Technical Aspects of Epstein-Barr Viral Load Assays

6

Jutta K. Preiksaitis and Catherine Burton

Introduction

Quantitative measurement of Epstein-Barr virus (EBV) DNA in peripheral blood, most often using assays employing nucleic acid amplification technology, has significantly impacted the management of both solid organ (SOT) and hematopoietic stem cell (HSCT) recipients at high risk for or with post-transplant lymphoproliferative disorders (PTLDs). Since the introduction of these assays two and a half decades ago, our understanding of the biology of acute and persistent EBV infection and its pathophysiologic role in the development of EBV-positive (+) PTLD has increased significantly [1, 2]. In addition, technologic advancements have made EBV viral load (VL) assays more sensitive and precise, and the development of a World Health Organization (WHO) International Standard (IS) has impacted result harmonization among assays [3]. Peripheral blood EBV VL assays have been extensively used by transplant clinicians for surveillance of patients at high risk for PTLD as part of preemptive programs for PTLD prevention, for PTLD and EBV disease diagnosis in symptomatic patients, and to monitor response to PTLD therapy [4]. They have also been used for safety monitoring in clinical trials of new immunosuppressive agents [5] and for tailoring immunosuppression in individual patients [6, 7]. However, result interpretation and the optimal matrix for testing (plasma vs peripheral blood mononuclear cells (PBMC) vs whole blood (WB)) remain uncertain.

J. K. Preiksaitis (✉)
Division of Infectious Diseases, Department of Medicine, University of Alberta, Edmonton, AB, Canada
e-mail: jutta@ualberta.ca

C. Burton
Division of Infectious Diseases, Department of Pediatrics, University of Alberta, Edmonton, AB, Canada
e-mail: cburton@ualberta.ca

V. R. Dharnidharka et al. (eds.), *Post-Transplant Lymphoproliferative Disorders*,
https://doi.org/10.1007/978-3-030-65403-0_6

This chapter summarizes current knowledge regarding EBV cell tropism, EBV DNA dynamics, and the biologic forms of EBV DNA in the cellular and acellular fractions of peripheral blood during acute and persistent EBV infection and in EBV+ PTLD. We highlight how this information influences choice of testing matrix, EBV DNA assay design, and result interpretation when using quantitative EBV DNA assays in specific clinical settings. The current status of result harmonization among assays is reviewed along with the impact of standards and calibrators, nucleic acid extraction methods, target and probe design, and other factors. Testing of non-peripheral blood samples and possible future enhancements to EBV VL measurement are also discussed.

What Are We Measuring When Quantifying EBV DNA in Peripheral Blood? Biological Form and Cell Tropism

How Are Biological Forms of EBV DNA and Cell Tropism Assessed?

The phenotype of EBV-infected cells has most commonly been studied by sorting cell subsets based on surface markers using fluorescence-activated cell sorting (FACS) followed by detection of EBV DNA in each subset [8]. More recently, some investigators have used ImmunoFISH techniques with infected cells detected by either flow cytometry [9] or cell counting on slides by fluorescent microscopy [10]. EBV DNA in latently infected cells exists in an extrachromosal episome (~170 kb) with a nucleosome structure similar to that of the host genome [11]. In addition, integration of subgenomic fragments of EBV into specific sites of the cancer genome has been observed in a subset of malignant cells in some EBV-associated malignancies [11]. Lytically infected cells also contain concatemeric DNA molecules as well as monomeric EBV DNA encapsidated in virions. EBV DNA in virions is free of nucleosomes. EBV-infected cells may have long half-lives depending on their rate of generation, homeostatic cell division, and cell death as well as immune-mediated killing (see Section "EBV VL Kinetics: Implications for Monitoring Algorithms").

EBV DNA in plasma could exist as naked free DNA released by apoptosis or necrosis of EBV-infected cells, encapsidated in virions or in exosomes. Naked EBV DNA in plasma has a very short half-life of ~2 hours [12], making dynamic changes in its measurement more rapidly responsive to treatment interventions than changes in cellular EBV DNA. Two primary techniques have been used to determine whether EBV DNA in plasma is encapsidated virion DNA or is "naked" EBV DNA released from cells. The first exploits the property that virions, but not free DNA, will be protected from DNase digestion; the second examines the proportion of EBV DNA in pellets versus supernatant after pelleting virions by ultracentrifugation [13]. The distribution of EBV DNA fragment sizes in plasma has historically been estimated using quantitative polymerase chain reaction (PCR) using different sized amplicons [13].

Circulating cell-free DNA (ccf DNA) is present at low levels in all human plasma and is composed of mono-nucleosomal DNA fragments originating from apoptotic and necrotic normal hematopoietic cells; ccf DNA levels rise in inflammatory states. During apoptosis, DNA is fragmented by caspase-activated DNase resulting in fragment lengths that are multiples of nucleosomal intervals (166 bps) forming a characteristic ladder on sizing gels. DNA fragment length is impacted by cause of cell death (necrosis longer fragments than apoptosis [14]) and varies by cell source resulting in a "nucleosome footprint" pattern that allows identification of very small amounts of non-hematopoietic tumor cell DNA in the background of normal DNA in plasma [15, 16]. Recently, target-capture deep sequencing to both count and profile EBV DNA fragment lengths in plasma has been used to study their origin, exploiting the non-nucleosomal profile that would be observed in EBV DNA originating from virions/lytically infected cells versus the nucleosomal pattern of latently infected cells [17].

Biologic Form of EBV DNA and EBV-Infected Cell Type Varies with Host Immune Status and Clinical Context

The number and type of cells either lytically or latently EBV infected in peripheral blood and the presence and biologic form of EBV DNA in plasma (naked vs. encapsidated in virions) vary and depend on clinical context. Because data in transplant populations are limited, we also extrapolate and glean important information from studies in immunocompetent hosts and other immunocompromised populations such as HIV-infected subjects; these data are summarized below and in Table 6.1.

Table 6.1 What biologic forms of Epstein- Barr virus (EBV) DNA are we measuring in the whole blood of immunocompetent subjects and immunocompromised patients?

Clinical setting	EBV DNA in plasma	EBV-infected cells
Immunocompetent		
Asymptomatic seropositive subjects with remote infection	Very small fragments of naked ccf** EBV DNA (predominantly <110 bp) Not encapsidated in virions May originate from apoptotic lytically infected cells in tissues [17] *Rarely detected and when present only transient* [17, 26] *Increased detection during critical illness and sepsis* [26]	Predominantly:* resting memory B cell IgG genes hypermutated, class-switched (CD10+, CD20+, CD3−, CD23−, CD80−, Ki67−, CD27+ IgD− CD5−) [18–21]; 2–5 genomes/cell (episomal form) [20]; latency 0 [22, 23] Fewer: other memory B cells CD27+ IgD+ IgM+ or CD27− IgA+ [24, 25] *Individual-specific stable "set point" VL* [66, 153, 198] *(estimates: in US 5–3000/10^7 B cells* [1]*, in UK median 79/10^6 PBMC infected)* [181]

(continued)

Table 6.1 (continued)

Clinical setting	EBV DNA in plasma	EBV-infected cells
Primary infection symptomatic subjects with infectious mononucleosis (IM) and asymptomatic subjects	ccf naked EBV DNA, not further characterized Presence of EBV DNA encapsidated in virions uncertain [30] *Almost always detected at symptom onset*; *duration 15–31 days* [29–33]	Predominantly: as above* [22] Fewer: other memory B cells CD27+ IgD+ [25] EBV-infected T cells in EBV-2-infected African infants [28]
EBV-HLH	Not available *Always detected (numbers studied small)* [32]	As in seropositive healthy adult plus EBV infection of activated CD8 T cell [39]
CAEBV	ccf naked EBV DNA, not further characterized Not encapsidated in virions [38] *Detected in 86% of patients* [37, 38]	As in seropositive healthy adult plus EBV infects a lymphoid progenitor cell with clonal evolution of a specific cell lineage or multiple lineages detectable in peripheral blood, predominantly T cell (CD4 or CD8) or NK cell in Japan [35, 38], B cell (CD20+ or CD20−) in North America [36]
EBV-associated malignancy	Very small fragments of naked EBV DNA, 87% fragments<181 bp, unique fragment length peak (150 bp in NPC) Not encapsidated in virions [13, 17, 41] *Tumor marker in this matrix*	Not available
HIV-infected patients		
Children with primary infection Adults with persistent high load/ set point	Not available *Infants: detected >3 months in most* infants [34] *Adults: variably detected with prevalence significantly lower than in WB* [51]	Children with primary EBV infection: B cells (not further phenotyped) plus small number of CD4 and CD8 T cells [52] Adults: B cells (not further phenotyped), EBV also detected in plasmablasts and plasma cells, monocyte cells not carrying B, T, or monocyte markers in some patients [10]
EBV+ lymphoma	ccf naked EBV DNA, not further characterized [30]	Not available
Transplant recipients		
SOT early <1 year post-transplant	ccf naked EBV DNA, not further characterized [30] *Pediatric primary infection: always detected, duration may be >1 year* [67, 75] *Adults seropositive pre-transplant: variably detected, prevalence proportional to quantitative levels in WB or PBMC* [60, 66, 68]	Adult population (presumably seropositive pre-Tx): as in seropositive healthy adult* [18] *Pediatric population experiencing primary EBV infection has not been studied during this phase*

Table 6.1 (continued)

Clinical setting	EBV DNA in plasma	EBV-infected cells
SOT >1 year post-transplant with persistent viral load/high set point	Not available *Rarely detected and when present only transiently; prevalence proportional to quantitative levels in WB or PBMC* [8, 42, 47, 75]	Pediatric with persistent low viral load: as in healthy seropositive adult (disproportionately IgM+) [42, 43] Pediatric with persistent high viral load: As in low load recipient plus up to 30% of infected cells highly atypical predominantly Ig null cells with 30–60 genome copies/cell (CD19+, CD5−, CD10−, CD27− CD23− CD38− and CD69− with variable expression of CD20 and CD40, often HLA class I and class II negative); may be transient and fall with decreasing viral load [43, 45] Predominantly CD20+ IgM+, IgD+CD27+ memory B cells, with~ 16.7 genome copies/cell [9] Some patients also have EBV-infected T cells and monocytes and EBV-infected cells lacking B, T, and monocyte markers [10, 47]
HSCT with early high-level reactivation	Not available *Variably detected, prevalence proportional to quantitative levels in WB or PBMC* [74]	Isotype-switched memory B cells (CD19+CD27+) of donor origin (median 19 genome copies/cell); significant proportion proliferating (Ki67+) rather than resting and express a plasmablastic (CD24− $CD38^{hi}$) phenotype and have latency III EBV gene expression [50]
EBV+ PTLD	ccf naked EBV DNA, not further characterized *Almost always positive (exception CNS PTLD and EBV+ mucocutaneous ulcer)* [48, 49, 68, 82]	Not available (patients not studied immediately before or at the time of PTLD diagnosis)

Abbreviations: *CAEBV* chronic active EBV infection, *ccf* circulating cell free, *HLH* hemophagocytic lymphohistiocytosis, *NPC* nasopharyngeal carcinoma

Immunocompetent Patients

Asymptomatic EBV Seropositive with Remote Infection When ultra-sensitive assays are used, EBV DNA can be found in the peripheral blood of all EBV-seropositive patients, predominately in long-lived resting B cells with the phenotypic hallmark of classical antigen-selected memory B cells (CD10+, CD20+, CD3−, CD23−, CD80−, Ki67−, CD27+ IgD− CD5−); Ig genes are hypermutated and class switched [18–21]. They are latently infected, express no EBV proteins (latency 0) [22, 23], and contain two to five genomes/cell [20]. Some investigators have also observed EBV DNA in a smaller number of CD27+, IgD+ IgM+, and CD27− IgA+ memory B cells, suggesting EBV can enter memory B cells without

germinal center transit [24, 25]. The number of infected cells among individuals varies significantly from 5 to 3000 /10^7 memory B cells, but each individual appears to have a unique relatively stable "set point" with respect to infected cell number. It is estimated that only ~1% of the systemic EBV VL in an individual is in peripheral blood [1].

EBV DNA is highly cell associated in healthy adult patients almost all of whom are EBV seropositive and is only rarely detected in plasma (0.6–5.5%); when detected it is usually transiently present (<4 weeks) [17, 26]. EBV detection prevalence increases significantly relative to similarly aged immunocompetent subjects in the setting of critical illness with rates higher in WB vs plasma as follows (15/127 (11.8%) vs. 3/55 (5.4%)); even higher rates are observed in immunocompetent critically ill patients with sepsis (275/522 (52.7%) vs. 75/235 (31.9%) [26].

Children and Adults with Primary EBV Infection (Symptomatic or Asymptomatic) The limited available data comes from studies of adolescents and young adults presenting with symptoms of infectious mononucleosis (IM). As in EBV-seropositive patients with remote infection, EBV DNA is found in a latent form in memory B cells (CD27+ IgD−) exclusively [22] or predominantly, with some CD27+, IgD+ cells also infected [25]. However, in IM, up to 50% of all memory B cells can carry EBV DNA [22]. Whether infectious virions are being produced is uncertain; limiting dilution RT-PCR studies of IM peripheral blood demonstrated the presence of a very low frequency of cells expressing lytic cycle gene BZLF in only two of five IM patients [22, 23].

Non-B cells can be infected during primary EBV infection, even in immunocompetent hosts. A recent study of the tonsils of IM patients suggests that approximately 9% of EBV-infected cells in this tissue express T cell antigens [27]. In a Kenyan study of HIV-uninfected mother/infant pairs, Coleman et al. observed that young infants infected with EBV-2 had EBV DNA in T cells while those infected with EBV-1 did not. Interestingly, T cell EBV infection was not observed in the EBV-2-infected mothers [28].

Using real-time PCR assays, EBV DNA is detected in plasma in almost all symptomatic adult and pediatric patients with IM at lower levels and for a significantly shorter period (<30 days from symptom onset) than in PBMCs [29–33]. Using a DNase assay to study the plasma of 20 IM patients, Ryan et al. [34] found 60% had only naked EBV DNA present. The remainder had incomplete degradation of the control β-globulin DNA as well as EBV DNA making the interpretation of results as demonstrating the presence of virion-associated EBV DNA uncertain (see discussion of this confounder by Chan et al. [13]). These results have not been validated by others. While the plasma of asymptomatic adults with primary EBV infection has not been studied, Slyker et al. studied EBV infection in Kenyan infants and found that 55% of EBV-infected, HIV-uninfected infants and 83.6% of HIV-infected EBV-infected infants had EBV DNA detected in plasma; EBV DNA remained

detectable in the plasma for >3 months in 62% of the HIV-infected infants but not in any of the HIV-uninfected infants [34].

Chronic Active EBV Infection (CAEBV)/Hemophagocytic Lymphohistiocytosis (HLH) This rare disorder appears to be a pre-malignant condition initiated by infection of a lymphoid progenitor cell from which malignant cells evolve by acquiring DDX3X and other driver mutations leading to monoclonal and less often oligoclonal evolution of EBV-infected T, NK, or B cells [35]. Patients exhibit persistent/recurrent IM-like symptoms as well as atypical symptoms and a high predilection for progression to lymphoma or leukemia; B cell depletion and hypogammaglobulinemia have been described in B cell CAEBV [36, 37]. EBV DNA in the form of naked EBV DNA, not encapsidated in virions, was detected in the plasma of 95/108 (86%) patients in a T/NK CAEBV cohort [37, 38]. In EBV-associated HLH, a hyper-inflammatory syndrome characterized by uncontrolled activation of T cells as well as macrophages, the EBV-infected cell appears to be an activated CD8 T cell [39]. EBV-associated HLH has been described in the transplant setting [40].

EBV-Associated Malignancies in Immunocompetent Patients EBV DNA detection in plasma appears to be preferred when compared to its detection in WB as a tumor marker. It has been evaluated as a screening tool for malignancy, as a prognostic marker, and to monitor response to therapy in settings such as NPC, diffuse large B cell lymphoma, extranodal T/NK lymphoma, and Hodgkin lymphoma [41]. In these settings, plasma EBV DNA appears to be naked DNA, not virion-associated, and fragments are very short (majority <180 bp) [13]. Recent studies using target capture sequencing and fragment length profiling of EBV DNA in plasma have demonstrated that NPC patients have a characteristic 150 bp peak unique to tumor cells and a nucleosome-bound fingerprint pattern suggesting latently infected cells as the source of plasma EBV DNA [17]. The size of the fragment length peak proposed as a tumor marker of NPC cells of epithelial origin cannot be extrapolated to PTLD which is of hematopoietic origin. However, this approach could be explored to determine whether EBV-associated smooth muscle tumors that occur after transplant also have a characteristic fragment length predominance.

Immunosuppressed Patients

Pediatric and adult SOT: Most studies examining the phenotype of infected cells in transplant recipients have studied patients with persistent VL elevation, usually later than 1 year after transplant, sometimes without reference to pre-transplant EBV serostatus (Table 6.1). While little is known about the type of cells infected in early primary EBV infection post-transplant, studies of adult kidney recipients and pediatric allograft recipients with persistent EBV VL, most of whom had experienced primary EBV infection with or without PTLD, suggest EBV is present primarily in resting memory B cells as in immunocompetent adults [18, 42, 43]. Schauer et al. [43] found that in pediatric SOT >1 year post-transplant with low persistent VLs, infected memory B cells contained one to two genomes/infected cells that were

disproportionately IgM+. In contrast, high EBV VL load carriers have a mixed population of EBV infected cells including the cell type found in low load carriers and EBV-infected cells containing 30–60 genome copies/cell which disproportionately contributed to total VL [43]. While the significance of this finding is not totally clear, the genome copy number/cell is known to reflect the number of replication cycles the EBV-infected cell has undergone [44]. These highly atypical cells were predominantly Ig-null. Surface immunoglobulin (sIg), when expressed, was disproportionately IgA+. These atypical cells appear transient, decreasing in number as VL falls in serially followed patients [45]. The observations related to these atypical high copy number cells have not been confirmed by others. As non-EBV-infected cells of this type are also found in pediatric transplant patients with undetectable VL, they are not solely the result of EBV infection [45]. Despite these observations, several investigators have found a reasonable correlation between total VL measured directly in peripheral blood of patients with persistent high loads and the number of EBV-infected cells detected by in situ hybridization [9, 10, 46]. An ImmunoFISH study of the peripheral blood of four pediatric Japanese liver transplant recipients with elevated VL found that the EBV-infected cells were predominantly CD20+ IgM+, IgD+CD27+ memory B cells, with an estimated 16.7 genome copies/cell [9]; this cell subset is usually only a minor infected population in the immunocompetent host [25].

Most investigators have found that in adult and pediatric SOT patients with elevated persistent VL, EBV infection is restricted to B cells. However, Greijer et al. [47] found EBV DNA in T cells and monocytes in two of six SOT recipients, and Calattini et al. [10] found EBV DNA in cells lacking B, T, and monocyte markers in two of three transplant recipients. The phenotype of EBV-infected cells in the blood of SOT patients at the time of or immediately prior to PTLD diagnosis has not been studied; whether they have circulating cells with any unique phenotypic features is unknown.

Most SOT transplant patients with chronic elevated EBV load studied late after transplant do not have detectable EBV DNA in plasma. When present, it is generally found in patients with higher VL in PMBC, is present in small amounts, and is transient [8, 42, 47]. EBV DNA is known to be present in the plasma of almost all cases of EBV+ PTLD in adult transplant patients, although it is not detected in EBV-negative PTLD and may miss EBV+ CNS disease and EBV+ mucocutaneous ulcers [48, 49]. Ryan et al. [30] studied the plasma of two transplant patients without PTLD and five with PTLD using a DNase 1 assay and found EBV DNA to be free DNA, not virion-associated in six, with uninterpretable results because of incomplete digestion of control DNA in one PTLD patient.

HSCT Recipients Burns et al. [50] recently studied the cellular tropism of EBV in HSCT recipients with high EBV load detected by WB monitoring within 3 months of transplant. They found that EBV resides almost exclusively in supranormal numbers of isotype-switched memory B cells (CD19+CD27+) of donor origin, prior to a time when memory B cell reconstitution is expected to occur, suggesting the

appearance of these cells is EBV-driven. Moreover, the EBV infection is latent (median, 19 genome copies/cell), but a significant proportion of infected cells are proliferating rather than resting and express a plasmablastic (CD24− $CD38^{hi}$) phenotype and have latency III EBV gene expression.

HIV-Infected Patients Adult EBV-infected HIV positive patients frequently have persistent high levels of EBV DNA detected in whole blood (65.5%) even on HAART therapy; detection in plasma is less frequent (4.8%) [51]. In addition to infected B cells, non-B cells of variable phenotype may be infected [10, 52] (Table 6.1). Using a DNase assay, Ryan et al. [30] found the EBV DNA in the plasma of 11 HIV-related lymphoma patients was free DNA, not encapsidated in virions in 10; in one results are uninterpretable because of failure to digest control DNA.

Conclusions Re: Biologic Form of EBV in Plasma and Phenotype of EBV-Infected Cells in WB

There is no clear evidence that the EBV DNA in plasma is encapsidated in virions at any time during acute or persistent infection or in EBV-associated malignancies in either immunocompetent or immunocompromised hosts. EBV DNA in plasma likely exists as fragmented free DNA, released predominantly from latently or lytically infected cells outside the circulation. Fragment length profiling of EBV DNA in plasma during different phases of infection and malignancy may be a useful tool to confirm this. EBV DNA appears in plasma only during a specific period during the course of primary EBV infection and only rarely after viral "set point" is reached in a stable patient. Whether the presence of EBV DNA in plasma could be used as a surrogate marker of immune control of EBV is unknown.

EBV DNA in the peripheral blood of transplant patients exists as complex mixtures of forms in the cellular and plasma fractions that may vary during stages of acute and persistent infection, inter-current illness, as well as evolving malignancy. There is evidence that EBV DNA may be found surprisingly often in atypical B cells as well as non-B cells in the peripheral blood, particularly in the immunocompromised host. In transplant patients with persistent viral elevated load detectable in WB, it may be important to differentiate patients who have only an altered viral set point from patients who carry clonally abnormal B, T, or NK EBV-infected cells in their circulation as is seen in CAEBV in the immunocompetent host.

Choosing Peripheral Blood Specimen Type and Reporting Units

Although there is general consensus that peripheral blood is the preferred sampling site for EBV VL assessments in transplant patients, the optimal sample type remains unresolved. Cellular specimen types including WB, leukocytes, PBMC, and isolated B cells (BC), as well as acellular fractions (plasma and serum), have been evaluated [47–49, 53–69]. In both SOT and HSCT recipients, high correlations have

been observed between quantitative EBV DNA measurements in different cellular specimen types (WB, PBMC, isolated B cells) when using the same assay [56, 61, 70, 71]. Moreover, normalization of results in cellular sample types to cell number or genomic DNA using either total DNA or housekeeping genes did not significantly change this correlation or alter dynamic trending in serially followed patients compared to the simpler method of reporting the results/volume (ml) [53, 56, 61]. However, the presence of severe lymphocytosis or leukopenia should be reviewed in interpreting dynamic changes in results [53, 72]. Because of reduced processing steps and lower blood volumes required, WB has become the preferred cellular specimen with VL reported in IU/ml, without normalization. Approximate conversion factors among historical reporting units are summarized in Table 6.2.

Serum is sometimes used as an alternative to plasma [49, 58–60, 64]. Although these acellular sample types have not been directly compared for EBV DNA detection, levels of genomic cell-free DNA in serum are known to be significantly higher than in plasma because white blood cells lyse during clotting [73]. On a theoretical basis, to avoid plasma contamination from EBV DNA in circulating cells, particularly when cellular VL is high, plasma is preferred over serum as the non-cellular fraction of choice.

Although, generally, EBV DNA becomes detectable in plasma or serum as EBV VL rises in matched WB or PBMC samples and a linear correlation exists for results in the two matrices [47, 56, 64], the correlation coefficient between quantitative EBV VL measured in WB or lymphocytes versus plasma is relatively low [54, 56, 60, 63, 64, 74]. When detection is discordant, the pattern most commonly observed is EBV DNA detection in WB or PBMC, while plasma is negative, particularly when VL in cellular sample types is low. Quantitative differences between WB and plasma may be >2 $\log_{10}$ copies/ml; differences appear smaller in symptomatic than asymptomatic patients [75]. However, extreme quantitative discordance in both directions has been described, particularly in the HSCT setting [47]. The relatively poor quantitative correlation between WB and plasma is not unexpected. Most

Table 6.2 Approximate conversion factors for historical reporting units of EBV viral load relative to copies/ml of whole blood

Units	Assumption	Conversion factor
IU/ml	Assay calibrated to WHO IS for EBV DNA	Assay specific
Copies/10^5 PBMC[a]		1
Copies /10^6 PMBC		10
Copies/10^7B cells	3–15% of PBMC, age dependent	667–3333
Copies/μg DNA	16–135 μg /whole blood, white cell count dependent, 2x10^6 PBMC/ml of whole blood	1.5–12.5
Copies/ml	Cell counts including B lymphocyte counts are stable over time, 1.5–2.0x10^6 PBMC /ml of whole blood	15–20

[a]*PBMC* peripheral blood mononuclear cells

studies comparing peripheral blood fractions were cross-sectional single-center studies of samples submitted to the laboratory and pooled for analyses. Transplant populations studied were in various stages of EBV infection and malignancy and included asymptomatic subjects experiencing primary infection or reactivation infection with or without inter-current illness as well as those diagnosed and being treated for PTLD; asymptomatic patients with persistent high VL are also often included. The biologic forms present and distribution of EBV DNA in the cellular component vs plasma differs over time in individual patients and in different clinical settings, likely explaining these observations. Available studies of longitudinally monitored patients comparing contemporaneous results in different sample types have limitations that make it difficult to determine the preferred sample type in specific clinical settings. These limitations include small patient numbers and few or no PTLD cases studied [56, 57, 60, 66, 74], monitoring of groups at very low risk of PTLD (seropositive adult SOT recipients) [60, 66], or sampling not starting at the time of transplant in patients in whom PTLD was documented [56].

Choice of Peripheral Blood Specimen Type in Transplant Recipients

The choice of specimen type may depend on the purpose for EBV DNA measurement; one size may not fit all settings. If the goal is to detect EBV infection/reactivation as early as possible in transplant populations at high risk for developing PTLD, with the goal of intervening to re-establish control of the infection assuming this will lower future PTLD risk, then the more sensitive WB sample may be preferred. EBV VL is most often significantly higher, usually by more than a $\log_{10}$ in cellular peripheral blood fractions than in acellular fractions [54, 55, 60, 64, 66, 74] and higher during primary infection than reactivation infection in both fractions after SOT [60, 76, 77]. Earlier temporal detection after transplant has been documented in WB compared to plasma in HSCT recipients [74] and seropositive lung transplant recipients [60]. In the small number of EBV-mismatched SOT for whom longitudinal contemporaneous monitoring in both specimen types was reported, EBV DNA was first detected in WB in only three of ten patients studied [57, 60, 67]. In a recent study of EBV-mismatched pediatric liver transplant recipients, all first detection of EBV DNAemia in WB was concordant with its detection in serum [75]. In HSCT recipients plasma monitoring alone has been used successfully in preemptive programs for PTLD prevention [78, 79]. Some investigators found results of testing in both WB and plasma/serum may be additive and increasing levels of EBV DNA in plasma/serum but not WB occur just before PTLD diagnosis; patient numbers however are small [56, 64]. Using an assay not calibrated to the WHO IS, Ruf et al. found 20,000 genome copies/ml of WB and 1000 genome copies in plasma had optimal sensitivity and specificity for PTLD prediction [56]. Using these values they found that the sensitivity, specificity, negative predictive value (NPV), and positive predictive value (PPV) were 100%, 87%, 19%, and 100% for WB; 88%,98%, 54%, and 100% for plasma; and 100%, 94%, 50%, and 100% when WB

and plasma values were combined for predicting PTLD in pediatric SOT and HSCT patients; this is not true in adult SOT populations where >50% of PTLD after the first year is EBV negative [48, 80]. Although either specimen type could be used in preemptive PTLD prevention programs, monitoring both would be optimal although associated with increased cost and more complex laboratory logistics. If the goal of EBV DNA testing is early EBV+PTLD diagnosis, treatment monitoring, or prediction of relapse, plasma may be the preferred specimen type, although this requires further validation. This would be in keeping with role of EBV DNA detection in these settings as a tumor marker in a naked cell-free form with a very short half-life.

The advocacy for WB over plasma as the preferred sample type in the transplant setting dates back to early studies that failed to detect EBV DNA in the plasma of a significant number of PTLD cases, despite detection in WB samples cases [58, 59]. However, these studies were performed using less sensitive pre- real-time PCR technology with large amplicons and older nucleic acid extraction methods that may have failed to identify the small EBV DNA fragments in plasma [81]. Studies using RT-PCR found detectable plasma EBV DNA in almost all cases of EBV+PTLD with the exception of CNS PTLD and EBV+ mucocutaneous ulcer [48, 49, 68, 82]. EBV DNA detection in plasma is also a more specific marker of EBV disease, including PTLD than WB or PBMC detection, when used as a diagnostic test in patients with signs or symptoms of PTLD [48, 54, 56, 68] and also discriminates EBV+PTLD from EBV- negative PTLD better [48, 68].

In the HSCT population, Kalra et al. [83] reported that persistently detectable EBV DNAemia in WB after rituximab treatment of PTLD in HSCT recipients had a 71% PPV and a 100% NPV for progression/relapse, though most of the PTLD in that study was not biopsy-proven. Similarly, in a multicenter study of 144 cases of rituximab-treated PTLD in HSCT recipients, Styczynski et al. [84] observed that persistent or increasing EBV DNAemia, from WB or plasma samples (in equal frequency with results pooled for analyses), after 1–2 weeks of therapy was a predictor of poor response and increased mortality. However, in both SOT and HSCT populations, the bulk of data suggest that plasma may be preferred over samples with cellular fractions for monitoring response to therapy and predicting relapse as VL correlations with clinical response appear better [48, 56, 68, 85–87].

The high prevalence of EBV DNA detection after transplant, particularly in WB in low-risk asymptomatic adult patients who are seropositive pre-transplant, raises questions regarding the cost- benefit and risks associated with routine screening of this patient group using either WB or plasma [80, 88]. EBV DNA detection in WB appears to increase with time after transplant and to be a poor marker of future PTLD risk in this low-risk setting [76]. Elevated and often sustained elevation in EBV loads in WB has been observed in many of these patients although peak loads are usually lower than those observed in primary infection and many EBV+ PTLD cases. Investigators have detected EBV DNA in 67–72% of adult liver [89, 90], 31–29% of adult kidney [76, 91], and 13–42% (assay dependent) [48] of adult lung transplant recipients almost all of whom were EBV-seropositive pre-transplant. EBV DNA prevalence appears to be lower when plasma is monitored as illustrated by the 13% prevalence in a longitudinal study of adult liver transplant patients [62].

In a recent cross-sectional study of 808 transplant patients without either EBV disease or PTLD, Kanakry et al. [68] detected EBV DNA in 24% and 7% when testing PBMC and plasma, respectively. Data regarding the prevalence of EBV DNA detection in the peripheral blood of EBV-seropositive pediatric transplant recipients are more limited. In two cohort studies of pediatric kidney transplant patients, EBV DNA was detected in WB above a "significant level (3,000 copies/ml)" in 19.9% [92] and at any level in 44.4% [77]. Children with non-intestinal transplants seropositive pre-transplant are at lower risk of PTLD than those who are seronegative, although the difference may be less marked in pediatric populations than in their adult counterparts [92, 93].

Result Harmonization

Calibration Standards, Traceability, and Commutability

Currently, most clinical laboratories use real-time PCR amplification and detection methods (RT-PCR) for the measurement of EBV DNA in peripheral blood after nucleic acid extraction [94]. RT-PCR assays, used extensively in the last decade, are more precise and less prone to effects of inhibitors and have a broader linear range (6–7 orders of magnitude) compared to earlier generations of competitive endpoint PCR assays, factors that should be considered when reviewing older literature reporting EBV VL results [94].

Even when RT-PCR assays are used, there can be extreme variability in results reported when the same sample is tested using different assays. In 2009, Preiksaitis et al. [95] found the variation of reported results on individual positive samples ranged from a minimum of 2.3 $\log_{10}$ to a maximum of 4.1 $\log_{10}$ with only 47% of all results falling within ±0.5 $\log_{10}$ of the expected results when 28 international transplant center laboratories tested a panel of 12 EBV DNA samples. Inter-laboratory variation was significantly higher than intra-laboratory variation suggesting that the use of an International Standard (IS) for calibration might improve result harmonization. This variability was concordant with observations in the period prior to 2011 using laboratory developed test (LDT) and commercial assays in both the transplant and NPC settings and was observed regardless of peripheral blood matrix tested, plasma [96, 97], PBMC [98], and WB [99–102]. It is not clear how much result variability using RT-PCR assays is achievable or clinically acceptable, but variation of ±0.5 $\log_{10}$ is often used, extrapolated from data in HIV [103].

In October 2011 the WHO Expert Committee on Biological Standardization approved a lyophilized whole virus preparation of the EBV B95-8 strain produced by the National Institutes for Biological Standards and Control (UK) as the first WHO IS for EBV DNA to be used in all nucleic acid testing as a calibrator [3]. The preparation was assigned a potency value in international units (IU), a consensus value not precisely related to genome copies. IS-calibrated assays report results in IU/ml rather than copies or genome copies/ml; the conversion factor from copies to IU will be specific for each assay. In order to improve result agreement, an IS must

be "commutable," a concept derived from clinical chemistry which is defined as "the equivalence of the mathematical relationships between the results of different measurement procedures for a reference material and for representative samples from health and diseased individuals" [104]. Commutability of a reference material is both assay and matrix specific [104–106], and when the IS is used and is non-commutable for the assays being tested, result agreement between assays can become worse [107]. The IS may be commutable for assays measuring EBV DNA in WB but not when the same assays are used in plasma [108]. Assays may demonstrate excellent result harmonization when proficiency panels created using whole virus preparations are tested but still show significant result variation when testing clinical samples, perhaps because the biologic form of EBV DNA in the tested samples is different [107, 108]. Therefore, to definitively determine the impact of WHO IS calibration on result harmonization among assays, clinical samples must be studied.

Laboratories have been slowly introducing EBV VL assays calibrated to the IS. When testing the 2013 College of American Pathologists (CAP) EBV DNA proficiency panel, only 9.4% of 319 laboratories reported results in IU [109], and in a survey of 71 transplant programs in 15 European countries in 2013, only 57.1% of virologists supporting these programs were aware of and had accessed information regarding the IS [110]. Most information regarding the impact of the WHO IS on result agreement comes from analysis of EBV DNA testing results of national or international proficiency panels that do not include clinical samples [109, 111, 112]. These studies all concluded that use of the IS for assay calibration significantly reduced result variability, with >80% results reported by multiple laboratories falling within ±0.5 $\log_{10}$ IU/ml, for WB and plasma samples [111, 112]. However, when four US laboratories participating in a clinical trial reported results for the WHO IS serially diluted in plasma, only 62% of values for dilutions above 4 $\log_{10}$ were within ±0.5 $\log_{10}$ IU/ml of expected values [113]. Data using clinical samples tested using assays calibrated to the WHO IS are limited and provide variable results, with studies often demonstrating excellent result agreement when only two assays are compared and others showing significant variability between specific assay pairs when a larger number of assays were studied [107, 117–120]. Other potential sources of variation summarized in Table 6.3 likely contribute to ongoing suboptimal result harmonization. Based on impact validation available to date, use of WHO IS-calibrated EBV DNA assays may have improved result harmonization and should be widely adopted to eliminate this source of variability at a global level.

Assays typically use secondary or tertiary standards often from commercial sources, rather than the primary WHO IS material for calibration. Variable results with different magnitudes of bias compared to nominal assigned values have been observed when commercial secondary CMV standards were tested by different RT-PCR and digital PCR assays, highlighting that these secondary or tertiary standards must also not only be traceable to the WHO material but also be commutable. Similar issues may be at play for EBV, and until the impact of the WHO IS and

Table 6.3 Factors that may need to be addressed to reduce variability of quantitative results of EBV DNA measurement using real-time RT-PCR assays

Calibration
Use of secondary and tertiary calibrators traceable to the WHO IS for EBV DNA is recommended
Commutability should be demonstrated for each assay/standard system
Method for nucleic acid extraction
If plasma tested, cell –stabilizing collection tubes that prevent contamination of plasma with cellular DNA may be warranted to increase purity and yield
Methods that reduce further fragmentation of DNA during the extraction process
Methods that improve isolation of small fragments <100 bp that may be significant component of plasma cell free EBV DNA
Primer and probe design
Target conserved sequence regions
Use of small amplicons <100 bp is recommended to allow quantitation of small fragments of cell –free EBV DNA likely present in plasma
Use of two gene sequences as targets may be beneficial
Other
Use of specific reagents
Probe chemistry
Instrument and software
Automated versus manual pipetting
General
Automation and standardization of all steps in the measurement procedure
Use of commercial versus laboratory developed tests would reduce number of assays used. Reduction in overall number of assays available would make global result harmonization easier

traceable commutable secondary standards on result harmonization among a wider array of assays is validated using clinical samples, inter-institutional result comparison requires formal cross-referencing of measurement results obtained from testing of the same clinical samples between institutions. In the interim, patients should be monitored using the same sample type and the same assay in a single laboratory. The precision of RT-PCR assays should be considered when interpreting results, recognizing that poorest precision occurs at low VLs. Changes in values should differ by at least threefold (0.5 $\log_{10}$) and as much as fivefold (0.7 $\log_{10}$) near the assay's limit of detection to be considered biologically important changes [103, 114].

Digital PCR (dPCR) has been proposed as alternate and perhaps better reference technology for EBV VL measurement as it provides absolute quantification without need for a standard curve [114]. This technology is more precise when quantifying low copy numbers. Other advantages and disadvantages of dPCR relative to RT-PCR have been reviewed [114]. Although it removes the calibrator as a source of result variability, dPCR does require nucleic acid extraction and design of primers and probes which may contribute to variability.

Nucleic Acid Extraction

Nucleic acid extraction procedures are a known source of variation in VL measurement with impact depending on the number and type of extraction systems and PCR systems studied [70, 81, 100, 115, 116]. An understanding of EBV DNA fragment length profiles, in plasma and WB, is important to inform the choice of DNA extraction system. As we have no information regarding EBV DNA fragment length profiles in the plasma and WB of transplant patients, we extrapolate from studies of plasma EBV DNA in NPC population-based screening, where EBV DNA fragments appear to be very short, often <110 bp [13, 17]. Cook et al. [81] recently studied the extraction yields of 11 commercial extraction methods commonly used in viral diagnostic laboratories and 4 new methods designed to isolate the shorter fragments of ccf DNA. Not only were a wide range of extraction yields observed across methods, but yields were especially inconsistent and poorer (<20%) for 50–100 bp fragments, even with two of the ccf extraction methods. If similar very short EBV DNA fragments (<110 bp) are also seen in plasma in the transplant setting, the specific extraction system yield of these very short fragments could significantly impact quantitation and result variability among systems. The DNA fragments in the cellular compartment of WB are likely predominantly episomal in origin and may be significantly longer. WB extraction systems are therefore challenged to extract longer fragments from the cellular fraction along with very small fragments from the plasma compartment. How well they do this is uncertain.

Gene Targets

Since most testing will occur in the setting of transplant PTLD prevention, diagnosis, and monitoring and in NPC screening (in some geographic locations), if possible, a single clinical assay that could be used in both clinical settings should be our goal [117]. Because of the impact of target amplicon size on both the sensitivity of the assay and measurement of VL, participants in a 2015 National Cancer Institute (NCI USA) workshop on harmonization of EBV testing for NPC recommended that the amplicon size for plasma EBV DNA assays should be <100 bp [117].

Advantages and disadvantages of targeting a multi-copy versus a single-copy gene in specific clinical situations should also be considered. Historically, in the NPC setting, plasma assays have amplified the BamHI-W (Bam W) fragment, a sequence found in the EBV nuclear antigen leader protein (EBNA-LP) region of the genome which has a variable tandem reiteration frequency of 5–11 copies in clinical isolates; the WHO IS has 11 copies. Assays using these targets have greater sensitivity than single-copy PCR target but may have more imprecision in quantifying EBV DNA [118, 119]. Although a “more sensitive” assay may be useful for plasma EBV DNA detection, it could prove too sensitive when WB testing is performed if it detects viral latency in healthy seropositive immunocompetent subjects with high viral set points. Current commercial WB assays have levels of detection (LOD) in the range of 1.6–2.5 $\log_{10}$ IU/ml and levels of quantitation (LOQ) in the range of 2.2–3.0 $\log_{10}$ IU/ml [120].

Theoretically, assays targeting highly conserved single-copy EBV genes should have comparable sensitivity and quantitation. However, Ryan et al. [121] and Tsai et al. [48] observed better performance with an EBNA-1 targeted assay with respect to sensitivity or quantitation compared to other single-gene targeted assays. However, it is difficult to attribute the sensitivity differences to gene targets alone as differences in amplification efficiency, amplicon size, calibrators, and polymorphisms in specific genes among clinical isolates may all have contributed.

Since 2013, EBV whole-genome sequences (WGS) available in GenBank have increased from <10 strains to more than 200, significantly increasing information regarding genome heterogeneity [122]. Historically, EBV strains are divided into two major groups that vary geographically, type 1 (type A) and type 2 (type B), based predominantly on polymorphisms in the EBNA-2 and EBNA-3 genes [123–125]. The EBV B95-8 WHO IS a type 1 isolate. Recent EBV WGS data analysis found large numbers of single nucleotide polymorphisms (SNPs) [124, 125], which were more common in latent than viral lytic genes [123, 126]. Although definitive links between specific gene polymorphisms and risk of EBV disease or EBV-associated cancer risk have not been definitively established, several variants are being further studied in this regard [122]. Mutations in lytic EBV genes, such as thymidine kinase, protein kinase, and DNA polymerase, have not been definitively demonstrated. However, these genes are involved in the mechanism of action of drugs with anti-EBV activity such as acyclovir and ganciclovir used frequently after transplant and known to induce resistance in other herpesviruses making it prudent to avoid these genes as targets for EBV DNA assays. Manufacturers of EBV DNA assays should regularly review EBV GenBank data to confirm that the gene sequences targeted remain conserved among clinical isolates. An approach currently commonly used to mitigate the problem of false-negative results due to genetic polymorphisms is to include two gene targets in the assay [117].

Toward Reducing Variability in EBV DNA Measurement Using Real-Time RT-PCR Assays

Factors such as the use of commercial products, specific commercial reagents, probe chemistry, automation, and robotics that have not been extensively evaluated to date [97, 116, 127] may also contribute to result variability and are summarized in Table 6.3. The problem of lack of quantitative nucleic result harmonization is not unique to EBV, and we should extrapolate lessons learned from viruses such as hepatitis B virus, hepatitis C virus, and human immunodeficiency virus infection. Assay result harmonization has significantly improved for these other viruses because a very few commercially produced and highly automated assays are in use and all have been cleared or approved by regulators. International quantitative standards have also been in place for these viruses for long periods of time, allowing continuous quality improvement in the assays [109]. While acknowledging that some redundancy is required with respect to available tests, significantly reducing the number of assays being used for quantitative EBV DNA testing would facilitate global result harmonization.

Measurements of EBV Gene Expression in Peripheral Blood

Several investigators have evaluated EBV gene expression of latent and lytic genes in the peripheral blood of patients as alternative or adjunct biomarkers of PTLD risk or for PTLD diagnosis in both the SOT [8, 18, 47, 128–133] and HSCT settings [47, 50, 129, 130, 134]. Results have been variable, likely due to a combination of factors. Reverse transcriptase-PCR assays are not standardized, and assays for individual genes may have variable sensitivity both within and among studies. Gene expression assays are often performed on pooled cells so the prevalence and distribution of specific expression patterns among individual cells in the pool are unknown [1]. Some investigators studied adult SOT recipients likely seropositive pre-transplant [18, 47, 50, 130, 132, 134], while others studied pediatric populations, a significant proportion of whom are likely experiencing primary infections [8, 128, 129, 131, 133]. Studies are both cross-sectional and longitudinal with variable sampling intervals post-transplant and sometimes included or were restricted to patients with persistent elevated loads late after transplant [8, 47, 128, 129, 131]; data from SOT and HSCT populations are sometimes pooled for analysis [130].

Initial gene expression profiling in the peripheral blood of a limited number of adult, likely seropositive, SOT patients without PTLD suggested that lymphoblasts were not seen, and latency 0 EBV gene expression was observed (EBER, BARTs, and sometimes LMP2) [18]. Although this latency 0 pattern has also been observed by others [8], many investigators also report less restricted latency expression patterns including latency III in some patients with both gene expression levels and latency levels varying significantly over time in individual patients [47, 128, 129, 132, 133]. Lytic gene expression has been observed with variable prevalence in conjunction with different latency expression patterns. No specific latency pattern has been clearly associated with quantitative EBV DNA measurement, although expression levels of LMP2 have been correlated with levels of EBV DNAemia by some investigators [129, 133]. No consistent peripheral blood EBV gene expression patterns have been found that are predictive for or diagnostic of PTLD although only small numbers of patients have been studied [47, 129, 130, 132]. Currently there is no evidence to support any additional benefit of EBV gene expression profiling over measurement of EBV DNAemia alone in transplant recipients.

Measurement of Host and Viral Micro-RNAs (miRNA) in Peripheral Blood

miRNAs are a family of small (18–25 nt) non-coding RNAs, with sequence complementarity to mRNAs that act as negative regulators of gene expression involved in the regulation of cellular differentiation, proliferation, and apoptosis. EBV infection not only impacts host miRNA expression, but EBV encodes ~44 miRNAs almost exclusively from two regions: BHRFI, expressed during latency III and lytic infection, and BART miRNAs expressed in all forms of latency. These viral miRNAs repress expression of both host and viral genes [135]. Because miRNAs are highly

stable and easily detected and quantified in either PBMC or plasma/serum using either commercial miRNA microarray analysis or reverse transcriptase-PCR techniques, miRNA profiling is attractive for study as a potential biomarker for PTLD. Kawano et al. [136] identified the plasma EBV miRNAs miBART2-5p, 13, and 15 as potentially more useful biomarkers than plasma EBV DNAemia for differentiating active from inactive CAEBV and for monitoring response to therapy in immunocompetent patients. Recently, investigators have also identified 215 differentially regulated host and viral miRNAs in the PMBC of college-aged immunocompetent students at presentation with IM that regressed to levels seen in age-matched controls over the subsequent 7 months [137]. Similar studies have not yet been performed in transplant recipients with primary EBV infection. However, preliminary studies have identified miR- BART22 in serum as a potential biomarker of EBV reactivation in pediatric liver transplant recipients [138] and plasma miBART2-5p, which targets the stress-induced immune ligand MICB to escape recognition and elimination by NK cells as possibly having a role in sustaining persistent high set points in pediatric kidney transplant recipients [139]. These interesting observations suggest further study of host and EBV miRNA profiling in peripheral blood is warranted as an alternative or adjunct marker for PTLD or PTLD risk.

Testing of Non-peripheral Blood Sample Types

Despite very limited data to inform result interpretation, in clinical transplant practice, CSF and bronchoalveolar lavage fluid (BAL) are often tested to diagnose EBV disease or PTLD in the CNS and lungs, respectively. EBV DNA assays have not been validated for these matrices.

CSF

Testing for EBV VL in the CSF of transplant recipients is used to assist in the diagnosis of CNS PTLD or EBV encephalitis, hoping to avoid the need for invasive biopsy procedures. Because CSF VL studies of SOT and HSCT recipients are extremely limited, data are extrapolated from the HIV-infected population where qualitative detection of EBV DNA in CSF is common in a wide variety of HIV neurological diseases and has poor PPV but good NPV for diagnosis of EBV+ CNS lymphoma [140–142]. Although assays were not calibrated to the WHO IS, quantitative cut-offs in the range of 3.3–4.0 $\log_{10}$ copies/ml in CSF improved both the specificity and PPV of the result for both primary CNS lymphoma and CNS involvement in systemic AIDS-related lymphoma when compared to qualitative results alone [140, 142, 143]. In a study that included five SOT recipients, Weinberg et al. [144] observed that patients with primary CNS lymphoma had high CSF VLs associated with low CSF leukocyte counts. In contrast patients with high leukocyte counts were more likely to be diagnosed with EBV encephalitis in the presence of high VL or post-infectious complications, when VLs were low. In HSCT recipients,

Liu et al. [145] found levels of EBV DNA were higher in CSF than in blood, in which EBV DNA was sometimes undetectable. CSF VL was also better at predicting CNS disease, and declining CSF VL correlated with clinical response.

BAL

In lung and heart-lung transplant patients, the lung is often the primary site of PTLD. Investigators initially suggested that high quantitative levels of EBV load in BAL fluid may be a more sensitive predictor of PTLD than EBV DNA measurement in peripheral blood [146]. However, EBV load in BAL was not predictive of PTLD in a larger multicenter study of pediatric lung transplant recipients [147]. Moreover, EBV DNA was detected in BAL fluid, often at high levels, of adult lung [148] and other transplant recipients [149] in the absence of PTLD.

Saliva

Studies of EBV DNA in saliva use a variety of techniques for specimen collection and assays of variable sensitivity making results difficult to compare. The most common collection method is an oral wash or gargle [150–153], but oral swabs [154] and chewing on cotton plugs [155] have also been used. A consistent observation is that there is more EBV DNA in the cell pellet than in the supernatant, suggesting EBV DNA is predominantly cell associated in saliva, presumably in epithelial cells [150–153, 156–158].

In adult EBV-seropositive healthy subjects with remote infection, the prevalence of EBV DNA saliva detection varies significantly from 24% to 90% tested using PCR technology either in cross-sectional [156, 157] or longitudinal studies [151, 153–155]. In longitudinal studies with follow-up varying from 18 weeks to 18 months, 96% to 100% of patients had detectable EBV DNA at least once [151, 153–155]. Historically, EBV-seropositive individuals have been classified as low-, intermediate-, and high-level saliva EBV shedders. However, Hadinoto et al. found seropositive individuals continuously shed virus in saliva and this reservoir is completely replaced approximately every 2 minutes. Although shedding levels in individuals appear stable over short periods of time, when studied over months to a year, the level of EBV DNA shedding by a given individual varies by 3.5 to 5.5 logs [153]. This rate of virus production in saliva could not be attributed to virus production by B cells alone, and the authors suggested virus must be amplified by epithelial cells.

Saliva excretion studies during acute primary infection are largely limited to patients with IM. EBV DNA appears in the saliva explosively at high levels approximately a week before onset of IM symptoms, peaks at 2 months after onset of illness, and remains markedly elevated over the first year after infection, levels that may persist for as long as 3.1 years [31, 158–160], Levels then decline over time to lower persistent levels seen in all EBV-seropositive subjects [151, 153]. In SOT recipients not receiving anti-viral agents with anti- EBV activity, both the detection

prevalence (83–96%) and quantity of EBV DNA in saliva increase significantly beginning in the second month post-transplant with persistence and then decline in the 6–12-month period [150, 161]. Saliva EBV DNA levels were higher with increasing immunosuppression, in primary infection and in patients with PTLD, demonstrating similar patterns to those described in peripheral blood as a predictor of PTLD [150]. In a recent study pre-transplant EBV DNA detection in either saliva or blood did not predict post-transplant EBV DNAemia [162].

The known *in vitro* effects of anti-herpesvirus drugs including IV ganciclovir, IV acyclovir, valacyclovir and valganciclovir have been confirmed *in vivo* by measuring saliva EBV DNA. Clearance or a significant reduction in VL is observed on treatment even in immunocompromised hosts, but rapid rebound occurs on drug withdrawal [150, 152, 154]. Lytic infection appears to be the major source of EBV DNA in saliva supported by the observation that 59–62% of EBV DNA in saliva is DNase resistant suggesting it is present in encapsidated virions [153].

Although, using older tissue culture techniques, Yao et al. [161] found a correlation between virus in saliva and peripheral blood in healthy seropositive subjects with remote infection, no correlation has been observed by others [153, 155, 156]. Patterns and quantitative levels of EBV DNA detection in saliva and peripheral blood have not been directly compared using current NAT technology as predictors of PTLD in transplant patients. Given the variability in sample collection and the frequent post-transplant use of anti-virals with EBV activity, saliva is not recommended as a matrix for VL surveillance in preemptive PTLD prevention programs. EBV VL detection in saliva may, however, prove to be a useful adjunct to serology in clarification of pre-transplant EBV infection status in patients with passive antibody and as an ongoing surveillance tool for community-acquired primary infection in children who remain EBV seronegative after the early post-transplant period, but this requires further study.

EBV VL Kinetics: Implications for Monitoring Algorithms

PTLD guidelines in SOT and HSCT suggest that dynamic changes in peripheral blood EBV VL may be as important as absolute quantitative levels of EBV DNA to inform implementation of preemptive PTLD prevention strategies and to monitor response to prevention and treatment interventions [4, 163]. Defining "abnormal" EBV VL kinetic patterns in transplant recipients requires an understanding of "normal" EBV VL dynamics in different fractions of peripheral blood during acute and persistent infection in immunocompetent people of varying ages. Comparing VL patterns in transplant versus immunocompetent populations is critical to inform optimal monitoring algorithms, interpret responses to interventions in PTLD prevention strategies, and identify possible predictors of PTLD. Since historically serology rather than EBV DNA measurement has been used for the diagnosis of acute EBV infection, data regarding VL dynamics in immunocompetent hosts are recent and limited. Primary EBV infection, assessed by EBV DNA measurements in peripheral blood, is characterized by an incubation or "eclipse phase," an acute and convalescent phase during which EBV VL accelerates to a peak characterized

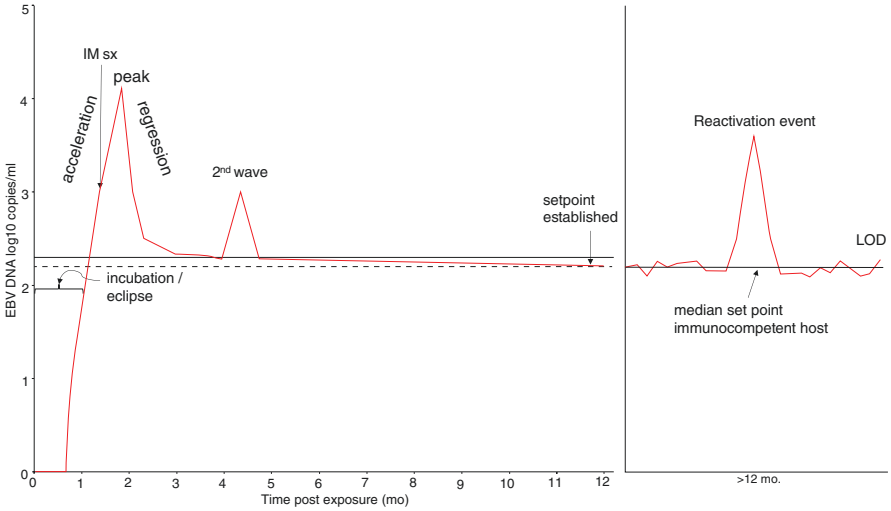

Fig. 6.1 A schematic of quantitative Epstein-Barr virus (EBV) DNAemia measured in whole blood in an immunocompetent subject experiencing primary EBV infection. Symptoms, if present, begin 32–49 days after exposure [158, 159, 164, 165]. EBV DNA can be detected approximately 3 weeks before symptoms using very sensitive research assays [160] becoming detectable by diagnostic assays 7–10 days before symptom onset and rising rapidly to peak over the next 2 weeks [158–160, 179]. EBV DNAemia then regresses in an exponential [166] or biphasic [178] pattern, with a rapid decline over the first 2 weeks and a much slower subsequent decline. EBV DNA is detected for a median of 17 days in adolescents and young adults [159] but may be detected for as long as 5–6 months in infants [192]. Recurrent episodes of EBV DNAemia with or without symptoms can occur [31, 158, 192], documented at 60–90 days after first symptom onset in young adults. EBV DNAemia does not approach a specific individual "set point" until approximately 1 year after symptom onset [22, 166, 180]. Reactivation events can occur after the set point is established in both asymptomatic subjects [17] and during critical illness and sepsis [26]. LOD = lower limit of EBV DNA detection by diagnostic assay [159] [dotted line]; set point [solid line] identified as median EBV viral load in healthy seropositive UK adults (79 copies/1 × 10^6 peripheral blood mononuclear cells or approximately 158 copies/ml whole blood)

by a doubling time and then regresses, followed by an equilibrium state with an individual "set point" marking persistent infection (Fig. 6.1). Time to first detection, time from first detection to peak, time from peak to set point, and area under the VL time curve (AUC) are additional parameters that might be important to study. Although it is challenging to compare studies because of differences in VL assays and the matrix used for testing, available data are summarized below.

Incubation or "Eclipse" Phase

Patients are asymptomatic and therefore rarely identified during the 32–49-day incubation period prior to development of IM symptoms determined using known or suspected expected exposure to EBV-infected saliva [158, 159, 164, 165]. In a

prospective study of 40 EBV-seronegative university students, Dunmire et al. [160] were able to detect very low levels of EBV DNA in WB as early as 22 days prior to IM symptom onset in the absence of both saliva EBV DNA detection and evidence of an adaptive immune response using a very sensitive nested PCR assay. This level of EBV DNA would not be detected by clinical diagnostic assays; investigators attribute the low level EBV DNA detection to relatively slow expansion of latently infected (latency 0) B cells in tonsillar tissues with spillover of these cells into the circulation. Lytic virus replication appears to have no role during this eclipse period aside from the initial transmission of infectious virus by saliva from another host to naïve B cells in the oropharynx of the recipient. There is an explosive increase in EBV DNA detection in both saliva (7 days) and peripheral blood (7–10 days) before IM symptom onset. These observations, particularly the late onset of oral viral shedding, contradict the model of acute IM proposed by Hadinoto et al. [166] who suggested that the VL increase in peripheral blood results from EBV reactivation in latently infected memory B cells that return to the oropharynx and infect new naïve B cells, a repetitive cycle that continues until a cytotoxic T lymphocyte (CTL) response is triggered.

The most comprehensive EBV VL data during primary EBV infection in infants is derived from two longitudinal studies in Africa where infection is almost universal before age 2. One studied EBV DNA in PBMC of infants (14–18 months) in Gambia over 6 months during a period of low malaria prevalence [167]. The other compared EBV VLs in WB in Kenyan infants (serially followed from 1 month to 2 years of age) from two regions, one with high and the other with low rates of both holoendemic malaria and Burkitt lymphoma [168]. While historical evidence suggested that infants may be protected from EBV infection during the first 6 months of life [169–171], mathematical modeling using data from the Kenyan infant study has made two interesting observations regarding the incubation/eclipse phase of primary EBV infection [172]. First, detection of early de novo EBV-specific serologic responses in the infant suggests that "infection" may occur as early as 1–2 months of life, but EBV DNAemia is not detected until maternal antibodies wane. The second is that the timing of EBV DNAemia is dependent on the initial level of maternal antibody and the rate of its decay. A recent Canadian study suggests that infant seropositive donors less than 6 months of age do not appear to transmit EBV to seronegative recipients (i.e., only have passive maternal antibody) and could be considered seronegative when risk stratifying for preemptive strategies [173].

SOT offers a unique opportunity to study the biology of primary EBV infection as the exact timing of EBV exposure is known, in the EBV donor positive/recipient negative scenario (EBV mismatch), and we are often monitoring VL during the eclipse phase. In an era prior to the routine use of anti-virals for CMV prophylaxis after SOT, the median time to EBV detection in saliva during primary donor-derived EBV infection after SOT was 6 weeks [150], similar to the IM incubation period. More recent studies in EBV-mismatched SOT patients monitored serially using RT-PCR EBV DNA assays noted later initial detection of EBV DNAemia at a

median of 84 days with only rare detection in the first post-transplant month [173]. The use of anti-viral prophylaxis appeared to delay the onset of EBV DNAemia in a cohort of adult kidney transplant recipients [174], but not in a similar pediatric cohort [175]. In the SOT setting, it is not known whether duration of the eclipse period is influenced by anti-viral therapy or by passive maternal or transfusion-derived antibody in the recipient given the mode of transmission. The low-level early EBV DNAemia described by Dunmire et al. [160] in IM has not been described in SOT recipients: the LOD of clinical assays may not be sensitive enough to detect this, even in immunocompromised hosts.

In three HSCT monitoring studies, the median time to EBV DNAemia post-transplant was 50 days (range 19–368 days) using plasma [176], 99 days (IQR 84–119 days) using WB [50], and 34 days (range 18–60 days) using either plasma or PBMC [54]. It may therefore be reasonable to delay the onset on post-transplant monitoring for preemptive interventions until after the first transplant month, particularly in SOT, to minimize costs; however, from a logistics and compliance perspective, this may increase the complexity of implementation.

Acute and Convalescent Infection Phase

*Acceleration phase and peak: S*everal investigators found that EBV VL in WB or PBMC may already be falling when immunocompetent adolescents and young adults first present with IM symptoms [22, 166, 177, 178]. However, in prospectively followed seroconverting university students undergoing frequent sampling, EBV VL continues to rise and peaks quickly within 2 weeks of symptom onset (median 8 days) [158, 159]. Median peak loads in WB reported in studies of IM patients were 3.0 $\log_{10}$ copies/ml [158], 3.9 $\log_{10}$ copies/ml [179], 7350 copies/10^6 PBMC or ~4.2 $\log_{10}$ copies/ml [180], and 6280 copies/10^6 PBMC or ~4.1 $\log_{10}$ copies/ml [181]. Peak VL is similar in both IM patients and asymptomatic patients [180, 182] supporting the concept that IM symptoms result from an exaggerated immune response rather than a higher VL. In the Kenyan infant study, infants in holoendemic vs. sporadic malaria areas were infected with EBV earlier in life (mean 7.28 months). Earlier infection was associated with higher and more persistent WB EBV VLs, suggesting infants infected earlier in life had poorer control of infection [168]. Mathematical modeling of the data from this study found very short EBV VL doubling times, 1.6 and 2.1 days in high and low malaria transmission regions, respectively [172]. Limited study of plasma EBV DNA in IM suggests that EBV DNA is detectable in the plasma of most patients at symptom onset at lower levels than in WB/PBMC and declines rapidly to undetectable levels 15–30 days later [29, 31–33].

Although there is overlap, transplant recipients with PTLD appear to have higher VLs measured in both plasma [68, 82] and PBMC [46, 68, 183, 184] than those observed in immunocompetent IM patients. The majority of historical studies suggest that high peak VL is a sensitive but not specific marker of EBV+ PTLD occurring early, usually in the first year after transplant, with VL most often peaking

before the onset of symptoms (reviewed by [185]). This is the basis of preemptive prevention strategies. Unfortunately, these studies have significant limitations. Most transplant studies are not natural history studies, as clinicians are implementing interventions based on available results. Almost all longitudinal studies attempting to determine EBV DNAemia levels predictive of PTLD are single-center studies, most often involving only pediatric SOT populations. In addition, result interpretation is complicated by the heterogeneity of the populations studied, with high- and low-risk populations and SOT and HSCT populations often pooled for analysis. The use of non-standardized assays and sample types precludes making recommendations regarding quantitative levels of EBV DNA that might be used as trigger points for interventions. The small numbers of PTLD cases limit the statistical analyses performed.

More recent studies of the association between high WB peak load and PTLD have been conflicting in both SOT and HSCT populations. No association was found in German pediatric kidney transplant recipients [77] or a Korean SOT population (predominantly pediatric liver recipients) [186]. However, Colombini et al. [92] identified peak VL as an independent predictor of PTLD, in a multivariate analysis of a multicenter study of pediatric kidney transplant recipients. Similarly early peak load, in univariate analysis, was a risk factor for PTLD in a single-center study of pediatric heart transplant recipients [187]. "EBV exposure" measured as area under the EBV viral load concentration-time curve (AUC) is an alternate potential biomarker of PTLD risk, although in a study of pediatric kidney transplant recipients, AUC of EBV VL during the first post-transplant year was not predictive of symptomatic EBV infection that included PTLD [77]. Although peak load was associated with increased PTLD risk in a HSCT cohort in the Cho et al. study [186], in a recent multicenter UK study of 69 PTLD cases, 45% and 23% of cases had VLs at diagnosis measured predominantly in WB that were lower than 40,0000 copies/ml and 10,000 copies/ml, respectively, commonly used as preemptive triggers in the HSCT setting [188]. Peak VL appears to be temporally concordant in plasma and WB in HSCT recipients [74]. Salano et al. [189] studying a mixed population of HSCT recipients at high and low risk for PTLD using plasma EBV DNA monitoring found that neither initial positive result nor doubling time predicted clinical features associated with high PTLD risk; these parameters also did not predict the need for preemptive rituximab therapy.

As observed during acute infection in immunocompetent hosts, in primary EBV infection after SOT and high-level reactivation of EBV in HSCT, the initial rise in EBV VL is explosive [50, 54, 58, 186]. In high-risk HSCT patients, Burns et al. [50] found the median time from first EBV DNA detection in WB to high-level DNAemia (median load 2.2×10^5 genomes/ml) was 7 days (IQR 0–14 days). Although doubling times as rapid as 56 hours have been described in the lung transplant setting [58], the time to peak has been less well described in the SOT setting. This very rapid initial VL rise makes the logistics of preemptive therapy challenging. Frequent monitoring of at least weekly for at least the 4 first post-transplant months is recommended in guidelines for high-risk HSCT recipients [163]. In SOT recipients, it can be argued that any detectable peripheral EBV VL in the setting of a primary EBV

infection should trigger review and minimization of immunosuppression when possible, to optimize opportunities for development of EBV-specific adaptive immune responses. Weekly post-transplant monitoring during the highest risk period for donor-derived infection (1–4 months) [173] is recommended for EBV-mismatched SOT recipients. However, there are no data to suggest that less frequent monitoring (i.e., biweekly or even longer intervals later in the first year after transplant) negatively impacts preemptive strategies [4]. In evaluating interventions during the acceleration phase of primary infection, reduction in doubling time or lower peak or set point may be positive endpoints; short-term absolute quantitative reduction in load may not be an appropriate measurement.

Regression from peak load: VL regression patterns in IM are variable among patients and may be dependent on patient age and the "immunosuppressive environment" in which primary infection occurs. In a study of adolescents and young adults, the median duration of WB EBV DNAemia was 17 days [159], but EBV DNA was detected as long as 202 days in individual patients. EBV DNA in PBMC studied by Fafi-Kremer in 20 IM patients progressively declined over 180 days with levels approaching those in EBV-seropositive patients by 30 days [31]. Investigators have observed that WB/PBMC viral load falls as adaptive immune responses develop in a biphasic pattern, with a rapid decline lasting 2–6 weeks (estimated half-life 1.5 days) followed by a more gradual decline thereafter (mean half-life 38.7 +/− 15 days) [177, 178]. After studying 24 children with IM (age 1–16 years), Nakai et al. [178] found two-thirds were slow regressors with PBMC EBV DNAemia still detectable at last follow-up (up to 90 days after onset of symptoms). VLs generally approach but do not achieve "set point" observed in EBV-seropositive asymptomatic children and adults until approximately 1 year after IM [22, 166, 180]. EBV sero-conversion rates due to community-acquired infection in infants and children are high [190, 191]. Some children awaiting transplant are likely recently infected (i.e., less than 1 year ago); this is particularly true for seropositive infants between 1 and 2 years of age. Although not been specifically studied, these children may be at intermediate PTLD risk, falling between high-risk seronegative and lower-risk seropositive children with remote infection. More extensive pre-transplant and waitlist serology profiling and WB EBV VL testing may be warranted to allow better risk stratification of children as seronegative, acutely (recently) infected, or remotely infected to inform monitoring algorithms for preventive measures. Viral kinetic data in infant EBV infection from a Kenyan study revealed a median duration of WB EBV DNAemia of 6.3 months and 4.9 months for infants in holoendemic and sporadic malaria areas, respectively [192]. The AUC of the first episode of EBV DNA detection, determined by both doubling time and duration, was greater in the high malaria exposure region but was not influenced by age. Malaria is thought to increase VLs either through promoting B cell proliferation [193], altering T cell responses [194], and/or decreasing maternal antibody transfer [195]. Potential analogies in the transplant setting include graft-associated chronic or recurrent B cell stimulation, even in the absence of observed acute rejection, and recurrent infection episodes, exogenous immunosuppression, and possibly more rapid passive maternal antibody elimination because of bleeding and transfusion.

In the transplant setting, observations regarding EBV VL regression kinetics in the absence of interventions are extremely limited. The half-life of the biologic form of EBV DNA being measured, in the order of hours for ccf EBV DNA in plasma versus weeks for latently infected cells, should be considered in interpreting dynamic changes observed. In HSCT patients who spontaneously resolved plasma EBV DNAemia, Solano et al. observed a median half-life of 4 days (range 0.04–123.7 days) and several patterns that included zero-order and first-order elimination kinetics as well as a biphasic "humped" pattern [189]. Unlike IM patients and asymptomatic non-HIV-infected African infants, EBV DNA detection in plasma in asymptomatic pediatric liver transplant recipients experiencing primary infection was prolonged, as long as a year after first detection [67, 75]. It is very difficult to assess the impact of interventions using viral kinetic data in the absence of a control group not receiving the intervention or controlling for the timing of the intervention relative to the onset of EBV DNA detection. When evaluating interventions implemented during the regression phase of acute primary infection after SOT, the regression half-life or time to clearance (for diagnostic assays) may be a better measurement of efficacy than an absolute quantitative reduction in VL. Kumar et al. [196] observed a decline in WB VL in 90.3% of 31 SOT patients experiencing primary EBV infection with median reductions of -0.49 $\log_{10}$ copies/ml and -0.87 $\log_{10}$ copies/ml 14 and 30 days, respectively, after either RIS or anti-viral therapy. The timing of the interventions relative to first EBV DNAemia or phase of infection was not specified in this study.

Recurrent episodes of EBV DNAemia: Acute primary EBV infection may include recurrent episodes or waves of EBV DNAemia that are not necessarily associated with short-term adverse events in either the immunocompetent subject or transplant recipient. In 20 IM patients, Fafi-Kremer et al. [31] observed rebound PBMC EBV DNAemia after initial regression between day 60 and 90 after symptom onset associated with recurrent symptoms in 20%. In Kenyan infants, patterns of WB EBV DNAemia varied with some infants demonstrating a single peak and others having multiple waves of detection [192]. However, a shorter time to subsequent waves that were characterized by both a slower doubling time and shorter duration of EBV DNAemia was seen when infection occurred at a younger age. Repeated waves of infection were more likely in high malaria exposure regions but were less likely with higher AUC with the first episode. Reinfection episodes could not be ruled out as a cause of multiple waves/episodes of infection in this environment. Rebound events after initial regression have also been observed in the SOT setting after primary infection managed by RIS or antiviral therapy [196, 197] with no apparent adverse effects [197].

Equilibrium or Set Point VL

All EBV-seropositive patients have persistent EBV infection in peripheral blood even though it is often below the level of detection of RT-PCR assays in specimen types used in clinical labs in the transplant setting. This host-virus equilibrium or set

point is estimated to take up to a year to achieve after acute infection [22, 166, 180] and is unique to an individual and relatively stable for at least 3.5 years [66, 153, 198]. There is some stochastic variation in values around the set point over time estimated at ±25%, and this should be considered when defining clinically meaningful VL kinetic outcomes if interventions in transplant patients are initiated after set points are established [153]. Peripheral blood VL may increase with aging in adults [199, 200] although results are not consistent [201]. Whether age-related increases represent more EBV reactivation events or altered "set point" associated with immunosenescence is uncertain. "Set point" VL may also be geographically variable. Healthy children and adults in Gambia have a set point (median 850 copies/ 10^6 PBMC) >10-fold higher than adults in the UK (median 79 copies/10^6 PBMC); some had VLs similar to those seen in UK IM patients [181]. It has been suggested that early age of infection may raise set point; recurrent exposure to malaria may further contribute [167]. It may be important to evaluate interventions occurring after set point has been established separately from those occurring earlier during acute primary infection after SOT as their impact may be significantly different in these settings that likely represent very different stages of host-EBV immune response development.

In the SOT setting, the time required to develop adaptive immune responses and EBV viral set point after donor-derived primary infection may be prolonged compared to the immunocompetent patient because of exogenous immunosuppression. Colombini et al. [92] observed that in the 38% of patients experiencing primary infection after kidney transplantation who "cleared" their EBV DNAemia during a median 5.4 years of follow-up, clearance occurred at a median of 22.1 months after detection. Similar protracted regression was observed by Kullberg-Lindh et al. [75] on withdrawal of all immunosuppression in pediatric liver transplant recipients with "set point" WB VLs sometimes not observed until 2 years after initial detection, often concordant with clearance of EBV DNAemia in serum. A significant proportion of SOT recipients experiencing primary EBV infection have detectable WB EBV DNA, sometimes at very high levels, that is sustained for many years and appears to represent an "abnormally high" viral set point; this is observed less frequently in SOT patients seropositive pre-transplant and has not been described in the HSCT setting [8, 77, 92, 128, 175, 202–206]. The immunopathogenesis and factors that influence setting and sustaining EBV viral set point after primary infection are unknown [207, 208]. Young age at infection, immunodeficiency, and intercurrent infections such as malaria are believed to elevate set points in African infants [209]; warm ischemia time and high graft to recipient weight ratio have been identified as factors in pediatric liver transplant recipients [205]. High EBV set point may be a form of neonatal tolerance also seen with other viral infections such as hepatitis B, CMV, and rubella acquired in utero or the early post-partum period. Examples of variable EBV set points after donor-derived primary EBV infection in three pediatric SOT recipients are illustrated in Fig. 6.2a–c.

Investigators have often defined SOT patients as having a chronic high VL phenotype (CHVLP) when having sustained (>50%) WB EBV VLs in the range of 5000–16,000 copies/ml for >6 months [8, 77, 92, 128, 175, 202–206]. High EBV

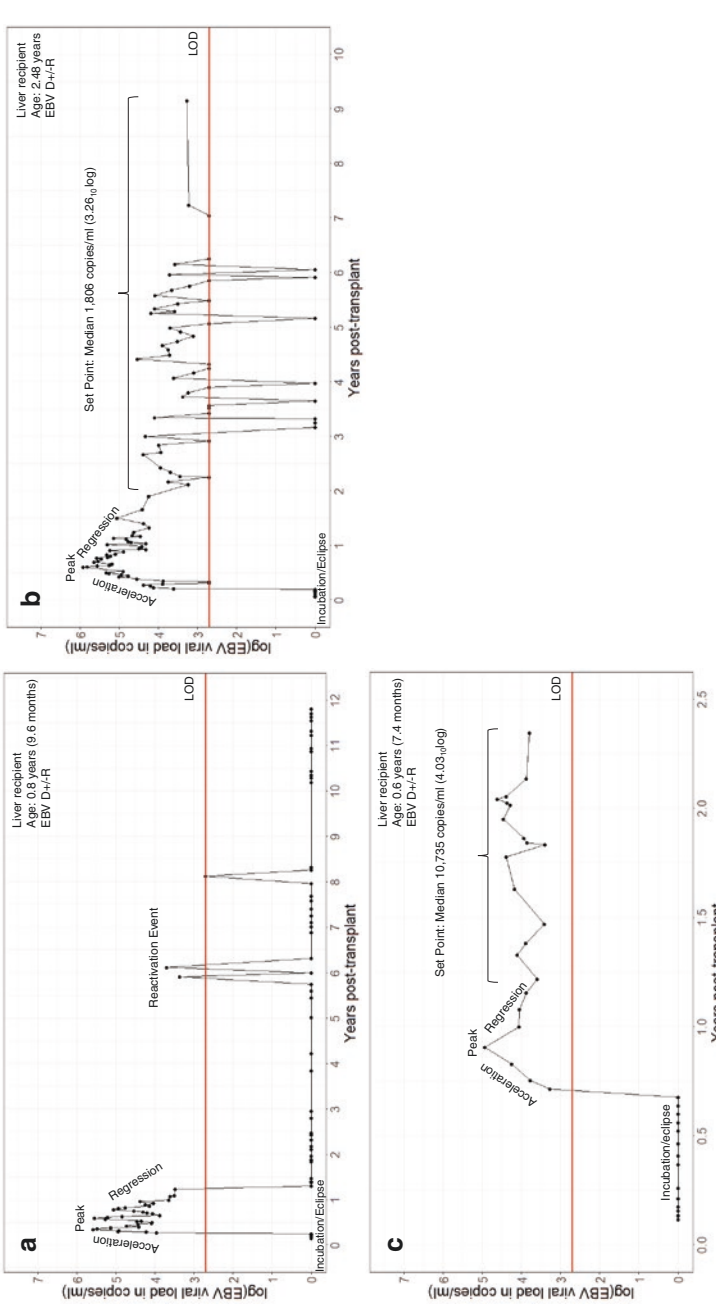

Fig. 6.2 (**a**–**c**) Kinetics of EBV DNAemia measured in the whole blood of solid organ transplant recipients with primary EBV infection. Post-transplant serial EBV DNAemia measurements in three EBV mismatched (donor seropositive/recipient seronegative) infants who developed an EBV viral load set point that was "undetectable" by the diagnostic assay (**a**), a set point at low detectable EBV DNAemia levels (**b**), and a set point at high detectable EBV DNAemia levels (**c**). None developed PTLD during follow-up. LOD = lower limit of EBV DNA detection by diagnostic assay [solid line]. A conversion of 0.5 can be used to convert from copies/ml to IU/ml (e.g., 2 copies/ml = 1 IU/ml)

viral set point may be a preferred terminology rather than CHVLP to describe this biologic state as it is the terminology that has been used to describe a similar state in the HIV setting [210]. However, it is not known whether 6 months after first detection is sufficient time for equilibrium or set point to be achieved and what specific quantitative set point, if any, is associated with increased later PTLD risk. Although studies of pediatric heart transplant recipients suggest that patients who are chronic high VL carriers may be at significantly increased risk of late-onset EBV-positive PTLD [202, 203], this risk appears in part to be organ-specific with intermediate risks observed in intestinal transplants [204] and low to negligible risk in pediatric liver [8, 205, 206] and kidney [77, 92, 175, 208] transplant recipients.

Whether any intervention such as immune reconstitution (i.e., RIS) or prolonged antiviral therapy can alter set point when established is not known. EBV-seropositive immunocompetent subjects develop increases in EBV viral "set points" that are sustained for up to 5 years after experiencing acute HIV infection; increases are proportional relative to their pre-HIV infection set points. These increased set points remain unchanged despite immune reconstitution by highly active anti-retroviral HIV therapy suggesting the increased set point is due to immune activation rather than immunodeficiency [210–212]. Similarly, Kullberg-Lindh et al. continued to see elevated "set point" loads even when immunosuppression was withdrawn in pediatric liver transplant patients experiencing primary EBV infection early after transplant [75]. Hoshino et al. [213] studied EBV-seropositive subjects receiving 1 year of valacyclovir prophylaxis for HSV infection and found a small but appreciable decrease in EBV set point when compared to a control group where no effect was seen. The authors estimated it would take 6 years of therapy to eliminate 99% of the systemic EBV load and 11.3 years to clear it if re-infection did not occur. This anti-viral effect has not been studied or confirmed by others.

Periodically EBV DNAemia can be detected using clinical assays even in asymptomatic seropositive subjects with remote infection; detection most often lasts <4 weeks in plasma [17, 26] and is detected at a higher rate in WB than plasma when contemporaneously samples are collected from the same patient [26]. Critical illness and sepsis appear to precipitate reactivation events in immunocompetent seropositive patients [26]. The duration of these "set point" disturbances is unknown. In acute malaria a five- to sixfold increase in PBMC EBV VL was observed that persists for at least 4–6 weeks [181]. Whether similar EBV dynamics occur in patients with other acute infections in either the transplant or non-transplant setting is not known.

Future Prospects for EBV VL Testing

Quantitative EBV DNA measurement in peripheral blood has a potentially significant role to play in PTLD prevention and management (see Chaps. 11 and 18). Calibration of assays to the WHO IS and, potentially, the use of dPCR have been important first steps toward result harmonization among EBV DNA assays. It is important that test manufacturers, national regulators, clinical virologists, transplant

physicians, and oncologists work collaboratively to continue to improve result harmonization through optimization of the commutability of secondary calibrators, nucleic acid extraction procedures, target design, and other factors that contribute to ongoing result variability. Clinical trials will be required to both validate result harmonization and evaluate the benefit of these assays in specific clinical settings. We must understand what we are measuring. This includes the biologic forms of EBV DNA and host and viral EBV miRNAs in plasma and the cellular blood fraction as well as the phenotype of infected cells and how these change during primary and reactivation infection after transplant and throughout the subsequent stages of PTLD development. This information is critical for assay improvement and identification of new biomarkers. New tools such as target-capture deep sequencing for EBV DNA fragment length profiling [17] in plasma and ImmunoFISH assays [9] that might allow analysis of EBV-infected cells at a single-cell level are potentially useful in that regard.

A high priority for research would be a multicenter trial comparing EBV DNA measurements in both plasma and WB to more definitively determine the optimal specimen type to use in specific clinical settings after transplant. Analyzing EBV DNA kinetics in both WB and plasma, including mathematical modeling of these dynamics [172], would increase our understanding of the biology of EBV transmission and infection in the transplant setting and also improve the definition of "abnormal" relative to natural community-acquired infection in the immunocompetent host. When EBV DNA dynamics in peripheral blood are used as surrogate markers of response to clinical interventions for prevention and treatment of PTLD, modeling of EBV VL data after transplant could also inform identification of VL parameters that could be used as better clinically relevant outcome measures.

References

1. Thorley-Lawson DA. EBV persistence—introducing the virus. Epstein Barr Virus. Curr Top Microbiol Immunol. 2015;390:151–209.
2. Shannon-Lowe C, Rickinson A. The global landscape of EBV-associated tumors. Front Oncol. 2019;9:1–23.
3. Fryer JF, Heath AB, Wilkinson DE, Minor PD. A collaborative study to establish the 1st WHO International Standard for Epstein–Barr virus for nucleic acid amplification techniques. Biologicals. 2016;44:423–33.
4. Allen UD, Preiksaitis JK. Post-transplant lymphoproliferative disorders, Epstein-Barr virus infection, and disease in solid organ transplantation: guidelines from the American Society of Transplantation Infectious Diseases Community of Practice. Clin Transpl. 2019;33:e13652.
5. Humar A, Michaels M. American Society of Transplantation recommendations for screening, monitoring and reporting of infectious complications in immunosuppression trials in recipients of organ transplantation. Am J Transplant. 2006;6:262–74.
6. Ahya VN, Douglas LP, Andreadis C, Arnoldi S, Svoboda J, Kotloff RM, et al. Association between elevated whole blood Epstein–Barr Virus (EBV)-encoded RNA EBV polymerase chain reaction and reduced incidence of acute lung allograft rejection. J Hear Lung Transpl. 2007;26:839–44.
7. Bakker NA, Verschuuren EAM, Erasmus ME, Hepkema BG, Veeger NJGM, Kallenberg CGM, et al. Epstein-Barr virus-DNA load monitoring late after lung transplantation: a sur-

rogate marker of the degree of immunosuppression and a safe guide to reduce immunosuppression. Transplantation. 2007;83:433–8.
8. Gotoh K, Ito Y, Ohta R, Iwata S, Nishiyama Y, Nakamura T, et al. Immunologic and virologic analyses in pediatric liver transplant recipients with chronic high Epstein-Barr Virus loads. J Infect Dis. 2010;202:461–9.
9. Ito Y, Kawabe S, Kojima S, Nakamura F, Nishiyama Y, Kaneko K, et al. Identification of Epstein-Barr virus-infected CD27+ memory B-cells in liver or stem cell transplant patients. J Gen Virol. 2011;92:2590–5.
10. Calattini S, Sereti I, Scheinberg P, Kimura H, Childs RW, Cohen JI. Detection of EBV genomes in plasmablasts/plasma cells and non-B cells in the blood of most patients with EBV lymphoproliferative disorders by using Immuno-FISH. Blood. 2010;116:4546–59.
11. Lieberman PM. Chromatin structure of Epstein-Barr virus latent episomes. Epstein Barr Virus: Springer International Publishing; 2015. p. 71–102.
12. To EWH, Chan KCA, Leung SF, Chan LYS, To KF, Chan ATC, et al. Rapid clearance of plasma Epstein-Barr virus DNA after surgical treatment of nasopharyngeal carcinoma. Clin Cancer Res. 2003;9:3254–9.
13. Chan KCA, Zhang J, Chan ATC, Lei KIK, Leung S-F, Chan LYS, et al. Molecular characterization of circulating EBV DNA in the plasma of nasopharyngeal carcinoma and lymphoma patients. Cancer Res. 2003;63:2028–32.
14. Jahr S, Hentze H, Englisch S, Hardt D, Fackelmayer FO, Hesch RD, et al. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer Res. 2001;61:1659–65.
15. Underhill HR, Kitzman JO, Hellwig S, Welker NC, Daza R, Baker DN, et al. Fragment length of circulating tumor DNA. Kwiatkowski DJ, editor. PLoS Genet. 2016;12:e1006162.
16. Snyder MW, Kircher M, Hill AJ, Daza RM, Shendure J. Cell-free DNA comprises an in vivo nucleosome footprint that informs its tissues-of-origin. Cell. 2016;164:57–68.
17. Lam WKJ, Jiang P, Chan KCA, Cheng SH, Zhang H, Peng W, et al. Sequencing-based counting and size profiling of plasma Epstein–Barr virus DNA enhance population screening of nasopharyngeal carcinoma. Proc Natl Acad Sci. 2018;115:E5115–24.
18. Babcock GJ, Decker LL, Freeman RB, Thorley-Lawson DA. Epstein-Barr Virus–infected resting memory B cells, not proliferating lymphoblasts, accumulate in the peripheral blood of immunosuppressed patients. J Exp Med. 1999;190:567–76.
19. Souza TA, Stollar BD, Sullivan JL, Luzuriaga K, Thorley-Lawson DA. Peripheral B cells latently infected with Epstein-Barr virus display molecular hallmarks of classical antigen-selected memory B cells. Proc Natl Acad Sci. 2005;102:18093–8.
20. Miyashita EM, Yang B, Babcock GJ, Thorley-Lawson DA. Identification of the site of Epstein-Barr virus persistence in vivo as a resting B cell. J Virol. 1997;71:4882–91.
21. Joseph AM, Babcock GJ, Thorley-Lawson DA. EBV persistence involves strict selection of latently infected B cells. J Immunol. 2000;165:2975–81.
22. Hochberg D, Souza T, Catalina M, Sullivan JL, Luzuriaga K, Thorley-Lawson DA. Acute infection with Epstein-Barr virus targets and overwhelms the peripheral memory B-cell compartment with resting. Latently infected cells. J Virol. 2004;78:5194–204.
23. Hochberg DR, Thorley-Lawson DA. Quantitative detection of viral gene expression in populations of Epstein-Barr Virus-infected cells in vivo. DNA viruses. New Jersey: Humana Press; 2005. p. 039–56.
24. van den Heuvel D, Jansen MAE, Bell AI, Rickinson AB, Jaddoe VWV, van Dongen JJM, et al. Transient reduction in IgA + and IgG + memory B cell numbers in young EBV-seropositive children: the generation R study. J Leukoc Biol. 2017;101:949–56.
25. Chaganti S, Heath EM, Bergler W, Kuo M, Buettner M, Niedobitek G, et al. Epstein-Barr virus colonization of tonsillar and peripheral blood B-cell subsets in primary infection and persistence. Blood. 2009;113:6372–81.
26. Walton AH, Muenzer JT, Rasche D, Boomer JS, Sato B, Brownstein BH, et al. Reactivation of multiple viruses in patients with Sepsis. Moldawer LL, editor. PLoS One. 2014;9:e98819.

27. Barros MHM, Vera-Lozada G, Segges P, Hassan R, Niedobitek G. Revisiting the tissue microenvironment of infectious mononucleosis: identification of EBV infection in T cells and deep characterization of immune profiles. Front Immunol. 2019;10:146.
28. Coleman CB, Daud II, Ogolla SO, Ritchie JA, Smith NA, Sumba PO, et al. Epstein-Barr Virus type 2 infects T cells in healthy Kenyan children. J Infect Dis. 2017;216:670–7.
29. Bauer CC, Aberle SW, Popow-Kraupp T, Kapitan M, Hofmann H, Puchhammer-Stöckl E. Serum Epstein-Barr virus DNA load in primary Epstein-Barr virus infection. J Med Virol. 2005;75:54–8.
30. Ryan JL, Fan H, Swinnen LJ, Schichman SA, Raab-Traub N, Covington M, et al. Epstein-Barr Virus (EBV) DNA in plasma is not encapsidated in patients with EBV-related malignancies. Diagn Mol Pathol. 2004;13:61–8.
31. Fafi-Kremer S, Morand P, Brion J, Pavese P, Baccard M, Germi R, et al. Long-term shedding of infectious Epstein-Barr Virus after infectious mononucleosis. J Infect Dis. 2005;191:985–9.
32. Kimura H, Nishikawa K, Hoshino Y, Sofue A, Nishiyama Y, Morishima T. Monitoring of cell-free viral DNA in primary Epstein-Barr virus infection. Med Microbiol Immunol. 2000;188:197–202.
33. Berger C, Day P, Meier G, Zingg W, Bossart W, Nadal D. Dynamics of Epstein-Barr virus DNA levels in serum during EBV-associated disease. J Med Virol. 2001;64:505–12.
34. Slyker JA, Casper C, Tapia K, Richardson B, Bunts L, Huang M-L, et al. Clinical and virologic manifestations of primary Epstein-Barr Virus (EBV) infection in Kenyan infants born to HIV-infected women. J Infect Dis. 2013;207:1798–806.
35. Okuno Y, Murata T, Sato Y, Muramatsu H, Ito Y, Watanabe T, et al. Defective Epstein–Barr virus in chronic active infection and haematological malignancy. Nat Microbiol Springer US. 2019;4:404–13.
36. Cohen JI, Jaffe ES, Dale JK, Pittaluga S, Heslop HE, Rooney CM, et al. Characterization and treatment of chronic active Epstein-Barr virus disease: a 28-year experience in the United States. Blood. 2011;117:5835–49.
37. Kimura H, Ito Y, Kawabe S, Gotoh K, Takahashi Y, Kojima S, et al. EBV-associated T/NK–cell lymphoproliferative diseases in nonimmunocompromised hosts: prospective analysis of 108 cases. Blood. 2012;119:673–86.
38. Kimura H, Hoshino Y, Hara S, Sugaya N, Kawada J, Shibata Y, et al. Differences between T cell–type and natural killer cell–type chronic active Epstein-Barr Virus infection. J Infect Dis. 2005;191:531–9.
39. Kasahara Y, Yachie A, Takei K, Kanegane C, Okada K, Ohta K, et al. Differential cellular targets of Epstein-Barr virus (EBV) infection between acute EBV-associated hemophagocytic lymphohistiocytosis and chronic active EBV infection. Blood. 2001;98:1882–8.
40. Weber T, Wickenhauser C, Monecke A, Gläser C, Stadler M, Desole M, et al. Treatment of rare co-occurrence of Epstein-Barr virus-driven post-transplant lymphoproliferative disorder and hemophagocytic lymphohistiocytosis after allogeneic stem cell transplantation. Transpl Infect Dis. 2014;16:988–92.
41. Kanakry J, Ambinder R. The biology and clinical utility of EBV monitoring in blood. Curr Top Microbiol Immunol. 2015;391:475–99.
42. Rose C, Green M, Webber S, Ellis D, Reyes J, Rowe D. Pediatric solid-organ transplant recipients carry chronic loads of Epstein-Barr Virus exclusively in the immunoglobulin D-negative B-cell compartment. J Clin Microbiol. 2001;39:1407–15.
43. Schauer E, Webber S, Green M, Rowe D. Surface immunoglobulin-deficient Epstein-Barr Virus-infected B cells in the peripheral blood of pediatric solid-organ transplant recipients. J Clin Microbiol. 2004;42:5802–10.
44. Roughan JE, Torgbor C, Thorley-Lawson DA. Germinal center B cells latently infected with Epstein-Barr Virus proliferate extensively but do not increase in number. J Virol. American Society for Microbiology. 2010;84:1158–68.
45. Schauer E, Webber S, Kingsley L, Green M, Rowe D. Increased Ig-null B lymphocytes in the peripheral blood of pediatric solid organ transplant recipients with elevated Epstein-Barr viral loads. Pediatr Transplant. 2009;13:311–8.

46. Kimura H, Morita M, Yabuta Y, Kuzushima K, Kato K, Kojima S, et al. Quantitative analysis of Epstein-Barr virus load by using a real-time PCR assay. J Clin Microbiol. 1999;37:132–6.
47. Greijer AE, Stevens SJ, Verkuijlen SA, Juwana H, Fleig SC, Verschuuren EA, et al. Variable EBV DNA load distributions and heterogeneous EBV mRNA expression patterns in the circulation of solid organ versus stem cell transplant recipients. Clin Dev Immunol. 2012;2012:1–10.
48. Tsai DE, Douglas L, Andreadis C, Vogl DT, Arnoldi S, Kotloff R, et al. EBV PCR in the diagnosis and monitoring of posttransplant lymphoproliferative disorder: results of a two-arm prospective trial. Am J Transplant. 2008;8:1016–24.
49. Limaye AP, Huang ML, Atienza EE, Ferrenberg JM, Corey L. Detection of Epstein-Barr virus DNA in sera from transplant recipients with lymphoproliferative disorders. J Clin Microbiol. 1999;37:1113–6.
50. Burns DM, Tierney R, Shannon-Lowe C, Croudace J, Inman C, Abbotts B, et al. Memory B-cell reconstitution following allogeneic hematopoietic stem cell transplantation is an EBV-associated transformation event. Blood. 2015;126:2665–75.
51. Amiel C, LeGoff J, Lescure FX, Coste-Burel M, Deback C, Fafi-Kremer S, et al. Epstein-Barr Virus load in whole blood correlates with HIV surrogate markers and lymphoma: a French National Cross-Sectional Study. JAIDS J Acquir Immune Defic Syndr. 2009;50:427–9.
52. Bekker V, Scherpbier H, Beld M, Piriou E, van Breda A, Lange J, et al. Epstein-Barr Virus infects B and non-B lymphocytes in HIV-1–infected children and adolescents. J Infect Dis. 2006;194:1323–30.
53. Bressollette-Bodin C, Coste-Burel M, Besse B, André-Garnier E, Ferre V, Imbert-Marcille B-M. Cellular normalization of viral DNA loads on whole blood improves the clinical management of cytomegalovirus or Epstein Barr virus infections in the setting of pre-emptive therapy. J Med Virol. 2009;81:90–8.
54. Kinch A, Öberg G, Arvidson J, Falk KI, Linde A, Pauksens K. Post-transplant lymphoproliferative disease and other Epstein-Barr virus diseases in allogeneic haematopoietic stem cell transplantation after introduction of monitoring of viral load by polymerase chain reaction. Scand J Infect Dis. 2007;39:235–44.
55. Wada K, Kubota N, Ito Y, Yagasaki H, Kato K, Yoshikawa T, et al. Simultaneous quantification of Epstein-Barr Virus, cytomegalovirus, and human herpesvirus 6 DNA in samples from transplant recipients by multiplex real-time PCR assay. J Clin Microbiol. 2007;45:1426–32.
56. Ruf S, Behnke-Hall K, Gruhn B, Bauer J, Horn M, Beck J, et al. Comparison of six different specimen types for Epstein-Barr viral load quantification in peripheral blood of pediatric patients after heart transplantation or after allogeneic hematopoietic stem cell transplantation. J Clin Virol. Elsevier B.V. 2012;53:186–94.
57. Ishihara M, Tanaka E, Sato T, Chikamoto H, Hisano M, Akioka Y, et al. Epstein-Barr virus load for early detection of lymphoproliferative disorder in pediatric renal transplant recipients. Clin Nephrol. 2011;76:40–8.
58. Stevens SJC, Verschuuren EAM, Pronk I, van der Bij W, Harmsen MC, The TH, et al. Frequent monitoring of Epstein-Barr virus DNA load in unfractionated whole blood is essential for early detection of posttransplant lymphoproliferative disease in high-risk patients. Blood. 2001;97:1165–71.
59. Stevens SJC, Pronk I, Middeldorp JM. Toward standardization of Epstein-Barr virus DNA load monitoring: unfractionated whole blood as preferred clinical specimen. J Clin Microbiol. 2001;39:1211–6.
60. Bakker NA, Verschuuren EA, Veeger NJ, van der Bij W, van Imhoff GW, Kallenberg CG, et al. Quantification of Epstein-Barr Virus–DNA load in lung transplant recipients: a comparison of plasma versus whole blood. J Hear Lung Transpl. 2008;27:7–10.
61. Hakim H, Gibson C, Pan J, Srivastava K, Gu Z, Bankowski MJ, et al. Comparison of various blood compartments and reporting units for the detection and quantification of Epstein-Barr Virus in peripheral blood. J Clin Microbiol. 2007;45:2151–5.

62. Loginov R, Aalto S, Piiparinen H, Halme L, Arola J, Hedman K, et al. Monitoring of EBV-DNAemia by quantitative real-time PCR after adult liver transplantation. J Clin Virol. 2006;37:104–8.
63. Wadowsky RM, Laus S, Green M, Webber SA, Rowe D. Measurement of Epstein-Barr Virus DNA loads in whole blood and plasma by TaqMan PCR and in peripheral blood lymphocytes by competitive PCR. J Clin Microbiol. 2003;41:5245–9.
64. Kullberg-Lindh C, Olofsson S, Brune M, Lindh M. Comparison of serum and whole blood levels of cytomegalovirus and Epstein-Barr virus DNA. Transpl Infect Dis. 2008;10:308–15.
65. Wagner HJ, Wessel M, Jabs W, Smets F, Fischer L, Offner G, et al. Patients at risk for development of posttransplant lymphoproliferative disorder: plasma versus peripheral blood mononuclear cells as material for quantification of Epstein-Barr viral load by using real-time quantitative polymerase chain reaction. Transplantation. 2001;72:1012–9.
66. Wagner H-J, Fischer L, Jabs WJ, Holbe M, Pethig K, Bucsky P. Longitudinal analysis of Epstein-Barr viral load in plasma and peripheral blood mononuclear cells of transplanted patients by real-time polymerase chain reaction. Transplantation. 2002;74:656–64.
67. Fafi-Kremer S, Brengel-Pesce K, Barguès G, Bourgeat M-J, Genoulaz O, Seigneurin J-M, et al. Assessment of automated DNA extraction coupled with real-time PCR for measuring Epstein–Barr virus load in whole blood, peripheral mononuclear cells and plasma. J Clin Virol. 2004;30:157–64.
68. Kanakry JA, Hegde AM, Durand CM, Massie AB, Greer AE, Ambinder RF, et al. The clinical significance of EBV DNA in the plasma and peripheral blood mononuclear cells of patients with or without EBV diseases. Blood. 2016;127:2007–17.
69. Tsai DE, Nearey M, Hardy CL, Tomaszewski JE, Kotloff RM, Grossman RA, et al. Use of EBV PCR for the diagnosis and monitoring of post-transplant lymphoproliferative disorder in adult solid organ transplant patients. Am J Transplant. 2002;2:946–54.
70. Fafi-Kremer S, Morand P, Barranger C, Barguès G, Magro S, Bés J, et al. Evaluation of the Epstein-Barr Virus R-gene quantification kit in whole blood with different extraction methods and PCR platforms. J Mol Diagn. 2008;10:78–84.
71. Wagner HJ, Jabs W, Smets F, Wessel M, Fischer L, Offner G, et al. Real-time polymerase chain reaction (RQ-PCR) for the monitoring of Epstein-Barr virus (EBV) load in peripheral blood mononuclear cells. Klin Padiatr. 2000;212:206–10.
72. Jabs WJ, Hennig H, Kittel M, Pethig K, Smets F, Bucsky P, et al. Normalized quantification by real-time PCR of Epstein-Barr Virus load in patients at risk for posttransplant lymphoproliferative disorders. J Clin Microbiol. 2001;39:564–9.
73. Lee T-H, Montalvo L, Chrebtow V, Busch MP. Quantitation of genomic DNA in plasma and serum samples: higher concentrations of genomic DNA found in serum than in plasma. Transfusion. 2001;41:276–82.
74. Lazzarotto T, Chiereghin A, Piralla A, Piccirilli G, Girello A, Campanini G, et al. Cytomegalovirus and Epstein-Barr Virus DNA kinetics in whole blood and plasma of allogeneic hematopoietic stem cell transplantation recipients. Biol Blood Marrow Transpl. Elsevier Inc. 2018;24:1699–706.
75. Kullberg-Lindh C, Saalman R, Olausson M, Herlenius G, Lindh M. Epstein-Barr virus DNA monitoring in serum and whole blood in pediatric liver transplant recipients who do or do not discontinue immunosuppressive therapy. Pediatr Transplant. 2017;21:e12875.
76. Morton M, Coupes B, Roberts SA, Johnson SL, Klapper PE, Vallely PJ, et al. Epstein-Barr virus infection in adult renal transplant recipients. Am J Transplant. 2014;14:1619–29.
77. Höcker B, Fickenscher H, Delecluse H-J, Böhm S, Küsters U, Schnitzler P, et al. Epidemiology and morbidity of Epstein-Barr Virus infection in pediatric renal transplant recipients: a multicenter. Prospective Study. Clin Infect Dis. 2013;56:84–92.
78. van Esser JWJ, Niesters HGM, van der Holt B, Meijer E, Osterhaus ADME, Gratama JW, et al. Prevention of Epstein-Barr virus–lymphoproliferative disease by molecular monitoring and preemptive rituximab in high-risk patients after allogeneic stem cell transplantation. Blood. 2002;99:4364–9.

79. Omar H, Hägglund H, Gustafsson-Jernberg Å, LeBlanc K, Mattsson J, Remberger M, et al. Targeted monitoring of patients at high risk of post-transplant lymphoproliferative disease by quantitative Epstein-Barr virus polymerase chain reaction. Transpl Infect Dis. 2009;11:393–9.
80. Peters AC, Akinwumi MS, Cervera C, Mabilangan C, Ghosh S, Lai R, et al. The changing epidemiology of posttransplant lymphoproliferative disorder in adult solid organ transplant recipients over 30 years. Transplantation. 2018;102:1553–62.
81. Cook L, Starr K, Boonyaratanakornkit J, Hayden R, Sam SS, Caliendo AM. Does size matter? Comparison of extraction yields for different-sized DNA fragments by seven different routine and four new circulating cell-free extraction methods. Tang Y-W, editor. J Clin Microbiol. 2018;56:1–13.
82. Niesters HGM, van Esser J, Fries E, Wolthers KC, Cornelissen J, Osterhaus ADME. Development of a real-time quantitative assay for detection of Epstein-Barr virus. J Clin Microbiol. 2000;38:712–5.
83. Kalra A, Roessner C, Jupp J, Williamson T, Tellier R, Chaudhry A, et al. Epstein-Barr virus DNAemia monitoring for the management of post-transplant lymphoproliferative disorder. Cytotherapy. Elsevier Inc. 2018;20:706–14.
84. Styczynski J, Gil L, Tridello G, Ljungman P, Donnelly JP, van der Velden W, et al. Response to rituximab-based therapy and risk factor analysis in Epstein Barr virus–related lymphoproliferative disorder after hematopoietic stem cell transplant in children and adults: a study from the infectious diseases working Party of the European Gro. Clin Infect Dis. 2013;57:794–802.
85. van Esser JWJ, Niesters HGM, Thijsen SFT, Meijer E, Osterhaus ADME, Wolthers KC, et al. Molecular quantification of viral load in plasma allows for fast and accurate prediction of response to therapy of Epstein-Barr virus-associated lymphoproliferative disease after allogeneic stem cell transplantation. Br J Haematol. 2001;113:814–21.
86. Yang J, Tao Q, Flinn IW, Murray PG, Post LE, Ma H, et al. Characterization of Epstein-Barr virus–infected B cells in patients with posttransplantation lymphoproliferative disease: disappearance after rituximab therapy does not predict clinical response. Blood. 2000;96:4055–63.
87. Oertel S, Trappe RU, Zeidler K, Babel N, Reinke P, Hummel M, et al. Epstein–Barr viral load in whole blood of adults with posttransplant lymphoproliferative disorder after solid organ transplantation does not correlate with clinical course. Ann Hematol. 2006;85:478–84.
88. Caillard S, Lamy FX, Quelen C, Dantal J, Lebranchu Y, Lang P, et al. Epidemiology of posttransplant lymphoproliferative disorders in adult kidney and kidney pancreas recipients: report of the French registry and analysis of subgroups of lymphomas. Am J Transplant. 2012;12:682–93.
89. Halliday N, Smith C, Atkinson C, O'Beirne J, Patch D, Burroughs AK, et al. Characteristics of Epstein-Barr viraemia in adult liver transplant patients: a retrospective cohort study. Transpl Int. 2014;27:838–46.
90. Schaffer K, Hassan J, Staines A, Coughlan S, Holder P, Tuite G, et al. Surveillance of Epstein-Barr virus loads in adult liver transplantation: associations with age, sex, posttransplant times, and transplant indications. Liver Transpl. 2011;17:1420–6.
91. Bamoulid J, Courivaud C, Coaquette A, Chalopin J-M, Gaiffe E, Saas P, et al. Subclinical Epstein-Barr Virus Viremia among adult renal transplant recipients: incidence and consequences. Am J Transplant. 2013;13:656–62.
92. Colombini E, Guzzo I, Morolli F, Longo G, Russo C, Lombardi A, et al. Viral load of EBV DNAemia is a predictor of EBV-related post-transplant lymphoproliferative disorders in pediatric renal transplant recipients. Pediatr Nephrol. 2017;32:1433–42.
93. L'Huillier AG, Dipchand AI, Ng VL, Hebert D, Avitzur Y, Solomon M, et al. Posttransplant lymphoproliferative disorder in pediatric patients: characteristics of disease in EBV-seropositive recipients. Transplantation. 2019;103:e369–74.
94. Gullett JC, Nolte FS. Quantitative nucleic acid amplification methods for viral infections. Clin Chem. 2015;61:72–8.
95. Preiksaitis JK, Pang XL, Fox JD, Fenton JM, Caliendo AM, Miller GG. Interlaboratory comparison of Epstein-Barr Virus viral load assays. Am J Transplant. 2009;9:269–79.

96. Perandin F, Cariani E, Pollara C, Manca N. Comparison of commercial and in-house real-time PCR assays for quantification of Epstein-Barr virus (EBV) DNA in plasma. BMC Microbiol. 2007;7:22.
97. Le QT, Zhang Q, Cao H, Cheng AJ, Pinsky BA, Hong RL, et al. An international collaboration to harmonize the quantitative plasma Epstein-Barr virus DNA assay for future biomarker-guided trials in nasopharyngeal carcinoma. Clin Cancer Res. 2013;19:2208–15.
98. Ruiz G, Pena P, de Ory F, Echevarria JE. Comparison of commercial real-time PCR assays for quantification of Epstein-Barr Virus DNA. J Clin Microbiol. 2005;43:2053–7.
99. Hayden RT, Hokanson KM, Pounds SB, Bankowski MJ, Belzer SW, Carr J, et al. Multicenter comparison of different real-time PCR assays for quantitative detection of Epstein-Barr Virus. J Clin Microbiol. 2008;46:157–63.
100. Germi R, Lupo J, Semenova T, Larrat S, Magnat N, Grossi L, et al. Comparison of commercial extraction systems and PCR assays for quantification of Epstein-Barr Virus DNA load in whole blood. J Clin Microbiol. 2012;50:1384–9.
101. Ahsanuddin AN, Standish MC, Caliendo AM, Hill CE, Nolte FS. Validation of an Epstein-Barr viral load assay using the QIAGEN Artus EBV TM PCR analyte-specific reagent. Am J Clin Pathol. 2008;130:865–9.
102. Ito Y, Takakura S, Ichiyama S, Ueda M, Ando Y, Matsuda K, et al. Multicenter evaluation of prototype real-time PCR assays for Epstein-Barr virus and cytomegalovirus DNA in whole blood samples from transplant recipients. Microbiol Immunol. 2010;54:516–22.
103. Yen-Lieberman B, Brambilla D, Jackson B, Bremer J, Coombs R, Cronin M, et al. Evaluation of a quality assurance program for quantitation of human immunodeficiency virus type 1 RNA in plasma by the AIDS Clinical Trials Group virology laboratories. J Clin Microbiol. 1996;34:2695–701.
104. Vesper HW, Miller WG, Myers GL. Reference materials and commutability. Clin Biochem Rev. 2007;28:139–47.
105. Tang L, Sun Y, Buelow D, Gu Z, Caliendo AM, Pounds S, et al. Quantitative assessment of commutability for clinical viral load testing using a digital PCR-based reference standard. McAdam AJ, editor. J Clin Microbiol. 2016;54:1616–23.
106. Tang L, Su Y, Pounds S, Hayden RT. Quantitative inference of commutability for clinical viral load testing. Tang Y-W, editor. J Clin Microbiol. 2018;56:1–3.
107. Hayden RT, Preiksaitis J, Tong Y, Pang X, Sun Y, Tang L, et al. Commutability of the first World Health Organization international standard for human cytomegalovirus. Loeffelholz MJ, editor. J Clin Microbiol. 2015;53:3325–33.
108. Preiksaitis JK, Hayden RT, Tong Y, Pang XL, Fryer JF, Heath AB, et al. Are we there yet? Impact of the first international standard for cytomegalovirus DNA on the harmonization of results reported on plasma samples. Clin Infect Dis. 2016;63:583–9.
109. Hayden RT, Sun Y, Tang L, Procop GW, Hillyard DR, Pinsky BA, et al. Progress in quantitative viral load testing: variability and impact of the WHO quantitative international standards. McAdam AJ, editor. J Clin Microbiol. 2017;55:423–30.
110. San-Juan R, Manuel O, Hirsch HH, Fernández-Ruiz M, López-Medrano F, Comoli P, et al. Current preventive strategies and management of Epstein–Barr virus-related post-transplant lymphoproliferative disease in solid organ transplantation in Europe. Results of the ESGICH questionnaire-based cross-sectional survey. Clin Microbiol Infect. Elsevier Ltd. 2015;21:604.e1–9.
111. Abbate I, Piralla A, Calvario A, Callegaro A, Giraldi C, Lunghi G, et al. Nation-wide measure of variability in HCMV, EBV and BKV DNA quantification among centers involved in monitoring transplanted patients. J Clin Virol. 2016;82:76–83.
112. Semenova T, Lupo J, Alain S, Perrin-Confort G, Grossi L, Dimier J, et al. Multicenter evaluation of whole-blood Epstein-Barr viral load standardization using the WHO international standard. Tang Y-W, editor. J Clin Microbiol. 2016;54:1746–50.
113. Rychert J, Danziger-Isakov L, Yen-Lieberman B, Storch G, Buller R, Sweet SC, et al. Multicenter comparison of laboratory performance in cytomegalovirus and Epstein-Barr virus viral load testing using international standards. Clin Transpl. 2014;28:1416–23.

114. Kuypers J, Jerome KR. Applications of digital PCR for clinical microbiology. Kraft CS, editor. J Clin Microbiol. 2017;55:1621–8.
115. Kim H, Hur M, Kim JY, Moon HW, Yun YM, Cho HC. Automated nucleic acid extraction systems for detecting Cytomegalovirus and Epstein-Barr virus using real-time PCR: a comparison study between the QIAsymphony RGQ and QIAcube systems. Ann Lab Med. 2017;37:129–36.
116. Hayden RT, Yan X, Wick MT, Rodriguez AB, Xiong X, Ginocchio CC, et al. Factors contributing to variability of quantitative viral PCR results in proficiency testing samples: a multivariate analysis. J Clin Microbiol. 2012;50:337–45.
117. Kim KY, Le QT, Yom SS, Pinsky BA, Bratman SV, Ng RHW, et al. Current state of PCR-based Epstein-Barr virus DNA testing for nasopharyngeal cancer. J Natl Cancer Inst. 2017;109:1–7.
118. Sanosyan A, Fayd'herbe de Maudave A, Bollore K, Zimmermann V, Foulongne V, Van de Perre P, et al. The impact of targeting repetitive BamHI-W sequences on the sensitivity and precision of EBV DNA quantification. Horwitz MS, editor. PLoS One. 2017;12:e0183856.
119. Le QT, Jones CD, Yau TK, Shirazi HA, Wong PH, Thomas EN, et al. A comparison study of different PCR assays in measuring circulating plasma Epstein-Barr virus DNA levels in patients with nasopharyngeal carcinoma. Clin Cancer Res. 2005;11:5700–7.
120. Baron A, Gicquel A, Plantier J-C, Gueudin M. Evaluation of four commercial extraction-quantification systems to monitor EBV or CMV viral load in whole blood. J Clin Virol. Elsevier. 2019;113:39–44.
121. Ryan JL, Fan H, Glaser SL, Schichman SA, Raab-Traub N, Gulley ML. Epstein-Barr virus quantitation by real-time PCR targeting multiple gene segments. J Mol Diagn. Association of Molecular Pathology. 2004;6:378–85.
122. Kanda T, Yajima M, Ikuta K. Epstein-Barr virus strain variation and cancer. Cancer Sci. Blackwell Publishing Ltd. 2019;110:1132–9.
123. Palser AL, Grayson NE, White RE, Corton C, Correia S, Ba Abdullah MM, et al. Genome diversity of Epstein-Barr Virus from multiple tumor types and normal infection. Longnecker RM, editor. J Virol. American Society for Microbiology. 2015;89:5222–37.
124. Neves M, Marinho-Dias J, Ribeiro J, Sousa H. Epstein-Barr virus strains and variations: geographic or disease-specific variants? J Med Virol. 2017;89:373–87.
125. Correia S, Bridges R, Wegner F, Venturini C, Palser A, Middeldorp JM, et al. Sequence variation of Epstein-Barr Virus: viral types, geography, codon usage, and diseases. Longnecker RM, editor. J Virol. American Society for Microbiology. 2018;92:e01132.
126. Santpere G, Darre F, Blanco S, Alcami A, Villoslada P, Mar Albà M, et al. Genome-wide analysis of wild-type Epstein–Barr Virus genomes derived from healthy individuals of the 1000 genomes project. Genome Biol Evol. 2014;6:846–60.
127. Navarro E, Serrano-Heras G, Castaño MJ, Solera J. Real-time PCR detection chemistry. Clin Chim Acta. Elsevier BV. 2015;439:231–50.
128. Moran J, Carr M, Waters A, Boyle S, Riordan M, Connell J, et al. Epstein-Barr Virus gene expression, human leukocyte antigen alleles and chronic high viral loads in pediatric renal transplant patients. Transplantation. 2011;92:328–33.
129. Ruf S, Behnke-Hall K, Gruhn B, Reiter A, Wagner HJ. EBV load in whole blood correlates with LMP2 gene expression after pediatric heart transplantation or allogeneic hematopoietic stem cell transplantation. Transplantation. 2014;97:958–64.
130. Kroll J, Li S, Levi M, Weinberg A. Lytic and latent EBV gene expression in transplant recipients with and without post-transplant lymphoproliferative disorder. J Clin Virol. Elsevier BV. 2011;52:231–5.
131. Qu L, Green M, Webber S, Reyes J, Ellis D, Rowe D. Epstein-Barr Virus gene expression in the peripheral blood of transplant recipients with persistent circulating virus loads. J Infect Dis. 2000;182:1013–21.
132. Hopwood PA, Brooks L, Parratt R, Hunt BJ, Maria B, Alero TJ, et al. Persistent Epstein-Barr virus infection: unrestricted latent and lytic viral gene expression in healthy immunosuppressed transplant recipients1. Transplantation. 2002;74:194–202.

133. Kasztelewicz B, Jankowska I, Pawłowska J, Teisseyre J, Dzierżanowska-Fangrat K. Epstein-Barr virus gene expression and latent membrane protein 1 gene polymorphism in pediatric liver transplant recipients. J Med Virol. 2011;83:2182–90.
134. Zawilinska B, Kosinska A, Lenart M, Kopec J, Piatkowska-Jakubas B, Skotnicki A, et al. Detection of specific lytic and latent transcripts can help to predict the status of Epstein-Barr virus infection in transplant recipients with high virus load. Acta Biochim Pol. 2008;55:693–9.
135. Münz C. Latency and lytic replication in Epstein–Barr virus-associated oncogenesis. Nat Rev Microbiol. Springer US. 2019;17:691–700.
136. Kawano Y, Iwata S, Kawada J, Gotoh K, Suzuki M, Torii Y, et al. Plasma viral MicroRNA profiles reveal potential biomarkers for chronic active Epstein–Barr Virus infection. J Infect Dis. 2013;208:771–9.
137. Kaul V, Weinberg KI, Boyd SD, Bernstein D, Esquivel CO, Martinez OM, et al. Dynamics of viral and host immune cell microRNA expression during acute infectious mononucleosis. Front Microbiol. 2018;8:1–9.
138. Bergallo M, Gambarino S, Pinon M, Barat V, Montanari P, Daprà V, et al. EBV-encoded microRNAs profile evaluation in pediatric liver transplant recipients. J Clin Virol. Elsevier. 2017;91:36–41.
139. Hassan J, Dean J, De Gascun CF, Riordan M, Sweeney C, Connell J, et al. Plasma EBV microRNAs in paediatric renal transplant recipients. J Nephrol Springer International Publishing. 2018;31:445–51.
140. Yanagisawa K, Tanuma J, Hagiwara S, Gatanaga H, Kikuchi Y, Oka S. Epstein-Barr Viral load in cerebrospinal fluid as a diagnostic marker of central nervous system involvement of AIDS-related lymphoma. Intern Med. 2013;52:955–9.
141. Ivers LC, Kim AY, Sax PE. Predictive value of polymerase chain reaction of cerebrospinal fluid for detection of Epstein-Barr Virus to establish the diagnosis of HIV-related primary central nervous system lymphoma. Clin Infect Dis. 2004;38:1629–32.
142. Corcoran C, Rebe K, van der Plas H, Myer L, Hardie DR. The predictive value of cerebrospinal fluid Epstein-Barr viral load as a marker of primary central nervous system lymphoma in HIV-infected persons. J Clin Virol. 2008;42:433–6.
143. Bossolasco S, Cinque P, Ponzoni M, Vigano MG, Lazzarin A, Linde A, et al. Epstein-Barr virus DNA load in cerebrospinal fluid and plasma of patients with AIDS-related lymphoma. J Neurovirol. 2002;8:432–8.
144. Weinberg A, Li S, Palmer M, Tyler KL. Quantitative CSF PCR in Epstein-Barr virus infections of the central nervous system. Ann Neurol. 2002;52:543–8.
145. Liu Q-F, Ling Y-W, Fan Z-P, Jiang Q-L, Sun J, Wu X-L, et al. Epstein-Barr virus (EBV) load in cerebrospinal fluid and peripheral blood of patients with EBV-associated central nervous system diseases after allogeneic hematopoietic stem cell transplantation. Transpl Infect Dis. 2013;15:379–92.
146. Michelson P, Watkins B, Webber SA, Wadowsky R, Michaels MG. Screening for PTLD in lung and heart-lung transplant recipients by measuring EBV DNA load in bronchoalveolar lavage fluid using real time PCR. Pediatr Transplant. 2008;12:464–8.
147. Parrish A, Fenchel M, Storch GA, Buller R, Mason S, Williams N, et al. Epstein-Barr viral loads do not predict post-transplant lymphoproliferative disorder in pediatric lung transplant recipients: a multicenter prospective cohort study. Pediatr Transplant. 2017;21:e13011.
148. Bauer CC, Jaksch P, Aberle SW, Haber H, Lang G, Klepetko W, et al. Relationship between cytomegalovirus DNA load in epithelial lining fluid and plasma of lung transplant recipients and analysis of coinfection with Epstein-Barr virus and human herpesvirus 6 in the lung compartment. J Clin Microbiol. 2007;45:324–8.
149. Costa C, Elia M, Astegiano S, Sidoti F, Terlizzi ME, Solidoro P, et al. Quantitative detection of Epstein-Barr Virus in Bronchoalveolar lavage from transplant and nontransplant patients. Transplantation. 2008;86:1389–94.
150. Preiksaitis JK, Diaz-Mitoma F, Mirzayans F, Roberts S, Tyrrell DLJ. Quantitative oropharyngeal Epstein-Barr Virus shedding in renal and cardiac transplant recipients: relationship to

immunosuppressive therapy, serologic responses, and the risk of posttransplant lymphoproliferative disorder. J Infect Dis. 1992;166:986–94.
151. Johnson KH, Webb C-H, Schmeling DO, Brundage RC, Balfour HH. Epstein–Barr virus dynamics in asymptomatic immunocompetent adults: an intensive 6-month study. Clin Transl Immunol. 2016;5:e81.
152. Balfour HH, Hokanson KM, Schacherer RM, Fietzer CM, Schmeling DO, Holman CJ, et al. A virologic pilot study of valacyclovir in infectious mononucleosis. J Clin Virol. 2007;39:16–21.
153. Hadinoto V, Shapiro M, Sun CC, Thorley-Lawson DA. The dynamics of EBV shedding implicate a central role for epithelial cells in amplifying viral output. Speck SH, editor. PLoS Pathog. 2009;5:e1000496.
154. Yager JE, Magaret AS, Kuntz SR, Selke S, Huang M-L, Corey L, et al. Valganciclovir for the suppression of Epstein-Barr virus replication. J Infect Dis. 2017;216:198–202.
155. Ling PD, Lednicky JA, Keitel WA, Poston DG, White ZS, Peng R, et al. The dynamics of herpesvirus and polyomavirus reactivation and shedding in healthy adults: a 14-month longitudinal study. J Infect Dis. 2003;187:1571–80.
156. Haque T, Crawford DH. PCR amplification is more sensitive than tissue culture methods for Epstein-Barr virus detection in clinical material. J Gen Virol. 1997;78:3357–60.
157. Ikuta K, Satoh Y, Hoshikawa Y, Sairenji T. Detection of Epstein-Barr virus in salivas and throat washings in healthy adults and children. Microbes Infect. 2000;2:115–20.
158. Balfour HH, Odumade OA, Schmeling DO, Mullan BD, Ed JA, Knight JA, et al. Behavioral, Virologic, and immunologic factors associated with acquisition and severity of primary Epstein–Barr virus infection in university students. J Infect Dis. 2013;207:80–8.
159. Grimm JM, Schmeling DO, Dunmire SK, Knight JA, Mullan BD, Ed JA, et al. Prospective studies of infectious mononucleosis in university students. Clin Transl Immunol. Nature Publishing Group. 2016;5:e94.
160. Dunmire SK, Grimm JM, Schmeling DO, Balfour HH, Hogquist KA. The incubation period of primary Epstein-Barr virus infection: viral dynamics and immunologic events. Ling PD, editor. PLOS Pathog. Public Library of Science. 2015;11:e1005286.
161. Yao QY, Rickinson AB, Gaston JSH, Epstein MA. In vitro analysis of the Epstein-Barr virus: host balance in long-term renal allograft recipients. Int J Cancer. 1985;35:43–9.
162. Verghese PS, Schmeling DO, Filtz EA, Grimm JM, Matas AJ, Balfour HH. Transplantation of solid organ recipients shedding Epstein-Barr virus DNA pre-transplant: a prospective study. Clin Transpl. 2017;31:e13116.
163. Styczynski J, Van Der Velden W, Fox CP, Engelhard D, De La Camara R, Cordonnier C, et al. Management of Epstein-Barr virus infections and post-transplant lymphoproliferative disorders in patients after allogeneic hematopoietic stem cell transplantation: sixth European conference on infections in leukemia (ECIL-6) guidelines. Haematologica. 2016;101:803–11.
164. Hoagland CRJ. The transmission of infectious mononucleosis. Am J Med Sci. Elsevier BV. 1955;229:262–72.
165. Svedmyr E, Ernberg I, Seeley J, Weiland O, Masucci G, Tsukuda K, et al. Virologic, immunologic, and clinical observations on a patient during the incubation, acute, and convalescent phases of infectious mononucleosis. Clin Immunol Immunopathol. 1984;30:437–50.
166. Hadinoto V, Shapiro M, Greenough TC, Sullivan JL, Luzuriaga K, Thorley-Lawson DA. On the dynamics of acute EBV infection and the pathogenesis of infectious mononucleosis. Blood. 2008;111:1420–7.
167. Jayasooriya S, de Silva TI, Njie-jobe J, Sanyang C, Leese AM, Bell AI, et al. Early virological and immunological events in asymptomatic Epstein-Barr virus infection in African children. Rochford R, editor. PLoS Pathog. 2015;11:e1004746.
168. Piriou E, Asito AS, Sumba PO, Fiore N, Middeldorp JM, Moormann AM, et al. Early age at time of primary Epstein–Barr virus infection results in poorly controlled viral infection in infants from Western Kenya: clues to the etiology of endemic Burkitt lymphoma. J Infect Dis. 2012;205:906–13.

169. Chan K, Tam JS, Peiris JS, Seto W, Ng M. Epstein–Barr virus (EBV) infection in infancy. J Clin Virol. 2001;21:57–62.
170. Jenson HB, Montalvo EA, McClain KL, Ench Y, Heard P, Christy BA, et al. Characterization of natural Epstein-Barr virus infection and replication in smooth muscle cells from a leiomyosarcoma. J Med Virol. 1999;57:36–46.
171. Biggar RJ, Henle G, Böcker J, Lennette ET, Fleisher G, Henle W. Primary Epstein-Barr virus infections in African infants. II. Clinical and serological observations during seroconversion. Int J Cancer. 1978;22:244–50.
172. Reynaldi A, Schlub TE, Piriou E, Ogolla S, Sumba OP, Moormann AM, et al. Modeling of EBV infection and antibody responses in Kenyan infants with different levels of malaria exposure shows maternal antibody decay is a major determinant of early EBV infection. J Infect Dis. 2016;214:1390–8.
173. Burton C, Mabilangan C, Preiksaitis J. Incidence of Epstein Barr Virus (EBV) DNAemia in EBV seronegative solid organ transplant (SOT) recipients with EBV seropositive donors. Am J Transplant. 2018;17:756.
174. Ville S, Imbert-Marcille B-M, Coste-Burel M, Garandeau C, Meurette A, Cantarovitch D, et al. Impact of antiviral prophylaxis in adults Epstein-Barr Virus-seronegative kidney recipients on early and late post-transplantation lymphoproliferative disorder onset: a retrospective cohort study. Transpl Int. 2018;31:484–94.
175. Yamada M, Nguyen C, Fadakar P, Ganoza A, Humar A, Shapiro R, et al. Epidemiology and outcome of chronic high Epstein-Barr viral load carriage in pediatric kidney transplant recipients. Pediatr Transplant. 2018;22:e13147.
176. Liu Q, Xuan L, Liu H, Huang F, Zhou H, Fan Z, et al. Molecular monitoring and stepwise preemptive therapy for Epstein-Barr virus viremia after allogeneic stem cell transplantation. Am J Hematol. 2013;88:550–5.
177. Hoshino Y, Nishikawa K, Ito Y, Kuzushima K, Kimura H. Kinetics of Epstein-Barr virus load and virus-specific CD8+ T cells in acute infectious mononucleosis. J Clin Virol. 2011;50:244–6.
178. Nakai H, Kawamura Y, Sugata K, Sugiyama H, Enomoto Y, Asano Y, et al. Host factors associated with the kinetics of Epstein-Barr virus DNA load in patients with primary Epstein-Barr virus infection. Microbiol Immunol. 2012;56:93–8.
179. Dunmire SK, Verghese PS, Balfour HH. Primary Epstein-Barr virus infection. J Clin Virol. Elsevier B.V. 2018;102:84–92.
180. Abbott RJ, Pachnio A, Pedroza-Pacheco I, Leese AM, Begum J, Long HM, et al. Asymptomatic primary infection with Epstein-Barr virus: observations on young adult cases. Longnecker RM, editor. J Virol. American Society for Microbiology. 2017;91:e00382.
181. Njie R, Bell AI, Jia H, Croom-Carter D, Chaganti S, Hislop AD, et al. The effects of acute malaria on Epstein-Barr Virus (EBV) load and EBV-specific T cell immunity in Gambian children. J Infect Dis. 2009;199:31–8.
182. Silins SL, Sherritt MA, Silleri JM, Cross SM, Elliott SL, Bharadwaj M, et al. Asymptomatic primary Epstein-Barr virus infection occurs in the absence of blood T-cell repertoire perturbations despite high levels of systemic viral load. Blood. 2001;98:3739–44.
183. Riddler S, Breinig M, McKnight J. Increased levels of circulating Epstein-Barr virus (EBV)-infected lymphocytes and decreased EBV nuclear antigen antibody responses are associated with the development of posttransplant lymphoproliferative disease in solid-organ transplant recipients. Blood. 1994;84:972–84.
184. Kenagy DN, Schlesinger Y, Weck K, Ritter JH, Gaudreault-Keener MM, Storch GA. Epstein-Barr virus DNA in peripheral blood leukocytes of patients with posttransplant lymphoproliferative disease. Transplantation. 1995;60:547–54.
185. Preiksaitis JK. Epstein—Barr viral load testing: role in the prevention, diagnosis and management of posttransplant lymphoproliferative disorders. Post-transplant lymphoproliferative disord. Berlin, Heidelberg: Springer Berlin Heidelberg; 2010. p. 45–67.

186. Cho Y-U, Chi H-S, Jang S, Park SH, Park C-J. Pattern analysis of Epstein-Barr Virus viremia and its significance in the evaluation of organ transplant patients suspected of having post-transplant lymphoproliferative disorders. Am J Clin Pathol. 2014;141:268–74.
187. Manlhiot C, Pollock-BarZiv SM, Holmes C, Weitzman S, Allen U, Clarizia NA, et al. Post-transplant lymphoproliferative disorder in pediatric heart transplant recipients. J Hear Lung Transpl. Elsevier Inc. 2010;29:648–57.
188. Fox CP, Burns D, Parker AN, Peggs KS, Harvey CM, Natarajan S, et al. EBV-associated post-transplant lymphoproliferative disorder following in vivo T-cell-depleted allogeneic transplantation: clinical features, viral load correlates and prognostic factors in the rituximab era. Bone Marrow Transpl. Nature Publishing Group. 2014;49:280–6.
189. Solano C, Mateo EM, Pérez A, Talaya A, Terol MJ, Albert E, et al. Epstein-Barr virus DNA load kinetics analysis in allogeneic hematopoietic stem cell transplant recipients: is it of any clinical usefulness? J Clin Virol. Elsevier. 2017;97:26–32.
190. Carvalho-Queiroz C, Johansson MA, Persson J-O, Jörtsö E, Kjerstadius T, Nilsson C, et al. Associations between EBV and CMV seropositivity, early exposures, and gut microbiota in a prospective birth cohort: a 10-year follow-up. Front Pediatr. 2016;4:93.
191. Balfour HH, Sifakis F, Sliman JA, Knight JA, Schmeling DO, Thomas W. Age-specific prevalence of Epstein–Barr Virus infection among individuals aged 6–19 years in the United States and factors affecting its acquisition. J Infect Dis. 2013;208:1286–93.
192. Reynaldi A, Schlub TE, Chelimo K, Sumba PO, Piriou E, Ogolla S, et al. Impact of Plasmodium falciparum coinfection on longitudinal Epstein-Barr Virus kinetics in Kenyan children. J Infect Dis. 2016;213:985–91.
193. Donati D, Espmark E, Kironde F, Mbidde EK, Kamya M, Lundkvist Å, et al. Clearance of circulating Epstein-Barr virus DNA in children with acute malaria after antimalaria treatment. J Infect Dis. 2006;193:971–7.
194. Chattopadhyay PK, Chelimo K, Embury PB, Mulama DH, Sumba PO, Gostick E, et al. Holoendemic malaria exposure is associated with altered Epstein-Barr virus-specific CD8 + T-cell differentiation. J Virol. 2013;87:1779–88.
195. Ogolla S, Daud II, Asito AS, Sumba OP, Ouma C, Vulule J, et al. Reduced transplacental transfer of a subset of Epstein-Barr virus-specific antibodies to neonates of mothers infected with Plasmodium falciparum malaria during pregnancy. Pasetti MF, editor. Clin Vaccine Immunol. 2015;22:1197–205.
196. Kumar D, Patil N, Husain S, Chaparro C, Bhat M, Kim SJ, et al. Clinical and virologic outcomes in high-risk adult Epstein-Barr virus mismatched organ transplant recipients. Clin Transpl. 2017;31(7).
197. Green M, Cacciarelli TV, Mazariegos GV, Sigurdsson L, Qu L, Rowe DT, et al. Serial measurement of Epstein-Barr viral load in peripheral blood in pediatric liver transplant recipients during treatment for posttransplant lymphoproliferative disease. Transplantation. 1998;66:1641–4.
198. Khan G, Miyashita EM, Yang B, Babcock GJ, Thorley-Lawson DA. Is EBV persistence in vivo a model for B cell homeostasis? Immunity. Cell Press. 1996;5:173–9.
199. Stowe RP, Kozlova EV, Yetman DL, Walling DM, Goodwin JS, Glaser R. Chronic herpesvirus reactivation occurs in aging. Exp Gerontol. 2007;42:563–70.
200. Smatti MK, Yassine HM, AbuOdeh R, AlMarawani A, Taleb SA, Althani AA, et al. Prevalence and molecular profiling of Epstein Barr virus (EBV) among healthy blood donors from different nationalities in Qatar. Pagano JS, editor. PLoS One. Public Library of Science. 2017;12:e0189033.
201. Vescovini R, Telera A, Fagnoni FF, Biasini C, Medici MC, Valcavi P, et al. Different contribution of EBV and CMV infections in very long-term carriers to age-related alterations of CD8+ T cells. Exp Gerontol. 2004;39:1233–43.
202. Das B, Morrow R, Huang R, Fixler D. Persistent Epstein-Barr viral load in Epstein-Barr viral naïve pediatric heart transplant recipients: risk of late-onset post-transplant lymphoproliferative disease. World J Transplant. 2016;6:729.

203. Bingler MA, Feingold B, Miller SA, Quivers E, Michaels MG, Green M, et al. Chronic high Epstein-Barr viral load state and risk for late-onset posttransplant lymphoproliferative disease/lymphoma in children. Am J Transplant. 2008;8:442–5.
204. Lau AH, Soltys K, Sindhi RK, Bond G, Mazariegos GV, Green M. Chronic high Epstein-Barr viral load carriage in pediatric small bowel transplant recipients. Pediatr Transplant. 2010;14:549–53.
205. Kamei H, Ito Y, Kawada J, Ogiso S, Onishi Y, Komagome M, et al. Risk factors and long-term outcomes of pediatric liver transplant recipients with chronic high Epstein-Barr virus loads. Transpl Infect Dis. 2018;20:e12911.
206. Green M, Soltys K, Rowe DT, Webber SA, Mazareigos G. Chronic high Epstein-Barr viral load carriage in pediatric liver transplant recipients. Pediatr Transplant. 2009;13:319–23.
207. Macedo C, Webber SA, Donnenberg AD, Popescu I, Hua Y, Green M, et al. EBV-specific CD8 + T cells from asymptomatic pediatric thoracic transplant patients carrying chronic high EBV loads display contrasting features: activated phenotype and exhausted function. J Immunol. 2011;186:5854–62.
208. Moran J, Dean J, De Oliveira A, O'Connell M, Riordan M, Connell J, et al. Increased levels of PD-1 expression on CD8 T cells in patients post-renal transplant irrespective of chronic high EBV viral load. Pediatr Transplant. 2013;17:806–14.
209. Moormann AM, Chelimo K, Sumba OP, Lutzke ML, Ploutz-Snyder R, Newton D, et al. Exposure to holoendemic malaria results in elevated Epstein-Barr virus loads in children. J Infect Dis. 2005;191:1233–8.
210. Piriou E, Dort KV, Otto S, Van Oers MHJ, Van Baarle D. Tight regulation of the Epstein-Barr Virus setpoint: interindividual differences in Epstein-Barr Virus DNA load are conserved after HIV infection. Clin Infect Dis. Oxford University Press (OUP). 2008;46:313–6.
211. Piriou E, Jansen CA, van Dort K, De Cuyper I, Nanlohy NM, Lange JMA, et al. Reconstitution of EBV latent but not lytic antigen-specific CD4 + and CD8 + T cells after HIV treatment with highly active antiretroviral therapy. J Immunol. The American Association of Immunologists. 2005;175:2010–7.
212. Piriou ER, van Dort K, Nanlohy NM, Miedema F, van Oers MH, van Baarle D. Altered EBV viral load Setpoint after HIV seroconversion is in accordance with lack of predictive value of EBV load for the occurrence of AIDS-related non-Hodgkin lymphoma. J Immunol. The American Association of Immunologists. 2004;172:6931–7.
213. Hoshino Y, Katano H, Zou P, Hohman P, Marques A, Tyring SK, et al. Long-term administration of Valacyclovir reduces the number of Epstein-Barr Virus (EBV)-infected B cells but not the number of EBV DNA copies per B cell in healthy volunteers. J Virol. 2009;83:11857–61.