Assessment of ELISA and real time PCR in diagnosis of Cytomegalovirus and Herpes Simplex Virus in Pregnant Women of Peshawar, Pakistan

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Abstract

Human Cytomegaloviruses and Herpes Simplex Viruses are the major cause of serious viral complications in pregnant women. Conventional screening methods that is ELISA for detecting Human Cytomegalovirus (HCMV) and Herpes Simplex virus (HSV) tend to be slow and insensitive. Therefore in this work, a rapid Real Time PCR-based assay was designed to detect CMV and HSV which are responsible for causing various viral infections among pregnant women in Khyber Pakhtunkhwa. The present study aimed to compare the specificity and sensitivity of the PCR-based assay with ELISA based assay in the diagnosis of HCMV and HSV infections in pregnant women in Khyber Pakhtunkhwa. In order to check the validity of Real time PCR technique in the early diagnosis of the infection, serological results were compared to the results of Real Time PCR based detection of HCMV and HSV from serum samples. Our study revealed that among the ELISA screened 175 positive sera samples i.e. 81 (70%) of CMV and 34 (30%) of HSV, while 17 (10%) had positive results for CMV DNA and 7(4%) had HSV DNA through real-time PCR. Real time PCR was more sensitive and reliable method in diagnosis of CMV and HSV infections in pregnant women in this comparative study.

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Introduction

Cytomegalovirus (CMV) and Herpes Simplex Virus (HSV) are species of viruses that belong to Herpesviridae or Herpesviruses family. These viruses are among the most ubiquitous viruses found in the adult population. This family has a characteristic of lifetime latency after primary infection and the latent virus can reactivate in infected individuals at any time (Ziyaeyan et al., 2007). CMV and HSV play important role in causing maternal infections and these are known to have an intrauterine route of transmission with significant mortality and morbidity (Surpam RB et al, 2006). In developing countries, CMV is the most common cause of congenital deformity which usually occurred during viral intrauterine infection (Gaytant et al., 2002). Due to virus reactivation during the child bearing age, Cytomegalovirus infection during pregnancy is more complex than other infections during pregnancy and can be transmitted to the fetus in spite of maternal immunity (Mukundan et al., 1977). On the other hand, HSV infection of the newborn can be acquired in ex utero intrapartum therapy and after child birth (Anzivino et al., 2009). These infections are usually asymptomatic and they are difficult to diagnose clinically (Sen et al., 2012).

Laboratory confirmation can be achieved using serological and molecular techniques. Conventional methods for detection of antibodies to CMV and HSV include various assays like Immunofluorescence assay (IFA), Enzyme-linked fluorescent assay (ELFA), Enzyme immunoassay (EIA), and Enzyme-linked immunosorbent assay (ELISA). These techniques have been used widely for both diagnostic and screening protocols for CMV, HSV and other viral infections (Berth. M et al, 2010). Recently Real time PCR due to its high specificity and sensitivity emerged as a novel approach in detection of molecular response to various infectious diseases (Binnicker et al., 2010). Real time PCR allows simultaneous detection and identification of multiple samples (Yasodhara et al., 2004).

In Pakistan, and especially in KPK rural and peripheral areas, along with other diseases, infectious diseases are becoming more day by day, due to no awareness, low health care facilities and low literacy rate. Due to lack of a national screening program, there is no baseline serological data regarding the seroprevalence of such infections in patients. Rare studies exist regarding occurrence and complications of these infections. No major study regarding RT-PCR based detection has not been done till now in Peshawar region. In Peshawar, Khyber Pakhtunkhwa, no study has been carried out to evaluate and compare different assays used for screening of these infections. Therefore, this comparative study was undertaken for the assessment of ELISA and PCR accuracy in detection of CMV and HSV exposure to pregnant women at high risk for miscarriages and other pregnancy related complications in Khyber Pakhtunkhwa.

Materials and methods

Blood samples collection and handling

The study population was 175 females having obstetrical problems and 5 females having no obstetrical problem as a control group. Blood specimen were collected from pregnant women during period of January 2016 to March 2016 from different hospitals in Peshawar. Inclusion criteria for all of these was; complications found during pregnancy or some other chronic diseases associated with pregnancy. All those female patients having other TORCH infections were excluded from this study. The blood samples were collected aseptically by using venipuncture techniques and centrifuged at 3000 rpm for 5 min. The sera collected were refrigerated (2-8 °C) upon collection or frozen (-20°C) if the test could not be performed within 7 days. For detection of CMV and HSV antibodies, Vircell, S.L (Spain) ELISA kit instructions were followed.

Detection of Cytomegalovirus IgM and IgG by ELISA

CMV IgG detection

Addition of 100 ul serum diluent to all wells and 5ul of each sample, 5 ul of negative control, 5 ul of cut off control (in duplicate) and 5 ul of positive control was added into the corresponding wells. Incubation was done at 37± 1°C for 45 mins followed by five times washing with 0.3 ml of washing solution per well.
100 ul IgG conjugate solution was then added and incubated for 30 min at 37±1°C. Washing was again done with 0.3 ml of washing solution. Immediately added 100ul substrate solution into each well. Room temperature incubation was then done for 20 mins and in the end 50 ul stopping solution was added immediately. Reading was done with a spectrophotometer at 450/620 nm within 1 hour of stopping.

**CMV IgM detection**

1/20 dilution of serum samples was prepared by adding 5ul of sample to 95 ul of sample dilution solution. 80 ul sample dilution solution was added into all wells except in control wells. 20 ul of the 1/20 dilutions of serum samples, 100 ul of positive control, 100 ul of cut off control (in duplicate) and 100 ul of negative control was added into the corresponding wells. Incubation was done at 37± 1°C for 60 mins. Five times washing with 0.3 ml of washing solution was done and then 100ul reconstituted conjugate was added in each well followed by incubation at 37± 1°C for 60 mins. Washing was again done with 0.3 ml of washing solution for five times. Immediately added 100ul substrate solution into each well. Room temperature incubation was then done for 20 mins and in the end 50 ul stopping solution was added immediately. Reading was done with an spectrophotometer at 450/620 nm within 1 hour of stopping.

**Detection of Herpes Simplex Virus IgM and IgG by ELISA**

For IgG test, addition of 100 ul serum diluent to all wells and 5ul of each sample, 5 ul of negative control, 5 ul of cut off control (in duplicate) and 5 ul of positive control was added into the corresponding wells.

For IgM test, 25 ul of VICELL IgG sorbent was added to each of the required well except for the wells where controls were dispensed. 5 ul of sample was added and then 75 ul of the serum diluent added to each well. Control wells were prepared by adding 100 ul of the serum diluent to each well and then 5 ul of positive control, 5 ul of cut off control (in duplicate) and 5 ul of negative control was added into the corresponding wells.

Incubation was done at 37± 1°C for 45 mins followed by five times washing with 0.3 ml of washing solution per well. 100 ul of IgG conjugate solution or IgM conjugate solution was then added to each well and incubated for 30 min at 37± 1°C. Washing was again done with 0.3 ml of washing solution. Immediately added 100ul substrate solution into each well. Room temperature incubation was then done for 20 mins and in the end 50 ul stopping solution was added immediately. Reading was done with an spectrophotometer at 450/620 nm within 1 hour of stopping. Validation protocol of ELISA and interpretation of results is shown as follows (Table 1 and 2).

**Molecular Detection of CMV&HSV By Real Time PCR DNA extraction**

DNA extraction kit of viral nucleic acid (Ampli Sens DNA Sorb-B, Russia) was utilized during the viral DNA extraction from serum according to the company protocol. The following protocol was adopted:

100 ul serum was added to eppendorf tubes which were having 300 ul lysis solution. Two to three times vortexing and incubation was done at 65 C for 5 mins. All tubes were then centrifuged for 5 seconds at 7000-8000 rpm. 25ul Universal sorbent was then added to each tube. Again vortexing and centrifugation was done for 1 minute at 5000 rpm. Supernatent was carefully removed using a vacuum aspirator without disturbing the pellet. 300ul washing solution was added, vortexed it and centrifugation was done for 1 min at 5000-6000 rpm and supernatant was discarded without disturbing the pellet. 500 ul washing solution 2 was then added, vortexing and centrifugation was done and supernatant was discarded. This step was repeated again and supernatent was completely removed. All tubes were put in heating block for 5-10 mins at 65 C for drying. 50ul TE-Buffer was added for DNA elution and tubes were vigorously vortexed. Again centrifugation at 12000 rpm was done for 1 minute and supernatant was collected which will have purified DNA. All the tubes were stired in freezer for PCR amplification. The purified DNA could be stored at 2-8 C weeks for 1 week and at ≤-16 C for 1 year.
**Real Time PCR Assay**

RT-PCR assay procedure was done according to the AmpliSens HSV/CMV-Multiprime-FRT PCR Kit (Moscow, Russia). This in vitro nucleic acid amplification kit was used for simultaneous detection of *Herpes simplex virus* and *Cytomegalovirus* DNA in clinical materials by using real-time hybridization-fluorescence detection. This qualitative test was used to identify possible reaction inhibition and that contained the internal control (IC) for controlling extraction process of each individual sample. This kit was used at 18-25°C. Following protocol was adopted: Centrifugation of PCR–mix-1-FL HSV/CMV, PCR-mix-2-FRT and polymerase (TaqF) was done for few seconds. For amplification of DNA from the test and control samples, required number of the tubes were prepared.

For N reactions (including 2 controls of amplification) mixed in a new tube:

\[ 10^\ast(N+1) \text{ ul of PCR-mix-1-FL HSV/CMV, 5.0}^\ast(N+1) \text{ ul of PCR-mix-2-FRT and 0.5}^\ast(N+1) \text{ ul of polymerase (TaqF)} \]

The prepared mixture was stirred and then centrifuged for about 1-2 s in order to remove all drops from the walls of tubes. About 15 ul of the prepared mix was transferred to each tube and 1 ul of extracted DNA obtained from the extraction test was added into the prepared tubes. Centrifugation was again done.

**Amplification of DNA**

According to Manufacturer’s manual, Guidelines and Table 3, the rmocycler was programmed. The tubes were inserted into the device reaction module cells. The amplification program was set with fluorescence detection. Results were analyzed after completion of the amplification program. In the end, fluorescent signal was detected in FAM, JOE and ROX channels. (Removed numbering).

**Data analysis (No software, its Real Time PCR detection results through different channels)**

HSV DNA was detected in the JOE Channel, CMV DNA was detected in the FAM Channel, and in the ROX fluorescence channel internal control DNA was detected [Figure 1]. (On last page figure of RT-PCR result is given).

**Results and discussion**

All blood samples from 175 patients in this study were tested using ELISA and PCR. According to this study, the results obtained from the serological test for 175 samples, 115 (66%) were seropositive while 60 (34%) were seronegative for CMV and HSV respectively.

**Table 1.** Validation protocol of ELISA.

<table>
<thead>
<tr>
<th>Control</th>
<th>Optical Densities (O.D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive Control</td>
<td>&gt;0.9</td>
</tr>
<tr>
<td>Negative Control</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Cut off Control</td>
<td>&gt;0.55</td>
</tr>
<tr>
<td></td>
<td>&lt;1.5</td>
</tr>
</tbody>
</table>

**Table 2.** Interpretation of Results.

<table>
<thead>
<tr>
<th>Index</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;9</td>
<td>Negative</td>
</tr>
<tr>
<td>9-11</td>
<td>Equivocal</td>
</tr>
<tr>
<td>&gt;11</td>
<td>Positive</td>
</tr>
</tbody>
</table>

Antibody index= (sample O.D / Cut off serum mean O.D) x 10.

The control group included 5 females which had normal delivery without any obstetrical complications. Among the 115 seropositive samples, 81 (70%) were positive for CMV and 34 (30%) were positive for HSV.

Furthermore, IgM and IgG positive samples among CMV positive sera samples were 14 (17%) and 67 (83%) respectively. Similarly this ratio for IgM and IgG in HSV positive sera was 10 (29%) and 24 (71%) respectively (Table 4) (Fig.2).

The results for PCR showed that 17 (10%) out of 175 patients were found to be CMV positive while 7 (4%) out of 175 patients were found to be HSV positive (Table 5). The control group did not detect any viral DNA presence.
Table 3. Ampli Sens-1 Program for RT-PCR.

| Step | Rotar-Type Instruments | | Plate-Type Instruments | |
|------|-----------------------|----------------------|-----------------------|
|      | Temperature | Time | Cycles | Temperature | Time | Cycles |
| 1    | 95          | 15min | 1     | 95          | 15min | 1     |
| 2    | 95          | 5s    | 5     | 95          | 5s    | 5     |
|      | 60          | 20s   |       | 60          | 20s   |       |
|      | 72          | 15s   |       | 72          | 15s   |       |
| 3    | 95          | 5s    | 40    | 95          | 5s    | 40    |
|      | 60          | 20s Fluorescence detection | | 60 | 30s Fluorescence detection | |
|      | 72          | 15s   |       | 72          | 15s   |       |

Table 4. Showing ELISA results of anti CMV and HSV IgG and IgM Antibodies.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Total Number</th>
<th>Anti CMV +ve</th>
<th>HSV +ve</th>
</tr>
</thead>
<tbody>
<tr>
<td>seropositive</td>
<td>175</td>
<td>81 (70%)</td>
<td>34(30%)</td>
</tr>
<tr>
<td></td>
<td>115(66%)</td>
<td>IgG+</td>
<td>IgM+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>67(83%)</td>
<td>14(17%)</td>
</tr>
<tr>
<td></td>
<td>60(34%)</td>
<td>24(71%)</td>
<td>10(29%)</td>
</tr>
<tr>
<td>seronegative</td>
<td></td>
<td>14(17%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>24(71%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10(29%)</td>
<td></td>
</tr>
</tbody>
</table>

In this study, CMV infection was comparatively more than HSV in pregnant women. In 2012, a study indicated the seropositivity of CMV and HSV in Babylon that was 57.2 % and 28.9 % respectively, thus indicating a higher prevalence of CMV infections (Ali et al., 2012).

Table 5. RT-PCR Results.

| No. of specimen | CMV | | HSV | |
|-----------------|-----|----------------------|-----|
| PCR+            | 17(10%) | 7(4%) |
| PCR-            | 158(90%) | 168(96%) |

This study correlates with our findings in which HSV IgM antibodies had lower percentage than HSV IgG antibody i.e, 24 (71%) of IgG antibodies and 10(29%) of IgM antibodies in pregnant women (Roziman et al., 2007). However the role of HSV in causing infections among pregnant women could not be denied as the incidence and prevalence of HSV infections are increasing rapidly globally (Duran et al., 2004).

Similarly, Festary et al. (2015) conducted a study in which they found that 89.5% pregnant women tested positive for CMV and 83.2% for HSV. Our study showed high rate of IgG (83%) antibodies to CMV as compared to IgM (17%) antibodies. In 2015, it was also reported that miscarriage women had highest percentage of seropositive to HCMV for IgG (40%) and (25%) for IgM out of 40 samples (Rehab et al., 2015).

There was also low prevalence rate of HSV among pregnant women as indicated by Hasan et al. (2013) in their seroprevalence study of HSV through ELISA. According to Roziman et al. in 2007, most people acquire HSV in childhood therefore HSV IgM is rarely found in adults and not all such patients have elevated HSV IgM.

In this study, real time PCR was run on all positive samples of extracted DNA obtained from pregnant women that have no history of hypertension, diabetes and other complications.
Fig. 1. RT-PCR data analysis.

The results for the RT-PCR showed that; 17 out of 175 (10%) pregnant women having miscarriages were found to be HCMV DNA Positive while 158 out of 175 (90%) were found HCMV DNA Negative. The RT-PCR results obtained were HSV DNA Negative. Festary et al. in 2015 detected CMV DNA in vaginal swab samples of pregnant women i.e 9(9.5%) and HSV DNA in 1(1.1%) among 95 sera samples. This finding is quite similar to our study; however we found CMV and HSV DNA in sera samples through real time PCR while they performed nested PCR for vaginal swabs.

In another study conducted to find out CMV DNA, it was found out that 10 out of 57 samples had positive CMV DNA which showed similarity with our work (Thikra et al., 2015). HSV genome confirmation by simple PCR was done by Sifakis et al. in 1998 in aborted material which gave positive DNA confirmation in 3 samples same as in our case we had only 7 HSV DNA in 175 pregnant women sera samples. These findings showed that most of the ELISA results were confirmed by PCR which means that seropositive results by ELISA were not specific or less significant due to probability of false positive results as a result of other microbial infection (Lenohova et al., 2011).

These results suggested that the best method to detect CMV and HSV was RT-PCR as Real time PCR was considered to be active, rapid and useful technique for diagnosis of active disease and monitoring response to therapy (Printice et al., 1997).

Conclusion
Severe life threatening complications of CMV and HSV in pregnant women may not be as rare as previously considered therefore proper diagnosis must be done before pregnancy in order to reduce miscarriage rate and different congenital infant infections.

The accurate diagnosis of Cytomegalovirus and Herpes Simplex virus must be done by sensitive molecular methods such as Real Time PCR while ELISA should be used as screening method as Real Time PCR is the best technique and has more sensitive and specific effect than conventional PCR and ELISA.

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References


