Paper

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 age[(1)]. Maternal infection plays a critical role in pregnancy out come 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 95ºC for 3 min; second denaturation at 94ºC for 30 sec was performed for 30 cycles, annealing at 50ºC for 30 sec and extension at 72ºC for 45 sec and the final extension was carried out at 72ºC 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 95ºC for 3 minutes; second denaturation at 94ºC for 30 seconds was performed for 30 cycles, annealing at 55ºC for 30 seconds and extension at 72ºC for 45 seconds and final extension was at 72ºC 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% agrose 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).

Table 1. Triple marker test and PCR

No.	Sample no.	Age	AFP	HCG	E2	TMT	CMV PCR
14 weeks			(n=26.26)	(n=35454)	(n=1)		
1	2FL66068	29	15.7	37779	0.53	Negative	Negative
2	2FL66244	28	32.2	27343	1.01	Negative	Positive
3	2FL 63097	35	15.5	59601	0.5	Positive	Negative
4	2FL 62082	29	29	22997	0.8	Negative	Negative
16 weeks			(n=33.52)	(n=27935)	(n=1.8)		
5	3FL006246	31	26.3	12770	0.91	Negative	Negative
6	3FL 006131	39	32.8	22120	2.7	Negative	Positive
7	2FL 069575	24	34.1	21881	2.5	Negative	Positive
8	2FL068132	34	28.1	25037	6.49	Negative	Negative
9	2Fl067373	31	13.8	20553	0.59	Negative	Negative
10	2FL067049	30	20.8	30246	1.45	Negative	Negative
11	2FL067010	25	28	28633	1.91	Negative	Negative
12	2fL066985	27	53.6	27374	0.88	Negative	Positive
13	2FL064190	31	27	35398	2.03	Negative	Negative
14	2FL064185	40	22.1	29701	2.96	Positive	Negative
15	2FL063736	36	49.4	69394	2.66	Negative	Negative
16	2FL059289	24	47.9	132810	1.45	Negative	Negative
17	2FL58881	35	68.2	29247	1.92	Negative	Negative
18	2FL58101	34	50	32120	1.96	Negative	Negative
19	2FL57639	31	73.7	6744	2.45	Negative	Negative
20	2FL57034	27	23.2	23369	2.45	Negative	Positive
21	2FL56964	36	38.8	35643	2.33	Negative	Negative
22	2FL55182	28	45	20748	1.17	Negative	Positive
23	2FL53615	25	33.4	14299	2.49	Negative	Negative
24	2FL53540	23	18.6	18486	2.15	Negative	Positive
25	2FL53536	27	46.9	28719	2.42	Negative	Negative
26	2FL52847	31	36.7	17513	1.28	Negative	Positive
27	2FL52641	39	31.9	28405	1.94	Positive	Negative
28	2FL48109	33	32.5	31882	0.87	Negative	Negative
29	9FL012164	30	27	26838	0.85	Negative	Positive
30	9FL10694	24	25.5	26838	0.85	Negative	Negative
31	9FL10689	34	48.4	41801	1.8	Negative	Negative
32	9FL10116	33	60.6	29944	1.42	Negative	Negative
33	9FL08607	40	31.2	27009	2.08	Positive	Negative

19 weeks			(n=51)	(n=15753)	(n=4.1)		
34	2GA001222	34	29.6	10010	4.16	Negative	Negative
35	2GA001196	28	53.7	18508	9.74	Negative	Negative
36	2FL062553	30	79.4	29406	4.97	Negative	Negative
37	2FL069197	30	44.3	13629	2.97	Negative	Negative
38	2FL069071	29	54.1	19323	6.28	Negative	Negative
20 weeks			(n=58.51)	(n=13662)	(n=4.6)		
39	9FL013719	33	72	25404	7.45	Negative	Negative
40	2FL68304	25	59	6902	5.17	Negative	Negative
41	2FL66136	23	77.2	14468	4.67	Negative	Negative
42	2FL61455	36	56.6	11331	5.76	Negative	Negative
21 weeks			(n=75.41)	(n=11263)	(n=5.70)		
43	2FL063055	31	42.1	14461	8.07	Negative	Negative
44	2FL062470	25	70.3	61023	4.19	Negative	Negative
45	2FL060770	36	35.5	14287	2.28	Negative	Negative
46	2FL053263	36	59.9	57733	5.89	Positive	Positive
47	FL051933	35	111	10526	5.7	Negative	Positive
48	2FL056385	26	45.6	18358	6.6	Negative	Positive
49	2FL048559	34	102	31012	6.6	Negative	Positive
50	2FL070420	24	33.9	76849	0.81	Negative	Negative

AFP= Alpha-fetoprotein, HCG=Human Chronic Gonadotropin, TMT= Triple Marker Test, PCR=Polymerase Chain Reaction

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	3FL 006131	39	32.8	22120	2.7	Negative	Positive
3	2FL 069575	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).

Table 3. CMV infection and age of pregnant women

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

Table 4. Age distribution CMV PCR positive samples

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

These findings correlated with a study[2] which states that the rate of CMV infection in pregnant women did not increase with the age of the 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 individuals who are CMV PCR positive were studied for serological examination. CMV IgG seropositivity was found in 92.30% (12/13) and CMV IgM seropositivity was found in 7.69% (1/13) (Table 6).

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 Non Immune : anti CMV conc. is < 0.8 IU/ml	Positive: ratio is >1.2 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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