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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 pregnanc 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°Cfor 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°Cfor 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 eppendoff 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 vaccum 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 supernantent 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 nucleicacid amplification kit was used for simultaneous detection of *Herpes simplex virus* and *Cytomegalovirus* DNA in clinical materials by using real-time hybridization-florescence 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*(N+1) ul of PCR-mix-1-FLHSV/CMV, 5.0* (N+1)ul of PCR-mix-2-FRT and 0.5* (N+1) ul of polymerase (Taq F).

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 15ul of the prepared mix was transferred to each tube and 10ul 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.

Control	Optical Densities (O.D)
Positive Control	>0.9
Negative Control	<0.5
Cut off Control	>0.55
	<1.5

Table 2. Interpretation of Results.

Index	Interpretation
<9	Negative
9-11	Equivocal
>11	Positive

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		95	5s	
	60	20s	5	60	20s	5
	72	15s		72	15s	
3	95	5s	40	95	5s	
	60	20s Fluorescence detection		60	30s Fluorescence detection	40
	72	15s		72	15s	

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

Samples	Total Number	Anti CMV +ve		HSV +ve	
	175	81 (70%)		34(30%)	
seropositive	115(66%)	IgG+	IgM+	IgG+	IgM+
seronegative	60(34%)	67(83%)	14(17%)	24(71%)	10(29%)

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+	PCR-	PCR+	PCR-
175	17(10%)	158(90%)	7(4%)	168(96%)

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.

This study correlates with our findings in which *HSV* IgM antibodies had lower percentage than *HSV* IgG antibodie 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).

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.

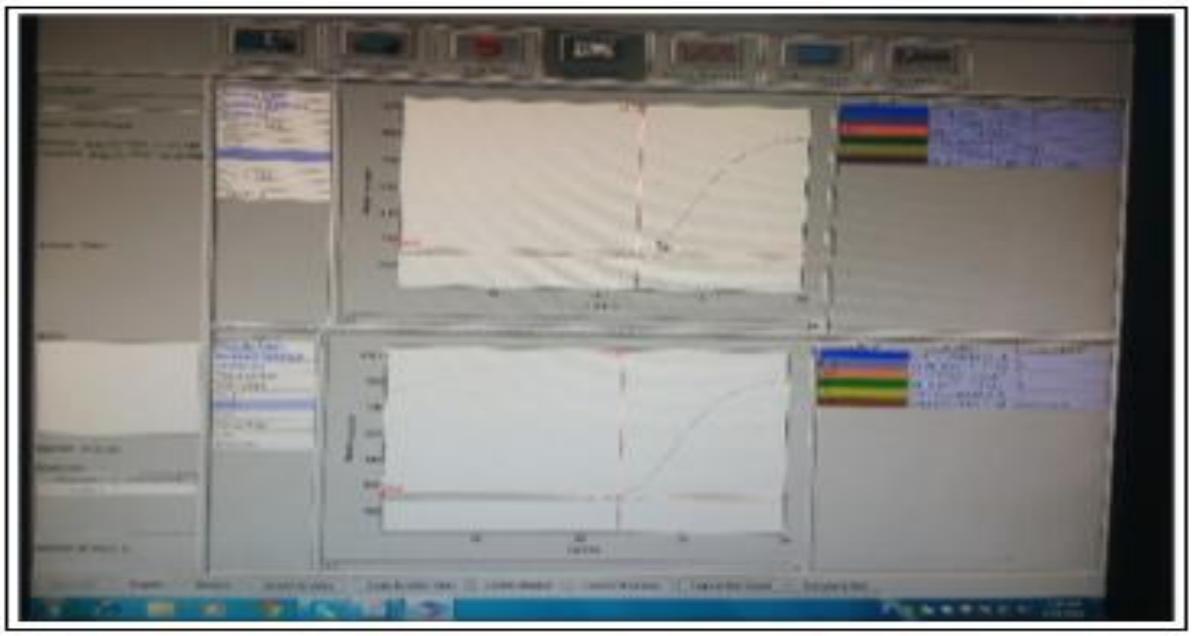

Fig. 1. RT-PCR data analysis.

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 (Lenochova *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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