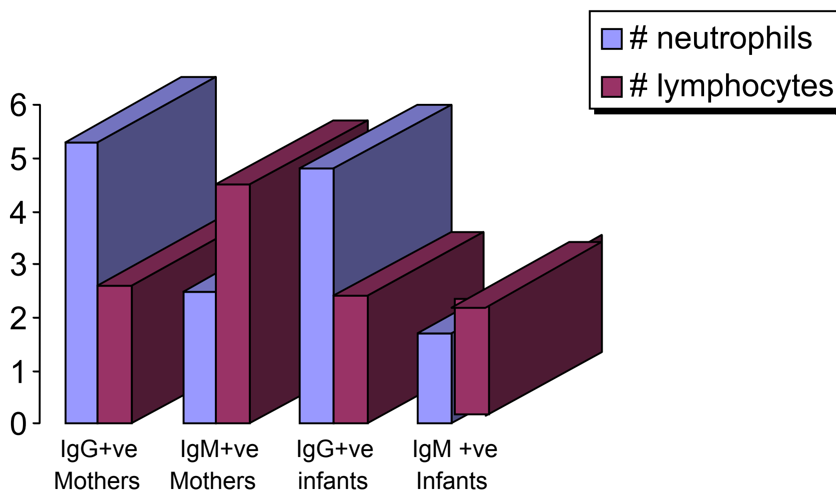

Figure 2. Neutrophils & lymphocytes in the studied groups.

As regards pregnancy outcome in all groups, the miscarriage rate in the study group was significantly higher (22%) in IgM +ve subgroup (5 cases), (30%) in rising IgG antibody titer subgroup (3 cases) and (1.34 %) in the group with low IgG antibody titer (<10 AU /ml; 11 cases) versus (2%) in the seronegative group (one case). Stillbirth rate also was significantly higher in the study group, (13.6 %) in IgM + ve sub group (3 cases), (10 %) in IgG rising titer subgroup (one case) and (0.12%) in low IgG antibody titer subgroup (one case) versus (0%) in the seronegative group. Normal outcome was significantly higher in the seronegative group (96%, 48 neonates) also in IgG +ve subgroup with low antibody titer (94.6%, 774 neonates) versus (9 %) in IgM +ve subgroup (2 neonates) and (10%) in IgG with rising titer subgroup (one neonate), as shown in table 4.

Table 4. Pregnancy Outcome in the Studied Groups.

	Group I (n=850)			Group II (n= 50) -ve IgM and IgG	*P-values
	Ig M + ve (n =22)	IgG +ve (n=828)			
		rising titer (n= 10)	no rising titer (n=818)		
Miscarriages	5 (22%)	3 (30 %)	11 (1.34%)	1 (2 %)	<0.05
Still Births	3 (13.6%)	1 (10 %)	1 (0.12%)	0	<0.05
Neonatal deaths	2 (9 %)	0	4 (0.49%)	1 (2%)	<0.05
IUGR/LBWT	7 (31.8%)	4 (40 %)	20 (2.44%)	0	<0.05
Microcephaly	0	0	2 (0.24%)	0	NS
Hepatosplenomegaly and jaundice	3 (13.6%)	1 (10 %)	3 (0.37%)	0	<0.05
Convulsions & delayed mentality	0	0	3 (0.37%)	0	NS
Normal outcome	2 (9 %)	1 (10 %)	774 (94.6%)	48 (96%)	<0.05

* $P<0.05$ = significant difference between each group and others NS= not significant

IUGR=Intrauterine growth retardation LBWT= Low-birth weight

Discussion

Cytomegalovirus is the most common congenital infection affecting about 0.2-2.4% of all live births. The virus can infect the fetus during primary and recurrent infection and at any stage of pregnancy (Flower & Pass 2006). Diagnosis of primary CMV infection during pregnancy is difficult because 90% are asymptomatic and 10% are present as a mononucleosis-like disease. Diagnosis of CMV infection can be made by serologic

assays or by detection of the virus DNA in different clinical specimens (Daniel *et al*, 1995). CMV antibodies were detected in 850 out of 900 pregnant women in the current study representing about 94.44 % of all cases. This may reflect the magnitude of the problem of CMV infection in high risk pregnant females in Sohag locality. However, Morris (1990), reported that 45% of European pregnant women are seropositive for CMV at the beginning of pregnancy. The incidence and risk of CMV infection in pregnancy found in our area, therefore, support the use of serological screening, certainly in the first trimester when the risk of infection is higher and, in the case of seronegative women, possibly also one screening in the second trimester and in the third one (Massimo *et al*, 2009). So serology is useful in checking for previous virus exposure and for identifying patients at risk. PCR is a very sensitive test for the detection of CMV as reported by Habbal *et al*., (2009). In our study, PCR testing was positive in 54.54% of mothers with positive IgM and 80% of mothers with rising IgG antibody titer (reactivated disease) which indicates that the presence of CMV IgM antibodies and/or presence of IgG antibodies with rising titers can predict the presence of the virus in 80% of cases with 20% false positive results. Most of the infants delivered to mothers with positive PCR and IgM antibodies have congenital CMV infection (diagnosed by serum PCR) and no infants of mothers with positive PCR and rising IgG titers proved to have the disease. This reflects that primary CMV infection during pregnancy (IgM +ve) is associated with higher fetal transmission rate than reactivated disease. The same results were mentioned in the literature of Inoue *et al.* (2001); Pass *et al.* (2006) and Malm & Engman (2007).

In the current study, ultrasonography was able to detect some CMV-related abnormalities such as intrauterine growth retardation (IUGR), fetal hydrops, and microcephaly. This agreed with that reported by Collinet *et al*. (2004).
In the current study cytomegalovirus infection was associated with liver and renal derangement, thrombocytopenia and anemia either in infected mothers or their infants, these results correlate with the results of Pass *et al.* (2006) and the review of Gaytant *et al.* (2002) and Kriss (2009).

Abortions, stillbirths, and neonatal deaths were higher in study group especially in IgM +ve patients than in the control group which reflects that CMV infection could be a cause of some of these deaths. This agreed with the review of Collinet *et al*. (2004), who reported that congenital CMV infection can lead to intrauterine fetal death and neonatal death. Intrauterine growth retardation and low birth weight rate was higher in study group than in the control group. Also neonatal and fetal morbidities were higher in the study group. These results correlate with that reported in the literature by Brown and Abernathy (1998), and Collinet *et al.* (2004) that CMV infection was associated with IUGR, hepatosplenomegaly, jaundice, thrombocytopenia, hemolytic anemia, and CNS, auditory and visual squeals.

Based on the presented data, we may conclude that detection of CMV specific IgM antibodies in maternal serum may preclude the need to the more expensive PCR testing and can predict congenital CMV infection in most cases. CMV infection should be looked for in women with poor obstetric history such as habitual abortions, congenital anomalies, and females with history of infants with neurological squeals. Repeated antenatal foetal sonography can help in the diagnosis of some CMV related foetal abnormalities in patients with positive CMV antibodies.

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