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Comparison between Serological and Molecular Diagnosis of Epstein Barr Virus Infection among Egyptian Renal Transplant Recipients

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ABSTRACT

Background: Kidney transplantation is the best treatment for end-stage kidney diseases**.** Lifelong immunosuppression preserves graft function. However, they usually lead to severe viral infections**;** as Epstein Barr Virus **(**EBV) which lead to development Post Transplant Lymphoproliferative Disorders (PTLD). **Objectives:** The aim of this study is to compare between serological and molecular assays as methods of detection of EBV infection. Presumed risk factors for development of EBV infection and its impact on transplant outcome were studied. **Patients and Methods**: A total of 50 Egyptian kidney transplant recipients received their renal allografts from living-related donors were studied. Recipients were tested for EBV infection by serological markers; anti-EBV viral capsid antigen (VCA) IgM and molecular assay by detection BamHI region by PCR. Different co-morbid risk factors for development of EBV infection (pre-transplant hemodialysis & blood transfusion, diabetes mellitus, CMV, and HCV infection) were studied. Likewise, the impact of EBV infection on transplant outcomes was evaluated. **Results:** Of the 50 patients, 66% were positive for VCA IgM and 42% were positive BamHI region of EBV. BamHI positivity was significantly correlated with duration of transplantation, and severity of rejections episodes. On the other hand, none of these risk factors were correlated with the positivity of VCA IgM. Among the studied recipients, EBV infection detected by either serological or molecular assay has no impact on the transplant outcome.
Conclusions: Although serological diagnosis for EBV infection is a simple method for screening and follow-up. Yet, molecular diagnosis seems to be more accurate diagnostic test.

Keywords: Epstein Barr Virus, Kidney transplantation, Serological Diagnosis, Molecular Diagnosis, Case series, Mansoura University.

INTRODUCTION

Renal transplantation is the best treatment option either for adults & children with chronic kidney disease **(1).** Unfortunately, opportunistic infections such as EBV and cytomegalovirus (CMV) are major complications to patients after transplantation, which have a negative impact on transplant outcomes and increase the risk of rejection**.** EBV infection occurs in more than third of renal transplant recipients and accounts for high morbidity and mortality rate, it was established after 1-6 months post-transplantation, after receiving high doses of immunosuppression **(2).**

EBV is a viral DNA belongs to herpesvirus family**.** It represented as a latent, asymptomatic infection in most cases **(3).** Most children in developing countries become positive at the age of 5, while it delayed in developed countries with high socioeconomic state. The main way of EBV spread is orally through the saliva. But it may be transmitted through many routes, such as blood transfusion, organ transplantation, sexual contact, and sharing infected personal objects **(4).**

After organ transplantation, the rate of infection increased in children compared to adults as the recipient is immunocompromised and exposed to seropositive donor leukocyte. The symptoms of infection include fever, enlarged lymph nodes, and hepatosplenomegaly **(5).** EBV serological diagnosis is common diagnostic test to evaluate EBV infectious state by detecting antibodies in at various stages of EBV life cycle, however, immunosuppression protocols may affect the interpretation of this test as they inhibit recipients' immune response**.** Therefore, molecular diagnosis is made to detect EBV DNA such as PCR **(6).** The classification of the EBV genome was designed according to a BamHI-restriction fragment map according to their sizes **(7).** The current study aimed to compare serological and molecular assays as methods for detection of EBV infection and studied the possible risk factors and impact on transplant outcome.

PATIENTS AND METHODS

The study was conducted on 50 Egyptian kidney transplant recipients who received their renal allograft in the period between 2010 and 2021. The selected recipients were having their first renal transplantation. Any recipients who had previously received a kidney transplant, another organ transplant or had cancer were excluded. They were subjected to pre- and post-transplantation evaluation as follows:

I) Pretransplant evaluations: age, sex, previous blood transfusion, pretransplant hemodialysis, associated comorbidities as diabetes mellitus, the degree of matching between recipient and donor, and history of CMV and HCV infections.

II) Post transplant evaluations: date of transplantation, laboratory investigations include

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CBC, differential leucocytic count, serum creatinine, eGFR using CKD EPI 2021 and MDRD equation, creatinine clearance, immunosuppression drugs (induction and maintenance), and histopathological examination of the graft biopsy in cases of graft dysfunction according to Banff classification were evaluated.

III) Specific investigations: ***A) Serological evaluation:*** EBV antibodies were detected in the serum of selected cases using commercial *DRG EBV-VCA IgM ELISA kits (DRG International, Inc., USA)*, according to manufacturer's kit as the color intensity is related to amount of VCA IgM Ab. The results were expressed as DU units according to the following equation $\frac{Patient\ absorbance\ value \times 10}{co}$,and classified according to their DU value as negative (<9 DU), grey zone (9-11), or positive (⩾11).

B) Molecular detection of EBV DNA:

BamHI region detection was done by conventional PCR as following: genomic DNA was extracted from whole blood obtained from EDTA-treated samples by using *QIAamp DSP Virus Spin Kit* according to kit instructions. PCR was carried to amplify 175-bp fragment of the EBV BamHI region, the primers used were forward:5'AACATGCTGTATGCCTCGCAGCG-3' and reverse: 5'AATTACTGGCGTGAATTGTGCCCA-3[(8)]. PCR reactions were done by 200 ng of DNA in a 25ml volume. The reaction contained 1U of Dream Taq polymerase, 1U PCR buffer, 0.25 mM of each dNTP and 0.2 mM of each primer. Settings of thermal cycle were an initial denaturation step 95°c for 5 min, followed by 25 cycles composed of denaturation 95°c, annealing at 55°c and extension 72 °c each for 30 sec then the last extension step for 10 min[(7)]**.** Amplification was done using (Gene Amp PCR System, Applied Biosystems). The amplified DNA were separated through electrophoresis containing 2% agarose gel stained with ethidium bromide and visualized in UV trans-illuminator. Samples with a single 175-bp band were recognized as positive samples.

Ethical Approval:

An approval of the study was obtained from Mansoura University Academic and Ethical Committee (IRB code number MD.20.01.268). Every patient signed an informed written consent for acceptance of participation in the study. This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

Statistical Analysis

The collected data were coded, processed, and analyzed using the SPSS (Statistical Package for Social Sciences) version 22 for Windows® (IBM SPSS Inc, Chicago, IL, USA). Qualitative data were described using number and percent. Quantitative data were described using median (minimum and maximum) and inter quartile range for non-parametric data and mean and standard deviation (SD) for parametric data after testing normality using Kolmogrov-Smirnov test. Chi-Square test for comparison of qualitative data of 2 or more groups, and Fischer exact test was used as correction for Chi-Square test when more than 25% of cells have count less than 5. Student's t-test or Mann Whitney U test was used to compare quantitative data of 2 independent groups. P value $\leq$0.05 was considered significant.

RESULTS

The median age of the studied recipients was 20.5, ranging from 3 to 54 years; 46% were pediatric (<18 years old) and most of them were males (64%). The majority of cases (70%) were transplanted within one year of transplantation (recent transplantation) while 30% were transplanted from 5 to 10 years (late transplantation).

Risk factor for EBV infection were assessed for all studied cases, 6% were diabetic, 6% were HCV positive, 30% developed CMV infection, the majority (90%) were maintained on hemodialysis prior to transplantation and 34% of them received blood transfusion

All the recipients were induced with IL-2 receptor antagonist (basiliximab) except one high immunologically risk recipient who received Anti thymocyte globin (ATG) as induction. All recipients were maintained on tacrolimus-based immunosuppression and 64% were enjoying steroid-free regimen.

Throughout the follow up period, half of the studied recipients developed graft dysfunction which necessitated graft biopsy. Histopathological examination of the biopsied graft evaluated by Banff classification revealed different grades of rejections.

All recipients were evaluated for hematological abnormalities; (66% anemic, 4% thrombocytopenia, 30% leukocytosis and 28% lymphocytosis).

Demographic data, risk factors, immunosuppression protocols, number of rejections and severity were correlated with positive EBV cases either estimated by VCA IgM or BamHI region of EBV. Also, the impact on both hematological abnormalities and graft function were studied for both markers.

It was found that 33(66%) were positive for VCA IgM. VCA IgM showed no statistically significant association with any parameters studied either demographic data, risk factors for EBV infection development, post-transplant immunosuppression protocol, number of rejections & severity or impact on transplant outcomes (table 1-5).

Table (1) Association of demographic data with positive E\\BV cases as estimated by VCA IgM

Parameter	Positive IgM	Negative IgM	P value
Age (in years): • Pediatric • Adult	22 (17-32) 13 (65.5%) 20 (74.1%)	16 (13-25) 10 (43.5%) 7 (25.9%)	0.071 0.157
Sex: • Male • Female	 20 (62.5%) 13 (72.2%)	 12 (37.5%) 5 (27.8%)	 0.353
Duration of transplantation: • Recent • Late	 21 (60%) 12 (80%)	 14 (40%) 3 (20%)	 0.148
HLA-A and HLA-B matching: • Zero% • 25% • 50% • 75% • 100%	 4 (66.7%) 3 (75%) 21 (72.4%) 4 (44.4%) 1 (50%)	 2 (33.3%) 1 (25%) 8 (27.6%) 5 (55.6%) 1 (50%)	 0.597
HLA DRB1 matching: • 50% • 100%	 28 (65.1%) 5 (71.4%)	 15 (34.9%) 2 (28.6%)	 0.554

Table (2) Risk Factors for development of EBV infection as estimated by VCA IgM

Risk Factors	Positive IgM	Negative IgM	P value
• Blood transfusion	13(76.5%)	4(23.5%)	0.212
• Pretransplant hemodialysis	28(62.2%)	17(37.8%)	0.112
• Diabetes	1(33.3%)	2(66.7%)	0.264
• CMV infection	11(73.3%)	4(26.7%)	0.353
• HCV infection	2(66.7%)	1(33.3%)	0.736

Table (3) Association of post-transplant immunosuppression protocol with positive EBV cases as estimated by VCA IgM

Parameter	Positive IgM	Negative IgM	P value
Induction Immunosuppression protocols			
Basilxumab	32 (65.3%)	17 (34.7%)	0.660
ATG	1 (100%)	0 (0%)	
Maintenance Immunosuppression protocols			
Steroid based	11 (61.1%)	7 (38.9%)	0.403
Steroid free	22 (68.8%)	10 (31.2%)	

Table (4) Association of number of rejections & severity with positive EBV cases as estimated by VCA IgM

Parameter	Positive IgM	Negative IgM	P value
Number of rejections			
No rejection	16 (48.5)	9 (52.9%)	0.500
One rejection episode	17 (51.5%)	8 (47.1%)	
Histopathological findings in recipients subjected to graft biopsy			
Borderline change	2 (66.7%)	1 (33.3%)	0.898
Acute cellular rejection	9 (69.2%)	4 (30.8%)	
Antibody mediated rejection	1 (100%)	0 (0%)	
Chronic allograft nephropathy	5 (62.5%)	3 (37.5%)	

Table (5) Impact of Positive EBV infection on hematological abnormalities and graft function as estimated by VCA IgM

Parameter	Positive IgM	Negative IgM	P value
Anemia* (<13.5gm/dl in male & <12gmldl in females)	20 (60.6%)	13 (39.4%)	0.212
Leukocytosis* (WBCs >11000 µl)	9 (60%)	6 (40%)	0.392
Lymphocytosis	7 (50%)	7 (50%)	0.261
Thrombocytopenia	2 (100%)	0 (0%)	0.239
Serum creatinine(mg/dl)	1.4 (1-1.7)	1.2 (0.9-1.6)	0.455
eGFR (CKD-EPI 2021)	76.4 (53.8-95.6)	85.5 (68.4-128.2)	0.235
eGFR (MDRD)	75.3 (53.6-93.2)	83.9 (71.2-126.9)	0.235
Creatinine clearance	66 (53-90)	64.5 (48.5-66.2)	0.429

BamHI region of EBV was positive in 21(44%) recipients (figure 1). BamHI region of EBV showed a statistically significant association with duration of transplantation as all late transplant recipients were positive for BamHI and 82.9% of recent transplant recipients were negative for BamHI (P<0.001). No significant association was observed with age, gender, HLA-A, -B, and -DR matching and risk factors (tables 6 and 7).

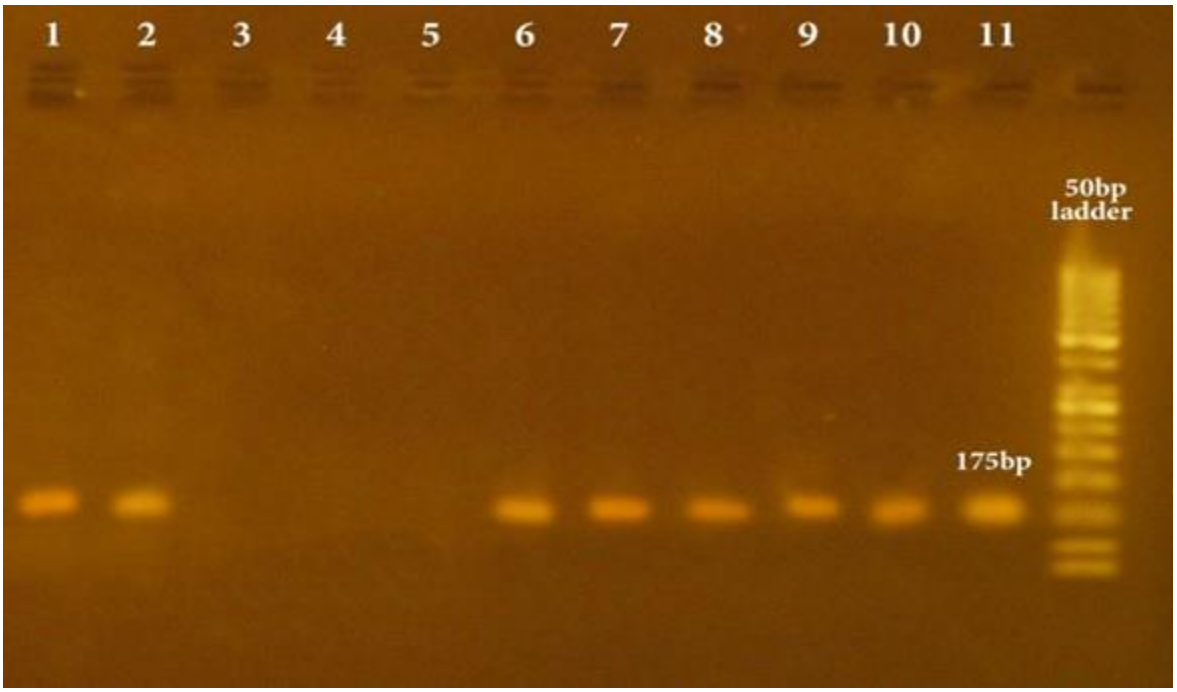

Figure (1): Agarose gel electrophoresis of BamHI region of EBV.

- **Lane 1, 2, 6, 7, 8, 9, 10, 11: PCR products of 175 bp.**
- **Lane3, 4, 5: show no bands indicating negative samples for EBV**

Table (6) Association of demographic data with positive EBV cases as estimated by BamHI

Parameter	Positive BamHI	Negative BamHI	P value
Age (in years):	20 (17-27)	22 (14-32)	0.723
• Pediatric	10 (43.5%)	13 (56.5%)	0.536
• Adult	11 (40.7%)	16 (59.3%)	
Sex:			0.488
• Male	14 (43.8%)	18 (56.2%)	
• Female	7 (38.9%)	11 (61.1%)	
Duration of transplantation:			**<0.001**
• Recent	6 (17.1%)	29 (82.9%)	
• Late	15 (100%)	0 (0%)	
HLA-A and HLA-B matching:			
• 0%	5 (83.3%)	1 (16.7%)	0.078
• 25%	3 (75%)	1 (25%)	
• 50%	10 (34.5%)	19 (65.5%)	
• 75%	3 (33.3%)	6 (66.7%)	
• 100%	0 (0%)	2 (100%)	
HLA DRB1 matching:			
• 50%	18 (41.9%)	25 (58.1%)	0.635
• 100%	3 (42.9%)	4 (57.1%)	

Table (7) Risk Factors for development of EBV infection as estimated by VCA IgM

Risk Factors	Positive IgM	Negative IgM	P value
• Blood transfusion	8(47.1%)	9(52.9%)	0.412
• Pretransplant hemodialysis	19(42.2%)	26(57.8%)	0.654
• Diabetes	2(66.7%)	1(33.3%)	0.379
• CMV infection	5(33.3%)	10(66.7%)	0.311
• HCV infection	1(33.3%)	2(66.7%)	0.621

Also, BamHI region of EBV showed no statistically significant association with post-transplant immunosuppression protocols, number of rejections and impact of EBV positivity either on hematological abnormalities or graft function (tables 8-10). While it showed a statistically significant correlation with the severity of rejection (P=0.007) (table 9).

Table (8) Association of post-transplant immunosuppression protocol with positive EBV cases as estimated by BamHI

Parameter	Positive BamHI	Negative BamHI	P value
Induction Immunosuppression protocols			
Basilxumab	20 (40.8%)	29 (59.2%)	0.420
ATG	1 (100%)	0 (0%)	
Maintenance Immunosuppression protocols			
Steroid based	8 (44.4%)	10 (55.6%)	0.512
Steroid free	13 (40.6%)	19 (59.4%)	

Table (9) Association of number of rejections & severity with positive EBV cases as estimated by BamHI

Parameter	Positive BamHI	Negative BamHI	P value
Number of rejections			
No rejection	8 (38.1%)	17 (58.6%)	0.126
One rejection episode	13 (61.9%)	12 (41.4%)	
Histopathological findings in recipients subjected to graft biopsy			
Borderline change	1 (33.3%)	2 (66.7%)	**0.007**
Acute cellular rejection	11 (84.6%)	2 (15.4%)	
Antibody mediated rejection	0 (0%)	1 (100%)	
Chronic allograft nephropathy	1 (12.5%)	7 (87.5%)	

Table (10) Impact of Positive EBV infection on hematological abnormalities and graft function as estimated by BamHI

Parameter	Positive BamHI	Negative BamHI	P value
Anemia* (<13.5gm/dl in male & <12gmldl in females)	13 (39.4%)	20 (60.6%)	0.412
Leukocytosis* (WBCs >11000 µl)	9 (60%)	6 (40%)	0.085
Lymphocytosis	7 (50%)	7 (50%)	0.734
Thrombocytopenia	1 (50%)	1 (50%)	0.930
Serum creatinine(mg/dl)	1.4 (1.1-1.9)	1.2 (0.9-1.4)	0.062
eGFR (CKD-EPI 2021)	68.9 (50.5-91.6)	85.5 (66.5-121.5)	0.080
eGFR (MDRD)	71.6 (51.9-90.9)	83.9 (65.6-120.9)	0.114
Creatinine clearance	65 (42.5-85)	65 (55.2-67)	0.625

DISCUSSION

Viral infections are frequent complications after renal transplantation. EBV infection is one of the serious infections which may be primary or reactivation due to immunosuppression therapy, and it could lead to the development of PTLD [(9)].

This study was designed to compare between serological testing (EBV VCA IgM) and molecular assay (BamHI region of EBV) for detection of EBV infection among kidney transplant recipients and to study the possible risk factors and impact on transplant outcome. In transplanted recipients, their immune system was dysregulated due to immunosuppressive drugs, so detection of antibodies is of less significance. So the use of molecular biology in diagnosis EBV DNA could provide a more accurate diagnostic option **[(6)].** We chose BamHI region of EBV as it is expressed in lytic phase and enhance cell survival**.** Moreover, it was reported that samples with negative EBNA-1 PCR were additionally tested for BamHI PCR to enable sensitive detection of EBV DNA **[(10)]**.

Several studies were carried out to diagnose EBV infection post renal transplantation using various serological markers in detection EBV antibodies. In the current study we preformed VCA IgM as one of the serological marker for detection EBV antibodies that indicate acute infection **[(6)].** It was found that 33 (66%) were positive for VCA IgM. **Byrne *et al.* [(11)]** found that VCA IgM was negative at time of transplantation, then it became positive at 5 months and their peak was at 9 months post-transplantation. Also, **Beader *et al.* [(12)]** examined the prevalence of EBV infection among different patients included renal transplant recipients either in adults or pediatric patients. They found that 9% were positive for VCA IgM indicating acute EBV infection.

Other studies used molecular assay to diagnose EBV infection among renal transplant recipients. In this study, the BamHI region of EBV was positive in 21 (44%) recipients. **Braz-Silva PH *et al.* [(13)]** indicated that EBV was more common in the oral mucosa of immunocompromised patients as 80% were diagnosed positive by detecting BamHI in their buccal mucosa using PCR.

In the current report, we studied the association of each marker with demographic data, different risk factors, immunosuppression protocols, number, and severity of acute rejection episodes. The impact on hematological abnormalities and graft functions were also studied. In positive VCA IgM cases, there was no association with

the studied risk factors or the transplant outcome. On the other hand, a significant association was found between the duration of transplantation and EBV infection diagnosed by BamHI region of EBV as all late transplant recipients were positive while 82.9% of recent transplant recipients were negative. This could be explained by late transplant recipients who were maintained on immunosuppression drugs for long duration were highly susceptible to EBV infection. In addition, BamHI positive cases experienced severe forms of acute rejections which required several courses of potent anti-rejection therapies in the form of high doses of steroid pulses.

EBV infection was observed in both adult and pediatric. Also, the onset of development of this infection is an important risk factor [(3)]**.** HLA matching is critical for the success of a kidney transplant and optimization of the immunosuppression protocols. Poorly matching recipients received higher dose of immunosuppressive drugs which impair their immune response and increase the susceptibility of infection [(14)]**. Bamoulid *et al.*** [(15)] found no association between positive EBV and recipients' gender, and age. **Beader *et al***. [(12)] found in their study that no significant association between VCA IgM and gender distribution. **Laurent *et al.*** [(16)] suggested that the risk factors for EBV infection were age <5 years, ≥5 HLA mismatches, and negative EBV at time of transplantation. **Morton *et al.*** [(17)] reported that the prevalence of EBV DNA increased significantly with time of transplantation from 16% in 1st year of transplantation, to 40% in 10th year post transplantation.

Several risk factors may contribute to the development of EBV infection post transplantation. They include blood transfusion which may transmit the infection, and pretransplant hemodialysis, which impair both adaptive and innate immune responses [(18)]. **Naraqi *et al*.** [(19)] showed that pretransplant hemodialysis was not significantly associated with EBV infection development. While **Beader *et al.*** [(12)] demonstrated that EBV is highly prevalent with hemodialysis patients (97.7%).

Other comorbid conditions that may predispose to EBV infection include diabetes, CMV and HCV infection were also studied. Diabetic patients are more prone to infectious complications [(20)]. **Dworzanski *et al.*** [(21)] reported that diabetic patients (35.9%) had a high incidence of EBV infection. In CMV and HCV infections, IL-10 was increased and in turn reduce the immune response and increase the exposure to infections. Moreover, co-infection with these viruses is associated with bad clinical outcome than single infection [(22)]. Similar to our study, **Bamoulid *et al.*** [(15)] detected that there was no significant correlation between EBV infection and HCV cases. In contrast to our findings, **Blazquez *et al.*** [(23)] found that CMV and EBV were significantly associated.

Immunosuppression protocols after renal transplantation was designed to combine between more than one agent with different actions to get the maximum benefit and provide long term graft survival with the least side effects. The current standard immunosuppression protocol include basiliximab as induction therapy and tacrolimus-based maintenance protocols except in low immunologic risk patients where steroid-avoidance regimens could be adopted[(24)]**.** Similar to our study, **Hocker *et al.*** [(25)] found that type of calcineurin inhibitors (tacrolimus and cyclosporine) didn't influence the incidence of EBV infection. For induction therapy, the rate of EBV infection didn't differ between patients received Basiliximab (63%) and patients didn't receive basiliximab (49%). **Bamoulid *et al.* and Blazquez *et al.*** [(15,23)] detected that EBV⁻positive cases were significantly associated with ATG, and rapid steroid withdrawal had a higher EBV prevalence, but the lowest EBV^+ prevalence was found with basiliximab and rapid steroid withdrawal.

Graft biopsies are the gold standard for diagnosis the allograft rejection, whether acute or chronic [(26)]. Frequency of acute rejection episodes, their severity, and time of rejection correlate with the transplantation outcome. Recipients with one rejection episode had better survival rates than who suffered more than one attack [(27)]**.** **Hocker *et al.*** [(25)] found no significant difference in the rate of biopsy-proven acute rejections between EBV positive cases and negative cases.

Post transplantation anemia is a main complication after renal transplantation due to various factors as immunosuppressive drugs, antiviral drugs, viral infections, and acute rejection[(28)]**. Mahmud *et al.*** [(29)] found that 76 % of renal transplant recipients of living related donor selected a group were anemic. Also, thrombocytopenia occurs in the first-year post-transplantation and it may lead to mild to severe bleeding, petechial hemorrhage, general weakness, and fatigue due to bone marrow suppression secondary to immunosuppressive drugs or viral infections[(30)]**.** In transplanted recipients, EBV infection causes a mononucleosis like syndrome, mainly present with lymphocytosis due to high doses of immunosuppressive drugs [(31)]**.**

The graft function was evaluated using estimated glomerular filtration rate (eGFR) Modification of Diet in Renal Disease (MDRD) equation and chronic kidney disease Epidemiology Collaboration (CKD-EPI) equation [(32)]. **Levi *et al.* and Morton *et al.*** [(17,33)] found negative significance between eGFR, rate of kidney function and EBV DNA infection. In contrast **Shams ELdin *et al.*** [(34)] found strong association between EBV positive cases and elevation of serum creatinine. So, elevation of creatinine is 20-time risk indicator of EBV development.

In our study, we found that BamHI was more accurate than VCA IgM evidenced by the significant correlation between the diagnosis of infection and both duration of transplantation and severity of rejection. This was also confirmed by **Chan *et al.*** [35] who found that EBV DNA detection in suspected primary EBV infection cases by targeting BamHI had a sensitivity & specificity (63% & 95% respectively), while serological diagnosis by VCA IgM had poor sensitivity & specificity (54% & 57%).

The advantage of this study, it provides a comparative analysis between serological assay (EBV VCA IgM) and molecular method (BamHI region of EBV) in diagnosis EBV infection among Egyptian live donor renal transplant recipients. However, the current study has some limitations; small number of recipients, we were not able to differentiate between primary or secondary infection, and molecular assay performed was qualitative not quantitative.

CONCLUSION

Despite that serological detection for EBV antibodies is of clinical importance in screening and follow up positive cases. Yet, it's accuracy is limited especially in immunocompromised renal transplant recipients. Though, molecular diagnosis by BamHI region of EBV is considered as an accurate diagnostic method which correlate with duration of transplantation and severity of acute rejection episodes.

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