Establishment of Real-Time Polymerase Chain Reaction-Based Assay for Quantitation of Epstein-Barr Virus DNA in Healthy Donors and in Patients with EBV Associated Lymphoma

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*Epstein-Barr virus (EBV) is associated with several malignancies including nasopharyngeal carcinoma and lymphoma in immunocompromised patients. Quantitative monitoring of EBV DNA in these patients has recently become essential for management of the disease. In the present study the authors developed a rapid and reliable real-time PCR to quantify the EBV DNA in peripheral blood mononuclear cell (PBMC) using hybridization probe technique. The real-time primers and probes in this real-time PCR system were designed based on EBNA-1 sequence. The newly-established real-time PCR demonstrated its high sensitivity (as few as 10 copies of EBV could be detected) and specificity. The intra- and inter-assay variations of the assay were shown to be within a 0.5-$\log_{10}$-difference range. A total of 2 EBV-seronegative, 14 EBV-seropositive healthy donors and 4 patients with PCNSL were enrolled into the study. Our results revealed the median of EBV-DNA in lymphoma patients (7,886 copies/10^6 PBMC or 15,150 copies /μg DNA) was higher than that of healthy donors (<10 copies/10^6 PBMC or <10 copies/μg DNA) with statistic significance (*P<0.01*). Assessment of this assay in larger number of donors and patients will provide clinical cut-off values which are essential for monitoring and diagnosis of EBV-associated diseases.*

Keywords: *EBV-DNA, Real-time PCR*

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Epstein-Barr virus (EBV) is a ubiquitous virus that infects more than 90% of the world adult population. In childhood individual primary infection is usually asymptomatic. However, if infection is delayed until adolescent, it may result in symptomatic Infectious Mononucleosis (IM)[1]. After the primary infection, the virus will remain as latency state in B lymphocytes for life[2]. In a majority of immunocompetent hosts, this 'harmless' latent infection is tightly controlled by EBV-specific cytotoxic T lymphocytes resulting in asymptomatic infection[3]. On the other hand, EBV infection is less efficiently controlled in immunocompromised patients and leads to development of lymphomas and other malignancies including Primary Central Nervous System Lymphomas (PCSNL) in HIV infected patients and Post Transplant Lymphoproliferative Disorder (PTLD)[4].

The quantitative analysis of EBV DNA has recently been used for monitoring and prognosis in a number of EBV-associated diseases. In contrast to semi-quantitative analysis, real-time Polymerase Chain Reaction (PCR) provides accurate and less-time consuming technique to quantify EBV DNA in patients. In the present study the authors successfully developed sensitive and highly specific *EBNA-1*-based real-time PCR and compared the level of EBV DNA in normal healthy donors and patients with Primary Central Nervous System Lymphoma (PCNSL).

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Material and Method

Donors

A total of 14 EBV-seropositive and 2 EBV-seronegative normal healthy donors and 4 clinically-diagnosed PCNSL from King Chulalongkorn Memorial hospital, Bangkok, Thailand were enrolled into the present study. The ethical approval of the present study was obtained from the Ethical Committee of Faculty of Medicine, Chulalongkorn University. The EBV sero-positivity of all donors and patients were confirmed by the presence of antibody to viral capsid antigen (Anti-VCA). The anti-VCA IgG detection was determined by Enzyme Linked Immunosorbent Assay (ELISA) purchased from Orkanon Tecnika, The Netherlands.

Standard EBV-DNA

In the present study the authors used standard EBV-DNA derived from Namalwa DNA (kindly provided by Associate Professor Dr. Apiwat Muthirangura, Department of Anatomy, Faculty of Medicine, Chulalongkorn University), B95-8 cells and newly-generated plasmid containing EBNA-1 DNA.

DNA extracted from peripheral blood mononuclear cells (PBMC)

PBMC were obtained from 7 ml of EDTA blood by ficoll-hypaque gradient centrifugation. The number of PBMC was counted. After centrifugation at 13,000 rpm for a minute, the DNA was extracted using DNA blood mini kit (Qiagen, Germany). The DNA concentration was quantified by spectrophotometer based on measurement value of optical density at wave length 260 and 280 nm.

Quantitative Real-time Polymerase Chain Reaction (PCR)

A 297 bp fragment of EBNA-1 gene was detected by using Real-time PCR based on the principle of Fluorescence Resonance Energy Transfer (FRET). The forward and reverse primer sequences were 5' -GAG GGT GGT TTG GAA AGC-3' (EB-30) and 5'-AAC AGA CAA TGG ACT CCC TTA G-3' (EB-40), respectively. The fluorescence probes were 5' -AGT CGT CTC CCC TTT GGA ATG GC-FITC-3' (3-FL) and 5'-LC-Red 640-CTG GAC CCG GCC CAC AAC CTG-3' (5-LC). All primers and probes were purchased from TIB MOLBIOL (Berlin, Germany). PCR reaction was carried out in a final volume of 17 ml consisting of 5 µl of sample DNA, 0.5 mM of each primer, 0.3 mM of each probe, 2 µl of LightCycler FastStart DNA Master Hybridization Probes (Roche Diagnostics, Germany) and 4 mM $MgCl_2$. PCR reaction was performed sequentially as follow 2 sec of UNG inactivation at 95ºC, 10 min of Taq DNA polymerase activation at 95ºC, 60 cycles of 0 sec of denaturation at 95ºC, 20 sec of annealing at 57ºC and 10 sec of extension at 72ºC with transition rates of 20, 20 and 2ºC respectively with a single fluorescence acquisition at annealing temperature. For melting analysis, a single cycle of 95ºC for 0 sec 51ºC for 20 sec and 95ºC for 0 sec was run with transition rates of 20, 5 and 0.2ºC, respectively with continuous fluorescence acquisition.

Results

Validation of the real-time PCR assay

The specificity of the assay was determined by amplification of B95-8 DNA together with the DNA from EBV-genetically-related viruses including DNA from cells infected with Cytomegalovirus, Herpes Simplex Virus (HSV)-1 and HSV-2 DNA by real-time PCR. The DNA extracted from EBV seronegative healthy donors was also performed in the same run as negative control. Whilst the 95-8 DNA was amplified by this system, the real-time PCR failed to amplify these genetically-related viruses. The system was also unable to amplify the DNA from EBV seronegative donors (Data not shown).

To construct the initial standard curve used to quantify the number of EBV-DNA copy, a serial 10-fold dilution of Namalwa EBV-DNA ranging from $1.4x10^4$ to 14 copies was amplified by the real-time PCR to generate the curve plotting Log concentration of EBV against cycle number (Data not shown). The EBV-DNA initial standard curve from previous experiment was used to quantify the number of EBV-DNA in plasmid containing 297 bp fragment of EBNA-1 gene. After that the plasmid containing EBV-DNA was used to establish the main standard curve for further analysis throughout the study (Fig. 1).

To determine the sensitivity of the assay, serial 10-fold dilutions of EBV recombinant plasmid ranging from 10^8 to 10 copies were quantified by the real-time PCR. The result showed as few as 10 copies of EBV-DNA could be detected by this assay (Fig. 2).

Evaluation of the intra- and inter-assay variation was carried out by amplifying EBNA1-recombinant plasmid at concentration 10^6, 10^4 and 10^2 copies in duplicate and for 2 independent experiments, respectively. All intra- and inter assay variations were shown to be within a 0.5 $\log_{10}$ difference (Table 1).

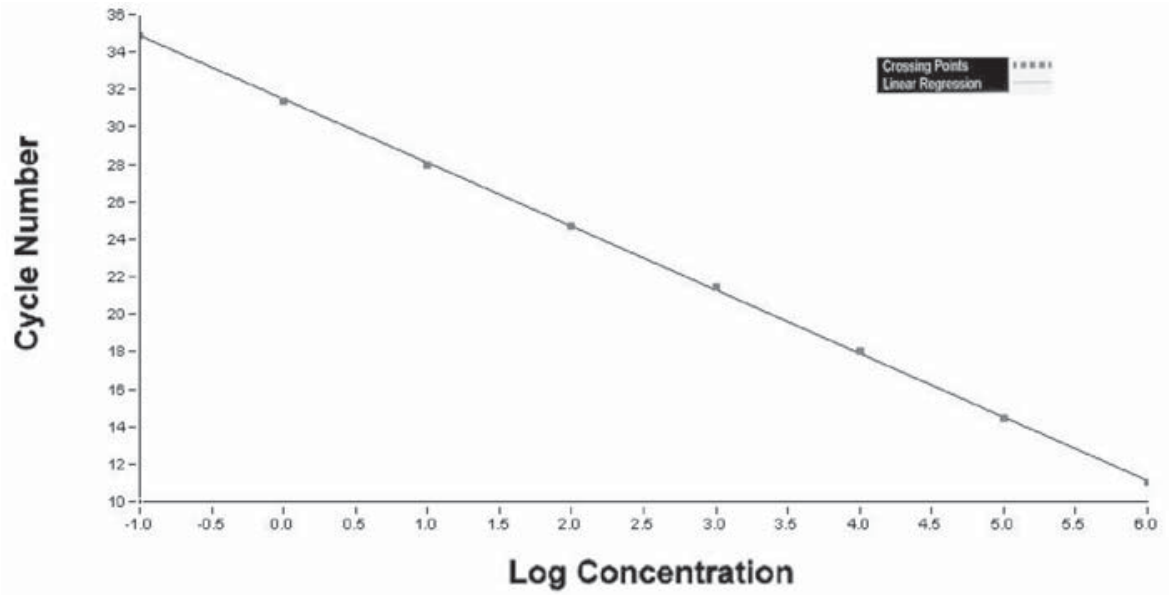

Fig. 1 Standard curve obtained by serial 10-fold dilutions of EBNA-1 recombinant plasmid. The curve plots cycle number versus $\log_{10}$ concentration of standardised plasmid with an error value of 0.0347 and a liner regression value (r^2) of –1.00

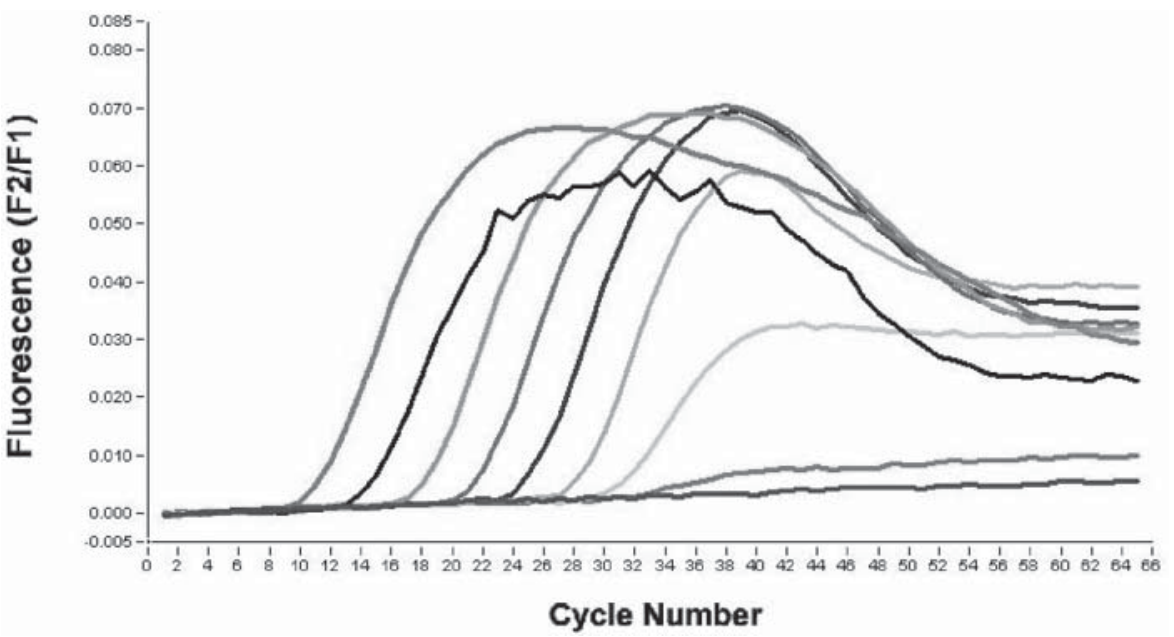

Fig. 2 Amplification curve of EBNA-1 recombinant plasmid serial 10 fold dilutions ranging from 10^8 to 10 copies per reaction

Quantitation of EBV-DNA in clinical samples

Whilst the EBV-DNA in 14 seropositive normal healthy donors was demonstrated to be in a range of <10 copies to 26 copies/10^6 PBMC (with a median of <10 copies/10^6) or <10 copies to 57 copies/μg DNA (with a median of <10 copies/μg DNA), the EBV-DNA of patients with PCNSL is significantly higher with a range of 315 to 21,811 copies/10^6 PBMC (with a median

Table 1. Intra- and Inter- variation assay of EBNA-1 recombinant plasmid

Dilution (copy number)	Intra Assay		Inter Assay	
	Range	Log_{10} difference	Range	Log_{10} difference
1,000,000	1,000,000 - 1,229,000	0.09	929,000 1,229,000	
	1,000,000 - 1,003,000	0.00	1,000,000 1,003,000	0.12
	Mean	0.05	1,229,000	
10,000	10,310 -11,600	0.05	9,830 10,310	
	10,000 -12,790	0.11	11,600 10,000	0.11
	Mean	0.08	12,790	
100	51 - 57	0.05	51 57	
	102 - 144	0.15	108 144	0.45
	Mean	0.10	101	

Table 2. Level of EBV-DNA in PBMC of healthy donors and patients with PCNSL

Sample	Sex	Age (yrs)	EBV load (copies per)		Mean of Age (yrs)	Median of EBV load (copies per)	
			10^6 PBMC	µg DNA		10^6 PBMC	µg DNA
Healthy donor							
1	F	23	26	53			
2	F	24	<10	<10			
3	F	22	<10	<10			
4	M	28	<10	16			
5	F	32	<10	<10			
6	F	24	<10	<10			
7	F	23	16	57	26±4	<10	<10
8	F	24	<10	<10			
9	F	23	16	36			
10	F	22	<10	<10			
11	F	31	<10	<10			
12	F	24	<10	<10			
13	M	28	<10	<10			
14	F	32	<10	<10			
PCSNL patients							
1	M	39	21,811	98,150			
2	F	27	12,673	21,726	33±12	7,886	15,150
3	M	20	315	1,039			
4	F	47	3,099	8,574			

of 7,886 copies/10^6 PBMC)or 1,039 copies/10^6 PBMC to 98,150 µg/ml (with a median of 15,150 copies/µg DNA, Table 2). The median of EBV-DNA in normal healthy donors was statistically higher than that in patients with PCNSL ($P<0.01$).

Discussion

EBV is associated with malignancies both in immunocompromised hosts and apparently immunocompetent hosts. Failure to control EBV replication, as evidenced by elevation of EBV-DNA, may be central to pathogenesis of the malignancies in these patients. Quantitative analysis of EBV-DNA may therefore become potentially standard technique for monitoring and predicting the outcome of treatment. In the present study, the authors established the assay to quantify EBV-DNA by real-time PCR which known to be a rapid and reliable technique for determining the level of

EBV-DNA[5-8]. The authors also demonstrated the difference of EBV load in PBMC from normal healthy donors and from patients with EBV-associated PCNSL. Various types of specimens including serum[9-11], plasma[12, 13], whole blood[14-16] and PBMC [5, 17] were previously used for quantification of EBV load. However, monitoring cell-associated EBV-DNA from PBMC was also reported as the effective way to predict the development of EBV PTLD in recipients of T cell–depleted transplants[18].

The presented newly-established real-time PCR was proved to be highly sensitive (as few as 10 copies of EBV-DNA could be detected), the sensitivity of which was comparable to the previously described real-time PCR system[5]. The real-time PCR system established in the present study also had high level of specificity whereby other genetically-related herpesviruses DNA was not amplified by this system. Moreover, the presented assay demonstrated high accuracy and reproducibility with intra- and inter assay variations within a 0.5 $\log_{10}$ difference.

To determine the clinical utility of the real-time PCR in clinical setting, the level of EBV-DNA in PBMC from healthy donors and from patients with PCNSL was estimated by the real-time PCR system. As previously expected, the EBV-DNA level was significantly higher in patients with PCNSL than in immunocompetent healthy donors ($P<0.01$). Indeed, whereas the EBV viral load in healthy donors ranged from <10 to 57 copies/μg DNA or <10 to 26 copies/10^6 PBMC, the EBV-DNA in patients with PCNSL ranged from 315 to 21,811 copies/10^6 PBMC or 1,039 copies/10^6 PBMC to 98,150 μg/ml. The relatively high EBV load was also demonstrated in HIV-infected patients[19], patients with NPC [12]and patients with lymphoma[20]. Estimation of EBV-DNA may therefore be important for monitoring and predicting clinical outcome of these EBV-associated diseases including NPC[12] which is one of the most prevalent cancers in Thailand. In addition to monitoring of the disease, the EBV load in cerebrospinal fluid may be diagnostically useful for EBV-associated diseases in the central nervous system (i.e., PCNSL) whereby the tissue for histological analysis is particularly difficult to acquire[21].

Despite the fact that the quantitation of EBV-DNA is beneficial for diagnosis and monitoring of EBV-associated diseases, the reference level of EBV-DNA should be established in Thai donors before the quantitative analysis of EBV-DNA is implemented in clinical practice. The present study has provided primary information of EBV-DNA level in Thai healthy donors and patients with PCNSL, albeit in a limited number of samples. Further assessment of this assay in larger number of donors is required to determine the reference level in normal healthy donors and in patients with various EBV-associated diseases.

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การหาปริมาณสารพันธุกรรมของเชื้อไวรัสเอปสไตน์-บาร์ ด้วยวิธี Real-time PCR ในคนปกติและผู้ป่วยโรคมะเร็งต่อมน้ำเหลืองที่มีความสัมพันธ์กับไวรัสเอปสไตน์-บาร์

พัชชา อินคำสีบ, ภาวพันธ์ ภัทรโกศล, วันล่า กุลวิชิต, วสันต์ จันทราทิตย์, ปกรัฐ หังสสูต

ไวรัสเอปสไตน์-บาร์มีความเกี่ยวข้องกับการเกิดโรคมะเร็งหลายชนิดรวมทั้งโรคมะเร็งของโพรงหลังจมูกและมะเร็งต่อมน้ำเหลืองในผู้ป่วยภูมิคุ้มกันบกพร่อง การวิเคราะห์ปริมาณไวรัสเอปสไตน์-บาร์เริ่มได้รับความสำคัญเพื่อช่วยในการดูแลรักษาโรคเหล่านี้ ในการศึกษานี้ผู้วิจัยได้พัฒนาเทคนิคที่รวดเร็วและเชื่อถือได้ในการหาปริมาณสารพันธุกรรมของไวรัสเอปสไตน์-บาร์ด้วยวิธีเรียลไทม์พีซีอาร์ และใช้ไฮบริไดเซชันโพรบ โดยได้ออกแบบไพรเมอร์และโพรบให้จำเพาะต่อจีนเอปนาร์วัน ระบบการวิเคราะห์นี้มีความไว (ปริมาณของไวรัสเอปสไตน์-บาร์ที่สามารถตรวจพบได้เท่ากับ 10 ก๊อบปี้) และความจำเพาะสูง ความแปรผันของค่าที่อ่านได้ทั้งในการทดลองเดียวและต่างกันมีค่าที่แตกต่างกันไม่เกิน 0.5 ล็อก การศึกษาปริมาณไวรัสเอปสไตน์-บาร์ได้วิเคราะห์คนปกติที่ติดไวรัส เอปสไตน์-บาร์จำนวน 14 ราย และผู้ป่วยมะเร็งต่อมน้ำเหลืองในสมองชนิดปฐมภูมิจำนวน 4 ราย ซึ่งผลจากการ วิเคราะห์พบว่าผู้ป่วยมะเร็งต่อมน้ำเหลืองในสมองชนิดปฐมภูมมีค่ามัธยฐานของปริมาณสารพันธุกรรมของไวรัสเอปสไตน์-บาร์ (7886 ก๊อบปี้ต่อล้านเซลล์พีบีเอ็มซี หรือ 15150 ก๊อบปี้ต่อดีเอ็นเอ 1 ไมโครกรัม) มากกว่าคนปกติ ที่ติดเชื้อไวรัสเอปสไตน์-บาร์ (น้อยกว่า 10 ก๊อบปี้ต่อล้านเซลล์พีบีเอ็มซี หรือ น้อยกว่า 10 ก๊อบปี้ต่อดีเอ็นเอ1ไมโครกรัม) อย่างมีนัยสำคัญ ทางสถิติ (ค่าพีน้อยกว่า0.01) การศึกษาปริมาณสาร พันธุกรรมของไวรัสเอปสไตน์-บาร์ในขนาดตัวอย่างที่มากกว่านี้ จะนำไปสู่การกำหนดค่าสาร พันธุกรรมของไวรัสเอปสไตน์-บาร์ซึ่งสามารถใช้เป็นปริมาณอ้างอิง ในการวินิจฉัย และติดตามผลการรักษาโรค ที่มีความเกี่ยวพันการติดเชื้อไวรัสเอปสไตน์-บาร์ต่อไป