Seroprevalence and real-time PCR study of Epstein–Barr virus and the value of screening in pretransplant patients

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Introduction

Epstein–Barr virus (EBV) is a member of the herpes virus family. Other members include herpes simplex I and II, varicella–zoster virus, cytomegalovirus (CMV), and human herpes virus (HHV)-6, 7, and 8 and EBV [1]. In 1968 the EBV was discovered to be the cause of infectious mononucleosis (IM), a usually self-limited condition [2]. Only about 5% of adults in western areas remain EBV uninfected; thus, antibody prevalence rates reach 95% or more in elderly individuals [3].

The EBV genome encodes a series of products interacting with different variants of antiapoptotic molecules, cytokines, and signal transducers, thus enhancing EBV infection immortalization and transformation [10,11]. EBV encodes for important proteins that show sequence to diverse human proteins. The proteins were BHRF1 (homologous to Bcl-2), BDLF2 (homologous to cyclin B1), BARF1 (homologous to intercellular cell adhesion molecule 1), and BCRF1 (viral IL-10, homologous to human IL-10) [12].

In immunocompetent individuals EBV infection is controlled by humoral and cell-mediated immunity, supported by the interferon system. However, in patients with EBV IgM positive sera, which in turn changes some concepts in organ transplantation.

Keywords:
EBV IgM VCA, Epstein–Barr virus, PCR, pretransplant recipient

Objectives

This study was performed to estimate the prevalence of Epstein–Barr virus immunoglobulin M virus capsid antigen (EBV IgM VCA) among healthy blood donors and to confirm the real risk of transfusion transmission by detection of virus load using PCR quantification.

Materials and methods

A total of 860 apparently healthy Egyptian blood donors were enrolled and tested for EBV IgM VCA. Quantitative PCR was performed for reactive cases for EBV IgM VCA.

Results

An overall 38 patients were reactive for EBV IgM VCA, constituting 4.4% of the sample. Reactivity of Epstein–Barr virus did not differ significantly as regards sex distribution, blood grouping, Rh factor positivity, and hemoglobin level, but it was significantly higher among upper Egypt participants than among those from other regions (P = 0.006). There was a very high statistically significant positive correlation between the titer of EBV VCA IgM reactive cases and age in the studied group (P = 0.0001 and r = 0.6). PCR was negative for all of the reactive cases.

Conclusion

Routine screening for Epstein–Barr virus in blood bags is not economical. Screening is highly recommended only for immunocompromised and pretransplant patients. Viremia is not the role in individuals with EBV IgM positive sera, which in turn changes some concepts in organ transplantation.

Keywords:
EBV IgM VCA, Epstein–Barr virus, PCR, pretransplant recipient

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with mononucleosis, cytotoxic T cells dominate over B cells. In contrast, in immunosuppressed patients B cells dominate over T cells [13].

The EBV has been involved in the pathogenesis of different chronic autoimmune conditions since the finding of elevated levels of antibody to the virus in systemic lupus erythematosus in 1971 [14]. However, in 2003 the EBV-infected autoreactive B-cell hypothesis of autoimmunity was proposed, which suggested that, in genetically susceptible individuals, EBV-infected autoreactive B cells seed the target organ where they produce pathogenic autoantibodies and provide costimulatory survival signals to autoreactive T cells, which would otherwise die in the target organ by activation-induced apoptosis [15]. Kannangai et al. [16] in India found an increased EBV activation among the autoimmune patient groups (rheumatoid arthritis and Hachimoto thyroiditis) compared with normal healthy controls.

Transfusion-transmitted infections are serious problems associated with blood transfusion. Hepatitis C virus (HCV), HIV, and hepatitis B virus are the most important agents responsible for transfusion-transmitted diseases. Others are CMV, EBV, human parvovirus, parvovirus B19, and Creutzfeldt–Jakob disease, which can be transmitted by transfusion of infected blood or its component [17]. Blood safety warrants strict screening measures to reduce the possibility of transmitting blood-borne pathogens. However, transfusion-transmitted infections for which testing is not currently performed continue to be a concern. Among these untested agents is EBV, which, in the transplant setting, is associated with the development of lymphoproliferative disorders [18].

The aim of the present work was to estimate the prevalence of Epstein–Barr virus immunoglobulin M virus capsid antigen (EBV IgM VCA) among healthy blood donors and to confirm the real risk of transfusion transmission by detection of virus load by PCR quantification.

**Materials and methods**

A total of 860 apparently healthy Egyptian blood donors (778 men and 82 women) were enrolled. They donated blood at The Blood bank of Cairo University Hospitals during the period from September 2012 until January 2013. Their ages ranged from 18 to 59 years. No history of any cardiovascular disease or diabetes mellitus was reported, nor a history of recent drug intake (of any kind) at least 14 days before sampling. They had different occupations and were from different Egyptian Governorates. The protocol of this study was approved by the Ethical committee and review board of the department of Internal Medicine according to the Declaration of Helsinki.

Samples were collected by venipuncture of the median cubital vein in closed sterile tubes using an EDTA vacutainer system. Any hemolyzed, icteric, or turbid sample was avoided. Testing was done within 8 h from the time of collection of samples. Samples were centrifuged at 3500 rpm for 10 min before the test to get clear pure plasma. Three milliliters of the plasma of any reactive sample were collected and frozen at −40°C for archiving.

All samples were tested first for:

1. Hepatitis B surface antigen (using the Bio RAD Monolisa HBsAg PLUS ELISA test, USA).
2. HCV antibody (using the ORTHO HCV 3.0 ELISA test, USA).
3. HIV types 1 and 2 antigen-antibody (using the BIO RAD GENSCREEN PLUS HIV Ag-Ab ELISA test, USA).
4. Treponema pallidum antibody (using the DiaMed ID-PaGIA SYPHILIS Ab test, Switzerland).
5. All samples that were confirmed to be nonreactive for the previous parameters were tested for EBV VCA IgM (using the BOUTY BEIA EBV VCA IgM Mab ELISA test, Bankasia, Australia).
6. Quantitative PCR was performed for all 38 cases proved to be reactive for EBV VCA IgM using the LightCycler EBV Quant Kit ROCHE Molecular diagnostics, USA, which is an in-vitro diagnostic assay that utilizes real-time PCR amplification of nucleic acids for quantitation of EBV DNA in human clinical samples. The kit is used with the LightCycler 2.0 Instrument with software version 4.05 or higher.
7. Hemoglobin level and ABO and Rh blood groups were determined.

**Statistical methods**

Precoded data were statistically analyzed with the statistical package of social science software program, version 21. Data were summarized using frequency and percentage for qualitative data or mean and SD for quantitative ones. Comparison between groups was performed using the χ²-test or Fisher’s exact test for qualitative data or the independent sample t-test for quantitative ones. Pearson’s correlation coefficient was calculated to clarify the association between quantitative variables. P values less than 0.05 were considered statistically significant and if less than 0.001 were considered highly significant.
Results

Descriptive statistics of the studied group showed that the number of EBV IgM VCA reactive cases was 38 and nonreactive cases was 822 (4.4 and 95.6%), their ages ranging between 18 and 59 years (25.5 ± 6.1). Men numbered 778 (90.5%), and women numbered 82 (9.5%). The number of donors coming from Great Cairo was 543/860 (63.1%), those from Delta was 200/860 (23.3%), from Sinai was 21/860 (2.4%), and from Upper Egypt was 96/860 (11.2%). Hemoglobin level ranged from 12 to 17 g/dl, with a mean ± SD of 14.6 ± 1.1. The distribution of patients on the basis of blood groups was as follows: blood group A, 299/860 (34.8%); blood group B, 215/860 (25%); blood group O, 256/860 (29.8%); and blood group AB, 90/860 (10.5%). Rh-positive patients numbered 836/860 (97.2%) and Rh-negative patients numbered 24/860 (2.8%) (Table 1).

Reactivity of EBV did not differ significantly as regard to age, sex distribution, blood grouping, Rh factor positivity, or hemoglobin level, but it was significantly higher among upper Egypt participants than among those from other regions \((P = 0.006)\). Reactivity to EBV did not significantly differ among participants of Great Cairo, lower Egypt, and Sinai regions (Table 2).

In our study, the number of male reactive cases for EBV was 37 (constituting 4.8% of the total number of men in the study), whereas female reactive cases for EBV VCA IgM was 1 (constituting 1.2%). The calculated percentage is between the total number and the positively reactive cases for EBV in both men and women in the study.

Analysis of the titer of reactive EBV VCA IgM (IU/ml) by ELISA in relation to different blood groups showed that there was a higher numerical value \((33.3 ± 13)\) in blood group O than in other blood groups (blood group A, 21.9 ± 2.7; blood group B, 20.7 ± 3.5; and blood group AB, 10.7 ± 1.7). But this difference was not statistically significant \((P = 0.4)\) (Table 3).

Comparison of titer of reactive cases for EBV VCA IgM in relation to different governorates in the studied group showed that there was a higher numerical value \((28.6 ± 7.7\) and \(27.8 ± 6.0)\) in Great Cairo and Sinai than in Delta and Upper Egypt \((18.1 ± 3.2\) and \(18.6 ± 3.5), respectively). But this difference was not statistically significant \((P = 0.7)\) (Table 4).

There was a very high statistically significant positive correlation between titer of EBV VCA IgM reactive cases and age in the studied group \((P = 0.0001\) and \(r = 0.6)\) (Fig. 1).

Quantitative PCR performed on all 38 cases that were reactive for EBV VCA IgM was negative.

Discussion

The risk of transmission of infectious diseases through transfusion is minimal because of effective preventive strategies including new laboratory tests. Well-recognized viruses including hepatitis A virus, hepatitis B virus, HCV, hepatitis D virus, hepatitis G

| Table 1: Descriptive statistics Description \((n = 860)\) |
|---|---|
| Age (years) range, mean ± SD | 18–59 | 25.5 ± 6.1 |
| Sex, n, % | M/F | 778/82 | 90.5\%/9.5\% |
| Region, n, % | Great Cairo | 543 | 63.1 |
| | Delta | 200 | 23.3 |
| | Upper Egypt | 96 | 11.2 |
| | Sinai | 21 | 2.4 |
| Blood grouping, n, % | A | 299 | 34.8 |
| | B | 215 | 25.0 |
| | AB | 90 | 10.5 |
| | O | 256 | 29.8 |
| RH factor, n, % | +VE | 836 | 97.2 |
| | −VE | 24 | 2.8 |
| HB (gm%) range, mean ± SD | 12.0–17.0 | 14.6 ± 1.1 |
| EBV, n, % | Reactive | 38 | 4.4 |
| Non-reactive | 822 | 95.6 |

| Table 2: Comparison between reactive and non-reactive cases for EBV VCA IgM |
|---|---|---|
| Reactive | Non-reactive | \(P\) value |
| Age (years) Mean ± SD | 25.5 ± 6.0 | 25.5 ± 6.1 | 0.9 |
| Sex, n, % | 37 | 4.8% | 741 | 95.2% | 0.3 |
| Male \((n = 778)\) | Male \((n = 822)\) | 1 | 1.2 | 81 | 98.8% |
| Regional, n, % | 19 | 3.5% | 524 | 96.5% | 0.1 |
| Great Cairo \((n = 543)\) | Great Cairo \((n = 860)\) | 8 | 4.0% | 192 | 96.0% | 0.8 |
| Delta \((n = 200)\) | Delta \((n = 860)\) | 10 | 10.4% | 86 | 89.6% | 0.006 |
| Upper Egypt \((n = 96)\) | Upper Egypt \((n = 860)\) | 1 | 4.8% | 20 | 95.2% | 0.6 |
| Sinai \((n = 21)\) | Sinai \((n = 860)\) | 13 | 4.3% | 286 | 95.7% | 1.0 |
| Blood grouping, n, % | 10 | 4.4% | 86 | 95.6% | 1.0 |
| A \((n = 299)\) | AB \((n = 90)\) | 11 | 4.3% | 245 | 95.7% | 1.0 |
| B \((n = 215)\) | AB \((n = 90)\) | 38 | 4.5% | 798 | 95.5% | 0.6 |
| AB \((n = 90)\) | O \((n = 256)\) | 0 | 0.0% | 24 | 100.0% | |
| HB (gm%) Mean ± SD | 15.0 ± 1.1 | 14.6 ± 1.1 | 0.052 |

\(P\)-value is significant if <0.05*.
virus/GB-C virus, HIV types 1 and 2, human T-cell lymphotropic virus types I and II, CMV, EBV, TT virus, HHV-6, SEN virus, and human parvovirus (B19) may pose a threat to the safety of blood [19]. Bacteria such as T. pallidum (the agent of syphilis), Yersinia enterocolitica, and Staphylococcus and Streptococcus spp. (common agents of bacterial contamination), and parasites such as Plasmodium spp. (the agent of malaria), Trypanosoma cruzi (agent of Chagas’ disease), and Babesia microti (agent of babesiosis) have also been reported to be transmitted through blood transfusion [20].

EBV can be transmitted through blood transfusion and usually presents as a clinical health hazard in high-risk recipients, such as immunosuppressed individuals [21].

Infection with EBV early in childhood is usually asymptomatic, whereas delayed primary infection is typically manifested by the signs and symptoms of IM. Once infection occurs, the viral genome is maintained for life in a small fraction of B lymphocytes. Systemic reactivation of an infection is normally kept in check by the healthy immune system that fights lytic replication using cytotoxic T lymphocytes, natural killer cells, and antibody-dependent cell cytotoxicity [22].

In immunocompromised states such as in allograft organ transplant recipients, especially in children with pretransplantation EBV seronegativity, there is a particular risk for developing post-transplantation lymphoproliferative disease (PTLD) during immunosuppressive therapy [23–25].

Babel et al. [26] aiming at decreasing PTLD in post-transplantation renal allograft children used pretransplantation minor infusions from seropositive donors for EBV in seronegative recipients aiming at inducing immunity to EBV. Follow-up for 5 years after transplantation showed negative cases for PTLD.

Aiming at detecting the seroprevalence of EBV infection in Egyptian blood donors, 860 blood bags were screened for EBV IgM VCA.

The results showed that the number of reactive cases for EBV VCA IgM was 38/860 (4.4%), whereas the number of nonreactive cases for EBV VCA IgM was 822/860 (95.6%).

A mass screening was performed in India between 1986 and 2000 comprising 1741 clinically suspected subjects for IM. All of them underwent the Paul-Bunnel antibodies test. The percentage of PB antibody-positive cases was found to be 11.1% in the studied group [27].

The difference between the percentage difference in our study and the Indian one is explained by the fact that our subjects were clinically free and there is a difference between the Paul-Bunnel test and IgM VCA in both sensitivity and specificity.

The difference between age and sex frequencies in relation to results of EBV VCA IgM were

Comparison between age distribution of reactive and nonreactive cases for EBV VCA IgM in the studied group showed that the mean age of the reactive group was 25.5 ± 6.0, whereas the mean age of the nonreactive group was 25.5 ± 6.1, with no detected statistically significant difference between the two groups (P = 0.9).

However, a very high statistical significance with moderate positive correlation was found between the titer of EBV VCA IgM reactive cases and increasing age in the studied group (r = 0.6). In addition, there was no statistical difference between sex frequencies in relation to results of EBV VCA IgM. The number of male reactive cases for EBV was 37 (constituting 4.8% of the total number of men in the study).

Table 4: Comparison between titre of reactive cases for EBV VCA IgM in relation to different Governorates

<table>
<thead>
<tr>
<th>Variables</th>
<th>Great Cairo</th>
<th>Delta</th>
<th>Sinai</th>
<th>Upper Egypt</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Titre of EBV VCA IgM (IU/ml)</td>
<td>mean ± SD</td>
<td>n = 19</td>
<td>mean ± SD</td>
<td>n = 8</td>
<td>mean ± SD</td>
</tr>
<tr>
<td></td>
<td>28.6 ± 7.7</td>
<td>18.1 ± 3.2</td>
<td>27.8 ± 6.0</td>
<td>18.6 ± 3.5</td>
<td>0.7</td>
</tr>
</tbody>
</table>

P-value is significant if <0.05*.
In rare cases VCA IgM antibodies persist longer even during the period when EBNA-1 IgG antibodies are still detectable. The persistence of VCA IgM antibodies after the resolution of EBV infection is well known and can last for weeks, months, or even years. It is important to note that the persistence of VCA IgM antibodies does not necessarily correlate with active EBV infection, as they can also be detected in asymptomatic carriers or in primary EBV infection. Therefore, the detection of VCA IgM antibodies alone should not be used as a diagnostic marker for active EBV infection. Instead, other diagnostic markers such as viral load measurements or the presence of EBV-induced lymphoproliferative disorders should be considered.

It is essential to understand the role of VCA IgM antibodies in the diagnostic evaluation of EBV infection. VCA IgM antibodies are produced early during the infection and can be detected in the serum of infected individuals. The presence of VCA IgM antibodies is a marker of recent infection, as they disappear within a few weeks after the onset of symptoms. Therefore, the detection of VCA IgM antibodies can help differentiate between recent and past EBV infection. However, the persistence of VCA IgM antibodies should not be used as a sole diagnostic marker for active EBV infection, as they can also be detected in asymptomatic carriers and in the late stages of primary EBV infection.

In conclusion, the detection of VCA IgM antibodies is a useful diagnostic marker for recent EBV infection. However, the persistence of VCA IgM antibodies should not be used as a sole diagnostic marker for active EBV infection. Other diagnostic markers such as viral load measurements or the presence of EBV-induced lymphoproliferative disorders should be considered to confirm the diagnosis of active EBV infection.
already produced. Therefore, a patient with a primary infection may exhibit the same serological profile as a patient with a past infection, and vice versa. In these cases further diagnostic approaches are required [36].

Transient immunosuppression of immunocompetent individuals may lead to EBV reactivation, whose detection requires molecular diagnostic methods such as PCR [37].

It is to be known that neither a test of EBV VCA IgM nor a test of the presence of VCA IgG in the absence of EBNA antibody is solely reliable for diagnosing primary EBV infection. PCR for EBV DNA in serum is a useful addition to the panel of tests available for this purpose, particularly if it is used as a confirmatory test in conjunction with serological tests [38].

Although EBV DNA presence is short lived after onset of symptoms, giving it a low negative predictive value, its detection in plasma has high sensitivity in primary EBV infection. An EBV PCR should be considered in cases of positive IgM VCA and negative heterophile antibody because it is difficult to exclude the possibility of a false-positive IgM VCA or false-negative heterophile antibody [39,40].

To avoid post-transplant lymphoproliferative disorder (PTLD) following organ transplantation, policy was changed at the United Network for Organ Sharing, giving priority to pediatric patients for kidneys from younger donors (ages ≤ 35 years), and prospective EBV testing of donors will be helpful in the appropriate allocation of these organs [41].

Nevertheless, a study performed by Trottier et al. [18] suggested an association between transfusions and post-transplant EBV infection in hematopoietic stem cell transplant recipients and they confirmed the necessity of strict screening measures for blood safety to minimize the risk of transfusion hazards.

Conclusion
We conclude from the above study that routine screening of EBV in blood bags is not economical as our results detected positive serology in 4.4% of the cases and the PCR performed for these reactive cases was negative, eliminating the risk of transfusing virus load to normal recipients and confirming that not all sera of individuals having positive EBV IgM harbor the virus, which in turn changes the protocol of organ transplantation.

We recommend the application of LR technique as a good measure minimizing the risk of transfusion hazards, not only at the level of EBV infection but also at the level of other multiple pathogens. Another matter of utmost importance is the strict application of serological and PCR screening for blood transfusion to immunocompromised individuals and patients prepared for transplantation to avoid PTLD.

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Conflicts of interest
There are no conflicts of interest.

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