Prognostic Value of Quantitative-PCR versus Serology for Detection of CMV in Pre- and Post- Transplantation Patients

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Two hundred-twenty six pre- and post- kidney transplantation patients were screened for CMV. They were categorized into three groups I: Eighty-five dialysis patients suffering from chronic renal failure, II: Sixty-two end stage renal disease patients prepared for transplantation, and III: Seventy-nine post-kidney transplant patients under immunosuppressive and cytotoxic therapy. The last group was subdivided according to clinical presentation into asymptomatic, mild, and invasive CMV disease subgroups. A control group comprised of fifty-two apparently healthy kidney and blood donors were also included. Serum and plasma samples were utilized for detection of anti-CMV antibodies by EIA, and determination of CMV viral load by quantitative -PCR respectively. QT-PCR results revealed high viral load; (4000-10.000 copy/ml) in 16(100%) kidney recipient with invasive CMV disease, While only 4 (25%) of them were positive for IgM anti-CMV. Another ten patients with symptoms of CMV syndrome were found CMV-PCR positive with low viral load (436-3070 copy/ml) and all were negative for IgM anti-CMV. On the other hand, no CMV viral load was detectable among end-stage renal disease patients, although 6/62(9.7%) were positive for IgM anti-CMV. All subjects were positive for IgG anti-CMV. It is concluded that QT-PCR rather than serology should be used for monitoring of CMV infection in pre- and post-transplantation patient.

CMV is considered a major pathogen for immunocompromised patients (Singh, 2006; Razonable, 2009, Lazzarotto, 2010, Stratta et al., 2010). Clinical diagnosis in CMV infection relay dominantly on laboratory detection of either CMV antigen or antibody, and molecular techniques(Tong et al., 1998). Tissue culture (TC), Immunofluorescent (IF), have met with many obstacles, time consuming, risk of contamination, low sensitivity, and low reproducibility regarding conventional cell culture assay. Antigenaemia assay needs special type of specimen, time consuming, does not lend itself to automation and specimens must be tested within a few hours of collection (Knipe et al., 2002; Lazzarotto, 2010). Immunoassays determine immune status and past infection. IgM antibody against CMV often lacks specificity in primary infection, because IgM false results may occur as false-positive due to rheumatoid factor interference, while false-negative IgM is obtained due to abundant IgG (Lazzarotto et al., 1999). Manual qualitative polymerase chain reaction is not usually positive in patients without CMV viremia and does not give any information regarding therapy. Quantitative polymerase chain reaction (QT-PCR) gives an early detection of CMV activation before symptoms, gives an important information regarding the kinetics of viral replication in transplant patients (Ljungman, 2006; Lazzarotto, 2010), allows for monitoring of antiviral treatment efficacy and specimen deterioration with time after sample collection is not a problem with PCR as other tests for CMV (Specter et al., 2000).

However, laboratory diagnosis of active CMV infection is not necessarily always associated with symptomatic CMV disease (Stratta, 1993). Serological tests (EIA), are of great importance in defining the clinical risk from CMV at the time of transplantation.
(Fishman & Rubin, 1998; Xue et al., 2009; Lazzarotto, 2010), and are useful to determine whether a patient has past infection (Tanabe et al., 1997). On the other hand QT-PCR, has the advantage that it can be utilized to detect CMV DNA in many types of samples: peripheral blood mononuclear cells, plasma, urine, cerebrospinal fluid, breast milk, bronchoalveolar lavage fluid, tissue samples (Clinque et al., 1992; Aspin et al., 1994; Lazzarotto, 2010). CMV viral load in plasma, correlates with the risk of disease in different at-risk populations, guiding the clinicians in deciding the start and dose of therapy (Specter et al., 2000; Xue et al., 2009; Lazzarotto, 2010).

The present study aimed at comparing sensitivity, specificity, and time consumption of two CMV diagnostic assays; the quantitative PCR for viral load determination, and the serological anti-CMV IgM & IgG detection assays.

**Materials and Methods**

**Experimental Design of the Present Study**

<table>
<thead>
<tr>
<th>Patients Groups</th>
<th>Control Group</th>
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<tbody>
<tr>
<td>E.S.R.D</td>
<td>Kidney donors</td>
</tr>
<tr>
<td>P.K.T.</td>
<td>Blood donors</td>
</tr>
<tr>
<td>C.R.F.</td>
<td></td>
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</table>

In the present study two-hundred and twenty six immunocompetent and immunocompromised individuals have been categorized into four groups: Group I (control): comprised apparently - healthy blood and kidney donors, Group II (E.S.R.D.): presented by end stage renal disease on dialysis, waiting for proper donated kidney, Group III (P.K.T.): presented post-kidney transplant patients, under immunosuppressive and cytotoxic therapy, Group IV (C.R.F.): presented patients suffering from chronic renal failure. Serum and plasma samples were collected and screened for both, the kinetics of antibody response to Cytomegalovirus by EIA of IgG and IgM, and the viral load determination by QT-PCR, to compare between the efficiency of these laboratory tests for rapid and accuracy detection to control HCMV infection in immunocompromised patients, and Determining the best tests to deal with CMV infection, which might help for perfect diagnosis. Thus control the development of disease.
Patients

Two hundred-twenty six pre- and post- kidney transplantation patients were screened along a period of one year at Jeddah Kidney Center (J.K.C.)-King Fahad General Hospital in Serology Lab and Molecular Diagnostic Lab. Patients consent were reported for medical investigation and approval of Lab director for using reagents and equipments for patients diagnostic services. Antibody response to Cytomegalovirus and viral load were investigated. Patients were chosen based on the following: Inclusion criteria: Age: ranged from twenty to sixty five years with the mean of forty seven years, Sex: males and females, Pathological Conditions: haemodialysis patients with prolonged renal diseases and transplantation patients. Exclusion criteria: we excluded other diseases e.g. HBV, HCV, and AIDS, seronegative by ELISA, active TB, no symptoms and negative X-ray.

Group I
Comprised eighty –five dialysis patients, suffering from chronic renal failure (C.R.F.).

Group II
Comprised sixty-two end stage renal disease patients (E.S.R.D.) prepared for transplantation.

Group III
Comprised seventy-nine post-kidney transplant patients (P.K.T.), under immunosuppressive and cytotoxic therapy, this group was subdivided into three subgroups according to their clinical presentation: A, B, C. (A) 53 Asymptomatic patients, (B) 10 with mild CMV syndrome, and (C) 16 with invasive CMV disease.

Group IV
The control group comprised fifty-two apparently healthy kidney and blood donors. From each patient and donor one serum sample and one plasma sample were collected and utilized for detection of antibodies IgM and IgG anti-CMV by EIA (Enzyme Immuno Assay), and determination of CMV viral load by QT-PCR respectively.

Materials

Reagents for immunoassay to detect anti-CMV IgG and IgM in patients sera (United Diagnostic Industry-UDI., K.S.A) include: microplate 12x8 wells coated with neat CMV harvest, calibrators from 1-5 diluted human serum, containing known concentrations of anti-CMV IgG antibodies, conjugate: Rabbit anti-human IgG monoclonal antibodies (UDI-K.S.A), or Rabbit anti-human IgM monoclonal antibodies, peroxidase labelled. IgM negative control, positive control, diluted human serum in phosphate buffer. Cut off control, diluted human serum in phosphate buffer, substrate TMB (tetramethylbenzidine), sulphuric acid 0.3M: stopping solution.

Venous blood samples were collected in plain tubes under aseptic conditions, centrifuged and then serum samples were stored at -20 c till use, while plasma samples were collected in EDTA tubes, centrifuged and stored at -20c till use.

Reagents for Detection and Quantitation of CMV-DNA

All reagents and equipments for quantitative PCR in virology molecular diagnostic lab-KFGH were from Roche- Switzerland. It is a closed system utilizing only its own manufacturing reagents.

DNA Extraction Reagents

Lysis buffer, Quantitation standard (QS), specimen diluent (DIL), Dextran blue reagent, controls: (negative), (low positive), (high positive) positive controls are E. coli plasmid containing CMV sequence as a quantitation standard, Negative human plasma as an internal control non-reactive to CMV.

DNA Amplification Reagents

Master mix, magnesium reagent(Mg 2+), specific detection reagent, Amplicon dilution reagent (AD3), probe suspension 1,2,QS probe suspension 1, QS probe suspension 2(VQ4).

DNA Detection Reagents-Generic Detection

Denaturation solution, Avidin-horseradish peroxide labeled conjugate, substrate A, B(SB3), wash buffer 10x-wash concentrate.

Methods

- Enzyme Immunoassay (EIA) for Detection of Anti-CMV IgG in Patients Sera

96- microtitre plate coated with the CMV antigen. The specific immunoglobulins were bound to the antigen through incubation with diluted human serum. After washing, an incubation step was performed with the conjugate. Washing was carried out for four times, then substrate was added, and incubated at room temperature. Finally stop solution was added, and absorbance was read at 450 nm within 30 minutes.

The anti-CMV IgG was expressed in ELISA Unit (EU/ml), or international unit (IU/ml) [WHO standard], by extrapolating the results of the 5 calibrators and comparing the O.D. of the sample with the curve obtained. When the anti-CMV IgG concentrations in
the sample was >12 EU/ml or 1.2 IU/ml, the sample considered positive. The ratio between the O.D. value of the sample and that of the cut off (calibrator 2) was calculated. The sample was considered positive if the ratio was >1.3, and negative if the ratio was <0.7.

- **Enzyme Immunoassay (EIA) for Detection of Anti-CMV IgM in Patients Sera**

The steps are almost the same as previously mentioned for IgG; except that the lyophilized CMV antigen in the vial was dissolved in the conjugate (immunocomplex), and was added to each well.

The sample was considered positive if the ratio of absorbance of the sample to that of cut-off value was >1.2 and negative if the ratio was <0.8.

- **Extraction, Amplification and Quantitation of CMV-DNA in Patients Plasma**

The Cobas Amplicor CMV Monitor test, is based on four major processes: Specimen preparation and DNA extraction, PCR amplification of target DNA using CMV specific primers, hybridization of the amplified products to oligonucleotide probes specific for the targets, and detection of the probe-bound amplified products by colorimetric determination. The procedure permits simultaneous PCR amplification of CMV and CMV quantitation standard DNA. The master mix reagent contains a primer pair specific for both CMV DNA and CMV quantitation standard DNA.

The quantitation of CMV DNA is performed using the CMV quantitation standard, which is non-infectious plasmid DNA that contains the identical primer binding sites as the CMV DNA target, and a unique probe binding region that allows CMV quantitation standard amplicon to be distinguished from target CMV amplicon. The CMV quantitation standard (Q.S.) is incorporated into each individual specimen at a known copy number, and is carried through the specimen preparation, PCR amplification, hybridization and detection steps along with the CMV target. The Cobas amplicor analyzer calculates the CMV DNA levels in the test samples by comparing the CMV signal to the Q.S. signal for each specimen. The CMV (Q.S.) compensates for effects of inhibition and controls for the amplification process to permit the accurate detection of CMV DNA in each specimen (Mullis & Faloona, 1987). Its analytical sensitivity (the minimal detection limit), is 99% and it agrees well with the clinical sensitivity (physicians opinion about the progress of patients symptoms and signs, whether improvement or regress.

**Statistical Analysis**

Our data were statistically analyzed utilizing the t-test to obtain P value. P values of 0.05 or less were regarded as significant.

**Results**

The serological data regarding IgM and IgG anti-CMV detected by enzyme immunoassay, and the viral load detected by QT-PCR have been reported in one table. According to the data reported in the table, the results of four groups studied with their corresponding serostatus versus their PCR results.

Table (1): shows the results of the four studied groups with regards to their CMV-serostatus versus PCR results. In group III-B, detectable CMV-DNA was reported by quantitative PCR, while the same samples were seronegative for CMV-IgM. In group III-C, only 25% of samples were seropositive for CMV-IgM, while 100% were positive by QT-PCR. Group III comprised seventy-nine post-kidney transplant patients (P.K.T), under immunosuppressive and cytotoxic therapy, were subdivided according to their clinical presentation—into three subgroups: A) fifty-three asymptomatic CMV P.K.T. patients, B), ten asymptomatic CMV P.K.T. patients, presented with mild CMV syndrome, and C), sixteen CMV P.K.T. patients suffering from invasive CMV disease.

The serological versus PCR results are shown in table (1). In the asymptomatic group I- (group I), all were positive for IgG 85(100%). Positive results due to their previous exposure and immunological memory to CMV. Meanwhile in group II, 6/62 (9.7%) patients with end stage renal disease on dialysis were IgM weak positive. In spite of being clinically-free from any CMV symptoms and signs, which indicates that IgM-anti CMV detection might be illusive especially in immunocompromised
patients due to production of cross-reactive errorious immunoglobulins, or otherwise the specificity of the detection reagents should be maximized to avoid false positive results due to overwhelming IgG. In group III-B, 10 kidney transplant recipients (P.K.T) presents with symptoms of CMV syndrome had CMV DNA viral load ranging from 436 to 3070 copy/ml. Whereas in group III-C, 16 of the (P.K.T.), presenting with invasive CMV disease, had a viral load ranging from 4000 to > 100.000 copy/ml. Notably, there is an impressive matching between the analytical sensitivity of QT-PCR results with the clinical sensitivity reported for the patients.

An alarming result was observed in group III-B, where the CMV viral load was detected in 10/10 (100%) symptomatic CMV patients with E.S.R.D., whereas their IgM-anti CMV failed to be detected 0/10(0.0%). In group III-C, the CMV high viral load ranging from 4000-100.000 copy/ml were detected in all patients with invasive CMV 16/16(100%) although only 4/16 (25%) were found positive IgM anti –CMV. Finally control group (IV) was asymptomatic carriers as they were positive IgG, negative for IgM, and PCR, which reflects immunological memory to CMV and the virus in a latent state according to their healthy immune system.

Statistical analysis of the results showed significant difference between PCR and serology results in group II and III-C, while no -significant difference was found when we compared - PCR with serological results in the control and other patient groups.

Table 1. Cytomegalovirus serostatus and PCR results in Patients with Renal diseases and controls

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total no.</th>
<th>EIA- IgM Anti-CMV POSITIVE</th>
<th>EIA- IgG Anti-CMV POSITIVE</th>
<th>Quantitative CMV-PCR</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic renal failure (C.R.F.)</td>
<td>85</td>
<td>Zero/85</td>
<td>0.0%</td>
<td>84/85</td>
<td>98.8%</td>
</tr>
<tr>
<td>End stage renal disease (E.S.R.D.)</td>
<td>62</td>
<td>6/62</td>
<td>9.7%</td>
<td>62/62</td>
<td>100%</td>
</tr>
<tr>
<td>Kidney transplant recipients A</td>
<td>53</td>
<td>Zero/53</td>
<td>0.0%</td>
<td>53/53</td>
<td>100%</td>
</tr>
<tr>
<td>Kidney transplant recipients B</td>
<td>10</td>
<td>Zero/10</td>
<td>0.0%</td>
<td>10/10</td>
<td>100%</td>
</tr>
<tr>
<td>Kidney transplant recipients C</td>
<td>16</td>
<td>4/16</td>
<td>25.0%</td>
<td>16/16</td>
<td>100%</td>
</tr>
<tr>
<td>Control Group: Healthy Kidney and blood donors</td>
<td>52</td>
<td>Zero/52</td>
<td>0.0%</td>
<td>52/52</td>
<td>100%</td>
</tr>
</tbody>
</table>

P<0.05 is significant, NS= not significant
Discussion

Human Cytomegalovirus (HCMV) is a serious pathogen for immunocompromised individuals (Singh 2006; Kinpe et al., 2007, Razonable, 2009, Lazzarotto, 2010; Stratta et al., 2010). CMV has been reported to be the most common viral infection in the first few months after transplantation (Blond et al., 1992; Esforzado et al., 1993; Caliendo, 2000; Brennan, 2001).

The immune defensive reactions rather than the virus determine the outcome by permitting or restricting viral replication and pathogenesis. Chronic renal failure is treated by regular haemodialysis which becomes insufficient after many years and the patient progresses into end-stage renal disease, where kidney transplantation is the only possible treatment. Post-transplantation patients normally receive corticosteroid and cytotoxic therapy which induces immunosuppresion, and the patient is left without any defense against pathogens.

In the present study we aimed at providing the best accurate method for CMV detection in four groups of immunocompetent and immunocompromised individuals. IgG anti-CMV was found positive in the four groups indicating past infection, but no conclusive information could be obtained. On the other hand, IgM anti-CMV was detected weak positive in six out of sixty-two (9.7%) of end-stage renal patients without any clinical manifestation of CMV activation, and no detectable viral DNA by QT-PCR, this may indicate elusive serological results. In agreement with these data Fischer & Mauser (1997) have recommended that the serostatus is not sufficiently informative, while molecular amplification assays are more reliable and to be the assay of choice in monitoring CMV seropositivity before transplantation (Boland et al., 1992; Tong, 1997; Lang et al., 2001; Xue et al., 2009; Lazzarotto, 2010).

The immunofluorescence, shell-vial, Antigenaemia methods (Xue et al., 2009; Lazzarotto, 2010) generally had high specificities but often gave late positive results and were not enough for use as prediction tools for this group of patients. However, Serology may be a very useful method to ascertain whether recipient, donors, and patients have a previous history of CMV infection or not. In the present study, serological testing of IgM-anti-CMV was detected positive in only four out of sixteen (25%) of kidney transplant recipients suffering from invasive Symptomatic CMV disease, while the remaining 75% were found negative, which questioned the reliability of IgM seronegativity. Mayers & Amestardam (1997) have reported a 20-30% failure of IgM production during acute CMV infection. No significant rise in IgG titer was observed before the onset of CMV Syndrome which indicates that serological markers could not help for the early diagnosis of CMV infection. Immunosuppression might be responsible for this poor immune response. Seropositivity and seronegativity did not correlate well with clinical Symptoms. Cross-reactive antibodies against Human Cytomegalovirus (HCMV) and Epstein-Barr virus (EBV) were reported by Lang et al., (2001) and Navalpotro et al., (2006). Eventually, serostatus seems to be useful only for detecting any previous CMV infection and for a retrospective confirmation of CMV infection.

Since the implementation of molecular diagnostic techniques twenty years ago, quantitative amplification of CMV viral genome proved to have high analytical sensitivity with very low detection limit (400 copy/mL), which correlated very well with the clinical sensitivity (Caliendo et al., 2000). In the present study all patients presented with mild CMV syndrome, were documented by quantitative PCR to have low viral load 436–3070 copy/mL, while sixteen patients
suffering from active invasive CMV disease were detected by QT-PCR to have high viral load 4000 to > 100,000 copy/mL reflecting 100% analytical Sensitivity of the technique.

It is concluded more than one parameter is required for monitoring CMV infection in transplant patients; serological markers for the pre-operative evaluation of the immune status of both donor and recipient, and QT-PCR for predicting CMV reactivation, and monitoring of - antiviral therapy.

References


