

Epstein-Barr Virus: Clinical Diagnostics

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Abstract

The vast majority of the human adult population is infected with Epstein-Barr virus (EBV), and the majority of the EBV-infected individuals tolerates the infection well, without any further symptoms after primary infection. In cases of individuals which undergo primary infection in the form of an infectious mononucleosis, or which have undergone primary infection in their past, it is sometimes important to appraise symptomatic disease or differentiate infectious mononucleosis from other conditions. In these cases, serological methods, i.e., immunofluorescence, ELISA, or Western blot, are the methods of choice to come to an unequivocal diagnostic conclusion, while the detection and quantification of viral DNA through PCR plays a minor role.

On the other hand, in a minority of the human population, EBV infection is associated or causally linked with autoimmune or malignant disease. Especially in the bone marrow or solid organ transplanted, or in otherwise severely immune-suppressed patients, prolonged EBV primary infection or EBV reactivation from latency may be a serious and life-threatening complication which needs to be diagnosed the faster the better, in order to take therapeutic steps in time. Determining the serostatus correctly is also important in these cases. However, the direct and quantitative detection of viral DNA are of importance for the diagnosis of serious EBV disease and its monitoring.

In the following, we give an overview of diagnostic methods to accurately determine EBV serostatus and viral load. We evaluate the advantages and disadvantages of each method and report on the diagnostic significance of each and how to resolve diagnostic problems in case of uncertainties. For practical procedures, we refer to the detailed instruction manuals of the respective test kit manufacturers which have to be closely followed for reliable results.

Key words Enzyme immunoassay (EIA), Immunofluorescence, Immunosuppression, Line blot, PCR, Posttransplant lymphoproliferative disease (PTLD), Serology, Transplantation, Western blot

1 Epstein-Barr Virus

Epstein-Barr virus was discovered by electron microscopy in cells from biopsies of Burkitt lymphomas more than 50 years ago [1]. Since then, the research on this multifaceted virus has tremendously stimulated tumor biology, tumor immunology, molecular virology, clinical virology, and virus diagnostics.

Initially, due to the history of its discovery, the focus was especially on the tumor biological aspects of EBV. The detection of the

EBV genome in Burkitt lymphomas was a milestone for human tumor virology. Thereby, EBV became the first established human tumor virus, and the discovery of EBV in nasopharyngeal carcinoma (NPC) biopsies [2], and specifically in the epithelial tumor cells of NPC [3], prepared for the discovery of further human tumor viruses in epithelial cancers from a conceptual perspective. The role of the virus in tumor development in humans was later expanded as a result of the detection of EBV genomes in gastric carcinomas, certain forms of classical Hodgkin's lymphoma, rare T-cell lymphomas, and parotid tumors. The PTLD (posttransplant lymphoproliferative disease)-related lymphomas, lymphomas in patients with Purtilo syndrome, and AIDS-related lymphomas are mechanistically related, but different in their pathogenetic details [4, 5]. This group of lymphomas directly highlights the importance of an intact immune system to control EBV-infected cells and points to the role of viral latency and to the impact of EBV-driven proliferation and antiapoptotic effects. The effects of EBV on transformation and cell proliferation, however, certainly do not represent all of the aspects which are important for the participation of EBV in tumor development [6]. With respect to the distinct viral functions involved in tumorigenesis in each case, the molecular pathogenesis of diverse EBV-associated tumors must not be lumped together [7]. Based on the discovery of a binding site for c -Myc in the central locus control region of the EBV genome [8], a clear distinction between transformative and antiapoptotic functions for the molecular pathogenesis of PTLD-like tumors and Burkitt tumors, respectively, has been made [9]. Because recent history-centered reviews [10, 11] keep ignoring the central viral myc-binding site [8] and the interconnected concept of alternative pathogenetic pathways [9], they have to engage in low-key workarounds and, thus, to approach the obvious solution to the Burkitt problem, at a slower pace. Possibly, more recent data [12–14], strongly supportive of the alternative BL model [7, 15], may finally deflate the older transformation-based Burkitt lymphoma models.

The *in vivo* binding site for the oncoprotein c -Myc in the locus control region of the EBV genome suggested a specific, antiapoptotic role for EBV in Burkitt lymphomagenesis and for the first time provided a direct link between oncogene function and the viral genome in a vulnerable phase of the germinal center precursor cell for a Burkitt lymphoma [8]. This was supplemented by the understanding of the role of epigenetic control [16] and the complex effects of reactive oxygen species (ROS) [17–19], the production of which is initiated by the latency protein EBNA-1.

Primarily, the interest in the tumor biological aspects of EBV and the availability of new methods in cell biology and molecular biology enhanced the characterization of latency-related proteins of EBV such as EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C, LMP-1, and LMP-2, immediate early antigens (IEA),

and early antigens (EA) that control the coordinated synthesis of the structural proteins VCA (viral capsid antigen) and MA (membrane antigen) and of the viral DNA in the replicative phase of the virus. This comprehensive pioneering work was of great advantage when the view on EBV was widened with respect to its importance in clinical virology.

Rather unexpected for a tumor virus, it was found that EBV infects the majority of the human population and that a distinct percentage of infected people develops mononucleosis for which EBV was proven to be the causative agent. Thereby the age at infection seemed to influence the probability of development of clinical symptoms. EBV-dependent mononucleosis was soon recognized as a clinical entity with a nonuniform appearance. Classical symptoms include large lymph nodes, pharyngitis, headache, fever, hepatosplenomegaly, hepatitis, mononuclear cells in the blood, and neurological symptoms. Due to the variability of the combination of symptoms, EBV-associated mononucleosis needs to be differentiated from primary infection with CMV, HHV-6, HIV, rubella virus, mumps virus, and classical hepatitis viruses HAV, HBV, HCV, and HEV. There is symptomatic overlap with toxoplasmosis, brucellosis, leptospirosis, and diphtheria. Clinically overt EBV infection can be misinterpreted as leukemia and lymphoma, and the neurological symptoms require distinction from a wide variety of neurotropic infections. It is less known that acute EBV infection may become overt just as a severe infection of the respiratory tract, especially in younger children (an age group, where clinical symptoms after primary infection with EBV are usually less expected).

The potential of EBV to drive the proliferation of lymphocytes has a strong side effect on the serological diagnosis of other infections that may lead to false interpretations. EBV infection can cause polyclonal stimulation of IgG-producing memory cells, but can also trigger the proliferation of IgM-producing cells without the need of presence of the respective specific antigen. This may lead to an increase in IgG titers directed against a random antigen and to detectable IgM responses without underlying specific interaction of the immune system with the respective agent. This situation may be especially problematic in cases where the symptoms of an EBV infection are misunderstood as being indicative of rubella infection. In this case, appearance of rubella-specific IgM due to polyclonal stimulation by EBV may be misinterpreted as indication for rubella infection. Therefore, in many cases (and all cases during pregnancy), the determination of the significance of IgM responses needs to be assured by parallel testing of serological EBV markers. If these exclude acute EBV infection, EBV-driven polyclonal stimulation is not likely. If, however, a primary EBV infection is diagnosed, further diagnostic measures need to differentiate between polyclonal stimulation due to EBV without infection with rubella virus and the less frequent, but occurring situation of double infec-

tion with both viruses. In the case of HHV-6, where IgG levels often drop below detectability a few years after primary infection, EBV infection later in life through polyclonal stimulation may cause a rise in IgG levels that even impress an experienced performer of viral diagnosis as seroconversion that seems to prove primary infection with HHV-6 [20]. In addition to the phenomenon of polyclonal stimulation, the transient immunosuppressive effect of EBV infection can lead to reactivation of other latent herpes viruses such as HHV-6, CMV, or VZV. This may lead to a strong increase in the level of VZV-IgA that is usually regarded as being indicative for primary infection with VZV or zoster [21].

Likewise, controlled latency of EBV can be influenced by iatrogenic immunosuppression or immunosuppression induced by infectious agents. As a consequence, the pool of cells that are latently infected with EBV may increase. This enhances the chance of induction of viral replication and increase the concentration of viral antigens, which in turn provokes an immunological response that may be misunderstood as indication of EBV being the cause of the clinical situation, while EBV is the target in this case. As massive immunosuppression may lead to the loss of anti-EBNA-1, an EBV-specific marker that is indicative of past infection, serological analysis may become rather ambiguous and require a broader approach for resolution.

2 EBV Serology Using Immunofluorescence

Classical EBV serology was based on the detection of VCA-IgM, VCA-IgG, and anti-EBNA-IgG, supplemented by the determination of anti-EA-IgG, using immunofluorescence techniques [22, 23]. If performed by experienced investigators, using excellent equipment and taking care of standardization, these methods were nicely reproducible and allowed quantitative measurements based on titration. VCA-IgG and VCA-IgM determinations were performed using indirect immunofluorescence with Burkitt lymphoma cell lines like P3HR-1 that were characterized by a small percentage of virus-producing cells surrounded by a surplus of latently infected, nonproductive cells that served as internal negative control. Due to the localization and relatively high local concentration of VCA in the nucleus, anti-VCA-IgG or anti-VCA-IgM was prominent as a bright stain of VCA-positive nuclei, surrounded by the surplus of negative nuclei (Fig. 1). EA-IgG determinations were performed by indirect immunofluorescence as well, using cell lines like Raji that showed no virus replication, but allowed induction of early antigen expression in a distinct percentage of cells. As early antigens are regulatory proteins, their concentration is much lower than that of structural proteins like VCA. Therefore, classical EA-IgG tests in contrast to VCA-IgG and VCA-IgM represented test systems with a suboptimal

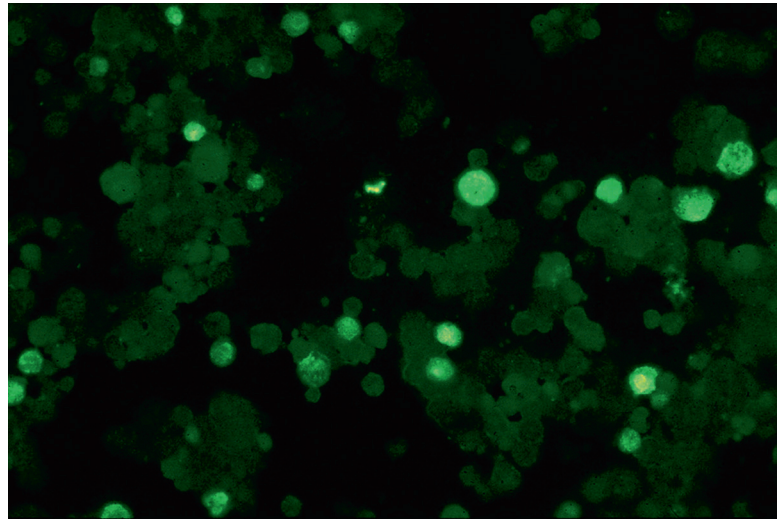


Fig. 1 Determination of VCA-IgG using indirect immunofluorescence

concentration of antigen and therefore only gave a positive response when very high concentrations of anti-EA antibodies were present. Therefore, using indirect immunofluorescence, EA-IgG was usually found positive in exceptional cases and was regarded as a marker for virus reactivation, often in connection with clinically problematic cases. Measurement of EA-IgG in healthy people with past EBV infection was rare. As EBNA proteins are found in extremely low concentrations in EBV-positive cells, detection of antibodies directed against EBNA was not possible when the indirect immunofluorescence technique was used. First of all, this is a good message, because otherwise VCA-antibody determination would not have been possible due to overlap with anti-EBNA reactivity. For the detection of anti-EBNA, an additional amplificatory step was required. This step took advantage from the intriguing finding that antibodies against EBNA always seem to be complement-binding antibodies, even years after primary infection, although complement-binding antibodies are usually shorter-lived. After binding of anti-EBNA to its target protein, the assays were washed, and the antibody complexed to EBNA was confronted with human complement that bound to the complex and built a platform for subsequent binding of many anticomplement IgGs labeled with the fluorescence label (Fig. 2).

A positive VCA-IgG and VCA-IgM finding was considered as evidence for an acute EBV infection. Negative VCA-IgM in case of positive VCA-IgG was considered as past infection. Retrospective analysis showed that this approach did not lead to correct conclusions in all cases and therefore had a high degree of insecurity when an individual case had to be diagnosed. The major problem was associated with IgM tests. Competition between IgG and IgM caused suppression of IgM detection, and therefore the determination of the true IgM status would have required the removal of

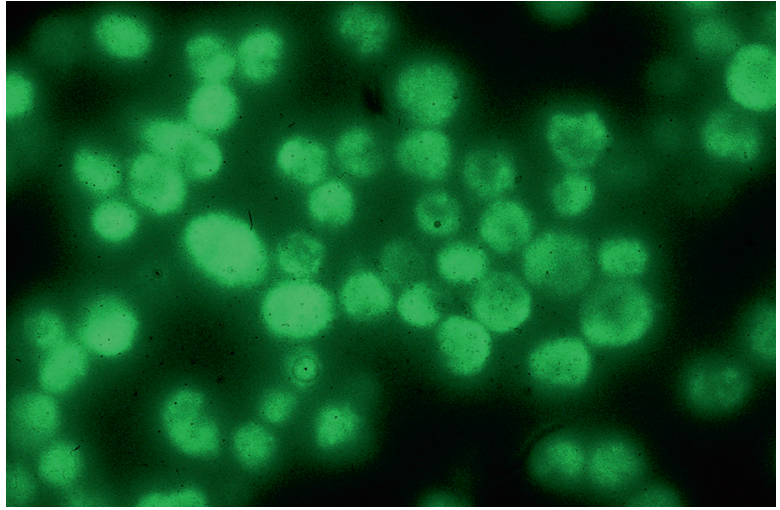


Fig. 2 Anti-EBNA-1 test using anticomplementary immunofluorescence

IgG in all serum samples before the IgM test. But even then, a significant percentage of sera from patients with secured primary EBV infection did not show positive IgM responses. In the study by Schillinger et al. (1993), nearly 20% of sera from acute infections were IgM negative. Several reasons sum up to this problematic number: (1) the IgM response is below the detection level; (2) the IgM response would have been detectable only for an extremely short period, and the first serum was taken too late; (3) the IgM test was not sufficiently sensitive; and (4) the competing IgG was not sufficiently removed. On the other side, VCA-IgM responses persisted for up to a year and more after acute infection, causing misinterpretation if only the late sera were tested [23, 24]. It was therefore neither possible to derive a reliable indication for an acute EBV infection from a positive VCA-IgM finding nor could an acute infection be ruled out with absolute certainty on the basis of the absence of an IgM response. Therefore, many laboratories used anti-EBNA testing as regular supplementary method, as anti-EBNA was regarded as a late marker. Therefore, its positivity was taken as clear exclusion of acute EBV infection. However, it was soon realized by many investigators that nonselective anti-EBNA testing by anticomplementary immunofluorescence was not as specific as initially anticipated. The reason for this is due to anti-EBNA-2 positivity in about 30% of acute infections at the onset of clinical symptoms [24]. The problem was solved through the use of Burkitt lymphoma cells with deleted EBNA-2 gene that allowed to focus on anti-EBNA-1 responses specifically. A positive anti-EBNA-1 response therefore was indicative of secured past infection with EBV, provided parallel testing with an EBV-negative Burkitt lymphoma cell line produced evidence for the absence of anticellular reactivities that mimicked anti-EBNA-1 responses in some cases. The problem of anticellular reactivity was especially

overt for anti-EBNA testing, as all cells contain EBNA, and therefore the test in its classical version has no internal negative antigen control. As anti-EBNA-1 is detectable at the earliest 4 weeks after the onset of the disease after acute EBV infection, it was possible to rule out with certainty an acute EBV infection in the case of positive anti-EBNA-1. Whereas a specifically positive anti-EBNA-1 response (especially in combination with a positive VCA-IgG) clearly excluded an acute EBV infection, negative anti-EBNA-1 in combination with VCA-IgG did not allow a conclusive interpretation, though this constellation was regularly found in acute infections. The reason for this failure is the fact that about 6% of healthy persons with past EBV infection never develop a detectable anti-EBNA-1 response and that previously anti-EBNA-positive people may lose anti-EBNA-1 selectively during massive immunosuppression due to iatrogenic intervention or immunosuppression based on infections, immune disorders, or tumors.

Therefore, even optimally performed and controlled EBV serology using immunofluorescence tests found its limitation by (1) about 5% of unresolvable cases due to anticellular reactivities and (2) insecure differentiation between negative anti-EBNA-1 due to primary infection from negative anti-EBNA-1 due to the failure to develop this marker or due to secondary loss of this marker. Therefore, a high degree of uncertainty with respect to the unequivocal diagnosis of acute EBV infections was not resolvable, whereas past EBV infections were diagnosed with high confidentiality. Though these cases represent the majority in routine diagnosis, the situation as a total was not bearable and required further steps for improvements. The use of avidity determination as an additional diagnostic tool in combination with immunofluorescence techniques will be discussed in a later section. The following sections discuss the use of recombinant viral proteins for further improvement of EBV serology.

The majority of diagnostic labs is not using the anti-EBV indirect immunofluorescence assay anymore. Among those labs, only a very small minority of experts are preparing their glass slides which carry infected cells expressing EBV antigens by themselves. Therefore, here we do not provide a step-by-step protocol for preparing glass slides and performing the indirect anti-EBV immunofluorescence assay. For those who choose to use it routinely, there are several manufacturers who sell ready-to-use assay IFT kits with detailed instruction manuals included.

3 The Use of Recombinant EBV Proteins in Western Blots and Line Assays

The development of immunoblots (Western blots) with recombinant antigens represents an important milestone in the improvement of EBV diagnostics [25]. In particular, p72 (EBNA-1, viral gene BKRF1, i.e., BamHI restriction fragment K, rightward frame 1),

p23 (BLRF2, a component of VCA), and the early proteins p54 (BMRF1) and p138 (BALF2) were used in the first generation of commercially available Western blots [26]. Later, p18 (BFRF3), another component of VCA, was added in various assays. It is important to note that the antibody response to unprocessed p18 is similar to that directed against p23, i.e., it is a marker that is detected relatively early in infection and that defines specific seropositivity. In contrast, Mikrogen patented a processed p18 that provided only epitopes that are recognized late in infection, and thus this marker is useful as a late marker, in analogy to p72. Sera from patients with acute EBV infection are regularly negative for p18(mod)-IgG during the first 3 weeks after onset of disease.

Compared to assays based on immunofluorescence, the blot had several advantages. (1) Because recombinant, highly purified antigens were used, the problem of anticellular reactivity (a problem in 5 % cases tested by immunofluorescence) was no longer an issue. (2) Due to the use of recombinant antigens, the test system now contained sufficiently high concentrations of antigens. Thus, the test result was solely dependent on the concentration of antibody and therefore their precise quantitation was relatively easy.

Whereas the patterns of antibodies directed at VCA and EBNA-1 were rather congruent, independently of whether they were detected by immunofluorescence or immunoblots, the determination of anti-EA-IgG showed major differences and was the cause of frequent misinterpretation. Due to the low concentration of EA proteins in immunofluorescence, only sera with very high titers gave a significant response with this method. Frequently, these were cases with acute infections or reactivations after immunosuppression. EA-IgG (detected by immunofluorescence) was therefore regarded as a marker indicative for recent active interaction between the immune system and EBV. In contrast, the high concentration of recombinant EA in immunoblots allowed to determine the true serostatus with respect to anti-EA. Depending on the EA markers used, at least 20 % of healthy people with past EBV infection were positive [27, 28]. Also, unexpectedly and in contrast to the experience obtained before with immunofluorescence, EA-IgG detectable by immunoblot very frequently stayed positive over very long periods. In addition, the kinetics of appearance of EA-IgG during acute EBV infection showed a major difference, depending on the method used. Whereas EA-IgG determined by immunofluorescence usually became detectable after VCA-IgG, EA-IgG detected by sufficiently high concentrations of recombinant EA usually was the first positive marker in acute EBV infection analyzed by immunoblot.

Whereas the molecular weight of viral proteins determined their position on the immunoblot (Western blot) stripes, the line assay, in which recombinant antigens were transferred onto nitrocellulose after extensive purification, allowed to group the antigens according to early and late antibody response with respect to the onset of disease. A

high degree of purification of proteins was necessary to ensure that the degree of specificity of responses determined by the line assay was the same as determined by immunoblot [29]. It has to be pointed out that not the blotting procedure itself determines the specificity reached by either method, but the degree of purity of the applied proteins.

The distribution of a specific protein within a band on immunoblots is characterized as Gaussian distribution, due to the movement of the proteins during electrophoresis. In contrast, purified proteins applied to the line assay show an equal concentration over the area of a distinct band. This difference has a major impact on the antigen/antibody reaction and its quantification. Antibodies interacting with a protein band on immunoblots face the highest antigen concentration in the middle of the band, with decreasing concentrations to both sides. Therefore, the reaction kinetics with a given antibody concentration is the highest in the middle and decreases within the band. This complex reaction is the basis for the finding that testing of serum dilutions in an immunoblot does not only lead to a decrease in staining but is paralleled by a change in the broadness of the band (Fig. 3). In contrast, testing dilutions of sera on line assays results in a decrease of the intensity, but not in the size or shape of the bands (Fig. 4). This is due to the equal

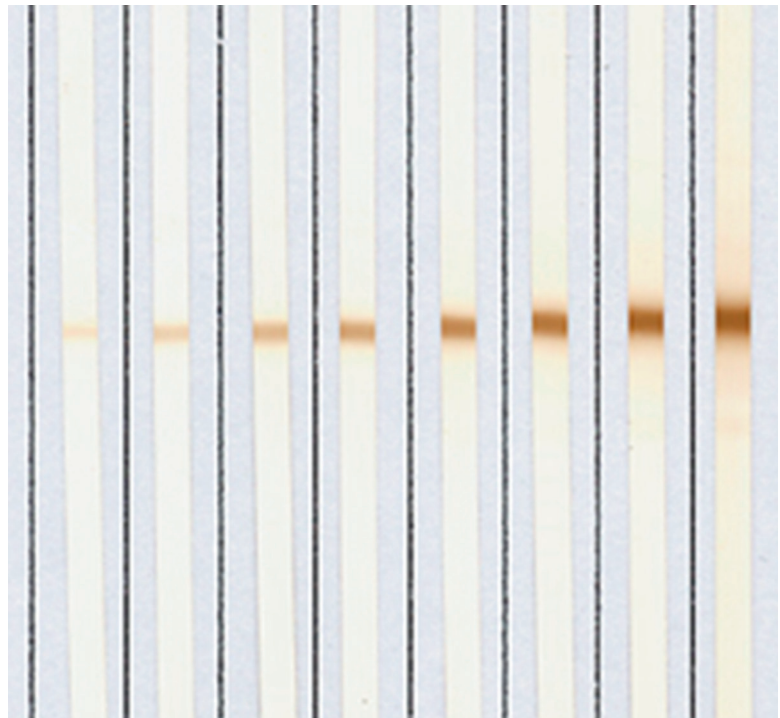


Fig. 3 Titration of a positive serum using immunoblot stripes. Dilution of the serum (from *right to left*) causes a decrease in intensity as well as a change in the size of the band

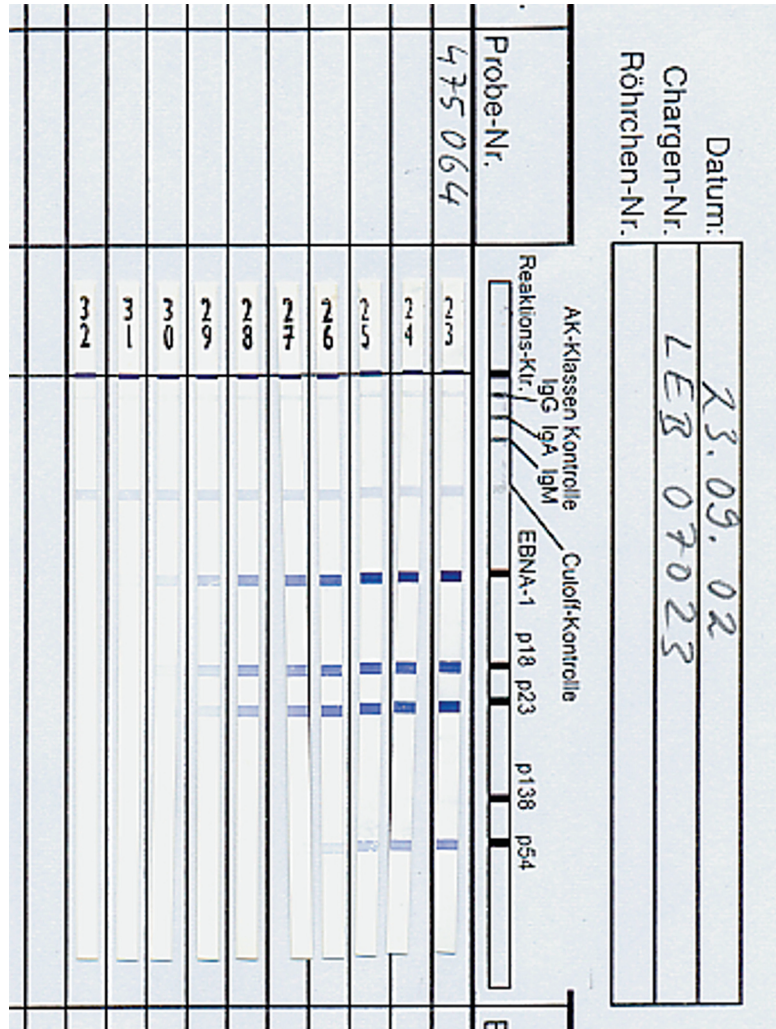


Fig. 4 Titration of a positive serum using a line assay. Dilution of the serum (from *right to left*) causes a decrease in intensity without a change in the size of the band

distribution of antigen within the band leading to homogenous reaction kinetics at all sites of the band. Therefore, tests that require rather precise measurements, like avidity determination, are preferably established by line assays.

For the practical blot procedure, we refer to the test manuals which are always included in the assay kits of the respective supplier and have to be followed in detail. Test strips, dilution and washing buffers, and conjugate and substrate solutions are always included in assay kits. In the following, we give an exemplary short description for a blotting procedure:

1. Pipet 2 mL of dilution or wash buffer into each well on an incubation tray. Wash buffer is a phosphate-buffered salt

solution containing skim milk powder to block nonspecific binding of serum antibodies to the test strips.

2. Using a plastic forceps, place a nitrocellulose test strip into the wells without skin contact and without damaging the strip.
3. Submerge the strip through gentle shaking.
4. Add 20 μL of the serum to be analyzed.
5. Cover the tray with a plastic lid and shake gently for 1 h on a horizontal shaker at room temperature.
6. After 1 h of serum incubation, pipet off the liquid, using a fresh pipet tip for each well or an aspirating device.
7. Wash off serum traces by three cycles of adding 2 mL of washing buffer, shaking 5 min, and pipetting off the washing buffer, always taking care to avoid cross contamination with serum antibodies between the wells.
8. Add 2 mL of freshly prepared conjugate solution and shake for 45 min at room temperature, while the tray is covered with a plastic lid. Conjugate contains an antihuman rabbit antibody labeled with horseradish peroxidase (HRP). Conjugates are specific for human IgG, IgM, or IgA. Thus, take care to choose the appropriate conjugate for the type of strip (IgG, IgA, or IgM) to be analyzed.
9. Pipet off the conjugate solution, using a fresh pipet for each well or aspirating device.
10. Wash off conjugate traces three times as above.
11. Add 1.5 mL of substrate solution and incubate for 5–10 min, by shaking gently at room temperature. Substrate solution contains the chromogenic substrate tetramethylbenzidine which is oxidized by the HRP that is conjugated to the secondary rabbit antibodies.
12. Pipet off the substrate solution or use an aspirating device.
13. Wash three times with deionized water as above.
14. Dry test strips for 2 h between two layers of absorbent paper.
15. Read out results according to the respective manufacturer's instruction manual.
16. Store the strips dry and protected from exposure to light.

4 Avidity Determination as an Additional Diagnostic Tool

Affinity maturation of the IgG response is known as a unidirectional and regularly occurring process during the establishment of an immune response [30]. The biological basis for this process is determined (1) by the need of IgG-expressing B-cell clones to

receive the specific antigen as stimulus for survival and proliferation and (2) by constant mutagenesis in the Fab structure. A few cell clones thus may eventually present IgG that binds the antigen with higher affinity. These clones are positively selected, whereas proliferation and survival of the parental clones is impaired. Stepwise cycles of mutagenesis and selection finally lead to clones that generate IgG with high affinity for the specific epitope structure. Plasma cells derived from these clones will be the source of high-affinity IgG. Affinity represents a complex biophysical principle that is not trivial to measure. For routine diagnosis, therefore, the determination is restricted to the strength of the binding between antigen and antibody. This measurement has been termed avidity determination and is representative for overall affinity. Please note that formerly the term avidity was also used to describe the complexity of antibody populations, e.g., against flagella of bacteria. The historic term has a different biological background and is not suitable in the context of avidity as defined above. Avidity determination has been applied to EBV serology and has been found to resolve certain problematic diagnostic cases [31, 32]. These initial studies used quantitative immunofluorescence and 6 M urea for the disaggregation of antibody/antigen complexes. Test slides were incubated with serum dilutions, and parallel slides were either treated with secondary fluorescent antibodies as usual or were treated with 6 M urea for 3 min, washed, and then processed like the control. The comparison between the titers was used to determine the avidity index. This procedure required a high standardization of quantitation of immunofluorescence titers. The maturation kinetics of VCA-IgG avidity was very fast. Only during the first 10 days after the onset of disease all sera from acute EBV infections showed low avidity. Thus, the application was more or less restricted to sera that were taken immediately after the onset of disease (a rare situation in present health management). Avidity determination was established for immunoblots and line assays in a patented application form. This approach has the advantage that the avidity of antibodies directed against several distinct antigens can be determined in one test. The major advantage was, however, that due to the larger concentration of recombinant antigen in the test system, the measured kinetics of avidity determination was much slower and with a later onset than the determination by immunofluorescence. This seemingly paradoxical finding is explained by less competition between antibodies with high, intermediate, and low affinity in the immunoblot and line assay. In the immunofluorescence test with its lower concentration of antigen, high-affinity antibodies will compete out antibodies with intermediate or low affinity and thus overestimate high affinity. When this competition is lowered through supply with higher concentrations of antigen (immunoblot and line assay), sera containing a mixture of low-, intermediate-, and high-affinity antibodies can be differen-

tiated from sera that only contain high-affinity antibody. In this way, the true status with respect to affinity is determined, and the kinetics of maturation allows conclusions even if the sera are not taken immediately after the onset of disease. It was found that the determination of avidity for the markers p23-IgG, p18-IgG, and p72-IgG was useful for discrimination between acute and past EBV infections, whereas avidity determination of markers from the EA-IgG complex was not suitable due to high variability.

5 Variability of the Serological Response After EBV Infections

The tests of non-preselected sera using a commercial line assay (Mikrogen) showed that the serological responses following EBV infections are defined by a certain basic pattern, but that a high degree of variability can also be observed regularly due to the stochastic processes inherent to the immune reaction. This is summarized in Tables 1 and 2.

From 1577 non-preselected sera, which were tested with the line assay, a relatively low percentage was identified as seronegative, and an even lower percentage was identified as acute infection. The majority of sera displayed the picture of a past EBV infection, whereby most sera showed the classical picture with both late markers (p72-IgG and p18-IgG). A very small percentage exhibited one or both late markers in a weakly pronounced way. In 7.4 % of cases, p72-IgG was negative, but the second marker p18-IgG (which exhibited high avidity) allowed the diagnosis of “past EBV infection” in these cases. Conversely, only 0.8 % of past infections exhibited negative p18-IgG and positive p72-IgG. This finding underlines the improvement of diagnostics through combining two late markers and thus preventing inaccurate findings. It shows in particular the stochastic component which plays an important part in serological responses. The finding allows the assumption that the immune system of an affected person incidentally failed to

Table 1

Main groups within 1577 cases tested in EBV serology using a line assay with recombinant antigens

	Number	Percent
Total number of sera	1577	100
Seronegative for EBV	90	5.7
Borderline	7	0.4
Acute EBV infections	42	2.6
Past EBV infections	1438	91.2

Table 2
Variability of the serological response in past EBV infections determined by a line assay with recombinant antigens

	Number	Percent
Past EBV infections	1438	100
Classical (p72-IgG and p18-IgG > +)	1154	80.2
p72-IgG and p18-IgG +	7	0.5
p72-IgG +, p18-IgG > +	86	6.0
p18-IgG +, p72-IgG > +	29	2.0
p72-IgG neg., p18-IgG > +	106	7.4
p18-IgG neg., p72-IgG > +	11	0.8
p23-IgG neg.	20	1.4
Isolated p23-IgG (high avidity)	5	0.3
Isolated p18-IgG (high avidity)	5	0.3
Isolated p72-IgG (high avidity)	3	0.2
Recent EBV infections (p18-IgG low avidity, p72-IgG neg.)	12	0.8

Using the cutoff band as reference, we differentiated between negative, +, ++, +++, and ++++ positive sera. Sera showing ++ to ++++ reactivity were grouped and named "> +". For simplicity, the term "p18-IgG" has been used for IgG directed against modified p18, which represents a late marker

form antibodies against p72 or p18. By accepting this point of view, it can be predicted that, according to the law of probability, a small number of cases should occur in which both late markers are not formed, despite past EBV infection. Five such cases have in fact been found within this study and they confirmed the plausibility of the preceding assumption. Of course, on a first glance, these p18-IgG/p72-IgG-negative cases of past EBV infections resemble acute infections, in which both late markers have not yet been formed. However, a differentiation could be achieved here with the aid of avidity determination, as the rare cases of past infection with missing p72- and p18-IgG showed p23-IgG of high avidity.

The stochastic problem of nonrecognition of epitopes of a certain antigen also arises in the case of p23. It is seen that in 1.4% of cases with past EBV infections, p23-IgG is not detected. However, these cases could still be identified with certainty as past EBV infection as they showed p72-IgG and p18-IgG of high avidity. The combination of the absence of certain markers in rare cases is the basis for the predictable and actually found constellation in which "isolated p72-IgG" and "isolated p18-IgG" occur. Finding such rare constellations is therefore not a shortcoming of the test system, but rather evidence of the precision with which the stochastic variability of the serological response is measured. It proves that a

reliable finding can be derived from it in every single case, despite being different from the basic pattern.

After all, a small fraction of cases exhibit the characteristic of a recent EBV infection in which only one of the two late markers, namely, p18(mod)-IgG, is positive, while p72-IgG is still absent. A differentiation between “recent” and “past (without p72-IgG)” is here achieved through the detection of a low-avidity p18-IgG in combination with an p23-IgG that already shows high avidity.

These cases demonstrate the efficiency of EBV serology by line assays and show the significance of the avidity determination with the aid of this method. The application of the *recomLine* EBV assay (Mikrogen) in avidity determination naturally allows determining the avidity of antibodies against several antigens in parallel. Figure 5 outlines the idealized progression (basic pattern) of the serological response and its avidity maturation following an acute EBV infection. At first, a successive occurrence of the markers BZLF1-IgG, p54-IgG, p138-IgG, and p23-IgG is noticeable in the beginning, before the late markers p18(mod)-IgG and p72-IgG appear. p23-IgG exhibits low avidity early on. The late markers normally appear successively, first p18-IgG and then p72-IgG. They too initially show low avidity, which matures to higher avidity later on. With it the approach to a rational and reliable appraisal is specified: if both late markers are present, an acute EBV infection can be ruled out. An additional investigation of the status with the aid of avidity determination will normally not be necessary in case of these sera. If one of the two late markers is missing, then the detection of the other late marker nevertheless allows, supported by the high avidity of the detected marker and the high avidity of p23-IgG, to reliably diagnose the infection as “past EBV infection.” An analogous procedure would be followed when in very rare cases both markers are present, while p23-IgG is missing.

Through the use of line assays with recombinant antigens in combination with avidity measurement in the same test systems, a high diagnostic reliability can be achieved for each individual case. Avidity determination is only necessary in cases which are seropositive for EBV, but have no detectable late markers. The additional time and effort required for an avidity determination is therefore reserved for a selected segment of sera, but then it will ensure an accurate diagnosis in the individual case.

6 Diagnostic Examples

Figure 6 exemplarily demonstrates the use of a commercial line assay with recombinant antigens (Mikrogen) for the serological analysis of acute EBV infection in Case #1. Cases #2–#4 are then discussed without showing the original line assays. *Case #1:* a 17-year-old girl showed the symptoms of infectious mononucleosis. The first serum sample was taken 1 day after the onset of disease.

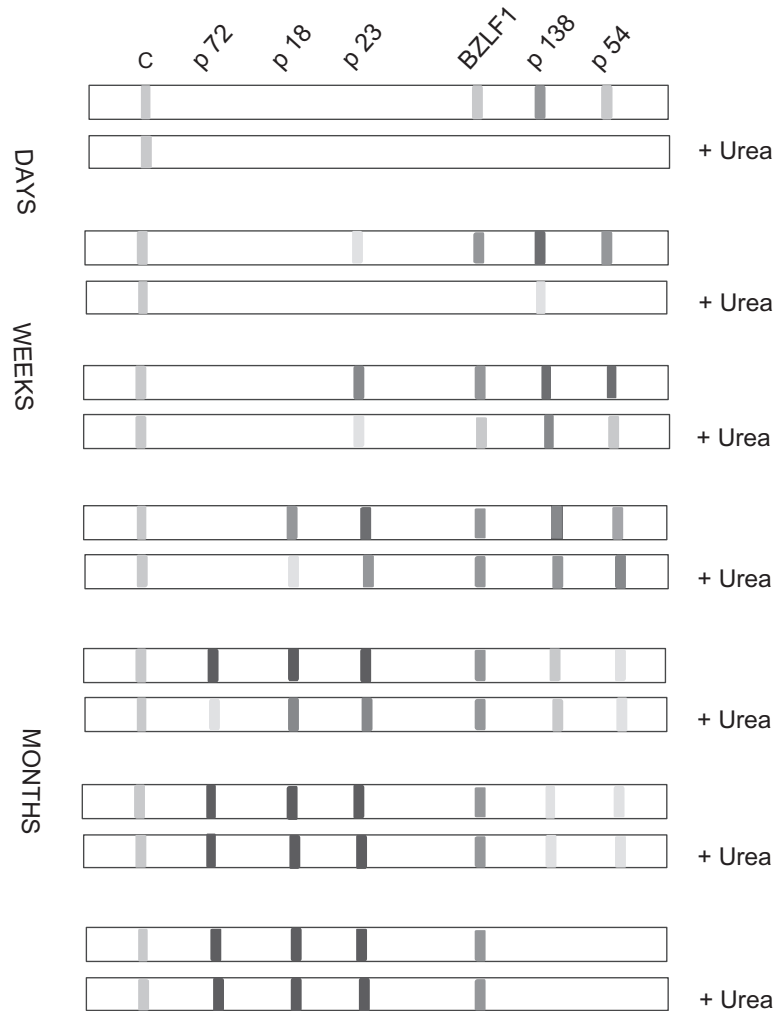


Fig. 5 Basic pattern of appearance of the IgG response after EBV infection measured by the recomLine assay. At the onset of disease, antibodies against BZLF1 (immediate early protein), p138 and p54 (early proteins), are the first ones to be detectable and usually still exhibit low avidity, as demonstrated by the urea-treated parallel test strip. Low-avidity IgG against p23 (viral capsid antigen) appears next. While the avidity of p23-IgG is maturing, the late markers p18-IgG (directed against the modified p18, viral capsid antigen) and p72-IgG (directed against EBNA-1) appear after 3–4 weeks after the onset of disease and show avidity maturation later on. In past infection, p23-IgG, p18-IgG, and p72-IgG exhibit high avidity. IgG directed against p138, p54, and BZLF1 may disappear or remain detectable. In contrast to p72-, p23-, and p18-IgG, IgG against the BZLF1, p54, and p138 may exhibit low avidity even in past infection. This finding is explained by the recognition of new epitopes during silent and clinically irrelevant reactivation of the virus. Therefore, avidity determination for the distinction between acute and past infection has to be restricted to p23-, p18-, and p72-IgG. As shown in Table 2, in a significant percentage of people, certain markers may be missing. This diagnostic problem can be resolved through the determination of two late markers, combined with avidity measurement.



Fig. 6 Application of a commercial EBV line assay, including avidity determination. Serum #1 was taken at the onset of EBV-related symptoms in a 17-year-old girl and was tested in the line assay with recombinant antigens. The next serum (serum #2) was taken 4 weeks later and final control was taken 8 months later (serum #3). Serums #1–3 are directly compared (positions 1–3). Positions 6–7, 9–10, and 12–13 show the avidity determination for sera #1–3. Positions 6, 9, and 12 are the control tests; positions #7, 10, and 13 are the tests treated with urea after first incubation. Positions 17–30 are repeats of the avidity determination and demonstrate the interassay reproducibility of the test system

It was positive for p138-IgG and p54-IgG. P23-IgG was below the cutoff. This constellation indicated seropositivity for EBV. Based on our knowledge of the variability of the serological response as outlined in the chapter before, the constellation was in line (1) with the assumption of acute infection and (2) with the less likely assumption that it was a past infection with missing p72- and p18-IgG and very low p23-IgG. Serum #2, taken 4 weeks later, showed the increase in the p23-IgG response that was still of low avidity and thus allowed the conclusive diagnosis of acute EBV infection. A control after 8 additional months confirmed this statement and showed the appearance of the late markers p72- and p18(mod)-IgG that showed high avidity, like p23-IgG.

In most cases, p23-IgG is present in the first sample and thus avidity determination is possible and useful to secure the

serological diagnosis. This is especially relevant in cases where the clinical symptoms are not convincingly clear or the age of the patient is not typical for primary EBV infection.

Case #2 is a 50-year-old male patient with lymphadenitis and fever. He was found positive for VCA-IgG and VCA-IgM using indirect immunofluorescence and anti-EBNA-1 negative in an ELISA test. Though this constellation is typical for acute EBV infection, in light of the untypical age of the patient for primary infection, the task was to exclude that positive VCA-IgM was due to reactivation, and negative anti-EBNA-1 was due to loss of anti-EBNA-1 under immunosuppression. The serum was found positive for p23-, p138-, and p54-IgG in the line assay with recombinant antigens. All three markers were of low avidity and thus allowed secure diagnosis of acute EBV infection.

Case #3 is a 36-year-old male patient from the oncological ambulance, showing multiple lymphomas. Testing his serum in the line assay with recombinant EBV antigens showed p23- and p54-IgG of low avidity and p138-IgG of high avidity. Low avidity of p23-IgG combined with the absence of late markers was taken as indication for acute EBV infection.

Case #4 is a 22-year-old male patient with symptoms of infectious mononucleosis. The serum showed isolated p23-IgG; thus, the missing late markers were indicative for acute infection, but the absence of p138- and p54-IgG was unusual. Therefore, avidity determination was used for clarification. The isolated p23-IgG was of low avidity and thus allowed the serological diagnosis of acute EBV infection. This case is contrasted by numerous cases (data not shown) where isolated p23-IgG was of high avidity and therefore did not allow the diagnosis acute EBV infection. Rather, they seemed to represent rare cases with both late markers missing despite past infection.

7 Additional Methods for EBV Serology

The section on immunofluorescence techniques has taught us the principles and problems of EBV serology; the subsequent sections have shown us how the use of recombinant antigens in combination with avidity determination can resolve most cases. Thereby the high variability of the serological response has to be kept in mind. Based on these facts, any format of a test system can be used that utilizes defined, purified antigens at sufficient concentration and combines several antigens to allow differentiation between early and late markers and to cope with the stochastics in the immune system that leads to unusual cases. Therefore, the evaluation of any new test system must not be based solely on selected “typical cases” for past and acute EBV infections as these only reflect part of reality (as shown in Table 2).

8 Enzyme Immunoassay (EIA) Testing

EIA tests by several manufacturers with purified recombinant antigens, native antigens, or synthetic peptides have been on the market for many years [33, 34]. Certainly, their advantage is their speed of operation and, especially in the case of robotic systems [35, 36], significantly less hands-on time, compared to the line assay. This fact and the pricing is certainly the reason that most commercial diagnostic virology labs use the EIA format for conducting EBV serology. All EIAs deal with the same restricted set of antibodies, i.e., anti-VCA-IgM, anti-VCA-IgG, and anti-EBNA1-IgG, some of them including also anti-EA [33, 34]. Principally, the three main test parameters should suffice to clearly determine seronegativity as well as past infection with classical serological markers and thus allow a secure conclusion in about 80 % of routine sera. Due to the variability of the serological response, these three markers are, however, not sufficient to unequivocally discriminate between acute infection on one side and past infection with either missing anti-EBNA-1 or secondary loss of anti-EBNA-1 on the other side. VCA-IgM is not discriminating between these cases, as it may be aberrantly missing during acute infection in some cases and may either persist or become reactivated in cases with past infection but negative anti-EBNA-1. Therefore, up to 20 % of cases from routine diagnosis can be expected to require other methods, like the line assay in combination with avidity determination in addition to EIA testing. In line with these findings, it is not surprising that, in a comparative analysis with IFA as the reference standard of the time, Gärtner et al. concluded that among four manufacturers of EIAs, due to the different composition of antigens and different interpretation criteria, only two of the tests were useful. Therefore, the authors emphasized the need for a standardization of the interpretative criteria between manufacturers [34]. A more recently developed robotic chemiluminescent microparticle assay (CMIA) system, which belongs to the larger EIA test group as well, compared favorably with the IFA and the line assay in two comparative analyses, with sensitivities and specificities for correctly categorizing primary infection, past infection, and seronegativity of well above 90 % [35, 36]. If sequential testing was applied, i.e., anti-EBNA-1 testing was done first, a substantial cost saving was obtained. In cases of positive anti-VCA-IgM, additional anti-CMV-IgM testing was recommended in one study, because cross-reactivity of anti-VCA-IgM in CMV primary infections was frequently observed [36]. The overall favorable performance of the CMIA accords with our own experience when we compared CMIA results for 90 routine sera with the results of the line assay as the reference standard of today. Only less than 5 % of our routine serum panel could not be clearly resolved by the CMIA (unpublished data). Therefore, it is reasonable to use a

suitable robotic EIA format for routine testing in the commercial diagnostic virology laboratory. However, a small percentage of more complex or problematic clinical cases which cannot be resolved by EIA tests should be followed up with the line assay which provides a more differentiated conclusion, including the two above-mentioned late markers and avidity testing.

9 PCR Testing

Besides CMV, EBV is the major viral pathogen in solid organ or bone marrow transplant patients demanding regular viral load determination in patient groups at high risk of acquiring posttransplant lymphoproliferative disease (PTLD). The risk to acquire EBV disease is particularly high, when the organ donor and recipient are discordant and the organ recipient is seronegative for EBV. Because serological reactivation, e.g., as documented by anti-VCA-IgM positivity or additional parameters, does not correlate with the viral load in immune-suppressed patients, EBV serological testing is not useful at all in this patient group, besides for establishing past infection [37]. Thus, while serological testing is applied to differentiating primary infection, past infection or seronegativity in individuals with a functional immune system, EBV testing in patients with immune suppression or EBV-associated malignancies is the domain of PCR testing. Together with quantitative anti-EBV-IgA antibodies as an adjunctive marker, blood viral load testing may be used for screening, for the detection of relapses, and for therapy monitoring of EBV-associated malignancies in high prevalence areas [38, 39].

Numerous commercial and in-house quantitative PCR formats are in use. If utmost sensitivity of virus detection is desired, the internal viral BamHI W-repeat which occurs between 7 and 11 times per viral genome is usually used as PCR target. For exact viral load quantitation, however, a viral single-copy gene must be the target of choice. Because many different viral genes are targeted by PCR assays, international standardization of quantitative PCR results may improve comparability in the future. Regarding the sample of choice, any clinical material may be analyzed for viral load, i.e., serum, plasma, whole EDTA blood, peripheral blood mononuclear cells (PBMCs), or tissue biopsies. Suspicion of EBV-positive PTLD must be raised, when the viral load rises between 10,000 and 100,000 copies/mL serum, plasma, or whole blood or between 1000 and 10,000 copies/million isolated PBMCs. However, it is important to keep in mind that there is considerable variation in viral loads, that also much lower viral loads can signify manifest PTLD, and that no clearly defined threshold and no single preferred type of clinical material exist in diagnosing PTLD. In

most laboratories, whole blood is used for initial screening [40]. In case of doubt, more than one type of sample have to be tested, and serial monitoring of the same sample type is helpful to notice dynamic changes also of low viral loads in time and thereby speed up therapeutic intervention [38, 41]. Particularly, PTLTD of the central nervous system sometimes coincides with zero viral copies/mL of peripheral blood. In such cases, in situ hybridization for EBV-encoded small nuclear RNAs (EBERs) in tissue sections from brain biopsies is frequently needed to unequivocally diagnose EBV-positive PTLTD [42].

In the following, we give a short description of the real-time PCR procedure, as it is used in the Regensburg Clinical Virology lab to quantify EBV DNA [43]:

1. Isolate nucleic acids from clinical material (serum, EDTA blood, EDTA plasma, PBMCs, or tissue biopsies) using an isolation kit according to the instructions of the manufacturer, e.g., QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany).
2. Prepare the master mix according to the instructions of the manufacturer. The master mix should contain dNTPs, heat-stable DNA polymerase enzyme, buffer, salts, primers, and fluorescent probe, according to the instructions of the manufacturer.
3. From a total elution volume of 200 μ L, add 5 μ L of isolated nucleic acids for each PCR reaction in a 96 well plate, yielding a total volume of 50 μ L per reaction.
4. Cover the 96 well plate and start the programmed PCR machine.
5. We use TaqMan probes and Step-One-Plus machines from ABI.
6. Sense primer: 5'-TGACCTCTTGCATGGCCTCT-3'.
7. Antisense primer: 5'-CCTCTTTTCCAAGTCAGAATTTGAC-3'.
8. Probe: FAM-5'-CCATCTACCCATCCTACACTGCGCTTCA-3'-TAMRA.
9. This primer-probe set amplifies a segment from the alkaline exonuclease gene of EBV (BGLF5).
10. The PCR profile consists of 45 cycles at 95 °C for 15 s and 60 °C for 60 s, preceded by a denaturation step at 95 °C for 10 min, then cooling to 25 °C, until the machine is opened.
11. Tenfold serial dilutions of plasmids containing the respective target sequence have to be amplified in each run, serving as quantitation standard and positive control. The detection limit of this quantitative EBV assay is at 2.5 plasmid copies per reaction, corresponding to 500 genome equivalents (641 IU/mL) per mL serum.

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