DEVELOPMENT OF REAL TIME PCR ASSAY FOR EARLY DETECTION OF CYTOMEGALOVIRUS INFECTION IN IRAQI WOMEN

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ABSTRACT

This study was constructed to discuss a Molecular Study of Cytomegalovirus isolated from women with repeated miscarriage in relation to immune response molecule Tall like Receptor 2. About (100) blood samples from women suffering from infection with Cytomegalovirus were collected from infertility clinic of Kamal Al – Sammarae hospital and (50) samples from normal subjects served as control for comparison. Test subjects were divided into two age groups: 20-30 years old and 31-40 years old. The women distributed as (60) samples of infertile and (40) samples as miscarriage women. This study included Enzyme Linked Immune Sorbent Assay (ELISA) test was used to detect anti- HCMV antibodies IgG and IgM in the patient serum samples. ELISA test result showed that the miscarriage women shown highest percentage of seropositive to CMV for IgG (40%) and (25%) IgM compared to infertile women IgG (20%) and (15%) IgM with a significant difference $P<0.05$. It has been concluded that there is high prevalence of anti- HCMV antibodies IgG and IgM in both miscarriage and infertile women compared with normal (healthy) women in Baghdad. The Seropositive of anti- HCMV IgG was higher in younger women (20-30) years old while the largest age classes (31-40) years showed higher level of IgM. The CMV primers were selected from highly conserved region of the major enveloped glycoprotein B (gB) and used probe labeled at the 5 end with FAM and the 3 end with TAMRA. The result shown amplification from the sixth cycle.
KEY WORD: Cytomegalovirus, miscarriage, infertile, ELASA, RT-PCR.

INTRODUCTION
Human cytomegalovirus (HCMV) is a ubiquitous member of the Herpesviridae family subfamily BetaherpesVirinae. HCMV is the major infectious cause of congenital infection and hearing loss in children as well as an important pathogen in immunocompromised patients. The viral nucleocapsid containing a linear double stranded DNA of 236 kb and is surrounded by a proteinaceous tegument which is itself enclosed by a loosely applied lipid bilayer. HCMV has an extremely broad tissue tropism that allows it to infect nearly every organ system in the body. It is a major cause of postoperative disease in chemically immunosuppressed transplant recipients and greatly increases the risk of graft rejection. HCMV is also a leading cause of congenital birth defect and infection during the first trimester of pregnancy often results in neurological and cognitive disorders in the developing child. HCMV can be transmitted by close personal contact and by blood transfusion or organ transplantation. HCMV is frequently transmitted from mother to child either in utero or during the perinatal phase. Perinatal transmission often results in severe disturbance of development and disease that may become manifest at birth such as thrombocytopenia, hepatitis, splenomegaly and microcephaly.

RT-PCR assays have been optimized by with improved extraction and amplification techniques, resulting in sensitive and specific assays. CMV DNA detection has highly become routine diagnostic tool reproducible, automated and quantitative nature.

METHODS
Patients Selection and Blood Sample Collection
Blood Samples were collected from one hundred (100) women suffering from CMV infection and miscarriage, 50 healthy women as a control (healthy), their ages ranged between (20-40) years. Samples were subjected to centrifugation at 2000 rpm for 10min. The serum was separated and stored at -20°C. All Samples were subjected for HCMV antibodies using ELISA techniques. In case of blood with EDTA, it was stored at -20°C until used for DNA extraction. The Samples were obtained from infertility clinic of Kamal Al-Sammarae hospital. The collection period extended from November 2013 to March 2014.
Serology (Antibody test)
This test is performed to find current active CMV infection, or past HCMV infection in people who are at risk for reactivation of infection. This technology relies on the utilization of the antibodies to detect virus encoded proteins or antivirus antibodies in infected persons [8]. The ELISA technique was performed using kit intended for estimation concentration of specific (CMV) IgM and (CMV) IgG markers. The kits were purchased from (Biokit, Barcelona, spain, the technique were performed according to the manufacture’s instructions.

DNA Extraction
Total cellular DNA was extracted from blood samples by using the Reliaprep Blood genomic DNA MiniPrep System from Promega USA, estimation the concatenation and purity of the extracted DNA were measured by using nanodrop (UVIS Drop\ACTGene\USA).

PCR Amplification

| Real -Time PCR sequence | 57.3 | 64.6 | FAM 5- AAC CCG TCA GCC ATT CTC TCG-3 TAMRA |

RESULT AND DISCUSSION
Distribution of the studied groups
The cytomegalovirus (CMV) is considered one of the opportunistic viruses with a worldwide distribution that can infect human at any stage of life[9] then the virus became dormant[10] and it is lifetime latency after primary infection and reactivation of the latent virus can reoccur in infected individuals at any time.[11] A majority of these infections are asymptomatic as others and they are difficult to diagnose clinically.[12] IgG and IgM antibodies to cytomegalovirus can be considered as an easy tool for selecting patients who are at risk of cytomegalovirus infection. IgG was reflected the previous infection, presence of it doesn't prevent the reinfection or re activation but may reduce the severity of pathogenesis. While IgM immunoglobulin was considered as evidence of recent or acute infection which is formed immediately after infection and disappeared after short period 16-20 weeks.[13] One hundred samples from infected women distributed as show in figure (1) as (60) infertile and (40) miscarriage women.
Among the two groups, the miscarriage women showed the highest percentage of seropositive to CMV for IgG (40%) and (25%) IgM compared to infertile women IgG (20%) and (15%) IgM as shown in figure (2).

There is a significant difference between two groups of patients for CMV antibodies, this result is in according with\textsuperscript{[14]} and\textsuperscript{[15]} that studied the importance of virus infection to cause miscarriage\textsuperscript{[16]} who studied the infertility importance of CMV also agreement with the study result.

**Seroprevalence of CMV in the miscarriage groups according to age**

Miscarriage is the spontaneous loss of a pregnancy between conception and 24 weeks into pregnancy.\textsuperscript{[17]} Some studies found high presence of CMV antigens in tissues from abortion\textsuperscript{[18]} and One prospective study also found a higher risk of pregnancy loss with CMV infection\textsuperscript{[19]} though results of other prospective studies did not approved it. Despite these reports on the role of CMV infection in spontaneous pregnancy loss, the role of CMV infection in recurrent losses is less clear (Roya et al., 2014) as shown in table (1).
Table (1): seroprevalence of CMV in the miscarriage groups according to age

<table>
<thead>
<tr>
<th>age</th>
<th>Total no.</th>
<th>Anti HCMV IgG</th>
<th>Anti HCMV IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>years</td>
<td></td>
<td>Positive%*</td>
<td>Negative%*</td>
</tr>
<tr>
<td>20-30</td>
<td>40</td>
<td>75</td>
<td>25</td>
</tr>
<tr>
<td>31-40</td>
<td>17</td>
<td>70</td>
<td>30</td>
</tr>
</tbody>
</table>

*P > 0.05.

The age of all patients' women arranged from (20 to 40) years old are shown in Table (1). The age (20-30) years old may be rearranged as the most age class which showed high prevalence of anti- HCMV antibodies IgG which represent 75%, while the age classes (31-40) years old showed the least prevalence of anti- HCMV IgG antibodies 70% without significant difference as shown in figure (3A). This result considered to be comparable with [16] who showed higher percentage of positively at ages (27-32) also [20] who showed 94% of positively at ages (25-34), this is because the chance of pregnancy is higher in younger ages (20-30) years than (31-40) years. Stated that 80% of IgG at age (35-40) years old women have 50-80% in younger ages. These results considered to be comparable with [21] who showed that an increase in seropositive CMV IgG in relation with abortion and infection, this might be due to the effect of CMV on cellular metabolism and activation of other viruses that co-infect the cells inducing subclinical inflammation.

![Figure (3A): IgG level in the miscarriage groups according to age.](image)

For IgM level among miscarriage groups it was clear from figure (3B) that the age (31-40) represent 20% and (20-30) years represent 10%.
Infertility is the inability of a couple to achieve pregnancy over an average period of one year in a woman under 35 years old or 6 months in a woman above 35 years old despite adequate, regular (3-4) times per week unprotected sexual intercourse. Infertility may also be referred to as the inability to carry a pregnancy to the delivery of a live baby.\textsuperscript{22} The sexual transmission of HCMV could be occurred among infertile couples this is assumed to be the most important route in adults.\textsuperscript{23} The Seroprevalence of anti- HCMV IgG in infertile women showed that 50% are Seropositive at (20-30) years old and 25% IgM while the largest age classes (31-40) years showed 40% and 35% respectively as shown in table (2) and figure (4A,B). These results considered to be comparable with\textsuperscript{21} who showed that the frequency of CMV seropositivity increases rapidly between the ages of 15 and 30 years when sexual contact is most active.

Table (2): Seroprevalence of HCMV in infertile groups

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Total no.</th>
<th>Anti HCMV IgG</th>
<th>Anti HCMV IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-30</td>
<td>26</td>
<td>50 Positive%*</td>
<td>50 Negative%*</td>
</tr>
<tr>
<td>31-40</td>
<td>34</td>
<td>40 Negative%*</td>
<td>60 Positive%*</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>25 Positive%*</td>
<td>75 Negative%*</td>
</tr>
</tbody>
</table>

*P> 0.05.
Real time PCR

Real time PCR is rapid, sensitive and useful technique for diagnosing active disease and monitoring response to therapy.[24] The CMV primers were selected from highly conserved region of the major enveloped glycoprotein B (gB) and used probe labeled at the 5 end with FAM and the 3 end with TAMRA. The result shown amplification from the sixth cycle as shown in figure (5). This result agrees with[25] which detection CMV DNA by real time PCR that using the gB primer and probe FAM and TAMRA.

REFERENCES


