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Epstein-Barr Virus Quantitation by Real-Time PCR Targeting Multiple Gene Segments

A Novel Approach to Screen for the Virus in Paraffin-Embedded Tissue and Plasma

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Epstein-Barr Virus (EBV) infects nearly all humans and then persists for the life of the host. In some people who later develop cancer, EBV DNA is present within malignant cells and circulates at elevated levels in the plasma. In the current study, we validated five novel quantitative polymerase chain reaction (Q-PCR) assays targeting disparate but highly conserved segments of the EBV genome (*BamH1W*, *EBNA1*, *LMP1*, *LMP2*, and *BZLF1*). Each assay was sensitive to as few as 50 copies of EBV DNA per reaction and was linear across at least four orders of magnitude. When applied to paraffin-embedded tissues in concert with EBV-encoded RNA (EBER) *in situ* hybridization, the *BamH1W* and *EBNA1* assays were the most informative, while use of the entire battery of EBV PCR assays may help identify genomic polymorphisms or deletions. Higher viral loads were found in the 17 EBER-positive compared with the 13 EBER-negative tumors (means 84,978 versus 22 copies of EBV per 100,000 cells, respectively). The five Q-PCR assays were also informative in plasma samples where EBV was measurable in all nine patients with lymphoma or infectious mononucleosis, whereas EBV was undetectable in all nine healthy controls. The findings suggest that Q-PCR is an effective method of distinguishing disease-associated virus from incidental virus in paraffin-embedded tissue and in plasma samples. *(J Mol Diagn 2004, 6:378–385)*

Epstein-Barr virus (EBV) has been implicated in the pathogenesis of several malignancies including non-Hodgkin and Hodgkin lymphomas and nasopharyngeal carcinoma.[1,2] Recent studies suggest that EBV DNA is present in tissues of more types of cancer than originally thought, including EBV-encoded RNA (EBER)-negative Burkitt and Hodgkin lymphomas, and breast and lung adenocarcinomas.[3–7] These data are controversial, in large part because some laboratory assays for EBV are not designed to specifically detect tumor-associated EBV or to distinguish it from the EBV normally carried in a small proportion of benign B lymphocytes by most humans.

The assay that many consider to be the gold standard for identifying tumor-associated virus is EBER *in situ* hybridization. Results are interpreted morphologically so that latent virus can be localized to malignant versus benign cell types. The accuracy of EBER *in situ* hybridization has recently been called into question by investigators who showed that EBV was present, based on positive molecular or immunohistochemical assays, in certain EBER-negative tumors.[3–5,7–11] To explain some of these discrepancies, the "hit and run" hypothesis has been put forward: it states that segments of EBV DNA or EBV gene products are undetected in certain cells or in entire tumors because portions of the EBV genome have been lost or rearranged or integrated into host chromosomal DNA.[4,5] An alternative possibility is that certain molecular or immunohistochemical assays for EBV are non-specific, resulting in false-positive interpretations of tumor-associated EBV.

In the current study, we developed and validated a battery of new quantitative real-time PCR (Q-PCR) assays targeting five different parts of the EBV genome. To assess their utility in detecting disease-related EBV, these assays were applied in a pilot series of paraffin-embedded tissues on which EBER *in situ* hybridization had also been performed. Furthermore, the assays

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were applied to several plasma samples to assess their suitability for use in studies in which patients are screened for an EBV-related disease.

Materials and Methods

Patient Samples and Cell Line Controls

Paraffin blocks ($n = 35$) and plasma samples ($n = 18$) were retrieved from the archives of our clinical and research laboratories under the supervision of our Institutional Review Board. Cases were selected to represent various EBV-related diseases and controls. EBER *in situ* hybridization was performed on paraffin sections using a fluorescein-labeled oligonucleotide probe targeting EBER RNA (Biogenex, San Ramon, CA) and the Innogenex ISH DAB Kit (Biogenex). A fluorescein-labeled oligonucleotide Oligo d(T) probe (Biogenex) served as a control for RNA preservation in the histological sections. A tumor was considered EBV-related if the EBER signal was localized to at least one definite malignant cell. A case was considered EBER-negative if EBER staining was undetected or was apparent only in benign-appearing lymphoid cells.

Sterile procedure was used during histological sectioning to prevent tissue carryover between cases. Before sectioning each block, the work area and forceps were wiped down with 10% bleach in water and rinsed with deionized water. Disposable blades were either discarded or moved to an unused edge before cutting each block. Two 10-μm thick sections from each block were placed into microfuge tubes for subsequent DNA extraction. Five-μm thick sections were placed on glass slides for histochemical staining procedures.

To extract DNA from paraffin-embedded tissue sections, deparaffination was performed using three washes in xylene for 10 minutes at room temperature followed by two washes in 100% ethanol to remove the xylene. Tissues were dried at 55°C and digested overnight at 55°C in 100 μl of TEN buffer (10 mmol/L Tris-HCl pH 8.0, 1 mmol/L EDTA pH 8.0, 20 mmol/L NaCl) containing 20 mg/ml proteinase K, and then the proteinase was inactivated at 95°C for 10 minutes. Undigested tissue remnants were pelleted by centrifugation at 14,000 rpm for 10 minutes. The supernatant containing extracted DNA was transferred to a new microfuge tube and stored at −20°C until use.

Plasma samples were selected from nine patients, four of whom had EBV-related malignancy as shown by EBER *in situ* hybridization on biopsy materials, and five with infectious mononucleosis confirmed by heterophile antibody positivity. Plasma was also obtained from nine healthy blood donors. Total DNA was extracted from 200 μl of plasma using the QIAmp Blood Kit (Qiagen Inc., Valencia, CA). Before extraction, the plasma was spiked with 2 μl of IPC (TaqMan Exogenous Internal Positive Control DNA, Applied Biosystems (ABI), Foster City, CA) to control for the effectiveness of extraction and amplification. Purified DNA was eluted into 50 μl of AE buffer (Qiagen).

DNA from the Namalwa cell line (two copies of EBV per cell, equivalent to 3×10^5 copies of EBV/μg DNA) was used as a standard by which EBV genomic DNA was measured (American Type Culture Collection, Rockville, MD). DNA from the Raji Burkitt lymphoma line and the P3HR-1 Burkitt cell line were used as additional EBV-related lymphoma cell line controls (American Type Culture Collection). DNA was extracted from fresh cell pellets using the Purgene DNA Isolation kit (Gentra, Minneapolis, MN). Serial 10-fold dilutions of these DNAs were made in nuclease-free water to examine the linearity of each quantitative PCR assay. Potential cross-reactivity with other viruses or genomic DNA was evaluated by BLAST sequence analysis. Specificity testing was performed on purified cytomegalovirus (CMV) and Kaposi's sarcoma-associated herpesvirus (KSHV) DNA (Advanced Biotechnologies, Inc., Columbia, MD) and on paraffin-embedded tissue from five patients diagnosed with other herpes family viruses (three with CMV and two with herpes simplex).

Development and Validation of Quantitative PCR Assays

EBV genomic sequences were downloaded from GenBank (www.ncbi.nlm.nih.gov). PCR primers and TaqMan probes targeting conserved portions of the reiterated *BamH1W* segment and the unique *EBNA1*, *LMP1*, *LMP2*, and *BZLF1* genes were designed using Primer Express 2.0 software (ABI). These sequences are shown in Table 1.

Primers and TaqMan probe targeting the cellular *ApoB* gene, as described by Sanchez and Storch,[11] served as a control for the efficacy of extraction and amplification of DNA from paraffin-embedded tissue. Using the Namalwa cell line as a standard, *ApoB* DNA was measured in each tissue sample. The quantity and quality of extracted DNA was considered adequate if *ApoB* measurement indicated that at least 50 cells were present in the PCR reaction. In the experimental samples, the minimum number of cells evaluated per PCR reaction was 94.

PCR was performed and products were detected using an ABI Prism 7900 Real-Time PCR instrument and Sequence Detection System software. Thermocycling conditions were: 50°C for 2 minutes; 95°C for 10 minutes; 95°C for 15 seconds and 60°C for 1 minute for 40 cycles. Each 50-μl reaction contained: 1X TaqMan Universal Master Mix, forward and reverse primer (15 ρmol each), and TaqMan probe (10 ρmol). DNA template volume was 1 μl for paraffin tissues, and 5 μl for plasma samples. The plasma reactions also contained 1X IPC Master Mix (ABI) to co-amplify the spiked IPC sequence. To improve assay sensitivity when targeting *LMP1* and *BZLF1* genes, 30 ρmol each of the forward and reverse primers was used. To check for amplicon contamination, every run contained at least two "no template" controls in which nuclease-free H_2O was substituted for template. A standard curve was generated using 10-fold dilutions of Namalwa DNA varying from 50,000 to 0.5 copies of EBV DNA, and this curve was considered acceptable if a difference of 3.3 +/−0.3 cycles was demonstrated between each of

Table 1. Sequences of Primers and TaqMan Probes Used for Real-Time PCR

BamH1W	Forward	5′-GCA GCC GCC CAG TCT CT-3′
	Reverse	5′-ACA GAC AGT GCA CAG GAG CCT-3′
	Probe	5′-(6FAM)AAA AGC TGG CGC CCT TGC CTG(TAMRA)-3′
	Amplicon size	83 bp
	Nucleotide #	47257 to 47340
EBNA1	Forward	5′-TAC AGG ACC TGG AAA TGG CC-3′
	Reverse	5′-TCT TTG AGG TCC ACT GCC G-3′
	Probe	5′-(6FAM)AGG GAG ACA CAT CTG GAC CAG AAG GC(TAMRA)-3′
	Amplicon size	78 bp
	Nucleotide #	107970 to 108048
LMP1	Forward	5′-CAG TCA GGC AAG CCT ATG A-3′
	Reverse	5′-CTG GTT CCG GTG GAG ATG A-3′
	Probe	5′-(6FAM)GTC ATA GTA GCT TAG CTG AAC(TAMRA)-3′
	Amplicon size	104 bp
	Nucleotide #	168117 to 168221
LMP2	Forward	5′-AGC TGT AAC TGT GGT TTC CAT GAC-3′
	Reverse	5′-GCC CCC TGG CGA AGA G-3′
	Probe	5′-(6FAM)CTG CTG CTA CTG GCT TTC GTC CTC TGG(TAMRA)-3′
	Amplicon size	69 bp
	Nucleotide #	679 to 748
BZLF1	Forward	5′-AAA TTT AAG AGA TCC TCG TGT AAA ACA TC-3′
	Reverse	5′-CGC CTC CTG TTG AAG CAG AT-3′
	Probe	5′-(6FAM)ATA ATG GAG TCA ACA TCC AGG CTT GGG C(TAMRA)-3′
	Amplicon size	91 bp
	Nucleotide #	102214 to 102305

Nucleotide # refers to location in the prototypic EBV B95.8 genome sequence (GenBank Accession No. V01555). The *BamH1W* reverse primer sequence matches EBV from the P3HR-1 cell line, and it is one nucleotide different from the prototypic B95.8 strain; this difference did not significantly affect viral loads.

the 10-fold dilutions, and if the correlation coefficient was at least 0.99. Quantification results for experimental samples were extrapolated from the standard curve. Experimental samples were run in duplicate and a mean viral load was calculated.

EBV viral load in paraffin tissue was calculated based on the ratio of copies of EBV to *ApoB* in a given volume of extracted DNA, with *ApoB* representing the number of cells in the sample. The resulting ratio was then multiplied by 100,000 to provide the number of copies of EBV per 100,000 cells. For plasma samples, EBV viral load was expressed in copies per ml of plasma. Plasma results were considered negative for EBV when the spiked IPC control sequence was amplifiable while EBV DNA was not. For purposes of data analysis, samples with no measurable EBV DNA were reported as having a viral load of zero.

Qualitative LMP2 Gene PCR Assay

To further investigate a case in which the *LMP2* segment selectively failed to amplify by Q-PCR, a second primer set was designed to amplify a 130-bp region of the *LMP2* gene encompassing the 69-bp segment that had been targeted by the standard *LMP2* Q-PCR primers. This qualitative PCR used the following primer sequences: LMP2Ext forward 5′-CTGTTTTGCAGCTGAGTCC-3′ and LMP2Ext reverse 5′-CAATGTTAAAAGGGCTGCACC-3′. The 50-μl PCR reaction consisted of: 1X PCR Buffer, 2 mmol/L $MgCl_2$ 2.5 units Platinum *Taq*DNA Polymerase (Invitrogen, Carlsbad CA), 0.2 mmol/L dNTPs (ABI), 50 ρmol of each primer, and nuclease-free water. Reaction conditions were: 95°C for 2 minutes; 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute; and then 72°C for 10 minutes. Products were electrophoresed in a 2% agarose gel containing 0.5 mg/ml ethidium bromide. DNA from Case 7 (EBER-positive AIDS lymphoma) and an EBER-positive gastric carcinoma were used as wild-type controls.

LMP1, LMP2, and BZLF1 Expression by Immunohistochemistry

In selected cases with atypical DNA amplification results, immunostains were used to assess viral gene expression. Immunohistochemical analysis for LMP1 and LMP2 was performed on paraffin sections using the Anti-EBV LMP1-clone CS1–4 cocktail of mouse monoclonal antibodies (Dako, Carpinteria, CA) and the TP/LMP2a-clone E411 rat monoclonal antibody (Asencion, Munich, Germany). Antigen retrieval (Biogenex Citrate Antigen Retrieval Buffer, HK086–9K) was performed for 30 minutes, and endogenous peroxidase was quenched for 10 minutes using Peroxidase Quenching Solution (Zymed, San Francisco, CA). Bound antibody was detected using the Zymed PicTure-Plus Kit with Polymer Detection System (Zymed).[12] Common antibody diluent (Biogenex HK156–5K) was used to dilute the LMP1 (1:100) and LMP2 (1 mg/ml) antibodies, and sections were incubated with primary antibody for 40 minutes. Bound antibody was detected with a ready-to-use α-rat-HRP polymer conjugate for 30 minutes (LMP1) or 20 minutes (LMP2), followed by diamino-benzidine (DAB) chromogen (Dako Liquid Dab and Substrate Chromogen System K3468). Tissues were counter-stained with hematoxylin (Dako). EBER-positive Hodgkin lymphoma slides served as pos-

itive controls. Results were interpreted microscopically by looking for cytoplasmic and membrane localization of the chromagen in neoplastic cells.

Immunohistochemical analysis of the EBV BZLF1 protein using the Zebra clone BZ.1 antibody (Dako, 1:25 dilution) was performed as described above except for the following modifications. All washes after antigen retrieval were performed in Automation Buffer (Biomeda Corp, Foster City, CA). Endogenous peroxidase was quenched using Peroxidase Block (Dako) for 10 minutes at 37°C. To reduce non-specfic binding, Avidin and Biotin blocks (Dako) were performed for 15 minutes followed by Protein Block (Biogenex) for 10 minutes at 37°C. Sections were incubated with primary antibody for 30 minutes at 37°C, and bound antibody was detected using the Biogenex StrAviGen Multi Link Kit (Biogenex). Sections were incubated with secondary antibody (1:20) for 8 minutes at 37°C followed by incubation with HRP label (1:20; diluted with Biogenex Streptavidin-Peroxidase Diluent) for 8 minutes at 37°C. An oral hairy leukoplakia section served as a positive control. Results were interpreted microscopically by looking for nuclear localization of the chromagen in neoplastic cells.

Results

Sensitivity of the Q-PCR Assays

Five different real-time PCR assays targeting various segments of the EBV genome (*BamH1W*, *EBNA1*, *LMP1*, *LMP2*, *BZLF1*) were developed and tested using 10-fold serial dilutions of Namalwa cell line DNA as a standard. All five assays consistently detected as few as 50 copies of DNA from the Namalwa, Raji, and P3HR-1 cell lines, and individual assays sometimes detected as few as 5 or 0.5 copies per PCR.

Assay Linearity

The *BamH1W* assay was linear across five orders of magnitude from 0.5 to 50,000 copies of Namalwa EBV DNA. The *EBNA1*, *LMP2*, and *BZLF1* assays were linear across four orders of magnitude (from 5 to 50,000 copies) and the *LMP1* assay was linear across three orders of magnitude (from 50 to 50,000 copies). The assays were efficient as shown by a cycle difference for each 10-fold dilution ranging between 3.3 and 3.4 (representing slope of the standard curve). The assays were linear as shown by a correlation coefficient greater than 0.99 (representing the linearity of the standard curve). Serial dilutions of DNA from the Raji cell line and the P3HR1cell line were also used to examine assay linearity. All five novel real-time PCR assays detected both Raji and P3HR1 EBV in a linear fashion (correlation coefficient >0.99), and cycle difference between each 10-fold dilution averaged 3.5 for Raji and 3.3 for P3HR1.

Application of the Viral Load Assays to Paraffin-Embedded Tissues

EBV viral load was measured on 30 paraffin-embedded tissues representing various EBV-related diseases (17 EBER-positive lymphomas or carcinomas) and controls (13 EBER-negative lymphomas) using each of the five validated real-time PCR assays. *ApoB*, a cellular gene, was co-amplified and used as a normalizer by which to control for the number of cells tested, and to check for inhibitors of amplification or failed extraction. None of the 30 tumors in this study had to be excluded because of inadequate *ApoB* control amplification. Results, expressed as copies of EBV per 100,000 cells, are shown in Table 2.

All 17 of the EBER-positive tumors had detectable EBV by at least two of the five Q-PCR assays, and 13 cases had measurable EBV by all five Q-PCR assays. EBER-positive carcinomas and non-Hodgkin lymphomas had the highest viral levels, while Hodgkin lymphoma cases had quite variable viral loads in keeping with the varying proportion of tumor cells in these tissues. Nine of the 13 EBER-negative tumors had low level EBV by at least one assay, consistent with EBV-carrier status.

When viral load was high by one assay, it tended to be high by the rest of the assays. Likewise, when viral load was low by one assay it was either low or undetectable by the other assays, suggesting varying lower limits of detection among the assays. The *BamH1W* assay was most sensitive, presumably because it targets a reiterated sequence that is present at approximately 10 copies per EBV genome. The *EBNA1* assay was the next most sensitive to EBV-related malignancy, while the *LMP1*, *LMP2*, and *BZLF1* assays were more prone to false-negative results.

Partial deletion or polymorphism of the EBV genomes could explain, at least in part, the differential productivity of the viral load assays in a given tumor. Case 8 had dropout of two EBV markers, suggesting that it had defective *LMP1* and *BZLF1* segments. An even more striking differential was seen in Case 6, where the *LMP2* gene was not amplified despite high levels of EBV DNA by the other four assays. When tested using an alternate primer set, Case 6 still had no amplifiable *LMP2* DNA, suggesting that this tumor had a global defect in the EBV LMP2 region such as gene deletion or chromosomal integration. Further support for this interpretation comes from immunohistochemical staining which revealed no LMP2 protein expression. Likewise, Case 8 did not express LMP1 or BZLF1 by immunohistochemistry, in keeping with our inability to amplify *LMP1* and *BZLF1* at the DNA level. While lack of protein expression is consistent with a genetic defect, it is certainly not diagnostic of a genetic defect. Further work is required to explore why selected segments of EBV DNA failed to amplify in these two AIDS lymphomas.

Human Plasma Samples

To test the versatility of these Q-PCR assays for an alternate sample type, we measured EBV viral load in plasma samples from nine patients with various EBV-related diseases and nine healthy donors (Table 3). Consistently

Table 2. Quantitative PCR Results on EBER-Positive and EBER-Negative Paraffin-Embedded Tumors

Case	Diagnosis	*ApoB* Control No. of cells	*BamH1W* copies/ 100,000 cells	*EBNA1* copies/ 100,000 cells	*LMP1* copies/ 100,000 cells	*LMP2* copies/ 100,000 cells	*BZLF1* copies/ 100,000 cells
EBER-positive cancers							
1	Nasopharyngeal carcinoma	6,935	250,937	370,310	562,653	80,404	477,520
2	Nasopharyngeal carcinoma	15,313	32,822	24,202	253,366	34,108	51,584
3	Nasopharyngeal carcinoma	69,917	23,047	62,701	126,005	3,948	45,545
4	Lymphoma, diffuse large cell	6,178	58,514	197,685	142,878	13,419	133,716
5	Lymphoma, diffuse large cell	1,501	41,173	45,969	181,346	42,638	82,545
6	AIDS lymphoma	2,228	631,239	1,431,059	1,314,183	0	783,214
7	AIDS lymphoma	592	349,493	1,168,581	1,299,831	735,473	696,453
8	AIDS lymphoma	209	2,153	11,005	0	2,871	0
9	Hodgkin, AIDS-related	979	3,575	9,295	2,451	102	1,328
10	Hodgkin, AIDS-related	3,633	2,697	7,514	13,075	3,165	2,780
11	Hodgkin, AIDS-related	4,032	397	893	744	124	109
12	Hodgkin, AIDS-related	421	95	0	0	0	143
13	Hodgkin lymphoma	18,058	2,226	5,394	2,426	244	3,156
14	Hodgkin lymphoma	94	319	106	0	0	0
15	Post-transplant LPD	2,778	25,054	116,847	38,661	21,274	15,623
16	Post-transplant LPD	6,559	15,612	22,702	39,549	6,342	23,052
17	Post-transplant LPD	2,733	5,269	12,880	21,661	6,659	114,453
		Mean viral load	84,978	217,946	285,631	67,912	162,081
		Qualitative totals	17/17	16/17	14/17	14/17	15/17
EBER-negative cancers							
18	AIDS lymphoma	60,348	5	3	8	0	5
19	AIDS lymphoma	50,966	57	84	47	0	33
20	AIDS lymphoma	41,928	0	0	0	0	0
21	AIDS lymphoma	32,544	34	38	55	4	9
22	AIDS lymphoma	18,094	6	11	33	0	0
23	AIDS lymphoma	14,180	21	42	0	0	21
24	AIDS lymphoma	12,626	8	16	0	0	8
25	AIDS lymphoma	691	0	0	0	0	0
26	Hodgkin lymphoma	11,660	21	13	21	0	6
27	Hodgkin lymphoma	4,203	2	0	0	0	0
28	Hodgkin lymphoma	1,182	42	59	0	0	0
29	Hodgkin lymphoma	1,551	0	0	0	0	0
30	Hodgkin lymphoma	643	0	0	0	0	0
		Mean viral load	22	33	33	4	14
		Qualitative totals	9/13	8/13	5/13	1/13	6/13

Each sample was tested in duplicate in each Q-PCR assay, and the values shown represent the mean EBV load for the specific assay normalized to *ApoB* control values, ie, mean EBV copy number/*ApoB* copy number × 100,000 = EBV DNA copies per 100,000 cells. Samples with no measurable EBV DNA are reported as having a viral load of zero. LPD, lymphoproliferative disorder.

high EBV loads were seen in the four patients with EBV-related malignancy and in the five infectious mononucleosis patients. In contrast, healthy individuals had no measurable EBV DNA by any of the five assays. It appears that the five Q-PCR assays are equally effective and virtually interchangeable in terms of their correlation with clinical status in this small cohort of plasma samples.

Assay Specificity

To test the extent to which the newly developed real-time PCR assays cross-react with other common herpesviruses, we tested them on pure CMV and KSHV DNA and on DNA extracted from paraffin-embedded lesional tissue of patients with active CMV or herpes simplex virus (HSV) infection (Table 4). No amplification was seen when pure CMV or KSHV DNA served as the template or when either of the herpes simplex lesions was assayed. All three of the CMV-related colon lesions had low-level EBV DNA by at least one assay. Since no amplification was observed with purified CMV DNA, the EBV signal in these colon biopsies could have emanated from rare EBV-infected lymphocytes in the tissue. There is no evidence for cross-reactivity with any of the viruses tested.

Assay Reproducibility

Reproducibility of the five Q-PCR assays and the *ApoB* control assay was examined by replicate testing of 11 paraffin tissue samples including Cases 2, 7 to 12, and 15 representing EBER-positive tumors that span a range of low to high viral loads, and Cases 21, 25, and 29 that were EBER-negative. Each sample was amplified by all six assays in duplicate on three different days for a total of 396 measurements, or six for each analyte on each sample. The coefficient of variance based on viral load was: 19% for EBNA1, 23% for ApoB, 28% for BamH1W, 33% for LMP1, 39% for LMP2, and 43% for BZLF1. The coefficient of variance based on cycle threshold (Ct) was: 1% for ApoB, 2% for BamH1W, 2% for EBNA1, 3% for LMP1, 4% for LMP2, and 4% for BZLF1. These findings suggest that the assays are quite precise and reproduc-

Table 3. EBV Viral Load by Real-Time PCR on Plasma Samples

Case	Diagnosis	*BamH1W* copies/ml	*EBNA1* copies/ml	*LMP1* copies/ml	*LMP2* copies/ml	*BZLF1* copies/ml
EBV-related disease						
A	Burkitt lymphoma	26,467	325,800	356,040	131,940	174,240
B	AIDS lymphoma	81,119	628,920	510,660	247,800	284,280
C	Post-transplant LPD	119,320	732,120	1,565,220	519,840	467,280
D	Post-transplant LPD	2,423	8,400	20,340	11,220	10,980
E	Infectious mono	13,003	35,940	99,420	16,140	10,500
F	Infectious mono	912	2,640	3,900	660	516
G	Infectious mono	676	2,040	4,020	318	780
H	Infectious mono	399	4,620	18,840	3,000	2,760
I	Infectious mono	351	780	4,320	402	198
	Mean viral load	27,186	193,473	286,973	103,480	105,726
Healthy controls						
J	Blood donor	0	0	0	0	0
K	Blood donor	0	0	0	0	0
L	Blood donor	0	0	0	0	0
M	Blood donor	0	0	0	0	0
N	Blood donor	0	0	0	0	0
O	Blood donor	0	0	0	0	0
P	Blood donor	0	0	0	0	0
Q	Blood donor	0	0	0	0	0
R	Blood donor	0	0	0	0	0
	Mean viral load	0	0	0	0	0

Each sample was tested in duplicate in each Q-PCR assay, and the values shown represent the mean EBV load for the specific assay. Samples with no measurable EBV DNA are reported as having a viral load of zero. LPD, lymphoproliferative disorder; Mono, mononucleosis.

ible. When these assays are used for serial monitoring of viral load in a given patient, changes in viral load beyond the analytic variance of the assay are presumed to be a consequence of change in clinical status.

Discussion

In this study, we validated five new real-time PCR assays, each targeting a different part of the EBV genome. The target genes were chosen because they encode products that are important in viral pathogenesis, and they represent sequences that are highly conserved across all EBV strains. We showed that the novel Q-PCR assays targeting these gene segments were sensitive, specific, linear, and reproducible in measuring EBV in cell lines as well as in paraffin-embedded tissues and plasma from patients with a wide variety of EBV-related diseases.

The *BamH1W* assay appears to be the most sensitive of the five for detecting low-level virus, in keeping with the fact that the target sequence is reiterated within each viral genome. A drawback of using this assay is that the number of reiterated *BamH1W* sequences varies among

Table 4. Specificity of EBV Viral Load Assays by Real-Time PCR

Case	Diagnosis	*ApoB* No. of cells	*BamH1W* copies/PCR	*EBNA1* copies/PCR	*LMP1* copies/PCR	*LMP2* copies/PCR	*BZLF1* copies/PCR
31	CMV DNA, purified	0*	0	0	0	0	0
32	Colon biopsy, CMV colitis	145	<1	0	0	0	4
35	Colon biopsy, CMV colitis	2,033	7	6	<1	1	9
36	Colon biopsy, CMV colitis	4,962	3	5	0	<1	11
33	Esophageal biopsy, HSV infection	191	0	0	0	0	0
34	Esophageal biopsy, HSV infection	584	0	0	0	0	0
35	KSHV DNA, purified (65,000 copies)	2.6†	0	0	0	0	0

*, Purified CMV DNA contains no human DNA, and therefore lack of *ApoB* DNA is expected.
†, Purified KSHV DNA contains low level *ApoB*, suggesting that residual human DNA emanated from the cell line from which this purified virus was prepared.
CMV, cytomegalovirus; KSHV, Kaposi's sarcoma associated herpesvirus; HSV, herpes simplex virus.

EBV strains, typically ranging between 7 and 11 repeats per genome. If the *BamH1W* copy number of a patient's EBV strain differs from that of the Namalwa Burkitt lymphoma standard, then we will overestimate or underestimate the viral genome copy number in proportion to that difference. This variability confounds our ability to precisely quantitate the number of viral genomes, but this deficiency might be outweighed by the assay's exquisite sensitivity to low-level virus, which could help detect early stage disease or help monitor residual disease burden after therapy. The *EBNA1* Q-PCR appears to be the next most sensitive assay among the five that were developed; it targets a single copy highly conserved gene that is thought to be essential for maintaining the virus long-term in dividing cells.

The true viral load in a given tumor could not be established because viral load varied, sometimes quite substantially, depending on which assay was used. The explanation for this variability was not always evident. If a partial inhibitor in a given tumor sample is assumed to inhibit all five assays equally, then inhibitors cannot explain the variance in viral loads by assay in a given tumor. The variance is especially striking given that the same Namalwa cell line was used as a standard for each of the five viral load assays. However, Namalwa DNA might contain a sequence variant that selectively affects binding of one primer or probe, thus rendering a given assay differentially efficient compared to the other assays.

Despite our efforts to target highly conserved gene sequences, two tumors (Cases 6 and 8, both AIDS lymphomas) had selective dropout of amplifiable EBV DNA at the *BZLF1*, *LMP1*, or *LMP2* loci. If a multiplicity of EBV assays had not been used in this study, then these cases might have been falsely characterized as negative for EBV. Furthermore, the selective failure of a given PCR assay suggests that a genetic defect is present, and this defect could potentially contribute to disease pathogenesis. For example, *BZLF1* encodes an immediate early lytic protein that is critical in controlling the latent versus replicative phases of viral infection. LMP1 protein has growth-promoting properties, which imply a role in tumor promotion or maintenance. LMP2, also located on the cell surface, acts to promote survival of B cells that are crippled by lack of functional antigen receptors. LMP2 is transcribed across the terminal repeat sequences, an area of the genome known to be involved in integration events. The Q-PCR assays described herein could provide a mechanism to screen for gene abnormalities including deletion, polymorphism, or integration. Such events can be further investigated by designing new up- or down-stream primers sets to map a putative deletion, and by sequencing the defective region to look for polymorphisms that interfere with primer or probe binding.

Another advantage of the Q-PCR assays described herein is that they are rapid and less labor intensive (3.5 hours total; 1 hour technologist time) than manual EBER *in situ* hybridization (7 hours total; 3 hours technologist time). Q-PCR is automated to facilitate high throughput, and is less subject to amplicon contamination than are traditional PCR assays in which product is detected by gel or by enzyme-linked immunoassay (ELISA). To improve these Q-PCR assays further, we recently showed that all components could be halved to make a 25-μl total reaction volume, and products could be amplified equally well on the ABI 7000 or 7700 instruments (Applied BioSystems) with little if any effect on outcome (data not shown).

Paraffin-embedded tissues representing EBV-related non-Hodgkin lymphomas and carcinomas had considerably higher EBV loads than did the EBER-negative tumors. These results suggest the possibility of using a rapid real-time PCR to screen for EBV, followed by EBER *in situ* hybridization on equivocal cases to distinguish tumor-associated virus from incidental viral infection. It appears that a threshold value of around 100 EBV copies per 100,000 cells distinguishes EBER-positive from EBER-negative cancers. This screening strategy is less likely to be useful for Hodgkin lymphoma cases, given the paucity of tumor cells and the associated low viral loads that approach the levels found in EBER-negative tumors.

Finally, the applicability of the five Q-PCR assays to plasma samples was demonstrated in a pilot series of cases. While all five assays yielded similar results in the nine cases we examined, we predict that future studies will show preferential amplification in certain strains of EBV, as was found in the biopsy samples we studied. The advantage of testing plasma as opposed to biopsy material is the less invasive sample collection and the ability to screen for lesions that are not yet clinically apparent. Prior studies suggest that EBV viral load testing of plasma samples is useful in at least two clinical situations. First, healthy allogeneic transplant patients usually have plasma levels below 700 copies per ml, whereas higher levels are suggestive of progression to post-transplant lymphoproliferative disorder.[13] EBV viral load is often elevated days to months before onset of symptoms, and levels quickly fall once effective therapy is initiated. Secondly, nasopharyngeal carcinoma patients often have elevated plasma levels of EBV, with advanced stage patients having higher levels than patients with localized disease.[14] After therapy, EBV is undetectable in patients who remain in remission, while it is measurable in those destined to relapse.[14]

The cell-free EBV DNA found in plasma or serum of patients with EBV-related neoplasia is thought to be unencapsidated, suggesting that it arose from apoptosis or necrosis of infected cells.[15,16] On the other hand, many infectious mononucleosis patients have a mixture of virion and un-encapsidated EBV DNA in their plasma, suggesting that viral replication contributes to the process by which EBV DNA accumulates in their plasma.[16] Further studies of infectious mononucleosis patients are warranted to determine the relative efficacy of EBV viral load and serological tests in confirming a clinical diagnosis; a recent study showed that EBV viral load was false negative in five of 30 heterophile antibody-positive cases (17%).[16]

In conclusion, the quantitative nature of the assays described herein helps distinguish EBV-related disease from incidental infection. While the BamH1W and EBNA1 assays seem most suitable for clinical applications, the full battery of assays can be used to screen for viral

genomic alterations. Their applicability to DNA extracted from paraffin sections allows them to be used retrospectively and in cases for which fresh tissue is not available. Their use in plasma may obviate the need for biopsy in selected clinical situations. Further work must be done on larger series of cases to confirm the results of this pilot study and to define the criteria for altering management based on viral load results. Furthermore, the incidence of and possible pathogenicity of genetic defects in the targeted gene segments must be explored.

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