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

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Objective

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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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 has been involved in the development of different types of B-cell lymphoproliferative disorders, including Burkitt's lymphoma, classic Hodgkin's lymphoma, and lymphomas in immunocompromised individuals (after transplant and HIV-associated lymphoproliferative disorders). T-cell lymphoproliferative disorders that have been reported to be EBV associated include a subset of peripheral T-cell lymphomas, angioimmunoblastic T-cell lymphoma, extranodal nasal-type natural killer/T-cell lymphoma, and other rare subtypes [4].

Many studies confirmed the association between EBV infection and the development of nasopharyngeal carcinoma with presence of EBV DNA in patients' peripheral blood [5–8]. Other studies revealed the association of EBV with about 10% of cases of gastric carcinoma worldwide [9].

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 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 χ^2-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

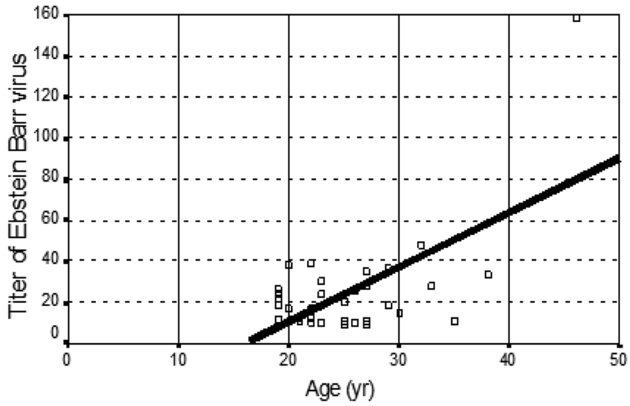
	Description (n = 860)	
Age (years) range, mean ± SD	18–59	25.5 ± 6.1
Sex n, %		
M/F	778/82	90.5/9.5
Region n, %		
Great cairo	543	63.1
Delta	200	23.3
Upper egypt	96	11.2
Sinai	21	2.4
Blood grouping n, %		
A	299	34.8
B	215	25.0
AB	90	10.5
O	256	29.8
RH factor n, %		
+VE	836	97.2
–VE	24	2.8
HB (gm%) range, 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

	EBV				P value
	Reactive (n = 38)		Non-reactive (n = 822)		
Age (years) Mean ± SD	25.5 ± 6.0		25.5 ± 6.1		0.9
Sex n, %					
Male (n = 778)	37	4.8%	741	95.2%	0.3
Female (n = 82)	1	1.2	81	98.8%	
Region n, %					
Great Cairo (n = 543)	19	3.5%	524	96.5%	0.1
Delta (n = 200)	8	4.0%	192	96.0%	0.8
Upper Egypt (n = 96)	10	10.4%	86	89.6%	0.006
Sinai (n = 21)	1	4.8%	20	95.2%	0.6
Blood grouping n, %					
A (n = 299)	13	4.3%	286	95.7%	1.0
B (n = 215)	10	4.7%	205	95.3%	0.8
AB (n = 90)	4	4.4%	86	95.6%	1.0
O (n = 256)	11	4.3%	245	95.7%	1.0
RH factor n, %					
+VE (n = 836)	38	4.5%	798	95.5%	0.6
–VE (n = 24)	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*.

Figure 1



Correlation between titer of EBV IgM VCA reactive cases and age in the studied group (P = 0.0001 and r = 0.6).

Table 3: Comparison between titre of EBV VCA IgM in relation to different blood groups

Variables	Blood group A mean ± SD n = 13	Blood group B mean ± SD n = 10	Blood group O mean ± SD n = 11	Blood group AB mean ± SD n = 4	P-value
Titre of epstein-barr virus (IU/ml)	21.9 ± 2.7	20.7 ± 3.5	33.3 ± 13	10.7 ± 1.7	0.4

P-value is significant if <0.05*.

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

Variables	Great cairo mean ± SD n = 19	Delta mean ± SD n = 8	Sinai mean ± SD n = 1	Upper egypt mean ± SD n = 10	P-value
Titre of epstein-barr virus (IU/ml)	28.6 ± 7.7	18.1 ± 3.2	27.8 ± 6.0	18.6 ± 3.5	0.7

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.

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 (P = 0.0001 and 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), whereas that of female reactive cases for EBV VCA IgM was 1 (constituting 1.2%). Miller in his study in 2002 reported that EBV antibodies have been isolated in all populations and appear to have no affinity for infecting one sex over the other. EBV antibodies are found in 90–95% of virtually all populations by the time they reach adulthood [28].

Reactivity of EBV was significantly higher among upper Egypt participants than among those belonging to other regions (total number: 96; reactive cases, 10, 10.4%; P = 0.006). Reactivity to EBV did not differ significantly among participants of Great Cairo, lower Egypt, and Sinai regions. Moreover, analysis of the titer of reactive cases for EBV VCA IgM in relation to different governorates in the studied group showed that there was a higher number (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). However, this difference was not statistically significant (P = 0.7). Those findings are against expectation as it is well known that the incidence of EBV prevalence is correlated with overcrowdness, which is more expected to be found in Cairo.

Those findings were different from those found by Silva and Pereira (2004) in Brazil when their detection of the overall prevalence of anti-Epstein–Barr virus nuclear antigen (EBNA) antibodies in different governorates corresponded well with that which was reported worldwide: progressively to higher prevalence of positive serology with increasing crowdness and without sex bias binding [29].

Our results were not in concordance with those of Hudnall and colleagues, who utilized real-time PCR assays for detection of all eight human herpes viruses. The prevalence of viral DNA load in 100 randomly selected healthy blood donors from the southeast Texas region in the USA was reported. Herpes simplex viruses 1 and 2, varicella-zoster virus, and HHV-8 DNA were not detected in any donor sample. In contrast, EBV (72%) and HHV-7 (65%) were commonly detected, HHV-6 (30%) was often detected (type B only), and CMV (1%) was rarely detected [30].

Infection caused by EBV usually present clinical diseases in high-risk recipients, such as immunosuppressed individuals; however, the transfusion transmission risk is now very small because of preventive strategies such as universal leukodepletion and solvent-detergent treatment [31].

A study was conducted for detection of the EBV genome on CD19+ B cells in 60 randomly selected fresh red blood cell (RBC) units before and after leukocyte reduction (LR). B lymphocytes from pre-LR specimens and mononuclear cells from post-LR specimens were assayed for EBV DNA with sensitive real-time PCR revealing that a 4-log reduction of EBV genomic copy number can be achieved with LR of RBC units and renders most RBC units EBV-negative by sensitive PCR [32].

A study performed by Wagner and colleagues in 1994 examined 15 EBV-seronegative pediatric patients who received EBV-seropositive red cell concentrates before and up to 11 weeks after transfusion. None of these children showed serological or clinical signs of active EBV infection. The use of modern leukocyte depletion systems dramatically reduced the number of EBV-positive cells in red cell concentrates, minimizing the risk of EBV infection [33].

It is well known that not all transmissible infectious agents are screened in donor blood and that yet-unrecognized, emerging pathogens cannot be detected. At least 15 European nations have adopted universal LR. Reporting at the recent US Food and Drug Administration Workshop on universal LR, the American Red Cross and America's Blood Centers, who provide the USA with more than 90% of all transfused blood products, presented data indicating that 80% of all donated blood in the USA undergoes LR. There is speculation by some that subjecting all donated blood to LR will become a universal standard of practice, as subjecting blood components to LR provides an additional and justified measure of caution [34].

Aiming at confirming EBV infection diagnosis in our subjects, PCR was performed in all seropositive cases of EBV IgM VCA to detect the virus load. The results were negative for all 38 cases.

Those results can be explained on the basis that our donors were clinically free and did not give any history of even minor flu-like illness or fatigue. This is an important factor because in order for PCR to be effective in detecting the viral load it must be performed early during infection before the immune system of the host eliminates the virus. Those results were similar to the results detected by Gartzonika and colleagues in their study carried out in 2012 testing the utility of PCR in the diagnosis of primary EBV infection. They concluded that real-time PCR is a reliable tool for diagnosis of primary EBV infection early in the course of disease and may especially serve as a useful diagnostic supplement in serologically unclear cases of EBV infection [35].

In rare cases VCA IgM antibodies persist longer even during the period when EBNA-1 IgG antibodies are 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 (age≤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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