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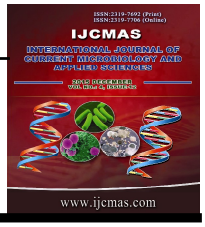

Original Research Article

A Study on the Feasibility of Identifying Rubella, Human Cytomegalovirus and Herpes Simplex Virus by Application of Polymerase Chain Reaction (PCR) on Fetal Samples

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Keywords

Amniotic fluid, Congenital infections, Fetal blood samples, Reverse-transcriptase Polymerase Chain Reaction

A B S T R A C T

To assess the feasibility of detecting Rubella virus (RV), Human cytomegalovirus (HCMV) and Herpes simplex virus (HSV) in fetal samples with antenatal suspicion of congenital infection and to correlate the results with ultrasonographic findings. A prospective study was carried out using amniotic fluid / cord blood samples obtained from pregnant women with fetuses suspected as possibly having an intra-uterine congenital infection ,. Samples were processed by PCR for the presence of RV, HCMV, and HSV. RV amplified products were further sequenced to identify the genotype. Out of 44 samples tested, 37 were included in this study for analysis as remaining 7 had an abnormal karyotype. A total of 6 samples tested positive for viral PCR (2 CMV and 4 RV; 5 AF and 1 FBS). The commonest indication for fetal testing for viral PCR was echogenic small b owel, followed closely by intra-uterine growth restriction and non-immune hydrops. Approximately half of the cases had more than one ultrasound marker at presentation. None of the samples were positive for HSV. It is feasible to test fetal samples for CMV, RV and HSV in India. No ultrasound marker appeared to be specific for a particular viral infection.

Introduction

Human cytomegalovirus (HCMV) and Rubella virus (RV) are common causes of intrauterine viral infections [1]. The incidence of fetal infection and the risk of congenital deformities are particularly high after RV infection in pregnant women. Despite the availability of specific vaccine, RV infection during pregnancy is still a problem and surprisingly, in several countries RV vaccination is still optional. The exact prevalence of viral infections in pregnant women or congenital infections in fetuses or neonates in India remains unknown owing to lack of a standardized approach and to the lack of standardized reference laboratories

in the country. This is compounded both by the lack of facilities to detect these infections and the fact that there is no requirement to report any detected case to a central database. Recently, a few groups have attempted to document the viral status in pregnant in their local regions in India and have demonstrated high prevalence of TORCH infections in women with bad obstetric histories[2].

Testing pregnant women for viral infections by the "TORCH" (Toxoplamosis, Rubella, Cytomegalovirus and Herpes Simplex) screening test is generally carried out on an ad hoc basis in most antenatal clinics in India. The indications used for such testing varies widely including recurrent miscarriages, rashes in the pregnant women, findings on routine and unwarranted ultrasound scans and also as part of an infertility work up. The vast majority of reports do not get translated into clinically relevant details on the patient records. It has been reported from selected regions in India that up to 30% of pregnant women may be identified as having IgM for Rubella in pregnancy [2, 3]. It is of note that this kind of screening is not universal and does not receive the attention it deserves. However, in cases where the results are noticed, the detection of RV-specific immunoglobulin M (IgM) during pregnancy does send panic waves and in many cases leads to termination of the pregnancy. Although there is a high risk of fetal transmission and embryopathy, it is well known that the presence of IgM does not always imply the occurrence of a recent infection nor do all RV infections during pregnancy cause embryopathy in the fetus[4].

Ultrasound has been used for nearly 30 years as the main modality to help diagnose fetal CNS anomalies [5]. Demonstration of characteristic ultrasound findings in the high-risk patient play a vital role and has a high predictive value for fetal infection and in case of the low-risk patient, fetal infection should be considered when multiple organ system anomalies, fetal growth restriction, placental enlargement, or abnormalities of amniotic fluid AF volume are demonstrated[6]. Rubella infection is generally is associated with a very few complications, but when acquired during first trimester of pregnancy by pregnant woman, the virus is transmitted to the foetus with 90 per cent chances of development of congenital malformations in the newborn[7].

In HCMV infections, fetal damage is mostly related to primary rather than recurrent maternal infection. In this case, the transmission of the virus to the fetus may occur in 20 to 50% of pregnancies [8, 9]. HCMV infection occurs in 0.2% to 2.2% of all live births and is the most common cause of intrauterine infection and the leading infectious cause of sensorineural hearing loss and mental retardation 10. It has been reported that HCMV transmission rates increase with gestational age and that the highest risk of transmission to the fetus is observed after seroconversion in late pregnancy[1].

Prenatal diagnosis of congenital human cytomegalovirus (HCMV) infection and Rubella virus nowadays is performed by rapid virus isolation from and/or Polymerase Chain Reaction (PCR) of viral RNA/DNA in amniotic fluid (AF) or fetal blood samples (FBS). In general, both techniques have been shown to provide high sensitivity (70 to 90%) and good specificity (96 to 100%) with the exception of a few reports from a single group showing low specificity and positive predictive value for PCR on AF samples[13].

In a previous study conducted by us at our tertiary care ophthalmic centre, we have

demonstrated RV by PCR technique in lens aspirates from 18% of infants with congenital cataract and HSV2 association with 18% infants and HCMV was not detected in any of the infants. CMV was not detected in any of these cases [7]. In the current study, our aim was to assess the feasibility of detection of these viral pathogens in fetal samples and to correlate this with the prenatal ultrasound findings.

Materials and Method

All pregnant women referred to a tertiary care centre from 2008-2010, with antenatal scan features suggestive of Congenital Viral Infections were included in the study if they opted for invasive testing as a confirmatory test.

All pregnant women with antenatal scan features suggestive of Congenital Viral Infections were referred to MediScan Systems, Chennai a tertiary care centre during a period of 3 years from 2008-2010, were included in the study if they opted for invasive testing as a confirmatory test.

The details of the findings at the time of the scan were noted, along with maternal age and gestational age at presentation.

After obtaining consent, amniocentesis or fetal blood sampling was carried out under ultrasound guidance and amniotic fluid / fetal blood was collected as described by Kerrigan and Goldberger [14]. Wherever available, maternal serology results were collated and attempts were made to correlate the results with the PCR results from the respective fetal samples.

Specimens were transported in ice in vials treated with Diethyl pyrocarbonate (DEPC) to L & T Microbiology Research Center, Vision Research Foundation, Sankara Nethralaya, Chennai for detection of HCMV, HSV and RV by Polymerase chain reaction. Ethics subcommittee of both the institutions approved the study.

Molecular Method of RNA Extraction

Rubella Virus (RV)

RNA was extracted from all specimens using Qiagen Viral RNA extraction kit (Cat no. 52904) procured from Qiagen, Hilden, Germany according to the manufacturer's instructions. The RNA was finally eluted in 60μl of AVE buffer and immediately frozen at -80º C until processed.

For Human Cytomegalovirus (HCMV) and Herpes Simplex Virus (HSV):

DNA was extracted from the amniotic fluid was extracted using QIAamp DNA Mini Kit (Cat no. 51304). The DNA eluted was stored at -20º C until processed.

Polymerase Chain Reaction

Reverse tTranscriptase PCR for RV

RV cDNA was generated using a one step RT-PCR kit (Qiagen, Germany) and nested RT-PCR for the detection of RV targeting E1 open reading frame was carried out from the RNA as described earlier [7].

PCR for Detection of HCMV

The nested PCR for the detection of human cytomegalovirus targeting *morphological transforming region II (mtrII)* was carried out as described earlier [15].

All PCR reagents used for amplification including primers were procured from Bangalore Genei, Ltd. Bangalore, India. All PCR amplifications were carried out using PCR thermal cycler Perkin Elmer Model 2700 (Applied Biosystems, Massachusetts, USA).

Real Time PCR for HCMV Quantification

The specimens that were positive by qualitative PCR were subjected to Quantitative Real time PCR. The sequences of the PCR primers and that of the probe used to quantify HCMV were selected from the *mtr*II of HCMV. The sequences of the forward (CMV PKSF3) and reverse primers (CMV- PKR 11) were 5'-TTACGCGACCAGATTGCAAGA-3' and 5'-TACCTACGTGACCTACCAACG-3' respectively. The *Taq*man probe used was 5' – (6 FAM) - CTCCGCGTCACCTTTC ATCGAGTAAA- (TAMRA) – 3'. The PCR product was detected as an increase in fluorescence with the Rotor-Gene 3000 (Corbett Research, Australia). PCR was performed with 30µl of HCMV primer probe mixture, each of the primers at a concentration of 30 picomoles, 0.006 nanomoles of Taqman probe, and 20µl of DNA in a total volume of 50µl. PCR was performed under the following conditions: after 10 min at 95^{o}C, the samples were subjected to 45 cycles, with each cycle consisting of a denaturation at 95^{o} C for 15 sec, followed by annealing at 55 ^{o} C for 30 sec, followed by extension at 72^{o} C for 20 sec. A plasmid containing the target sequence of 74 bp was used as standard. A standard graph of the cycle threshold (C_T) values obtained from serial dilutions (10 to 10^5 copies) of the plasmid was constructed for HCMV. The C_T values from unknown samples were plotted on the standard curve, and the number of HCMV genome copies/ml was calculated with Rotor gene 3000 Sequence detector (Corbett Research, Australia). As a control for cross-contamination, a reagent control containing Milli Q water in place of template DNA was included. Samples were considered negative if the C_T values exceeded 45 cycles. The intra-assay and inter-assay reproducibility were evaluated using triplicates of plasmid dilutions (10^1, 10^3 and 10^5) corresponding to an input of $2.5x10^3$, $2.5x10^5$, $2.5x10^7$ copies/ml per reaction in the same and four independent runs respectively.

Presence of inhibitors was ruled out by including spiked control in each run of the PCR reaction.

PCR for Detection of HSV

The nested PCR for the detection of Herpes simplex virus *targeting* Glycoprotein D was carried out as described earlier [16].

DNA Sequencing Reaction

Cycle sequencing reaction was carried out for the Rubella virus positive amplified products. The products were purified according to standard protocol, loaded onto ABI 3130 Genetic Analyzer (Applied Biosystem, USA) with polymer POP7 and sequences were analyzed as described earlier [17].

Results and Discussion

A total of 44 samples (42 amniotic fluid and 2 fetal blood samples) were available during the study period. Of these, 37 were included in this study as the remaining seven had an abnormal karyotype and were excluded in the final analysis. The data presented here includes only these 37 cases. Amongst these, four had an abnormal karyotype that was thought of as a normal variant. Of the two blood samples one was positive for rubella and the other was negative. The commonest indication for fetal testing for viral PCR was echogenic small bowel, with intra-uterine growth restriction following closely and non-immune hydrops being third in line (Figure 1). About 50 % of the fetus had more than one feature noted in the figure. The median maternal age was 26

(Range 18 – 37), whilst the median gestational age at sampling was 22 weeks (Range 18 to 27 weeks).

All 37 samples were good enough to be tested for molecular detection of RV, HCMV and HSV. In other words, none of the samples were rejected or failed owing to quality issues in the samples. Six of these samples tested positive for viral PCR, four for Rubella reverse transcriptase PCR and two for CMV by qualitative PCR. The CMV positive samples were further analyzed by real time PCR and they showed 130 and 200 copy numbers of HCMV respectively. None of the samples tested positive for HSV. The commonest fetal indication in the PCR positive group was echogenic bowel (Figure 2). Maternal serum antibody titers were not available for all the 37 and in the ones that were available; no particular trend was noted in the presence of IgG or IgM antibodies. In particular, of the six samples that showed positive viral particles, only three had additional maternal serology test results. No meaningful association could be drawn between maternal serology and viral PCR in the fetal samples. Of the six pregnancies that were positive for viral particles, 5 underwent termination of pregnancy. The remaining one continued the pregnancy despite testing positive for Rubella. Out of four rubella cases only one gave autopsy and that was suggestive of rubella.Out of two CMV one came for autopsy and that was positive with CMV inclusion bodies seen in histology. All others did not come for autopsy. One foetus was not terminated and is doing well. Outcome of the remaining pregnancies was identifiable only in a small number of patients and have not been included in this report.

DNA sequencing results of the amplified products of RV from the amniotic fluid specimens were multalined with sequences of reference strains Therien, Cendehill and BRD II obtained from GenBank showed significant identity (98%) with genotype IE.

This study shows that it is feasible to identify viral particles using PCR technique in India. It is also of note that all of the 37 samples included in the study were analyzed successfully with no quality issues. Six out of the 37 samples from fetuses tested positive for viral particles. These were at high risk for possible fetal infection and it was possible to rule out viral infection with certainty in the remaining 36 cases. Identifying a viral agent as the causative agent for an abnormal fetus would help the prospective parents in understanding the probable outcome for their baby. Obviously that would lead to making an informed choice to be made in any given pregnancy. Equally, ruling out a viral infection would allow the focus to shift on to other possible etiological agents.

The spectrum of findings seen on ultrasound in these fetuses is quite varied. None of the findings were specifically associated with a particular viral infection. However, the strength of such associations may not be delineated with a small sample size as in this study. The findings in this study are not different from the features described by Degani in 2006. This review also highlights the non-specific nature of the findings on ultrasound along with the fact that many intra-uterine viral infections may present with similar findings on antenatal ultrasound. However, the risk of congenital infection may usually be estimated by establishing the gestational age at the time of maternal infection [14.]

HCMV is common in all socio-economic groups and all age groups but congenital infection with significant impairment is reported to be highest among the women of higher age group [10] while in case of RV infection such an age criteria doesn't seem

to play an important role since the infection was seen at the age of even 25. Although the sample size was small in the current study, the median maternal age was 28 with a range extending from 18 to 37 years. No specific conclusions may be drawn from this, especially when there is no specific protocol or practice in India, to routinely screen the pregnant population for viral infection, which is likely to yield prevalence data.

This leads on to the next issue of interpreting IgG antibodies identified in the second trimester. In developed countries, where all pregnant women book for antenatal care in the first trimester, identification of IgG antibodies would award them with an immune status for that particular virus. This is usually carried out for Rubella and implies that she is unlikely to contract the rubella infection in the current pregnancy. In the absence of the security of such first trimester booking bloods, it becomes difficult to interpret the presence of IgG antibodies in the second trimester. Although, one could argue that further avidity tests or serial increase in titres may clarify the issue, the cost incurred by the patient and the time spent waiting for results from further tests are precious commodities. This takes further significance when taking into consideration that the law in India does not allow terminations beyond 20 weeks of gestation and therefore waiting for indefinite periods of time before performing a diagnostic invasive procedure would be not be clinically acceptable. Maternal screening for CMV is not universal worldwide, as the presence of IgG does not confer immunity against re-infection during the pregnancy. Standardization of antenatal care with guidelines for minimum requirements on a national level to include a standard set booking blood tests including status of Rubella immunity is an urgent need of the hour.

This was a prospective observational study that did not specify assessing maternal serology status as a requirement for inclusion. Not surprisingly, in a significant number of cases, maternal serology was either not done or not available for review. Requesting and interpreting viral PCR tests on fetal samples become easier in the presence of maternal serology results. Firstly, if the serology from the mother is negative for IgG and IgM for a particular infection, it then doesn't make sense to request a PCR test on the fetal sample for that particular causative agent. We have now modified the current protocol in our unit so that maternal serology is done as a first step whenever congenital infections are suspected and then an invasive procedure is carried out with a plan to test only for the appropriate virus as dictated by the maternal serology.

The PCR done on fetal samples were 100% specific. The sensitivity PCR for the detection of RV is 10 fg of RVRNA equivalent to 10 viral particles [6]; sensitivity of the HSV PCR was determined as 0.02 attograms of DNA of both HSV 1 and 2 [15] and the sensitivity of HCMV is 1.5μg/ml [16]. None of the fetal samples in our study tested positive for HSV and none showed co-existent HCMV and RV. However, this is different from the reports on maternal serology, which have shown that up to 38% of the seropositive samples show co-existing IgM for infections such as Toxoplasmosis, CMV and Rubella. However, the limited number in the current study is perhaps the reason for not detecting co-existent viral particles.

Figure.1 Graph Showing the Most Common Indication for Testing the Fetal Specimen for Viral PCR

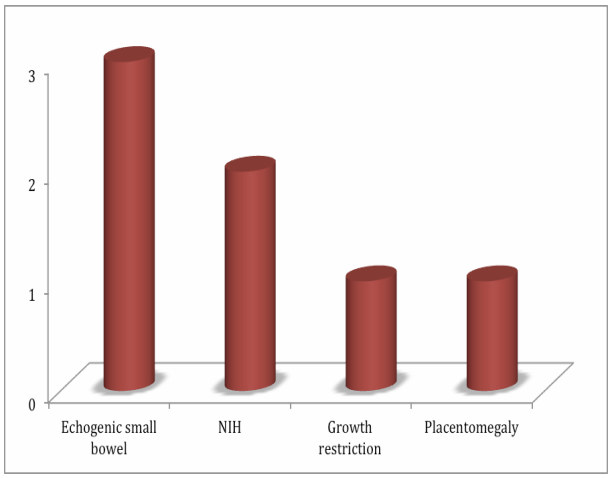
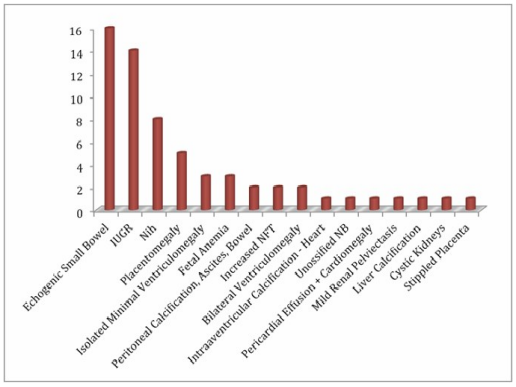


Figure.2 Graph Showing the Most Common Indications in the PCR Positive Group Specimen



The limitations of the current study include firstly, the small number of patients and hence samples which primarily limits any major conclusion being drawn. Nevertheless, it undoubtedly establishes the feasibility of detecting viral particles using the PCR technique in fetal samples. The small number of positive results may be a true reflection of the fetal viral status or due to wrong timing of the procedure, i.e too early for the virus to be detected. This is not possible as only fetuses with some ultrasound finding suggesting possible fetal affect were included and would therefore imply that adequate time had elapsed from the time of fetal infection to sampling to expect viral particles to be excreted into the amniotic fluid. The second limitation is that we could not get adequate outcome details to ascertain false negatives in the study group. This is however true of most diagnostic centers, where the population investigated is a migrant one that is extremely difficult to keep track of.

This study establishes for the first time in India that it is feasible to perform Viral PCR studies on fetal samples, both amniotic fluid and fetal blood. This would give patients and clinicians a chance to rule out or

confirm suspicions about possible fetal infection especially in the presence of ultrasound abnormalities. Further studies need to be undertaken with larger numbers and standardized specific protocols to establish the sensitivity and specificity of the PCR tests in India.

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