Molecular detection of cytomegalovirus in pregnant women referred for the triple marker test

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Abstract

The study was carried out to correlate and analyze the cytomegalovirus (CMV) infection in pregnant women who had come for triple marker screening. Fifty blood samples were collected from women in the age group of 20 to 40 years and were tested for the detection of CMV by serum analysis with PCR and serological examination by ELISA technique. PCR and serological examination can detect CMV in serum samples and distinguish primary infection and recurrent infection. Twenty six per cent (13/50) blood samples from women showed PCR positivity for CMV infection. Serological examination of a 27 year old woman showed a negative IgG and a positive IgM, indicating a recent infection with CMV.

Keywords: Cytomegalovirus, Maternal infection, Triple marker test, Pregnant women

Introduction

Maternal infections are being increasingly recognized as a major cause of birth defects in new born babies. Cytomegalovirus (CMV) infection is probably one of the most common intrauterine infection in humans. It is characterized by mild, self limiting infection with fever in healthy individual. The prevalence of CMV infection varies from 0.3% to 2.4% and about 90% of congenitally infected infants had no clinical signs. The disease ranges from no apparent clinical signs to pre-maturity, encephalitis, deafness, hemolytic disorders and death. In India, serological surveys in different parts of the country had shown the prevalence of 80-90% seropositivity for CMV IgG antibodies in women of child bearing age¹. Maternal infection plays a critical role in pregnancy outcome especially in patients with a bad obstetrics history (BOH).

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Generally pregnant women with BOH or having abnormal sonographic findings are referred for triple marker or quad marker screening, or amniocentesis. The triple marker screening panels are tests for alpha-fetoprotein (AFP), human chronic gonadotropin (HCG) and unconjugated estriol. Inclusion of Inhibin A which is a relatively new marker, turns the triple test into quad test. During the second trimester it is expected that the level of AFP and unconjugated estriol will increase, while the amount of HCG will decrease and the amount of inhibin A will stay relatively constant. The triple test can detect approximately 60% of the pregnancies affected by trisomy 21 with a false positive rate of about 5%.

The level of each serum marker is measured and reported at a multiples of median (MoM) for women with pregnancies of the same gestational age as that the patient. A negative screening result may falsely reassure many women who are carrying an affected foetus. Conversely, a false positive result may culminate in termination of a normal pregnancy. The main aim of a screening test is to identify a group of women at significantly high risk of having an affected child and to justify the offer of a diagnostic test. Screening strategies for the detection of women infected during pregnancy have not been implemented yet. Only women considered at risk are tested serologically during the 1st trimester of pregnancy. This implies that, in many cases, sonographic examinations performed during pregnancy may be the only tool available to identify an affected foetus. Even if the entire pregnant population is screened by immunologic studies in the 1st trimester, the clinical and laboratory evidence may prove that some of fetal infections are due to maternal reinfection. In such cases, the sonographic examination may be the first means of raising suspicion of intrauterine CMV infection.

It is of great concern if a woman develops CMV infection during pregnancy. Even though the infected woman herself may not become ill, she may pass viruses to her unborn baby. In some infants the signs of CMV infection are evident at birth, in others consequences of CMV infections, such as hearing loss or mental retardation may not become apparent until later in childhood. Some children do not develop these serious effects because their mothers also pass protective antibodies to them and these children remain well, however, they may excrete the virus for several months.

Primary infections results due to acquisition of the virus during pregnancy and is seen by conversion from seronegative to seropositive for IgG antibodies to CMV. The presence of both IgG and IgM antibodies to CMV may be considered presumptive evidence of a primary maternal infection. IgG antigen avidity has been used to clarify primary or non primary infections by measuring the binding affinity of IgG antibodies\(^{(2)}\). Infants born to
mothers who are infected early in pregnancy are more likely to be small for respective gestational age and may have microcephaly and intracranial calcification, whereas those infants who are born to mothers infected later in pregnancy are more likely to have acute disease with hepatitis, pneumonia, purpura and severe thrombocytopenia. Most of the women when infected with CMV have no symptoms and very few have a disease resembling mononucleosis.

Transmission of CMV from mother to the foetus can occur throughout gestation and infection during the first 16 weeks of pregnancy has been associated with a higher incidence of damage. Congenital CMV infection can be the result of either exogenous or endogenous maternal infection. The exogenous infection can be primary or non primary as it can occur in both seronegative and seropositive women. Endogenous infection is the result of reactivation of latent virus (3).

It has been reported that the prevalence of human CMV was generally high in the developing countries and those with lower socio-economic status in developed countries (4). Human CMV prevalence was generally high among pregnant women and women of childbearing age which can have severe consequences in the offspring (5-11). Australia, Belgium, France, Germany and USA had a low seroprevalence of 40%-60%. A high seroprevalence of CMV (> 90%) was reported from Brazil, Qatar, Saudi Arabia, Taiwan and Turkey (12-19). A varying degree of human CMV seroprevalence among different ethnic groups were found in Israel and USA (20-22). Adequate studies pertaining to human CMV have not been carried out in India. An avidity index above 65% during the first trimester of pregnancy could reasonably be considered a good indicator of past CMV infection (23). An IgG avidity assay in combination with an IgM ELISA could be used for monitoring pregnant women for primary CMV infection.

The aim of this study was to determine the incidence of CMV infection in pregnant women, who were referred for triple marker test (as routine screening/or due to BOH) and to correlate CMV infection with the age of pregnant women and the gestational age.

Materials and methods

A total of 50 serum samples were randomly collected from blood samples sent for Triple Marker study. They were collected and analysed from February 2007 to April 2007. The study subjects were between the age group of 20 to 40 years. The samples were selected from patients with a period of gestation of 13-27 weeks. Serology was done for CMV IgG, IgM. CMV DNA detection was done by Nested PCR.
Nested PCR amplification

DNA from blood-serum was extracted by using QIAamp DNA Mini Kit as per manufacturer’s protocol. The PCR was performed as a Nested PCR with two sets of primers. Master mixed was prepared by using PCR reaction mixtures. The final volume was adjusted up to 50 µL by using distilled water. Nested PCR was performed using automated, computerized and thermal cycles.

The first round of amplification was performed with initial denaturation at 95°C for 3 min; second denaturation at 94°C for 30 sec was performed for 30 cycles, annealing at 50°C for 30 sec and extension at 72°C for 45 sec and the final extension was carried out at 72°C for 5 min. After the amplification of DNA in the first round of nested PCR, the amplified PCR product of the first round served as the template for the second round of nested PCR.

The second round of amplification was performed with initial denaturation at 95°C for 3 minutes; second denaturation at 94°C for 30 seconds was performed for 30 cycles, annealing at 55°C for 30 seconds and extension at 72°C for 45 seconds and final extension was at 72°C for 5 minutes. The second round PCR product which was obtained was subjected to electrophoresis. DNA extracted from CMV positive patients served as positive controls. Negative controls consisted of PCR reaction mixture with no template DNA. A 5 µL of loading dye and 10 µL of amplified PCR product was loaded in a well of 2% agarose gel. Electrophoresis was performed at 90 mA current, till the dye reached 1 cm above the lower end of the gel. Products were visualized under UV trans-illuminator.

Serological examination

Serological tests in the screening of pregnant women with CMV IgM, CMV IgG and CMV IgG avidity led to a more accurate diagnosis of CMV infection. When serological screening was performed in early gestation, it was possible to identify women at risk for intrauterine transmission of the virus, i.e., women with a primary CMV infection, who should be enrolled in prenatal diagnosis. CMV specific IgM and IgG antibodies were detected by using DIESSE ENZYWELL CMV IgG/IgM kit. Optical density (OD) was measured at 450 nm on ELISA microplate reader.

The CMV IgM and IgG profile in the 13 individuals who were CMV PCR positive were determined. The results indicated that CMV IgG seropositivity was found in 92.30% (12/13) and CMV IgM seropositivity was in 7.69% (1/13). Thus it indicated that the early detection of CMV antibody, before 21 weeks, can be a helpful tool to identify women at risk of transmitting infection.
Results and discussion

In the study group, a total of 50 serum samples of women of child bearing age of 20 to 40 years, were included. The DNA was extracted and CMV PCR was carried out. Of which 26% (13/50) were positive for CMV PCR. One out of 13 positive CMV PCR samples also showed triple test positivity (Tables 1 and 2).

| No. | Sample no. | Age | AFP  | HCG   | E2  | TMT  | CMV PCR |
|-----|------------|-----|------|-------|-----|------|---------|
|     |            |     | (n=26.26) | (n=35454) | (n=1) |      |         |
| 1   | 2FL66068   | 29  | 15.7 | 37779 | 0.53| 0.25 | Negative |
| 2   | 2FL66244   | 28  | 32.2 | 27343 | 1.01| 0.25 | Negative |
| 3   | 2FL63097   | 35  | 15.5 | 59601 | 0.5 | 0.25 | Positive |
| 4   | 2FL62082   | 29  | 29   | 22997 | 0.8 | 0.25 | Negative |
|     |            |     | (n=33.52) | (n=27935) | (n=1.8) |      |         |
| 5   | 3FL006246  | 31  | 26.3 | 12770 | 0.91| 0.25 | Negative |
| 6   | 3FL006131  | 39  | 32.8 | 22120 | 2.7 | 0.25 | Negative |
| 7   | 2FL069575  | 24  | 34.1 | 21881 | 2.5 | 0.25 | Negative |
| 8   | 2FL068132  | 34  | 28.1 | 25037 | 6.49| 0.25 | Negative |
| 9   | 2FL067373  | 31  | 13.8 | 20553 | 0.59| 0.25 | Negative |
| 10  | 2FL067049  | 30  | 20.8 | 30246 | 1.45| 0.25 | Negative |
| 11  | 2FL067101  | 25  | 28   | 28633 | 1.91| 0.25 | Negative |
| 12  | 2FL066985  | 27  | 53.6 | 27374 | 0.88| 0.25 | Negative |
| 13  | 2FL064190  | 31  | 27   | 35398 | 2.03| 0.25 | Negative |
| 14  | 2FL064185  | 40  | 22.1 | 29701 | 2.96| 0.25 | Negative |
| 15  | 2FL063736  | 36  | 49.4 | 69394 | 2.66| 0.25 | Negative |
| 16  | 2FL059289  | 24  | 47.9 | 132810| 1.45| 0.25 | Negative |
| 17  | 2FL58881   | 35  | 68.2 | 29247 | 1.92| 0.25 | Negative |
| 18  | 2FL58101   | 34  | 50   | 32120 | 1.96| 0.25 | Negative |
| 19  | 2FL57639   | 31  | 73.7 | 6744  | 2.45| 0.25 | Negative |
| 20  | 2FL57034   | 27  | 23.2 | 23369 | 2.45| 0.25 | Negative |
| 21  | 2FL56964   | 36  | 38.8 | 35643 | 2.33| 0.25 | Negative |
| 22  | 2FL55182   | 28  | 45   | 20748 | 1.17| 0.25 | Negative |
| 23  | 2FL53615   | 25  | 33.4 | 14299 | 2.49| 0.25 | Negative |
| 24  | 2FL53540   | 23  | 18.6 | 18486 | 2.15| 0.25 | Negative |
| 25  | 2FL53536   | 27  | 46.9 | 28719 | 2.42| 0.25 | Negative |
| 26  | 2FL52847   | 31  | 36.7 | 17513 | 1.28| 0.25 | Negative |
| 27  | 2FL52641   | 39  | 31.9 | 28405 | 1.94| 0.25 | Negative |
| 28  | 2FL48109   | 33  | 32.5 | 31882 | 0.87| 0.25 | Negative |
| 29  | 9FL012164  | 30  | 27   | 26838 | 0.85| 0.25 | Negative |
| 30  | 9FL10694   | 24  | 25.5 | 26838 | 0.85| 0.25 | Negative |
| 31  | 9FL10689   | 34  | 48.4 | 41801 | 1.8 | 0.25 | Negative |
| 32  | 9FL10116   | 33  | 60.6 | 29944 | 1.42| 0.25 | Negative |
| 33  | 9FL08607   | 40  | 31.2 | 27009 | 2.08| 0.25 | Positive |
### Table 2. Details of women positive for CMV PCR

| No. | Sample no. | Age | AFP  | hCG   | E2   | Triple Marker Test | CMV PCR |
|-----|------------|-----|------|-------|------|--------------------|---------|
| 1   | 2FL66244   | 28  | 32.2 | 27343 | 1.01 | Negative           | Positive |
| 2   | 3FL006131  | 39  | 32.8 | 22120 | 2.7  | Negative           | Positive |
| 3   | 2FL069575  | 24  | 34.1 | 21881 | 2.5  | Negative           | Positive |
| 4   | 2FL066985  | 27  | 53.6 | 27374 | 0.88 | Negative           | Positive |
| 5   | 2FL57034   | 27  | 23.2 | 23369 | 2.45 | Negative           | Positive |
| 6   | 2FL55182   | 28  | 45   | 20748 | 1.17 | Negative           | Positive |
| 7   | 2FL53540   | 23  | 18.6 | 18486 | 2.15 | Negative           | Positive |
| 8   | 2FL52847   | 31  | 36.7 | 17513 | 1.28 | Negative           | Positive |
| 9   | 9FL012164  | 30  | 27   | 26838 | 0.85 | Negative           | Positive |
| 10  | 2FL053263  | 36  | 59.9 | 57733 | 5.89 | Positive           | Positive |
| 11  | 2FL051933  | 35  | 111  | 10526 | 5.7  | Negative           | Positive |
| 12  | 2FL056385  | 26  | 45.6 | 18358 | 6.6  | Negative           | Positive |
| 13  | 2FL048559  | 34  | 102  | 31012 | 6.6  | Negative           | Positive |
This showed a reliable distinction between primary and non-primary CMV infection in pregnant women and for the identification of pregnant women at risk of transmitting the virus to their foetus. The study also indicated that the incidence of CMV infection was higher in women less than 30 years of age i.e., 34.8% (8/23) as compared to women above 30 years of age 18.5% (5/27) (Tables 3 and 4).

| Age of pregnant women | CMV infection (%) |
|-----------------------|-------------------|
| < 30 years            | 34.8% (8/23)      |
| > 30 years            | 18.5% (5/27)      |

Table 3. CMV infection and age of pregnant women

| No. | Sample no. | Age | Gestational week |
|-----|------------|-----|------------------|
| 1   | 2FL53540   | 23  | 16th week        |
| 2   | 2FL069575  | 24  | 16th week        |
| 3   | 2FL056385  | 26  | 21st week        |
| 4   | 2FL57034   | 27  | 16th week        |
| 5   | 2FL066985  | 27  | 16th week        |
| 6   | 2FL55182   | 28  | 16th week        |
| 7   | 2FL66244   | 28  | 14th week        |
| 8   | 9FL012164  | 30  | 16th week        |
| 9   | 2FL52847   | 31  | 16th week        |
| 10  | 2FL048559  | 34  | 21st week        |
| 11  | 2FL051933  | 35  | 21st week        |
| 12  | 2FL053263  | 36  | 21st week        |
| 13  | 3FL0063131 | 39  | 16th week        |

Table 4. Age distribution CMV PCR positive samples

These findings correlated with a study which states that the rate of CMV infection in pregnant women did not increase with the age of patient. However, it was consistently high in women of less than 30 years of age. The presence of 315bp PCR product indicated a positive result, whereas absence of the 315bp band indicated that the sample was negative for CMV. When a correlation of the rate of CMV infection with gestational age was done it was found that at 21 weeks of gestation, a higher percentage of women were infected i.e., 50% (4/8). (Table 5).
### Table 5. CMV infection and gestational age

| Gestational age/week | % of CMV infection |
|----------------------|--------------------|
| 14th week            | 25% (1/4)          |
| 16th week            | 27% (8/29)         |
| 21st week            | 50% (4/8)          |

The result was in concordance with a study by (2), which stated that, ‘women infected with CMV during late gestation are more likely to transmit the virus to their unborn child than women who are infected in early gestation’. The result was in concordance with a study by (2), which stated that, ‘women infected with CMV during late gestation are more likely to transmit the virus to their unborn child than women who are infected in early gestation’. The result was in concordance with a study by (2), which stated that, ‘women infected with CMV during late gestation are more likely to transmit the virus to their unborn child than women who are infected in early gestation’. The result was in concordance with a study by (2), which stated that, ‘women infected with CMV during late gestation are more likely to transmit the virus to their unborn child than women who are infected in early gestation’. The result was in concordance with a study by (2), which stated that, ‘women infected with CMV during late gestation are more likely to transmit the virus to their unborn child than women who are infected in early gestation'.

### Table 6. Serological examination of CMV positive individuals

| No. | Sample no | Age | Gestational week | CMV IgG result IU/mL | CMV IgM result in ratio |
|-----|-----------|-----|------------------|----------------------|------------------------|
| 1   | 2FL053540 | 23  | 16th week        | Positive (10.21)     | Negative (0.66)        |
| 2   | 2FL069575 | 24  | 16th week        | Positive (18.31)     | Negative (0.30)        |
| 3   | 2FL056385 | 26  | 21st week        | Positive (7.25)      | Negative (0.27)        |
| 4   | 2FL057034 | 27  | 16th week        | Negative (0.18)      | Positive (1.32)        |
| 5   | 2FL066985 | 27  | 16th week        | Positive (12.04)     | Negative (0.25)        |
| 6   | 2FL055182 | 28  | 16th week        | Positive (4.50)      | Negative (0.33)        |
| 7   | 2FL066244 | 28  | 14th week        | Positive (12.43)     | Negative (0.29)        |
| 8   | 9FL012164 | 30  | 16th week        | Positive (17.19)     | Negative (0.34)        |
| 9   | 2FL052847 | 31  | 16th week        | Positive (5.33)      | Negative (0.62)        |
| 10  | 2FL048559 | 34  | 21st week        | Positive (5.43)      | Negative (0.33)        |
| 11  | 2FL051933 | 35  | 21st week        | Positive (18.79)     | Negative (0.61)        |
| 12  | 2FL053263 | 36  | 21st week        | Positive (13.73)     | Negative (0.30)        |
| 13  | 3FL0063131 | 39 | 16th week        | Positive (5.44)      | Negative (0.29)        |

**Interpretation of CMV IgG and IgM**

| Interpretation- CMV IgG | Interpretation- CMV IgM |
|-------------------------|-------------------------|
| Immune: anti CMV IgG conc. is > 1.2 IU/ml | Positive: ratio is >1.2 |
| Non Immune: anti CMV conc. is < 0.8 IU/ml | Negative: ratio is <0.8 |
The women who were CMV PCR positive were also IgG positive and one individual was IgM positive indicating recent infection.

The results showed a very high percentage (26%) of pregnant women who were CMV positive. The high incidence of CMV in pregnant women who have come for triple marker test implies the importance of screening for CMV in those women who have some BOH or had shown abnormal findings on sonography. Since there is no treatment or vaccination available for CMV, more emphasis needs to be laid upon educating women to maintain good hygiene. It is therefore recommended that all pregnant women should be routinely screened for this infection. Early diagnosis will help in proper management of these patients.

**Conclusion**

CMV is one of the most important intrauterine infections in pregnant women. A higher percentage of CMV PCR positivity 26% (13/50) was observed in women who were referred for triple marker test. The rate of CMV infection was found to be higher in women of less than 30 years of age i.e. 34.8% (8/23) as compared to 18.5% (5/27) women over 30 years of age. The rate of women infected with CMV at 21 weeks of gestation was higher i.e. 50 % (4/8). In serological examination one of the pregnant women (27 years) at 16 weeks of gestation showed IgG negativity and IgM positivity, indicating recent CMV infection.

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