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Determination of Epstein-Barr virus (EBV) DNA Load as A Biomarker to Follow up EBV Related Hodgkin's and Non Hodgkin's Lymphoma Patients Using Quantitative Competitive Polymerase Chain Reaction Technique

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Abstract

Background The Epstein -Barr virus (EBV) is the first human virus implicated in the carcinogenesis. EBV contributes to the carcinogenesis like Hodgkin's Lymphoma (HL) and Non Hodgkin's Lymphoma (NHL).

Objective Quantitative Competitive Polymerase Chain Reaction (QC-PCR) and ELISA was used to quantitate the EBV DNA load in blood samples of HL and NHL patients pre and post therapy.

Methods EBV DNA extracted from blood samples of 18 HL and NHL patients pre and post therapy, 9 apparently healthy controls used to quantify the EBV DNA load. Quantitative Competitive Polymerase Chain Reaction (QC-PCR) and ELISA were used to quantify EBV DNA load. Wild EBV DNA (WT) obtained by Transformation of Escherichia coli MM 294 with Wild type (WT) DNA plasmid *pGEMBamHI-K*.

Results EBV DNA load in controls was found to be 7-1.99 × 10^3 in HL and NHL patients, while in patients it's ranged from zero to 1.936×10^9 copy numbers per ml of blood. High EBV load with the range of 10715(1.071×104) to 1936421960 (1.936×109) above cut-off value was detected in 66.7% of HL and 5861(5.86×103)-50118(5.01×104) copies/ml blood in 44.5 % of NHL patients pretherapy. After chemotherapy, 60% of HL patients and 100% of HL patients with high EBV load showed significant response. Low viral load was found in 44.45% of patients. Only 55% of lymphoma patients with high EBV load, after chemotherapy 16.6% of them continue to have high EBV DNA load compared to the control group, 38.3% of the patients showed response to chemotherapy when their viral load decreased below cut off value. While 11.1 % continue to have high DNA load. One patient (5.5%) showed an elevated EBV load after completion of chemotherapy.

Conclusions EBV DNA load estimated by Quantitative Competitive Polymerase Chain Reaction considered as valuable promising tumor biomarker in the diagnosis and monitoring of EBV related HL and NHL patients.

Key words Quantitative Competitive Polymerase Chain Reaction (QC-PCR), Epstein-Barr virus (EBV), Viral DNA load, Hodgkin's (HL) and non Hodgkin's Lymphoma (NHL) Patients.

Introduction

The Epstein Barr virus (EBV) is a lymphotropic virus that infects and immortalizes B-lymphocytes, which maintained their viral genome in a non-

replicating latent form EBV is mainly B-cell tropic but capable of infecting T-cells and epithelial cells. EBV is the first human virus implicated in the pathogenesis of lymphoid and epithelial malignancies which reach 80% in developing countries; these malignancies including Burkitt's lymphoma (BL), undifferentiated nasopharyngeal carcinoma (UNFC), HL, NHL, post-transplant lympho-proliferative disease (PTLD), some T-cell lymphoma and more recently certain cancers of stomach and smooth muscles. Hodgkin's lymphoma is uncommon malignant tumor of the lymphatic system where approximately 40% of HL were shown to contain clonal EBV [(2)]. NHL are associated with EBV, like nasal T / natural killer (NK) cell lymphoma and angioimmunoblastic lymphadenopathy [(3)]. Due to EBV associated malignancies, recent advances in PCR technology called Quantitative Competitive PCR (QC-PCR) permit precise measurement of EBV DNA level in clinical samples called EBV viral load. QC-PCR is used to quantify PCR products; it's a method to quantitatively measure DNA amount, and number of its copies in the sample [(4)]. Epstein -Barr virus (EBV) viral load assays able to distinguish low-level infection in carriers from higher levels associated EBV diseased patients. The patients affected by EBV often have high levels of EBV DNA in their body fluids like blood, plasma or serum and this is used as specific marker for EBV carcinogenesis. The QC-PCR co-amplify EBV DNA and a spiked or endogenous control sequence called Internal standard (IS), the relative amount of EBV and control product was measured in EBV related disease for early diagnosis and for monitoring the efficiency of therapy [(5)]. QC-PCR used to quantitate, EBV-DNA in plasma of all EBERs-positive AIDS lymphoma patients; they concluded that QC-PCR is very promising in diagnosis and management of EBV related lymphoma [(6)].

Aim of the study: Utilizing EBV DNA load as molecular biomarker to predict the prognosis and to check response to chemotherapy in HL and NHL patients.

Methods

Patients and samples preparation: Peripheral blood samples, were taken before and 3-4 months after chemotherapy from Eighteen HL and NHL patients at Baghdad Medical City Teaching Hospital. Sampling extended from Feb 2005 to Nov 2005. Nine apparently healthy individual were enrolled in this study as control group.

Viral DNA extraction: Fresh whole blood from healthy group and patients were diluted 10 times in NASBA lysis buffer contains 5M guanidine thiocyanate, 0.75% Triton X-100, 1 M Tris -HCL, stored at –20 °C until DNA extracted by silica based extraction method as described previously by Boom et al, 1990.

EBV DNA: EBV DNA obtained by Transformation of Escherichia coli MM 294 with Wild type (WT) DNA plasmid *pGEMBamHI-K* and according to those reports by Kushner, 1978.

Plasmid DNA used: WT DNA *pGEMBamHI-K* plasmid which has the prototype EBV B95-8 EBNA1 sequence, used as positive control and *PQPCR8* plasmid DNA as in Gene bank was used as Internal Standard (IS) which compete viral DNA in QC-PCR, both obtained from Dr. Stevens, S.J.C, University Hospital Vrije, the Netherlands.

Plasmid DNA extraction: Plasmid DNA extracted from *E. coli* cells as described by Pospiech and Neuman, 1995, number of WT and IS DNA copies was determined.

Primers and Probes: The nucleotide sequences and localization of primers are listed in Table 1.

Table 1: Primers and Probes used for QC-PCR (*)

Primers or probe	Sequence (5´ – 3´)	Localization (EBV B95-8 genome)
QP1	GCCGGTGTGTTCGTATATGG	109462-109482
QP2	bio-CAAAACCTCAGCAAATATATGAG	109652-109675
WT probe	dig-TCTCCCCTTTGGAATGGCCCCTG	109563-109563
IS probe	dig-CTATATGCCTGCTTCCTCCTCCGGCG	

* Stevens et al, 1999 [10]

Amplification reactions were carried out at a reaction volume of 50 µl containing PCR reaction buffer (50 Mm kCl, 1.5 Mm $MgCl_2$, 10 Mm Tris (pH 8.2), 200 µM (each) deoxynucleoside triphosphate, and 1U of Taq DNA polymerase (Roch, USA). 25 pmole of Primer QP1. A 25 pmole of antisense primer QP2, 5 µl of DNA elute was added and amplified as follows: Denaturation at 95 °C for 4 min. and subjected to 40 cycles, each cycle consist of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, extension at 70 dC for 1 min. Samples maintained at 72 dC for 3 min [10].

Qualitative EBNA-1 PCR:

Standard dilution curve of the wild type (WT) DNA of known copy number was constructed by making serial dilution of WT DNA (106, 105, 104, 103, 102, 101, and 100). Each one of these dilutions was amplified separately. The PCR cocktail preparation performed in a 50 µl volume reaction, Primers and probes are listed in table 1 [10].

The PCR product of each dilution was quantified by ELISA detection method and optical density (O.D) was read at 405 nm.

Standard curve was blotted between O.D. of each PCR product of each dilution and the number of WT copies present in each dilution (Figure 3).

Prescreening method for patients and healthy control samples:

DNA eluted from blood samples of each patient before treatment and controls DNA was amplified as mentioned previously. By comparison of the results obtained to the standard dilution curve, the number of copies of EBV present in each sample was estimated.

Quantitative competitive PCR assay:

Five µl elute of DNA extracted from blood samples of each patient before and after treatment was amplified with IS DNA copies (104, 103, and 102) separately. Primers and probes used as in Table 1.

Quantification of PCR products by enzyme immunoassay (EIA):

EIA with a modified procedure Jacobs, 1996 used Density. Five µl of biotinylated PCR products were added to 50 µl of 1 x hybridization buffer, denatured by 0.2 M NaOH. A 50 pmol/ml WT Digoxigenine (DIG) labeled oligonucleotide probe was added to one of the wells then IS DIG labeled oligonucleotide probe was added to the other well. Antidigoxigenine – conjugated antibodies (75 mU/ml hybridization buffer) added to all wells followed by 100 µl of 2, 2-Azino-di (3-ethyle benzthioazoline sulphonate 6) diammonium salt (ABTS) substrate (Roch. USA). The color intensity was measured at 405 nm and the runs included positive control PCR product, where as distilled water (D.W) was used as negative control [11].

Results

Prescreening method for DNA extracted from patients:

The extracted DNA which obtained from whole blood samples was found to have the optical density $O.D_{260}$. (1.6-1.8). The size of the DNA fragments separated compared to the DNA Marker was found to be 5148bp (Figure 1).

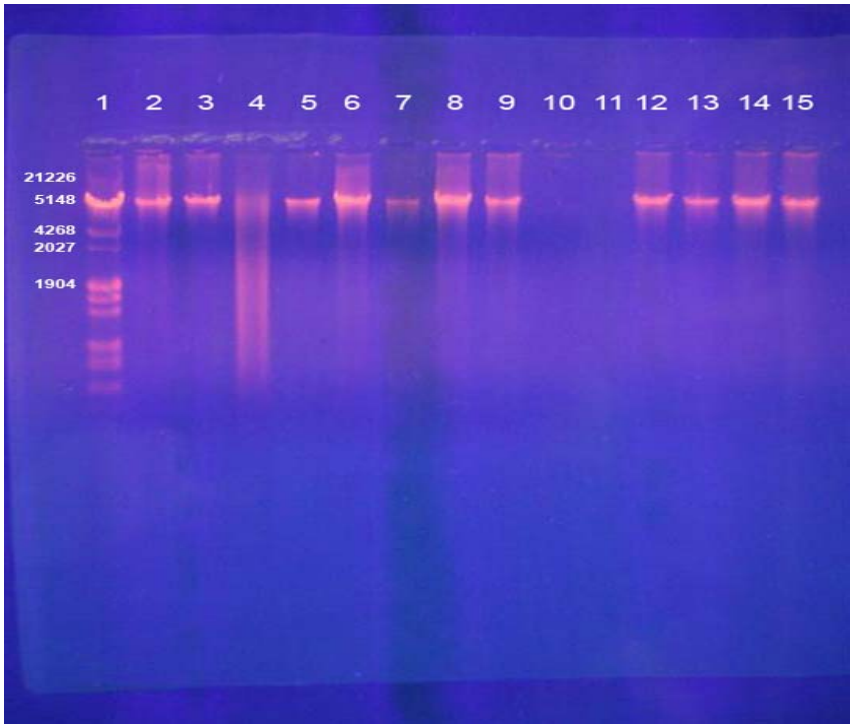

Figure 1: Ethidium bromide stained Agarose gel electrophoresis for the DNA extracted from the blood of the lymphoma patients, shows DNA fragments extracted from blood samples.
Lane (1): lambda DNA/ECOR 1+HindIII Marker 3.
Lane (2, 3, 4, 5, 6, 7, 8, 9, 12, 13, 14, and 15): DNA extracted from patient blood samples.
Lane (11): negative control.

Transformation:

Plasmid WT DNA *pGEM Bam HIK* (WT) was extracted from transformed E. coli MM294 by salting method compared to DNA Marker. The size of the prepared plasmid DNA was determined by comparison of their relative prepared position to that of the DNA ladder appeared in lane 8 (Figure 2).

Plasmid quantification:

WT plasmid DNA concentration:

The concentration of WT plasmid DNA was 635ng/µl and the number of copies of WT plasmid DNA was 0.075×10^{12} copies/µl.

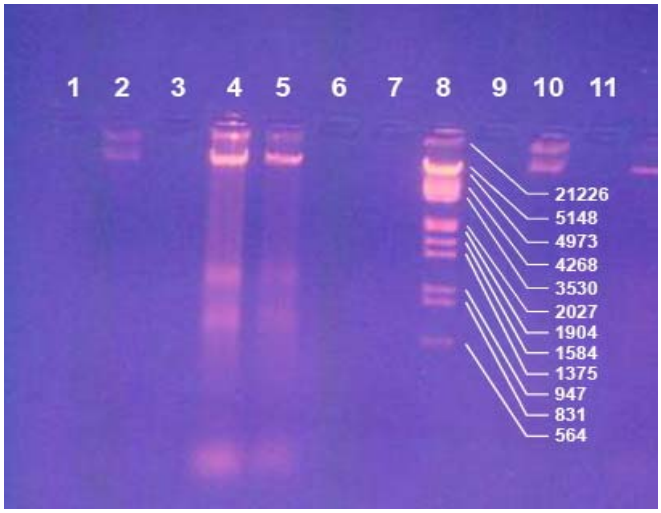

Figure 2: 0.5% agarose gel electrophoresis stained with ethidium bromide showing the Wild type (WT) DNA extracted from plasmid pGEMBam HIK extracted Lane 2, 10: WT DNA plasmid pGEMBam *HIK* purchased from Dr. Stevens S.J.C.

Lane 4: WT DNA plasmid pGEMBam *HIK* prepared high concentration.
Lane 5: WT DNA plasmid pGEMBam *HIk* prepared diluted.
Lane 2, 10: WT DNA plasmid pGEMBam *HIK* purchased from Dr. Stevens S.J.C.
Lane 7: negative control.
Lane 8: lambda DNA/*ECOR 1+Hind III*.

Standard dilution curve o f WT DNA was constructed by plotting optical density Product of each WT dilution against the number of WT copies present in each dilution (figure 3). It was observed that patient's samples have qualitatively EBV DNA copy numbers range from 102.4 to 10^6 DNA copy/µl.

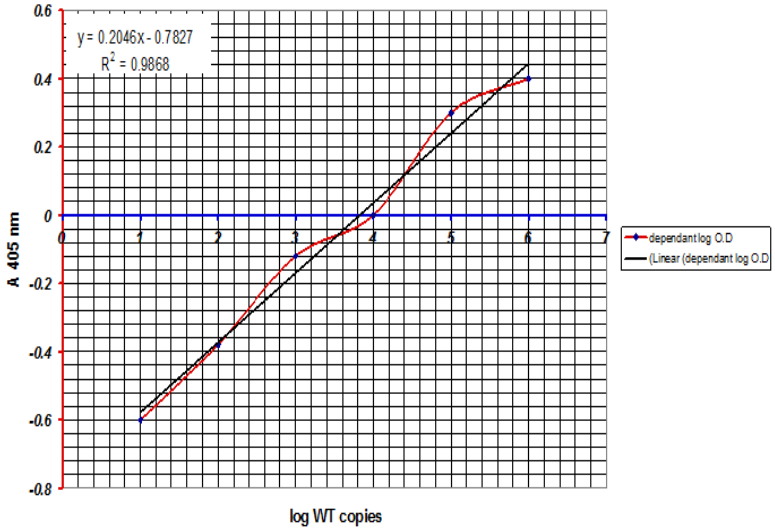

Figure 3: Standard curve of Wild type (WT) EBV DNA

Figure 4 reveals the Analytical sensitivity and amplification equivalence of QC-PCR for both WT and IS plasmid DNA. Five µl (10^1-10^5) /µl of each of WT and IS plasmid DNA copies were amplified separately. The amplified PCR products were analyzed using 1.5% gel electrophoresis and then visualized by ethidium bromide stain where 10^1 copies could be detected for both WT DNA and IS DNA (figure 4).

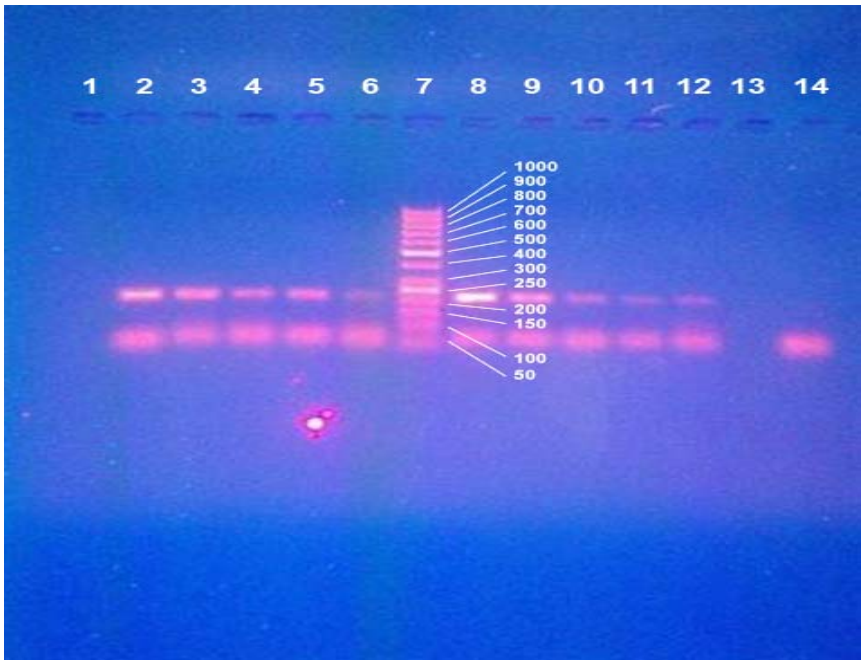

Figure 4: Ethidium bromide gel electrophoresis showing the analytical sensitivity of EBNA-1 QC - PCR for WT and IS plasmid DNA target

Lane 2, 3,4,5,6 having 10^5-10^1 copies of the WT PCR products
Lanes 8, 9, 10, 11, and 12 having 10^5-10^1 copies of the IS PCR products
Lane13 negative control (D.W)
Lane 14 positive control

As shown in figure 5, 10 fold serial dilution of 10^1-10^5copies of WT DNA were spiked with increasing amounts of IS 10^1-10^5 DNA copies in separate reaction. Equivalent amplification and true competition was observed between two DNA template (WT and IS). This was confirmed by the production of equal signals when similar amounts (10^3) of WT and IS DNA were present in the reaction mixture.

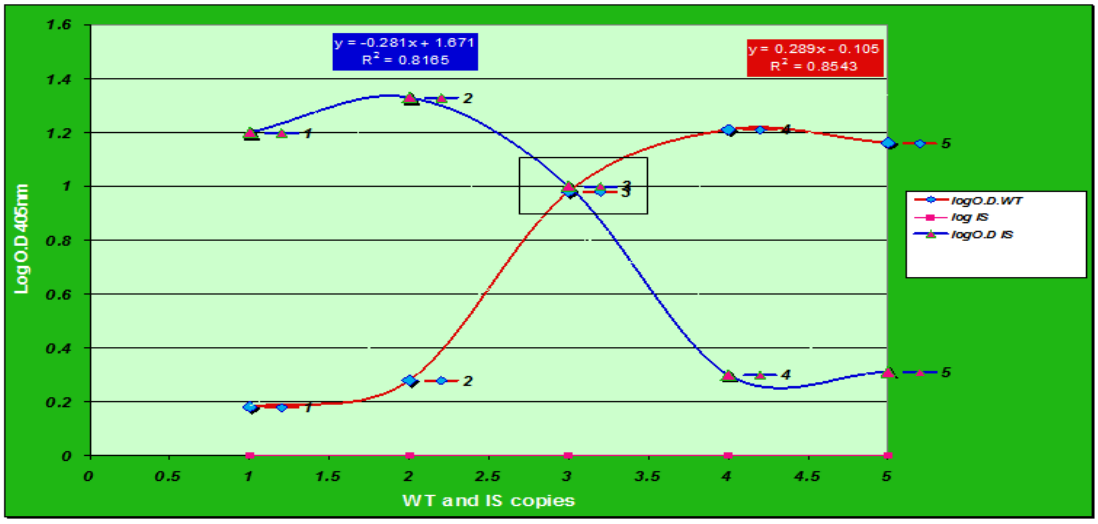

Figure 5: Serial 10 fold dilution of 10^1-10^5 WT copies were spiked with increasing amount of IS copies (10^5-10^1) in separate reaction. PCR products were detected by ELISA detection system.

The validation of accuracy of QC-PCR was reflected in Figure 6, where WT DNA estimated from linear regression curve was 822.2 which are nearly equal WT DNA copies added in the mixture amplified and this reflect the validation of accuracy of test since we have already added 1000 copies of WT DNA to the amplified reaction mixture.

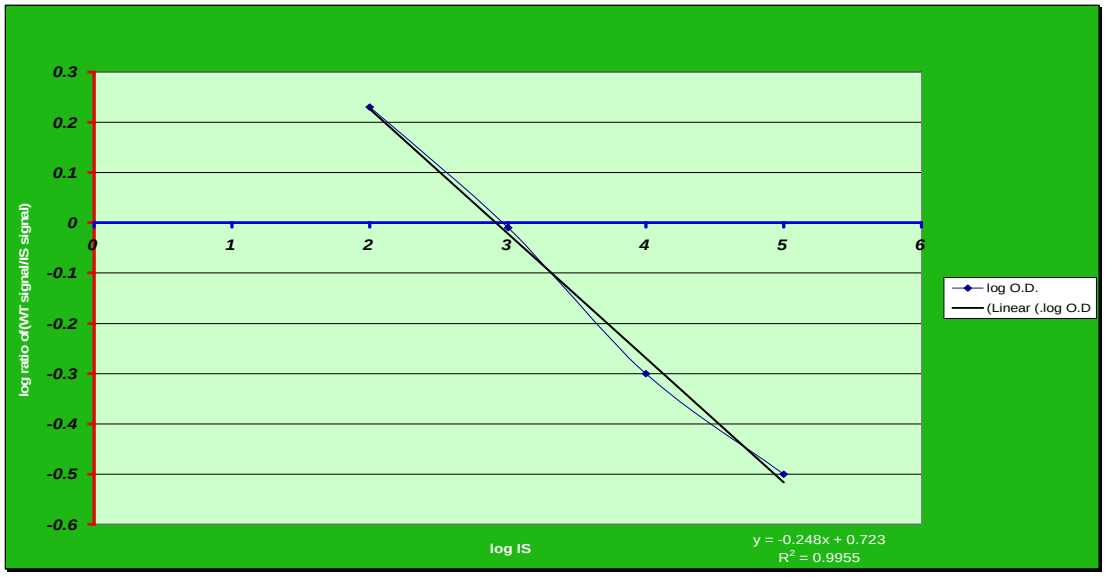

Figure 6: Quantification of WT EBV DNA .1000 copies of WT DNA were spiked with 10^2, 10^3, 10^4, 10^5 copies of IS in four separate reaction

Quantitative Competitative PCR assay:

The EBV DNA load in whole blood samples of patients and control was quantified through the estimation of linear regression curve between the logarithmic ratio of WT signal/IS signal produced by ELISA using WT and IS probe against the logarithmic values of IS copies added. The quantification of each sample estimated from the linear equation Y = ax + b pre and post therapy. The EBV load in those samples ranged from 0–1.936 × 10^9, while the EBV viral load in healthy controls were ranged from 7–1.9×10^3 there is significant difference between the viral load of patient and controls as shown in table 2 where P is 0.011 .

Cut off value: Healthy controls screened by QC-PCR, it was found that cut off value have EBV copy number ≤1.9× 103 EBV DNA copies/ml.

Follow up samples were available in 18 patients with active HL and NHL. Figure 7 showing the distribution of viral load values in patients from 0-1936421960 (1.936×109) EBV DNA copies/ml of blood and in controls from 7 to 1990 (1.99×103) EBV DNA copies/ml of blood .

Table 2 shows the EBV load in blood of patients at time of diagnosis and after completion of chemotherapy where 66.7% of HL Patients have viral load above cut off value while 44.5% of those patients with NHL have high viral load above cut off value. Regarding HL AND NHL patients response After completion of chemotherapy in table 3, the viral load declined in the group I, where 38.3% have high viral load above cut off value shows decline below cut off value while group II still have viral load above cut off value and elevated viral load above cut off value was detected in Group III.

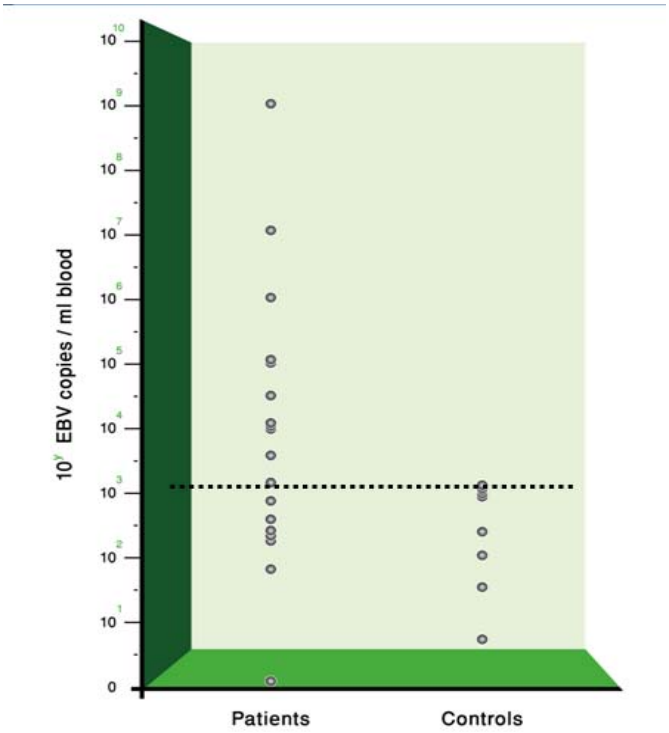

Figure 7: Distribution of EBV load in peripheral whole blood of lymphoma patients and controls.

Table 2: Epstein Barr virus load in lymphoma patients as determined by EBNA-1 QC-PCR

Patient lymphoma type and No.	EBV viral load (copies/ml) pre therapy	EBV viral load above or below cut off value pre therapy	EBV viral load below cut off value percentage pre therapy	EBV viral load above cut off value percentage pre therapy	EBV viral load (copies/ml) post therapy	EBV viral load above or below cut off value post therapy	EBV viral load below cutoff value percentage post therapy	EBV viral load above cut off value percentage post therapy
HL 1	121060	Above	3(33.3%)	6 (66.7%)	5010	Above	6(66.7%)	3(33.4%)
HL 3	1778280	Above			501187	Above		
HL 6	340	Below			160	Below		
HL 7	16982436	Above			1280	Below		
HL 8	327	Below			1595	Below		
HL 9	840	Below			zero	Below		
HL 11	10715	Above			630960	above		
HL 17	109650	Above			10	Below		
HL 18	1936421960	Above			32	Below		
NHL 2	5861	Above	5(55.5 %)	4 (44.5%)	400	Below	9 (100.0%)	0(0%)
NHL 4	1860	Below			1000	Below		
NHL 5	80	Below			85	Below		
NHL 10	600	Below			zero	Below		
NHL 12	11220	above			45	Below		
NHL 13	50118	above			300	Below		
NHL 14	275	Below			130	Below		
NHL 15	Zero	Below			94	Below		
NHL 16	13500	Above			50	Below		

Table 3: Patients groups according to their viral load and their response to chemotherapy

Groups	Number (%)	EBV DNA load pre therapy in copies/ml	EBV DNA load post therapy in copies/ml	Response
I (High viral load)	7 (38.3%)	5861-1936421960 (Range)	10-1280 (Range)	Low viral load (Below cut off value)
П (High viral load	2 (11.1%)	121060-1778280	5010-501187	High viral load (Above cut off value)
Ш (High viral load	1 (5.5%)	10715	630960	Elevated viral load (Above cut off value)
IV (Low viral load	8 (44%)	0-1860	0-1595	Low viral load (Below cut off value)

Table 4: Quantitative Competitive Polymerase reaction (QC-PCR) Comparison of frequency between pre and post therapy

	Frequency of high viral load (Percentage)	Frequency of low viral load (Percentage)	Significance	
Pre therapy	10 (55.55%)	8 (44.45%)	Pre therapy *Vs* post therapy	0.036 *
Post therapy	3 (16.66%)*	15 (83.34%)	Pre therapy *Vs* control	0.011*
Control	0 (0%)	9 (100%)	Post therapy *Vs* control	0.202

Mann-Whitney Test: * Pre therapy *Vs* post therapy ($p < 0.05$)
* Pre therapy *Vs* control ($p < 0.05$)
The significance of QC-PCR assay results were highlighted and summarized in Table 4.

Discussion

The Epstein-Barr virus is a herpes virus which establishes a life-long persistent infection in over 90% of human adult population worldwide based upon its association with a variety of lymphoid and epithelial malignancy, EBV considered as group 1 carcinogen by the international agency for researches on cancer and it has a precise roles in development of virus associated human malignancies [12]. Patients viral load are included within the range of healthy controls.

In this study highly advanced molecular method was used to provide rapid and highly productive amplification of specific DNA sequence for achievement of an accurate and highly reproducible EBNA-1 QC-PCR which is important in diagnostic laboratories for diagnosis and monitoring of diseases [13]. The EBV load in patient blood samples ranged from 0– 1.936×10^9 while the EBV viral load in healthy controls were ranged from 7– 1.9×10^3 (Figure 7).

Table 2 shows that 66.7 % of HL patients and 44.5% of patients with NHL having EBV load above cut off value at time of diagnosis and before chemotherapy after starting of chemotherapy the EBV DNA load decreased below the cutoff value by 66.7% in HL patients and by 100 % in NHL patients indicating good response to chemotherapy. These results come in agreement with Gandi et al, 2004 who stated that half of those patients having HL will respond to therapy and can be cured with conventional modality treatment also others found that after treatment no EBV genome were found in plasma of 6 HD patients which are stable with complete remission of the disease [15].

In group I HL patients with high EBV load at time of diagnosis with a range of 10715 (1.071×10^4) to 1936421960 (1.936×10^9) copies/ml blood, 50% of them showing response to chemotherapy with decline of viral load below cutoff value or to undetectable values, these results are in agreement with Gandi et al., 2006 who concluded that plasma EBV DNA have excellent sensitivity and could be used as a biomarker for EBV associated HL when he reported the presence of detectable viral load in 50% of EBV positive HL patients prior to therapy and after therapy EBV was undetectable unless in one patient under study.

In group I NHL the four patients who have a high EBV load above cut off value 5861 (5.86×10^3) -50118 (5.01×10^4) copies/ml blood

at time of diagnosis, they show 100% response and their viral load lowered below cutoff value, these results are in agreement with Josting et al, 2002 who observed continuously low or undetectable level of EBV in serum samples of NPC patients who reported that EBV DNA load is a valuable tool for monitoring of NPC patients against tumor recurrence.

Our results obtained confirm reports that patients with HL have an excellent prognosis with modern chemotherapy even if the disease is far advanced at diagnosis [18,19].

In studies assaying QC-PCR in lymphoma AIDS patients, our results come in agreement with others who reported that the viral load in 17 EBERS-positive lymphoma patients ranging from 34-1,500.000 copies per ml, this viral load fall rapidly upon initiation of lymphoma therapy and remaining undetectable except in two patients with persistent tumor [20] .

In group II (Table 3) QC-PCR shows that Patients viral load decrease after chemotherapy but still above cutoff value, similar results obtained by Fan et al. 2004 who work with EBV viral load of lymphoma in AIDS patients , they found that the viral load usually falls upon initiation of chemotherapy except in two patients with persistent tumor. It is reported that when analyzing the EBV status in the peripheral blood of pediatric patients with HD where no EBV DNA was detected in plasma of HD with complete remission while 2 of 5 HD patients will relapse and are positive for EBV DNA [15].

In group Ш (Table 3) who have high viral load above the cutoff value and increase in the EBV load after chemotherapy. The presence of high viral load at diagnosis time is in agreement with Gallagher et al. 1999 who reported that EBV DNA was detected in 91% of serum samples from patients with EBV positive HL. The increase in the EBV load after completion of treatment are in consistent with Drouet et al. 1999, that this viral DNA was probably a consequence of viremia which is related to increased viral replication in a non neoplastic compartment such as oropharynx which explain the increase in the viral load during the period of treatment.

In the field of EBV associated NPC, the same finding of persistence in the viral load after chemotherapy was observed, workers in this field explained that this persistence of viral load following primary therapy was predictive of relapse [23,24,25,16].

Other worker with EBV viral load of lymphoma in AIDS patients, found that the viral load usually falls upon initiation of chemotherapy except in two patients with persistent tumor [26], others analyze the EBV status in the peripheral blood of pediatric patients with HD where no EBV DNA was detected in plasma of HD with complete remission while 2 of 5 HD patients relapse and were positive for EBV DNA [15].

For group IV of patients with initial viral load at the time of diagnosis below cutoff value, some of them are EBV negative, after the completion of the chemotherapy they showed slightly increase in the viral load but still below the cutoff value. The absence of elevated EBV DNA load in those patients who constitute 33.3% in HL and 55.5% in NHL as shown in Table 2 and falls in group IV which constitute 44 .45% in table 4, This absence of elevation of EBV load in these groups of patients might be explained by the fact that those patients developed an EBV-negative lymphoma and these results could be debated by that some patients have lymphoma not due to oncogenic potential of EBV this is consistent with [10]. These results are also consistent with studies done [10,21], they reported that EBV DNA was detected in 23% of EBV negative HL patients. Others

found EBV DNA in 24% of EBV negative lymphoma [24].

The molecular nature of EBV DNA in these tumors elucidated by Fan and Gullay 2001, they found that circulating EBV DNA exists as short fragments of less than 200bp. This implies that the increase of EBV DNA is due to tumor release of EBV DNA fragments instead of virion reactivation and these fragments are naked molecules not protected by viral protein coat [27], They found a relationship between circulating EBV DNA and apoptosis that DNA is fragmented by caspase-activated DNAase and resulting in DNA fragments with length in multiple of nucleosomal DNA.

It's concluded that EBV could be considered as a target in the effective diagnosis of EBV associated tumors. EBV DNA load could be a promising marker for the patients who express EBV load above the cutoff value. The QC-PCR assay allow accurate quantification of EBV load and show promise as a tool to assist in diagnosis and management of EBV related lymphoma patient, it is potentially useful in the diagnosis and follow up as well as in the assessment of the efficiencies of chemotherapeutic regimens consequently, in these cancers EBV DNA may be considered as a real tumor biomarker.

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