Simultaneous detection of Epstein - Barr virus and Cytomegalovirus DNA in Mini-pooling of Whole blood Samples from Iraqi blood donors by Multiplex Real-Time PCR Assay

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Abstract
A qualitative multiplex real-time PCR assay was used for simultaneously detection of Epstein-Barr virus (EBV), cytomegalovirus (CMV) DNA within blood donors by using 10 sample mini-pools (MP10) of whole blood from blood donors using a commercial multiplex PCR kit. The multiplex assay saved time and was cost effective through using blood mini-pools. A total of 1000 whole-blood specimens were collected from blood donors and pooled in 100 MP10 and were categorized to 50 pools after 6h from blood withdrawal and the rest (50 pools) after 12h from blood withdrawal then analyzed by multiplex qPCR kit (Sacace/Italy) . The results were that 42% of MP10 whole blood from blood donors was positive for EBV and 4% positive for CMV; in addition, NAT assay for CMV DNA detection was shown to be better after 12 hrs from blood withdrawal. It is concluded that the activated EBV in a whole blood was high in Iraqi blood donors while the activated CMV was moderately low and the multiplex PCR assay was found to be sensitive for CMV and EBV DNA detection using whole blood specimen within mini pools. EBV and CMV detection by NAT is essential to reduce complications that could be associated with blood transfusion.

Keywords: blood transfusion, multiplex NAT, EBV, CMV


Introduction
Epstein-Barr virus (EBV) and cytomegalovirus (CMV) are human herpesvirus which are ubiquitous in human population and often become reactivated in latently infected immunocompromised patients [1]. Human Cytomegalovirus (HCMV) is the most significant cause of transfusion-associated morbidity and mortality [2]. On the other hand, EBV transmission through transfused blood has also been reported [3] HCMV and EBV are strictly cell-associated, and transmission through blood appears to be due to reactivation of latent virus in white blood cells [2]; hence, the detection of these viruses (qualitatively) or quantitatively aids in the clinical management, allowing for early treatment in order to prevent disease or complications that could contribute to transfusion of reactivated or recently infected viruses through blood [4].

The problem of Human Herpes viruses (HHV)infections that they differ from other transfusion-transmitted infections in which only certain groups of patients require HHV-free blood or blood components, like pregnant women, premature infants of low birth weight who are born to seronegative mothers, seronegative recipients of allogeneic bone marrow transplants from seronegative donors, seronegative AIDS patients, Seronegative immunosuppressed patients in general herpes-naïve immunocompromised recipients. Real-time PCR has become the standard test for viral detections because it is fast, sensitive, and could detect the targets quantitatively or qualitatively. Moreover, the recent PCR assays are capable for the detection of more than one target per well with the use of different fluorochromes through application of multiplex PCR. Considerable time and effort can be
saved by simultaneously amplifying multiple sequences in a single reaction; Multiplex PCR requires that primers lead to amplification of unique regions of DNA, therefore it is becoming a rapid and convenient screening assay in both the clinical and the research laboratory [5]. In addition, the application of multiplex PCR to pool samples improved the cost-effectiveness. Until now, EBV and CMV reactivation has been diagnosed only by serology; however the serological tests reflect reactivation only and do not represent the actual event, especially under immune suppression, and serologic responses may be delayed [6]. To the best of our knowledge, there is no previous study on EBV and CMV circulatory DNA level in blood donors in Iraq by using whole blood samples, therefore this study aimed to estimate the rate of blood donors with reactivation or recent infection of EBV and CMV through detection of EBV and CMV DNA simultaneously in pooled whole blood samples by multiplex PCR assay.

Materials and Methods

Study population and sample collection

Across sectional study was designed to collect 20 ml of whole blood from apparently healthy Iraqi blood donors in EDTA tubes. Whole blood was collected for the detection of EBV and CMV through NAT test by commercial kits for pooled whole blood. One thousand blood donors were included in this study most of them were male (98%) and the rest were female (2%), 95% were from Baghdad and, just (5%) from different Iraqi provinces (except Kurdistan). Blood was taken from donors at the National center of blood bank in Baghdad, Bab-ALmuadham. Five hundred (500) of whole blood samples were collected after 6hrs from blood withdrawal and the rest (another 500) were collected after 12hrs from blood withdrawal. From each blood donor 1 ml of whole blood in EDTA tubes was pipette and put it in 1.5 ml micro centrifuge tube and stored in -80°C until further use.

Pooling of samples

Mini-pooling for stored donor’s whole blood in 1.5 ml micro centrifuge tube was conducted by pupating 150 ul from 10 whole blood samples and put them together in one 1.5 ml micro centrifuge tube i.e. mini-pool 10 (MP10) so the total volume for one MP10 was 1.5 ml.

Nucleic acid extraction

The DNA was extracted from MP10 whole blood by QIAamp® DNA Mini and Blood Mini 250(Lot no. 151043756 Qiagen/Germany) and the steps of extraction were carried out following the manufacturer's instructions. The concentration and the purity of all extracted DNA samples were measured using Quantus Florometer (promega/USA); then, DNA extract were stored at -20°C.

EBV and CMV DNA detection by multiplex real time PCR:

CMV/EBV/HHV6 Quant Real-TM kit (Lot no.27H181705 Sacace/Italy) was used for the detection and differentiation of Cytomegalovirus (CMV) and Epstein Barr Virus (EBV) using 10 μl of DNA as per manufacturer's instructions [7]. The principle of the detection in this kit is based on using real-time amplification with fluorescent reporter dye probes specific for EBV and CMV DNA polymerase genes. This kit also contained an endogenous internal control (IC) that amplifies β-globin gene. ABI® 7500 Real-Time PCR (Applied Biosystems, USA) was used. The positive extraction, positive amplification, negative extraction and non-template negative controls and samples were prepared according to the manufacturer instructions. Thermal profile for plate-type instruments was: 1 cycle for 15 min at 95°C (step1), 5 cycles for 5 sec at 95 C°, 20 sec at 60 C° and 15 sec at 72 C°(step 2), then 40 cycles for 5 sec at 95 C°,30 sec at 60 (fluorescence detection) ,15 sec at 72 C° (step 3).The interpretations of the controls and tested samples results were depended on tables mentioned in manufacturer instruction. B-Globin gene DNA (IC) is detected in the FAM/Green channel, EBV DNA is detected in the JOE/HEX/Cy3/Yellow channel, and CMV DNA is detected in the ROX/Texas Red/Orange channel [7].

Results:

The results of NAT assay by multiplex Real-Time PCR revealed that4% of mini-pools were CMV positive whole blood while 42% of mini-pools of whole blood were positive for EBV within Iraqi blood donors’ figure (1). NAT positive pools were resolved by testing the individual donation samples through multiplex Real-Time PCR, the positive pools showed that each positive MP10 for EBV contained 3-5 positive Individual samples while the positive MP10 for CMV included one Individual positive sample, in addition to that 2 mini-pools were positive for both EBV and CMV were included 3 EBV positive individual samples and one CMV positive individual whole blood samples.
Figure 1: show the percentage of positive MP10 whole blood samples of blood donors that detected by commercial kit multiplex PCR for EBV and CMV.

The samples were categorized into two groups; 500 samples (50 mini-pools) were collected after 6 h of blood withdrawal and the rest of samples (500 samples, 50 mini-pools) were collected after 12 h of blood withdrawal. The results showed that the detection rate by multiplex qPCR of CMV and EBV after 6 h and 12 h from blood withdrawal through Sacace (commercial kit) revealed that CMV detection was significantly better after 12 h than 6 h (P value < 0.05) as in figure 2.

Figure 2: Percentage of positive detection of CMV and EBV by Sacace kit within the first 6 hours versus 12 hours from withdrawing blood. The difference in detection rate of CMV is significantly better in 12 hrs compared to 6 hrs withdrawal of blood. (P value > 0.05)

Discussion

Transfusion-transmitted CMV infections (TT-CMV) were first described by Kaariainen and co-workers in 1966 [8]. Traditionally all CMV seropositivity donors have been regarded as potentially infectious for at-risk patients, so provision of blood products from seronegative donors and leukoreduction of blood products were introduced to reduce the incidence of TT-CMV in risk populations [9]. And the risk of CMV infection during window period may increase the possibilities of TT-CMV infections for seronegative risk patients. On the other hand, there are 80-90% of blood donors are seropositivity [10] that’s why the obtaining of sufficient seronegative blood donors may pose a problem, so many scientists were directed toward the estimation of CMV-DNA in whole blood or plasma of blood donors by PCR as an attempt to assess the presence of CMV-DNA which may result in developing TT-CMV infection in seronegative blood recipients [11]. Regarding to EBV in ability to transmit through blood transfusion the leukoreduction procedure can pointedly reduce the number of EBV genome; it has been found that EBV can still be detected in leukoreduced products [12]. Stanley David et al. detected and quantified all eight human herpes viruses on white cell-enriched blood from 100 randomly selected blood donors from the southeast Texas region in 2008 by utilizing a set of newly developed real-time polymerase chain reaction assays, their results may represent some similarities to our results they reported (EBV) (72%) and CMV (1%) [13]. Furui Y and his colleagues in 2013 in Japan tested blood donors in different age groups for CMV-DNA by using polymerase chain reaction in the cellular fractions of all samples they found that CMV DNA in the cellular fraction of 4.3% of samples from donors in their 60s and in 1.0% of samples from donors younger than 60 years [14]. We depended on many scientific aspects in designing of our study, the first was using whole blood specimens rather than serum or plasma based on the concept that EBV developed a latent infection in B-cells lymphocytes and CMV developed latency in monocytes/macrophages; so we assumed whole blood samples may reflect the absolute viral burden of CMV and EBV latent infection and reactivation in circulation. Colleen S. Kraft et al revealed that CMV DNA is detected more frequently and viral load values are often higher in whole blood compared with plasma [15].
assumption also comes close with Kaoru Wada, Naomi Kubota et al. in Tokyo in 2007 when they used multiplex assay to quantify the EBV, CMV, and HHV-6 of 303 whole-blood and plasma specimens, the results revealed that all forms of viral DNA were detected more frequently in whole blood than in plasma. During the symptomatic period, EBV DNA was detected in all whole-blood specimens but not in all plasma specimens. Servi J. C. Stevens et al. studied the EBV-DNA level in Post-transplant lymph proliferative disease (PTLD) patients by unfractionated whole blood; they found that increased EBV DNA loads in PTLD patients were restricted to the cellular blood compartment, as parallel serum samples were all below cut-off value, which indicates absence of lytic viral replication [16]. The second aspect of our study design was utilizing the multiplex qPCR for simultaneous detection of EBV-CMV in blood donors screening tests and give insight about the usefulness of this assay in developing the approaches of blood screening assays in blood banks by simplifying the workflow, reducing sample turnaround time and costs. In addition to multiplex qPCR, we tried in this study to estimate using mini-pool system in the detection of CMV-EBV within blood screening assay, like Markoulatos P et al. used sensitive multiplex PCR assay that detects simultaneous herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), varicella-zoster virus (VZV), human cytomegalovirus (CMV), and Epstein-Barr virus (EBV) on 86 cerebrospinal fluid (CSF) specimens from patients which had the clinical symptoms of encephalitis, meningitis or meningoencephalitis, the sensitivity of the multiplex PCR was determined to be 0.01 and 0.03 [17]. Gunson et al. in 2008 in Scotland developed an adenovirus–CMV–EBV triplex assay that uses one set of five pooled quantitative standards, demonstrated sensitivity and an accuracy of Quantitation equivalent to previous single tests and was shown to be able to detect mixed infections with no loss in sensitivity. The low rate of reactive CMV within apparently healthy blood donors may related to viral dynamic, that could impact the viral level in blood circulation, for CMV the median doubling time was 4.3 days was estimated and considerably long [18]. While EBV doubling time was estimated by Simona Sica et al. as shorter as 46-56 h observed EBV loads in healthy donors are very low or undetectable related to low frequency of EBV positive B cells in the circulation [19]. Our study results for CMV and EBV in whole blood represented that the detection of CMV after 12h was significantly more than 6 h (P value < 0.05) while for EBV there were no difference. Shafer et al. reported that delayed separation of plasma increased the frequency of positive PCR results for CMV in latently infected patients. Those authors suggested that lysis of latently infected leukocytes released CMV into the plasma and thus increased the number of positive results [20].

Conclusion
We concluded that the rate of activated EBV in a whole blood was high in Iraqi blood donors while the activated CMV was moderately low so there were a significant risk of activated EBV transmission through blood transfusion even for serologically positive recipients. In addition, the whole blood was more preferable sample for the detection of EBV and CMV by NAT assay than plasma or serum and pooling system application was successfully done with dependable results. The using of qPCR multiplex that amplified the 3 or 4 targets at the same amplification reaction with mini-pooling of samples was cost effective, saving time and reduced the cross contamination problems. NAT assay for CMV DNA detection was better after 12 h from blood withdrawal.

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