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CMV BY RT-PCR IN PRENATAL DIAGNOSIS THE DETECTION OF CMV IN AMNIOTIC FLUID AND CERVICOVAGINAL SMEAR SAMPLES BY REAL-TIME PCR ASSAY IN PRENATAL DIAGNOSIS

Aydan BİRİ[*], Gulendam BOZDAYI[**], Banu ÇİFTÇİ[*], Bora DOĞAN[**], Aysegul YÜCE[***], Seyyal ROTA[**], Ozdemir HİMMETOĞLU[*]

* Gazi University School of Medicine, Department of Obstetrics & Gynecology, Ankara, Turkey
** Gazi University School of Medicine, Department of Medical Microbiology, Ankara, Turkey
*** Gazi University School of Medicine, Department of Immunology, Ankara, Turkey

SUMMARY

Objective: *Prenatal diagnosis has a critical role in the management of pregnancy complicated by CMV infection. The identification of reliable prognostic markers of fetal disease remains the main purpose and a major challenge on this issue.*
Design: *In this study, we investigated the prevalence and clinical consequences of CMV infection from cervicovaginal smear and amniotic fluid samples of pregnant women by using RT-PCR assay.*
Setting:*The study was performed in Gazi University, Faculty of Medicine, Obstetrics and Gynecology outpatient clinic. Patients: Two hundred and six samples, of which 135 was cervicovaginal smear and 71 was amniotic fluid, were enrolled in the study.*
Main outcome measures: *Clinical outcomes of CMV RT-PCR positive pregnancies and reliability of RT-PCR assay in prenatal diagnosis of this infection.*
Results: *CMV DNA was found to be positive in 1.5% (2 in 135) of cervicovaginal smear and 1.4% (1 in 71) of amniotic fluid samples by RT-PCR. IgM and IgG were found to be negative in all of the cervicovaginal smear samples by both MEIA and ELISA, while IgG antibody was found to be positive in only one of the amniotic fluid samples by MEIA.*
Conclusions: *The fact that, the clinical consequence of the newborn whose amniotic fluid evaluation revealed CMV infection by RT-PCR made us think that this molecular diagnosis method may be a reliable assay in prenatal diagnosis of this pathogen.*

Key words: *CMV, intrauterine infections, real time PCR (RT-PCR)*

ÖZET

Prenatal Tanıda, Amniyotik Sıvı ve Servikovajinal Smear Örneklerinde, Real-Time PCR Assay Tekniği ile CMV'nin Tespiti

Objektif: *CMV infeksiyonu ile komplike olmuş gebeliklerin yönetiminde, prenatal tanının kritik bir rolü vardır. Fetal infeksiyonu gösteren güvenilir prognostik belirleyicilerin tanımlanması bu konudaki asıl çelişki ve amacı oluşturmaktadır. Planlama: Bu çalışmada, RT-PCR tekniğini kullanarak, gebelerin servikovajinal smear ve amniyotik sıvı örneklerinde, CMV infeksiyonunun prevalansını ve klinik sonuçlarını araştırdık.*
Ortam: *Çalışma, Gazi Üniversitesi, Tıp Fakültesi, Kadın Hastalıkları ve Doğum polikliniği'nde gerçekleştirildi.*
Hastalar: *Yüz otuz beşi, servikovajinal smear, 71 i amniyotik sıvı olmak üzere, 206 örnek çalışmaya alındı.*
Değerlendirme parametreleri: *CMV RT-PCR positif olan gebeliklerin klinik sonuçları ve RT-PCR tekniğinin bu infeksiyonun prenatal tanısında güvenilirliği araştırıldı.*

Adress for Corresponding: Banu ÇİFTÇİ. Gazi Üniversitesi Tıp Fakültesi Kadın Hastalıkları ve Doğum Anabilim Dalı Beşevler, ANKARA
Tel: (312) 202 59 43
Fax: (312) 215 77 36
e-mail:banuciftci@hotmail.com
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Sonuç: *CMV DNA, RT-PCR tekniği kullanılarak, servikovajinal smear örneklerinin %1.5 (2\135) ve amniyotik sıvı örneklerinin %1.4 (1\71) inde pozitif bulundu. Hem MEIA hem de ELISA, yöntemiyle, IgM and IgG tüm servikovajinal smear örneklerinde negatif bulundu. Amniyotik sıvı örneklerinin ise sadece birinde IgG pozitif bulundu.*

Yorum: *RT-PCR tekniği ile amniyotik sıvısında CMV infeksiyonu pozitif bulunan yenidoğanın klinik sonuçları, bize, bu moleküler metodun, patojenin prenatal tanısında güvenilir bir teknik olabileceğini düşündürdü.*

Anahtar kelimeler: *CMV, intrauterin infeksiyon, real time PCR (RT-PCR)*

BACKGROUND

Human cytomegalovirus (CMV), a member of the herpes virus family is the most common cause of intrauterine viral infection and sensory neural deafness, affecting 0.2–2% of live births[1]. Unlike most viral infections, CMV may be transmitted to the fetus during either primary infection or reactivation[2]. Following primary infection, the virus is transmitted to the fetus in approximately 40% of cases (21–68%)[3, 4, 5, 6, 7, and 8]. In case of recurrent infection, fetal infection is rare (2%)[2]. Ten percent of infected fetuses are symptomatic at birth, consisting mainly of central nervous system and multiple organ involvement; 1/3 of them may die and most survivors suffer serious neurological and systemic sequels[9 and 1]. The remaining 90% of infected fetuses are asymptomatic, but 10–15% will develop sequels within the first year of life, including progressive deafness, visual impairment, learning disability, and delayed development[10]. In the absence of specific antiviral therapy or a vaccine which could be safely administered to the pregnant women with primary human cytomegalovirus infection, prenatal diagnosis has a critical role in the management of pregnancy complicated by this virus. Prenatal diagnosis may be performed for several reasons: routine prenatal screening; recent maternal exposure to an infectious pathogen; maternal symptoms of infection or abnormal sonographic findings (e.g. ventriculomegaly or intracranial calcifications)[11]. A combination of tests, including serology, avidity, and polymerase chain reaction (PCR), may be necessary to improve accuracy of the diagnosis. The interval between exposure to an infectious agent and prenatal testing can be critical to the interpretation of the test result. Available methods used to detect CMV include, culture, shell vial assays, antigenemia assays (which quantify positively stained blood leukocytes), PCR to detect and quantify CMV DNA, and more recently, nucleic acid sequence-based amplification techniques to detect messenger RNA of specific CMV proteins[12]. The objective of the present study is the diagnosis and quantification of CMV DNA, together with investigation of the congenital CMV infection in the CMV positive cases. Additionally, correlation of the amniotic fluid and cervicovaginal smear CMV results with sonographic findings has been evaluated.

MATERIAL AND METHODS

Samples

Two hundred and six pregnant women were included in the study and 135 cervicovaginal smear and 71 amniotic fluid samples were studied. The cervicovaginal smear samples were obtained from asymptomatic women with no history of any accompanying diseases to the pregnancy, in their first antenatal visit. Amniotic fluid samples were achieved from cases by amniocentesis that had advanced maternal age (22 cases) or positive second trimester screening test results (28 cases). Additionally in 21 cases amniocentesis was performed because of abnormal USG findings; oligohydramnios (4 cases); intrauterine growth restriction (IUGR) (3 cases); both IUGR and oligohydramnios (3 cases); ventriculomegaly (4 cases); bilateral choroid plexus cysts greater than 7mm (1 case); both echogenic bowel and choroid plexus cyst (1 case); polyhydramnios (2 cases); omphalocele (1 case); multiple structural abnormalities (1 case), and hydrops fetalis (1 case). CMV DNA was detected in these 71 amniotic fluid samples and was found to be positive in one case by Real Time Polymerase Chain Reaction assay (RT-PCR) of which viral load was 1.309 105 copy/ml.

Cervicovaginal smear samples were transported in phosphated buffer saline (PBS) solution and amniotic fluid samples were taken and transported in sterile syringes. The samples, which were transported to the laboratory, were liquated and while the samples, which

are to be studied, were taken to study, the others were stored at –80 Co until they were studied.

Real Time Polymerase Chain Reaction (RT-PCR) DNA extraction: 200_l of cervicovaginal smear or amniotic fluid was used to extract DNA of CMV by using High Pure Viral Nucleic Acid Kit (Roche diagnostics Germany) according to the manufacturer's instructions.

Amplification of DNA by RT-PCR and Quantification: Metis Biotechnology (Ankara, Turkey), projected quantitative CMV primers and probe. By using primer premier 5.0 software from pp65 gene (gene bank locus HSPPBC region) (primer 1:5' –ATATCGAAA AAGAAGAGCGC, primer 2 : 5' – GGTAACCT GTTGATGAACG , probe: 5' – FAMGGGATCGT ACTGACGCAGTTCCACTAMRA 3'). The presence of PCR products was detected by an increase in fluorescence signal in Light-Cycler System (Roche Diagnostic, Germany) 3_l of extracted DNA, 10pmol from each primer, 4pmol of probe, 4,5mM of MgCl and 1 unit Taq DNA polymerase and 1X Reaction Hybridization mix from Light-Cycler Master Hybridization Probe Kit (Roche Diagnostics, Germany) were used in Real time PCR reactions. Amplification was carried out in capillary tubes containing 10 _l total reaction volume in stages of incubation at 95 Co for 10 minutes following 45 cycles of 95 Co for 10 second and 60 Co for 10 second. In each study, HCMV AD 169 DNA containing 2 x 102 – 2 x 106 CMV DNA copy with four serial dilutions was used and quantitative results were analyzed by using Light-Cycler software version 3.5.3. A capillary tube containing distilled water instead of DNA was used a negative control in each study. The determining limit was established as 200 CMV DNA / ml in blood.

Serology

Anti-CMV IgM antibody: Specific IgM responding to CMV in amniotic fluid and cervicovaginal smear samples was studied with two different methods: microparticle enzyme immunoassay (MEIA) system and its appropriate anti-CMV IgM kit (Imx System, Abbott Laboratories, Abbott Park, ILL,USA) and a commercial diagnostic anti-CMV IgM enzyme-linked immunosorbant assay (ELISA) kit (Radim SPA, Pomezia-Rome, Italy).

Anti-CMV IgG antibody: Specific IgG responding to CMV in amniotic fluid and cervicovaginal smear samples was studied with MEIA system and its appropriate anti CMV IgG kit (Axsym System, Abbott Laboratories, Abbott Park, ILL,USA).

CMV IgG avidity test: The amniotic fluid sample with positive anti-CMV IgG and the cervicovaginal smear sample with 4U/ml of anti-CMV IgG were studied with a commercial diagnostic CMV IgG avidity EIA (Enzyme immunoassay) kit (Radim SPA, Pomezia-Rome, Italy) according to the manufacturer's instructions.

RESULTS

RT-PCR: One (1/71) of the amniotic fluid samples (1,309 x 105 copy/ml) and two (2/135) of the cervicovaginal smear samples (corresponding to 2,937 x 101 and 2,124 x 102 copy/ml viral loads) were found to be positive in favor of CMV.

Serology

Anti CMV IgM results: Anti CMV IgM was found to be negative in all of the amniotic fluid and cervicovaginal smear samples.

Anti CMV IgG results: In all of the samples studied, one amniotic fluid was found to be anti CMV IgG positive (96 U/ml) and one cervicovaginal smear sample was found to be 4U/ml, with negative result. Anti CMV IgG and IgM results were 0 U/ml in the other samples. Because both tests for anti CMV IgM, and CMV IgG were designed for only serum evaluation, the cut off values for amniotic fluid and smear may be lower than the cut off values (10 U/ml) accepted for serum. In this respect, the cervicovaginal smear sample with 4 U/ml of anti CMV IgG value was also approved as positive and studied with CMV IgG avidity test.

CMV Ig G Avidity test results: According to the kit that we used, we considered the values below 35% as a low avidity, values between 35% - 45% as a gray zone and above 45% as a high avidity. Low avidity is a finding in favor of a primary acute infection. The amniotic fluid sample with positive anti-CMV IgG was studied with CMV IgG avidity test to evaluate a primary acute infection and, low avidity (25%) was found. This finding was in favor of a primary acute infection.

The cervicovaginal smear sample with 4 U/ml of anti-CMV IgG value was also studied with CMV IgG

avidity test and again low avidity (21%) was found. This situation was also suggested a primary acute infection and supported the hypothesis that anti-CMV IgG cut off limit could be lower than serum in these kind of samples.

DISCUSSION

Although, CMV screening during pregnancy has been greatly discussed for many years, there has been no consensus on this issue yet. If the mother seroconverts during pregnancy and no maternal or fetal clinical manifestations are seen, the fetal management will be difficult to predict, as its outcome is unknown. Several factors such as gestational age at the time of maternal CMV infection, the level of viral replication in both mother and fetus, possible differences in viral virulence and the immune response might influence the outcome of fetal infection[13, and 14]. Most of these newborns will never develop any type of complication. Moreover, if any sequel occurs, there has been no specific treatment to reduce its incidence or severity yet. We should also, state that, routine testing would lead to unnecessary therapeutic abortion.

Prenatal diagnosis of fetal CMV infection is important for informed decision-making regarding pregnancy management and for planning of strategies for follow-up of newborns at risk. Transmission is not affected by the stage of pregnancy[10, 15, and 16]. However, some authors reported an increased rate of transmission with increased gestational age[17, and 18]. Bodeus et al. reported transmission rates of respectively 36, 45 and 77% for the first, second, and third trimester[18]. Viral isolation from amniotic fluid by shell-vial assays or conventional cell cultures, although reliable indicators of fetal infection with 100% specificity and positive predictive value[1, 4, 6, 8, 19, 20, 21, 22, 23, 24, 25, 26, and 27], has low sensitivity and can lead to misdiagnosis in up to 50% of cases[19, 20, 23, 25, and 26]. PCR detection of viral DNA in amniotic fluid can detect the presence of viral DNA in 7–50% of culture negative amniotic fluid samples[6, 8, 21, 23, and 26]. Viral isolation, the classic method for diagnosing congenital CMV infection, presents technical limitations to its large-scale use. PCR assays provide some advantages, being particularly fast and practical techniques, as well as allowing the use of stored samples[28]. RT-PCR provides an accurate means of quantifying viral DNA; with the major advantage of avoiding post-PCR handling that can be the source of DNA carryover[29]. However, PCR sensitivity is still sub-optimal, ranging between 71% and 100% and resulting occasionally in false-negative results in amniotic fluid[3, 4, 5, 6, 7, 21, 23, 26, and 29].

Three major variables were suggested to cause the false-negative PCR results: gestational age at the time of amniocentesis, elapsed time between maternal infection and amniotic fluid sampling, and the intrinsic sensitivity of the PCR protocol used for diagnosis. Sampling of amniotic fluid before 21 weeks of gestation and less than 6–9 weeks following maternal infection was associated with low PCR sensitivity[5, 6, 19, 20, and 29]. All our amniotic samples were taken before this gestational age (16-18 weeks); nevertheless, sampling time is not sufficient to explain all misdiagnosed cases using PCR. Amniotic fluid supernatant is inhibitory to PCR, which was proposed as one of the factors to reduce the intrinsic sensitivity of this assay. In addition, PCR diagnosis of congenital CMV fetal infection is currently carried out in clinical laboratories by a wide range of PCR protocols, mostly, non-standardized assays. There are two PCR-based approaches, which are commonly used for clinical diagnosis: the first is the nested technique, which includes two amplification stages, of single-round PCR followed by semi-nested or nested PCR, and the second is the probe hybridization based technique, which includes single-round PCR amplification, followed by probe hybridization. Of these two, the latter is standardized, non-labor-intensive, and allows minimal opportunity for contamination, thereby making it the preferred method for diagnosis. We used RT-PCR techniques; Real time PCR is the first choice method allowing as to evaluate the quantative measurements of viral load and to follow up the treatment efficacy. The association of viral load of CMV and the rate of affected fetus was well documented previously[29]. Therefore, we used real-time PCR method to determine the viral load of CMV to evaluate the patients if the intrauterine infections may cause on fetal anomaly.

The only positive amniotic sample for CMV PCR had been obtained from a pregnant woman presented in the 23rd week's gestation. Her ultrasound examination revealed intrauterine growth restriction and severe oligohydramnios. Estimated fetal weight was less than

5 percentile. There were no abnormalities in the chromosomal evaluation of the amniotic fluid, but high viral load (1,309 x 105 copy/ml) was found with RT-PCR. Therefore, CMV infection was thought to be a reason of intrauterine growth restriction. Detection of virus in the amniotic fluid does not necessarily correlate with symptomatic congenital infection[21]. The challenge is to determine the clinical consequences of fetal infection. In a study performed by Revello et al[30] . although there was no statistical difference between symptomatic and asymptomatic babies of CMV DNA (+) mothers in amniotic fluid with RT-PCR, CMV viral load was found to be higher in amniotic fluid of the mothers of symptomatic babies. Guerra et al[21] indicated that CMV DNA viral load higher than 105 copy/ml in amniotic fluid could give information about symptomatic infection. Gouarin et al[29] also detected a higher CMV viral load in symptomatic cases than in asymptomatic ones in amniotic fluid. Our results seem to be parallel to those of all authors.

Two week after diagnosis, the fetal death occurred, in 25th week. In ELISA studies from amniotic fluid, anti-CMV IgG was positive and IgM was negative. CMV IgM in amniotic fluid demonstrates fetal IgM because IgM isotype antibodies cannot be transported from mother by placental way. In this case, amniotic fluid was investigated with CMV IgG avidity test and, low avidity was found. In the maternal serum both anti-CMV IgM, and IgG were positive and IgG avidity was low. The help of these findings established primary acute CMV infection in mother and fetus. Nevertheless, the virus had to be demonstrated in fetal and placental tissues in order to mention an absolute fetal infection. We also found CMV DNA positivity in fetal and placental tissues. This finding confirmed primary acute CMV infection. All these results, with combination of clinical and laboratory findings made us think that the cause of the fetal death was primary acute infection of the mother and fetus.

It is indicated that virus spread is frequent in pregnant women with asymptomatic infection and virus can be isolated from 2-28% of pregnant women in cervical mucus and urine[28]. Latent CMV infection has an affinity to cervical and renal cells[31, and 32]. Probably because of this latency and transient humoral and cellular immunity, suppression during pregnancy and hormonal changes cervical CMV secretion increases [33]. It is demonstrated that uterine tissues can be infected via cervical or hematogenous way. In the studies done about this subject, a sexual transport of CMV infection is shown in sexually active women[34, 35, and 36]. Virus can spread from cervix to uterus ascendantly in primary or reactivated infections of CMV[34]. There are some studies supporting this idea about CMV secretion from cervix in young non-pregnant women[35, and 37]. It is demonstrated that CMV secretion from cervix increases during pregnancy (35%)[37, and 38]. In comparison with lung, liver kidney and blood vessels (15% positive) CMV DNA level in uterine tissue and cervical smear was found to be higher (50%)[39]. These findings show that, CMV replicates continuously and is secreted from cervix in sexually active women. As a result, all these data show that, CMV infection spreads to placenta and then to embryo or fetus before spreading to uterine tissues[34].

In the 135 smear samples included in our study, two of them were found to be positive with RT-PCR with a quantification of $2,937x10^1$ and $2,124x10^2$ copy/ml. Both of these quantification results seem to be very low. However, a possible viral spread to these babies during delivery and a sexual transport to partners must be taken into consideration. There were no clinical findings and no abnormalities on USG examinations in these pregnant women. Both cases delivered healthy neonates at term, one with vaginal delivery, the other with cesarean section due to an obstetrical reason. No clinical or laboratory abnormalities have been observed in newborns and CMV DNA could not to be detected in the urine samples of these newborns by RT-PCR. ELISA was studied for both of these pregnant women in cervicovaginal smear samples. While anti-CMV IgM was found to be negative in both of the smear samples, CMV IgG was 4 U/ml in the first smear sample and non-detectable in the second one. Both test systems studying anti-CMV IgM and IgG were designed for serum evaluation and probably, the cut off values for cervicovaginal smear may be lower than the value considered for serum. In this respect, the smear sample with 4U/ml of anti- CMV IgG was accepted to be positive and CMV IgG avidity test was performed for this sample and avidity was found to be low (21%). This situation was compatible with primary acute CMV infection and supported the hypothesis that anti-CMV IgG cut off limit of these kinds of samples could be lower than serum limit. We should also state that CMV IgG avidity testing is currently the most widely used

technique for differentiating between primary and past CMV infection[4].

However recently, to distinguish between CMV primary or past infection, an enzyme immunoassay (EIA) test was developed to assess the IgG response to the CMV glycoprotein B (Biotest, Dreieich, Germany)[40]. Despite the fact that CMV is isolated as the most frequent causative agent in congenital infections and despite the augmentation of CMV carriage in cervix during pregnancy, we found CMV DNA positivity in only two of 135 cases (1.5%). This may be a characteristic of our pregnant group. Nevertheless, since the results are lower than the ones studied before, they are not fitting to our main goal such as detecting CMV DNA in cervical smears by RT-PCR and comparing with anti-CMV IgM and IgG results. Low viral load and CMV DNA positivity were found in only two cases, and maternal or fetal complications did not occur. Besides, in only one of these cases anti-CMV IgG positivity was found. Since we cannot say that demonstration of CMV DNA with RT-PCR in cervix is more sensitive than serology with limited data in this case, advanced studies are needed for more accurate predictions.

To demonstrate CMV existence in smear with RT-PCR guides us to make a decision about type of delivery, since it has a risk of newborn infection by ascendant spread. In addition, a low avidity in CMV IgG avidity test may be an important sign for perinatal outcomes. While the advances in immunologic, sonographic and molecular biological techniques have improved the clinicians' ability to diagnose both maternal and fetal infections, the clinicians' knowledge of the usefulness and limitations of these tests is essential to avoid unnecessary maternal anxiety and to prevent potentially adverse obstetric interventions.

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