

## Serological diagnosis of Epstein-Barr virus infection: Problems and solutions

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### Abstract

Serological tests for antibodies specific for Epstein-Barr virus (EBV) antigens are frequently used to define infection status and for the differential diagnosis of other pathogens responsible for mononucleosis syndrome. Using only three parameters [viral capsid antigen (VCA) IgG, VCA IgM and EBV nuclear antigen (EBNA)-1 IgG], it is normally possible to distinguish acute from past infection: the presence of VCA IgM and VCA IgG without EBNA-1 IgG indicates acute infection, whereas the presence of VCA IgG and EBNA-1 IgG without VCA IgM is typical of past infection. However, serological findings may sometimes be difficult to interpret as VCA IgG can be present without VCA IgM or EBNA-1 IgG in cases of acute or past infection, or all the three parameters may be detected simultaneously in the case of recent infection or during the course of reactivation. A profile of isolated EBNA-1 IgG may also create some doubts. In order to interpret these patterns correctly, it is necessary to determine IgG avidity, identify anti-EBV IgG and IgM antibodies by immunoblotting, and look for heterophile antibodies, anti-EA (D) antibodies or viral genome using molecular biology methods. These tests make it possible to define the status of the infection and solve any problems that may arise in routine laboratory practice.

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### INTRODUCTION

Epstein-Barr virus (EBV) or human herpesvirus 4 is ubiquitous, and about 90% of adults throughout the world have antibodies against it<sup>[1]</sup>. Acute infection is usually asymptomatic in immunocompetent children, and manifests itself as mononucleosis in 30%-50% of immunocompetent adolescents and adults<sup>[2,3]</sup>. Especially in immunocompromised patients, EBV is associated with various lymphoproliferative disorders and some neoplastic diseases, including Burkitt's lymphoma and nasopharyngeal carcinoma.

Like other herpesviruses, EBV has a productive lytic cycle and a latent phase. B lymphocytes are infected after the viral envelope glycoprotein gp350/220 has bound to the CD21 cell receptor, which is also the receptor for the C3d component of complement<sup>[4]</sup>. During the lytic cycle, regulatory proteins belonging to the immediately early antigen (IEA) and early antigen (EA) groups are synthesized to allow the production of viral DNA (EBV-DNA), the virion structural proteins (viral capsid antigen, VCA) and membrane proteins (MA). The lytic cycle leads to the destruction of infected cells and the production of viral particles, but EBV can also persist in host cells without complete virus production by replicating ex-

trachromosomal nucleic acids (episomes) following the expression of a few selected viral genes<sup>[1]</sup>. The expression of these genes leads to the immortalization of B cells and their transformation into proliferating blasts<sup>[5,6]</sup>. In immunocompetent patients, cytotoxic T lymphocytes and NK cells control the growth of transformed cells during primary infection, particularly the CD8+ T cells directed against antigens of the lytic cycle<sup>[7]</sup>. These cells are also directed against antigens of the latent phase<sup>[8]</sup>, but the response is insufficient to ensure their complete eradication, and the virus can persist throughout life with low or intermittent levels of virion production<sup>[9]</sup>. After recovery, it has been estimated that about 1 in 10 000-100 000 memory cells contain EBV DNA in episomal form<sup>[10]</sup>. During the latent phase, EBV nuclear antigens (EBNAs) and three latent membrane proteins (LMPs) are expressed in infected cells. EBNAs represent a complex of at least six proteins (EBNA 1-6). EBNA-1 is responsible for maintaining the episomal state of EBV DNA in infected cells, and EBNA-2 seems to be involved in the immortalization of B lymphocytes<sup>[11]</sup>. The LMPs (LMP1, LMP2A and LMP2B) may also play a role in the process of immortalization, and oncoprotein LMP-1 appears to be responsible for most of the effects altering the growth of infected B cells<sup>[12]</sup>. Depending on the expressed antigens, it is possible to distinguish four types of latency, each of which is typical of the diseases associated with EBV (Table 1). As EBNA-3 is a target of CD8+ lymphocytes<sup>[7]</sup>, latent cells are normally eliminated by cytotoxic T lymphocytes in immunocompetent patients<sup>[1]</sup>, whereas the transformed cells can proliferate and cause various lymphoproliferative disorders in immunosuppressed patients<sup>[13]</sup>.

Reactivation of the lytic cycle may occur during latency, possibly because of the recirculation of infected memory B cells in lymphoid tissues<sup>[14]</sup> which, stimulated by their natural antigens, differentiate into plasma cells. The beginning of the replication cycle is characterized by the initial expression of the BZLF1 gene initial expression and the production of Z Epstein-Barr replication activator protein (ZEBRA), an IEA that starts a cascade process which, by transactivating the expression of itself and other IEAs, regulates the production of the viral DNA polymerase and thymidine kinase required for viral replication<sup>[15]</sup>, and leads to the production of VCA and MA, and the release of infectious virions<sup>[16]</sup>. Various studies have associated this reactivation with episodes of transplant rejection<sup>[17,18]</sup>, post-transplant lymphoproliferative diseases (PTLDs)<sup>[19-21]</sup>, and clinical diseases in patients with multiple sclerosis<sup>[22]</sup>.

The humoral response includes antibodies against antigens of both the lytic cycle and the latent phase. However, only a few are widely studied and used for diagnostic purposes. Anti-EA antibodies (EA IgG) reflect two patterns, a diffuse (D) and a restricted pattern (R), that were originally observed by means of immunofluorescence on the basis of their distribution inside cells and their differential denaturation by means of fixation and

proteolytic enzymes. Although not always present, EA (D) IgG increases during the first 3-4 wk and is no longer detectable after 3-4 mo (approximately 85% of the patients with acute infection are positive for up to 3 mo after symptom onset)<sup>[23,24]</sup>, even though in some cases they can still be detected years after a primary infection<sup>[23]</sup>. Approximately, 20%-30% of healthy subjects who have previously been infected by EBV have EA (D) IgG<sup>[2,23,25]</sup>. High titers have also been seen during reactivation<sup>[26]</sup> and in patients with nasopharyngeal carcinoma<sup>[27]</sup>, who also have high titers of VCA and EA IgA<sup>[28]</sup>. EA (R) IgG levels may remain high for up to 2 years and, in cases of protracted disease, can be detected after the disappearance of EA (D) IgG<sup>[29]</sup>. EA (R) IgG has been found in children aged less than 2 years with silent infection, in patients with Burkitt's lymphoma, and also in the previously infected subjects at low levels<sup>[28]</sup>. High levels of EA (D) and/or EA (R) IgG can also be seen in cases of reactivation and in immunocompromised patients<sup>[29]</sup>.

Anti-MA antibodies seem to be particularly important in limiting the spread of infection and preventing reinfections<sup>[30,31]</sup>. The membrane antigen consists of three glycoproteins (gp 350, gp 250 and gp 85), which are present on the viral envelope and the membranes of infected cells, and mediate the cell binding of the virus.

Antibodies against the capsid antigen IgG (VCA IgG) typically appear at the time of the onset of the clinical symptoms of acute infection, and remain positive for life<sup>[32]</sup>, whereas IgM antibodies (VCA IgM) usually appear at the same time as VCA IgG and disappear within a few weeks<sup>[32-37]</sup>, although they may persist for several months<sup>[35]</sup>. Children and adults with primary infection are not always positive for VCA IgM<sup>[32,38]</sup>.

Anti-EBNA-2 IgG (EBNA-2 IgG) appear early, and may be present in up to 30% of the patients in the course of disease<sup>[2,23]</sup>, whereas anti-EBNA-1 IgG (EBNA-1 IgG) is usually undetectable during the first 3-4 wk after the onset of clinical symptoms<sup>[32,39]</sup> and is therefore indicative of past infection. Furthermore, most patients with chronic infection and immunosuppressed patients are negative for EBNA-1 IgG or have only low levels<sup>[40]</sup>.

Searching for all of these antibodies is a means of defining infection status and can also help in the differential diagnosis as mononucleosis syndrome may be caused by other pathogens such as rubella, mumps, HHV 6, HCMV, HIV, *Toxoplasma gondii*, *etc.*<sup>[2,41]</sup>.

Using only three parameters (VCA IgG, VCA IgM and EBNA-1 IgG), it is generally easy to distinguish acute and past infections in immunocompetent patients<sup>[42,43]</sup>. The presence of VCA IgG and VCA IgM in the absence of EBNA-1 IgG indicates acute infection, and the presence of VCA IgG and EBNA-1 IgG in the absence of VCA IgM is typical of past infection (Table 2). However, some cases may have different profiles that can create diagnostic doubts, such as the presence of VCA IgG in the absence of VCA IgM and EBNA-1 IgG, the simultaneous presence of VCA IgG, VCA IgM and EBNA-1 IgG, and the presence of EBNA-1 IgG in the absence of VCA IgG

**Table 1** Types of latency in Epstein-Barr virus-associated diseases

Type 0 latency (EBERs, BARTs)	AIDS-related plasmablastic lymphoma
Type I latency (EBNA1, LMP2, EBERs, BARTs) (BamHI A rightward fragments)	Burkitt's lymphoma
Type II latency (EBNA1, LMP1, LMP2, EBERs, BARTs)	Hodgkin's lymphoma, AIDS-related Burkitt's lymphoma or primary effusion lymphoma Peripheral T cell lymphoma NK/T cell lymphoma, nasal type Nasopharyngeal carcinoma (plus BARF1) Gastric adenocarcinoma (plus BARF1)
Type III latency (EBNA1, -2, -3A, -3B, -3C; LMP1, LMP2, EBERs, BARTs)	Post-transplant lymphoproliferative disorder AIDS-related immunoblastic or brain lymphoma Infectious mononucleosis Chronic active EBV infection Lymphoblastoid cell lines <i>in vitro</i> X-linked lymphoproliferative disease

**Table 2** Interpretation of Epstein-Barr virus serological profiles in immunocompetent patients

Anti-EBV antibodies			Interpretation
VCA IgM	VCA IgG	EBNA-1 IgG	
Negative	Negative	Negative	No immunity
Positive	Negative	Negative	Acute infection or non-specificity <sup>1</sup>
Positive	Positive	Negative	Acute infection
Negative	Positive	Positive	Past infection
Negative	Positive	Negative	Acute or past infection <sup>1</sup>
Positive	Positive	Positive	Late primary infection or reactivation <sup>1</sup>
Negative	Negative	Positive	Past infection or non-specificity <sup>1</sup>

<sup>1</sup>Further testing required.

and IgM (Table 2). In such circumstances, in addition to following up the patients to assess any changes in the antibody profile, it is also useful to perform other laboratory tests<sup>[43]</sup>.

The detection of antibodies is less useful in immunocompromised patients because of their immune system dysfunctions, and the fact that the type of antibody and its maintenance may vary over time depending on the dynamics of the disease, thus leading to atypical profiles<sup>[44]</sup>. There is generally an increase in the titers of VCA IgG and EA (D) or EA (R) IgG, with a decrease in the titer or loss of EBNA-1 IgG; there may also be increases in other antibody classes such as EA IgA and, although less frequently, VCA IgM. The variability observed in different patients (this may be complicated by the therapeutic use of immunoglobulins) indicated that a search for EBV DNA by molecular biology methods is useful for the diagnosis and follow-up of patients at risk of developing EBV-related lymphoproliferative disorders<sup>[45]</sup>.

Serology is generally not decisive also in patients with EBV-associated tumors as high values of VCA IgG and EA IgG may persist with low titers of EBNA-1 IgG (Table 3)<sup>[15,16]</sup> and, once again, a search for EBV DNA is essential. There is one type of EBV-related cancer whose

serology is characteristic: nasopharyngeal carcinoma typically leads to high VCA and EA IgA levels as a result of its origin on the nasopharyngeal mucous membrane<sup>[46]</sup>.

## LABORATORY METHODS

Various laboratory tests have been used to diagnose EBV infection. In addition to tests for other diagnostically useful parameters (leukocytosis, lymphocytosis with atypical lymphocytes, abnormal liver function test, *etc.*), there are tests for detecting non-specific heterophile antibodies and specific anti-EBV antibodies, as well as molecular biology methods used to detect EBV DNA.

### Heterophile antibodies

Heterophile antibodies are antibodies that agglutinate cells of other animal species that are mainly associated with mononucleosis due to EBV but may be, albeit rarely, also detected in other diseases<sup>[47]</sup>. They peak 2-5 wk after symptom onset and then decline rapidly, although they may rarely persist for 6-12 mo<sup>[35,48]</sup>. Between 85% and 90% of adolescents and adults are positive during the course of EBV infection: about 50% in the first week, and 60%-90% in the second and third<sup>[29]</sup>. However, only 50% of children aged 2-5 years are positive at any time during the course of infection, and only 10%-30% of those aged less than 2 years<sup>[2]</sup>. The rate of false negative results can therefore be high in young children, whereas false positive results are observed in only 2%-3% of the patients with autoimmune diseases<sup>[2]</sup>.

Despite these limitations, detecting heterophile antibodies can be helpful in the case of primary infection mainly because of the simplicity of the tests. In routine clinical practice, these include agglutination tests using sheep, goat or horse red blood cells as antigens after absorption with guinea pig kidney extracts in order to remove natural antibodies against Forssman antigen (the Paul Bunnell reaction), and newer tests based on latex particles with adherent specific bovine antigen for heterophile antibodies (monospot assays). Recently, multiplex flow immunoassays (MFI) have been proposed because

**Table 3 Serological profiles in Epstein-Barr virus reactivation and some Epstein-Barr virus-associated diseases**

Diseases	VCA IgM	VCA IgG	VCA IgA	EA (D) IgG	EA (R) IgG	EA IgA	EBNA1 IgG
Chronic active infection	+/-	++	+/-	+	++	-	+/-
Burkitt's lymphoma	-	++	-	+/-	++	-	+
Nasopharyngeal carcinoma	-	++	+	++	+/-	+	+
Hodgkin's lymphoma	-	++	-	+	-	-	+
Reactivation	+/-	++	+/-	+	+/-	+/-	+/-

they are more sensitive in the case of acute infections<sup>[49]</sup>. However, given the level of false negative results, negative findings need to be followed by a search for specific antibodies<sup>[2,48]</sup>.

### Specific EBV antibodies

The specific tests for anti-EBV antibodies use different substrates or antigens and various technologies, of which those commonly used for the routine screening of EA IgG, EBNA-1 IgG, VCA IgG and IgM are immunofluorescence assays (IFAs) or enzyme immunoassay (EIAs) with enzyme-linked immunosorbent assays (ELISA) and chemiluminescence immunoassay (CLIA) versions or newer multiplex flow immunoassay (MFI). IFAs usually use EBV-transformed B cell lines from patients with Burkitt's lymphoma (e.g. the P3HR-1 or Raj cell lines)<sup>[2,23,45,50-52]</sup>, whereas EIAs use purified native or recombinant proteins, synthetic peptides or fusion proteins (complete proteins or fragments of the proteins encoded by the EBV genes)<sup>[42,48]</sup>. The type and preparation of the antigens used are probably responsible for the differences in the sensitivity and specificity of the various assays<sup>[42,53,54]</sup>. IFA has been used as the reference method, although its sensitivity is the same as or less than that of EIAs<sup>[43]</sup>, automated versions of which allow a large number of samples to be tested and are commonly used in laboratories with a large routine workload. The latest CLIAs using synthetic peptides with different cut-off values for VCA IgM and EBNA-1 IgG can better distinguish the stage of infection<sup>[55]</sup> and, in the case of samples that are simultaneously EBNA-1 IgG, VCA IgG and IgM positive, may help distinguish recent (transient phase) and past infection or reactivation<sup>[55,56]</sup>.

### Immunoblotting

In order to confirm the screening assays, various immunoblotting tests have been developed using viral lysates of EBV-transformed cells and recombinant antigens (line blots)<sup>[57]</sup>. It is considered that the latter is unaffected by antibodies against the cell material that can be found in patients with mononucleosis<sup>[23,58]</sup>. Some line blots use recombinant antigens coated on the solid phase, such as EBNA-1 (p72), VCA (p18 and p23), EA (p54 and p138) and MA (gp 350/250); the most recent also use IEA (ZEBRA). It has been reported that the sera of patients with acute infection show anti-p23 IgG, anti-p55 IgG and anti-p138 IgG, but not anti-p72 IgG, whereas the sera from patients with past infection show anti-p23 IgG, anti-p72 IgG and anti-p18 IgG<sup>[23]</sup>. Kinetic studies

have found that a strong IgG response to p72 is not observed until 20 d after disease onset. As anti-p72 IgG and anti-p18 IgG are present in patients with past infection, but not in those with acute infection<sup>[23]</sup>, anti-p18 IgG can be considered equivalent to EBNA-1 IgG in terms of significance. In addition, as anti-p18 IgG is not lost in the case of immunosuppression<sup>[23]</sup>, immunoblotting is especially useful in distinguishing acute and past infections in cases that are VCA IgG positive, but EBNA-1 IgG and VCA IgM negative.

Immunoblots for IgM have also been developed to detect VCA IgM in patients with acute infection, but not anti-p72 IgM<sup>[23]</sup>, which may be useful for confirming the specificity of the VCA IgM detected by screening assays.

### IgG avidity

The IgG avidity test can assess the degree of IgG maturation. Avidity is low at the beginning of an acute infection, but increases when the immune response matures<sup>[59-61]</sup>. For example, the maturation kinetics of VCA IgG lasts several weeks and in some cases up to 3 mo after symptom onset<sup>[62,63]</sup>. Avidity can be measured using an EIA, IFA or immunoblotting<sup>[23,57,63]</sup>. Two aliquots of the same sample are tested in parallel for the presence of IgG antibodies: one is not treated, and the other is treated with substances that dissociate the antibodies from the antigens (usually 8 M urea). Since the dissociation depends on antibody avidity, the ratio between the treated and untreated sample defines the degree of avidity. A search for avidity can therefore be used to estimate the duration of a primary infection, and differentiate acute and past infection<sup>[23,57,63-65]</sup>.

The published data depend on the examined antibody and therefore the type of antigen used for the test. In addition to a mix of antigens, it is possible to use the whole or specific VCA (p23 or p18), IEA (ZEBRA), EA (p138 or p54) or EBNA (p72). Avidity varies depending on the kinetics of the various antibodies<sup>[63,66]</sup>, e.g. in VCA IgG, it has been reported that avidity is low in the samples collected during the first 12 wk after symptom onset, thus indicating recent infection<sup>[67]</sup>. With the passing of time, the avidity of VCA IgG may become borderline or high when the avidity of EA IgG is still low<sup>[63]</sup>. Immunoblotting, which has various antigens coated on the solid phase, can be used to visualize the avidity of various antibodies simultaneously (Table 4), although the maturation of anti-IEA and EA antibodies (BZLF1, p138 and p54) does not seem to indicate immune status as antibodies with a low degree of avidity can be found even during reactivation. Consequently, the manufacturer points

**Table 4 Interpretation of IgG avidity test with immunoblotting**

IgG	IgG avidity	Interpretation
Negative	Not observed	No infection
Positive BZLF1, p138, p54	Low-high avidity for BZLF1 and/or p138 and/or p54	Acute infection
Positive p23, BZLF1, p138, p54	Low avidity for p23	Acute infection
Positive p23, BZLF1, p138, p54	High avidity for p23	Recent infection
Positive p18, p23, BZLF1, p138, p54	Low-intermediate avidity for p18 and p23	Recent infection
Positive EBNA-1, p18, p23, BZLF1, p138, p54	Low-high avidity for EBNA-1 and/or p18, and possible high avidity for p23	Past infection

out that a reduction in the intensity of the EA and IEA bands alone is not an index of recent infection<sup>[68]</sup>.

The limitations of avidity testing are the individual maturation rates of antibodies and the fact that the tests cannot be used in newborns because of the presence of maternal antibodies.

### Molecular biology

A number of different methods, techniques and protocols have been used to determine the presence of EBV DNA and measure viral load<sup>[69-72]</sup>. Dot blotting, Southern blotting, PCR and *in situ* hybridization have all been applied to various materials, but their differences in sensitivity and specificity have led to the results that need to be considered cautiously<sup>[28]</sup> as they vary from laboratory to laboratory<sup>[73,74]</sup>. More recent studies indicate that real-time PCR is particularly sensitive<sup>[28]</sup>, and very useful for defining infection status, especially in immunocompromised patients<sup>[45,75,76]</sup> and those at risk of developing EBV-related disorders<sup>[45]</sup>. However, there is still no consensus concerning the best material to use, units of measurement, or the quantitative levels requiring intervention or predicting prognosis<sup>[16,74,77-79]</sup>. This means that particular care is necessary when comparing the data of different studies<sup>[73]</sup>: for example, the units of measurement include copies per milliliter, copies per microgram of DNA, copies per 100 000 leukocytes, and copies per positive cell<sup>[77]</sup>. The targets used may also vary from one method to another: LMP2, BKRF1 or BamHI-W (EBNA-1), BNRF1 (membrane protein), BXLF1 (thymidine kinase), BZLF1 (ZEBRA), BALF5 (viral DNA polymerase) or BHRF-1 (transmembrane protein).

Furthermore, there is much debate concerning the material that should be used to search for EBV DNA, such as whole blood, peripheral blood mononuclear cells (PBMCs), plasma or serum<sup>[13,80]</sup>. There is also the problem that incorrectly stored whole blood can cause EBV DNA to leave the intracellular compartment and give rise to false positive results in plasma or serum, and false negative results may be due to nucleases that are capable of partially degrading plasma EBV DNA<sup>[81]</sup>.

In general, the best material used to search for EBV DNA depends on where it is, and varies during the course of the disease<sup>[13]</sup>. The virions produced during

primary infection spread in peripheral blood<sup>[82,83]</sup>, and it is also possible to determine the EBV-free or fragmented DNA coming from apoptotic cells<sup>[83]</sup>, and the B cells transformed during the latent phase also pass into the bloodstream. EBV DNA can therefore be determined in serum or plasma as well as in PBMCs<sup>[84]</sup>. In patients with primary infection, it is frequently detected in whole blood (PBMCs and plasma/serum) within 14 d of symptom onset<sup>[85-89]</sup>. After the initiation of an immune response, viral load decreases slowly in PBMCs, but rapidly in plasma/serum, and it becomes undetectable after 3-4 wk<sup>[90-92]</sup>, whereas memory cells with EBV may remain latent for a long time in blood. However, it must be kept in mind that there may be individual variations due to individual differences in kinetics, and viral load may increase after an initial decline, and in some cases, it may take as long as a year or more before it reaches stably low levels. Finally, even when this level is reached, the blood of a healthy carrier contains 1-50 copies of EBV DNA per million white blood cells, whereas EBV-DNA is almost always undetectable in plasma or serum<sup>[82,85,93-96]</sup>. The presence of plasma/serum EBV-DNA is therefore considered a sign of primary infection<sup>[13]</sup> or reactivation, and the viral load correlates with disease severity<sup>[85,88,92]</sup>. A search for EBV DNA may be more sensitive than serology in the early stages of the disease<sup>[89]</sup>, and some studies have found that it correlates better with clinical acute infection than the avidity of VCA IgG<sup>[86]</sup>. However, in immunocompetent patients with acute infection, it is not usually necessary to look for EBV DNA as serology is sufficient except in cases with negative or doubtful serological findings in which there is a strong clinical suspicion of infection<sup>[89,97,98]</sup>.

A search for EBV DNA is particularly important in immunocompromised patients with an incomplete humoral response and patients who have received transfusions or immunoglobulins that confound serological test results<sup>[28]</sup>. It has been reported that immunocompromised patients have higher baseline viral levels than healthy carriers<sup>[99,100]</sup>, which decline after treatment. A search for EBV DNA is also useful in patients with EBV-related tumors, except for those with AIDS-related brain tumors in whom the blood levels are low because of the blood-brain barrier<sup>[99,101]</sup>. In EBV-related cancers, episomal or naked EBV DNA from apoptotic tumor cells is found

**Table 5** Additional tests in the case of an isolated viral capsid antigen IgG pattern

Tests	Advantages	Disadvantages
EBV IgG immunoblotting	Useful in distinguishing acute from past infection	Individual antibody production; expensive
IgG avidity	Useful in distinguishing acute from past infection	Individual maturation. Not useful in newborns
Molecular biology	Useful in distinguishing acute from past infection	Uncorrected conservation of blood sample, presence of nucleasis; expensive; organisational problems
Heterophile antibodies	Useful in distinguishing acute from past infection if positive; inexpensive and simple	Not very sensitive (especially in children)
Anti-EA(D) IgG	Of some use in distinguishing acute from past infection; costs the same as a screening test	Not useful in at least 10% of cases

EBV: Epstein-Barr virus.

in serum and plasma<sup>[84,102]</sup>, which may also contain tumor cells with latent EBV infection<sup>[13]</sup> and virions from a small number of tumor cells undergoing lytic infection. The most suitable material varies with the tumor<sup>[13]</sup> and depends on where EBV DNA is mainly found during the course of the disease (PBMCs or serum/plasma).

## ATYPICAL PROFILES IN IMMUNOCOMPETENT PATIENTS

The presence of VCA IgG and IgM in the absence of EBNA-1 IgG indicates acute infection, whereas the presence of VCA IgG and EBNA-1 IgG in the absence of VCA IgM is typical of past infection. These profiles cover the vast majority of situations found in routine laboratory practice. However, the profile of a minority of cases may give rise to doubts or require confirmation.

### Isolated VCA IgM

VCA IgM usually appears at the same time as VCA IgG, but because they can be detected earlier, a profile of isolated VCA IgM is usually thought to indicate an early stage of acute infection. Nevertheless, it is recommended to assess the specificity of the result because it may be made aspecific by interfering rheumatoid factor and autoantibodies, or cross-reacting factors such as HCMV or parvovirus B19<sup>[67]</sup>. A search for these factors and immunoblotting for IgM may be as helpful as the determination of other parameters of acute infection such as heterophilic antibodies or HBV DNA<sup>[98]</sup>. A search for anti-EA (D) IgG can also be useful as about 85% of the patients with acute infection are positive for these antibodies for up to 3 mo after the onset of symptoms<sup>[23,24]</sup>.

### Isolated VCA IgG

In some cases, VCA IgM may not be produced or appear 1-2 wk after VCA IgG, or present for a very short time or at low concentrations and it may not be detected by conventional tests<sup>[2,32,103,104]</sup>. In addition, about 5% of the patients do not produce EBNA-1 IgG after EBV infection<sup>[103,105]</sup> or their levels remain below the limit of detection<sup>[37,105]</sup>. Furthermore, even when they are produced, they may be lost over time particularly, but not exclusively, in immunocompromised patients<sup>[23,103,106]</sup>. Conse-

quently, isolated VCA IgG may be found in cases of past infection with the loss or disappearance of EBNA-1 IgG, as well as in cases of acute infection with the delayed or early disappearance of VCA IgM. This pattern can be found in approximately 7% of cases in routine laboratory practice and in about 8% of all subjects with at least one marker of EBV infection; it also tends to become more frequent with advancing age<sup>[107]</sup>.

Such cases require further diagnostic investigations (Table 5), including immunoblotting for IgG, avidity tests for VCA IgG, searches for viral genome, or heterophile antibodies or EA (D) IgG<sup>[43]</sup>, or a repetition of the test after about 30 d in order to identify any change in the antibody profile. However, this last option inevitably delays the diagnosis until the second sample is collected, and physicians tend to avoid it if the symptoms improve over time, especially in the case of children, they may find it traumatic, which means the second sampling usually involves only a small number of patients<sup>[107]</sup>.

Immunoblotting for IgG allows the interpretation of cases with isolated VCA IgG<sup>[23]</sup>. Some tests use recombinant p72 antigen (EBNA-1), p18 and p23 (VCA), p54 and p138 (EA) and gp 350/250 (MA). As anti-p18 IgG can be considered equivalent in meaning to EBNA-1 IgG (because they are both produced late during EBV infection), their presence indicates past infection, and their absence indicates an acute infection. Immunoblotting can therefore distinguish cases with the loss or non-appearance of EBNA-1 IgG, which however develop an anti-p18 IgG response, from those with primary EBNA-1 IgG negativity (acute infection)<sup>[23,108]</sup>. Using this method to examine cases of isolated VCA IgG, about 20% was identified as acute infections and 80% as past infections<sup>[107]</sup>.

A VCA IgG avidity test can also be used to distinguish acute and past infection<sup>[64,67]</sup>, which are respectively characterized by low and high levels of avidity; studies of patients with isolated VCA IgG have found low levels in about half of the cases, and high levels in the remaining half<sup>[75,86]</sup>. When using avidity test, it is important to have all the patients' temporal clinical data in order to interpret the results more appropriately.

The detection of EBV DNA is particularly useful in the case of serologically indeterminate EBV infec-

**Table 6** Additional tests in the case of a simultaneous Epstein-Barr virus nuclear antigen 1 IgG, viral capsid antigen IgG and IgM positive pattern

Tests	Advantages	Disadvantages
EBV IgM immunoblotting	Useful only in verifying the specificity of EBV IgM	Not useful in distinguishing late primary infection (transient) from reactivation; expensive
HCMV IgM Parvovirus IgM	Useful in verifying the specificity of EBV IgM	Not useful in distinguishing late primary infection (transient) from reactivation
EBV IgG immunoblotting	Only useful in verifying the specificity of EBNA-1 IgG	Not useful in distinguishing late primary infection (transient) from reactivation; expensive
IgG avidity	Useful in distinguishing primary infection (transient) from reactivation	Individual maturation
Molecular biology	Useful for EBV reactivation follow-up	Difficult to distinguish late primary infection (transient) from reactivation in a single sample; expensive; organisational problems
Heterophile antibodies	Useful in distinguishing late primary infection (transient) reactivation when positive; inexpensive and simple	Not very sensitive (especially in children)
Anti-EA(D) IgG	Useful for EBV reactivation follow-up	Not useful in distinguishing late primary infection (transient) from reactivation in a single sample
CLIA for EBV antibodies with differential cut-off values	Useful in distinguishing primary infection (transient) from past infection; can be used for screening	Requires further study

EBV: Epstein-Barr virus; EBNA: EBV nuclear antigen; CLIA: Chemiluminescence immunoassay.

tion<sup>[75,86,98]</sup> because its presence in serum or plasma indicates primary infection or reactivation<sup>[13]</sup>. Some studies have found that the presence of EBV DNA in patients with isolated VCA IgG is usually associated with a low degree VCA IgG avidity<sup>[75,86]</sup> (especially when the sample is taken early in the course of suspected infectious mononucleosis), and in some cases it has been found to correlate better than VCA IgG avidity<sup>[86]</sup>. Furthermore, in cases of past infection diagnosed on the basis of a high level of VCA IgG avidity, none of the serum samples were positive for EBV DNA<sup>[86]</sup>.

A search for heterophile antibodies may be useful in patients with isolated VCA IgG if the result is positive, although there have been reports of their presence for 6-12 mo<sup>[28,35,48]</sup>. About 60% of patients with acute infection and isolated VCA IgG present heterophile antibodies, as against none of those with past infections<sup>[107]</sup>. However, nothing can be said in the absence of heterophile antibodies, especially in children aged less than 5 years, and other tests must be used.

One possible alternative is to search for EA (D) IgG because about 85% of patients with acute infection are positive for EA (D) IgG for up to 3 mo after symptom onset<sup>[23,24]</sup>. However, as high titers can be found during EBV reactivation<sup>[26]</sup>, as well as in 20%-30% of healthy subjects with past infection<sup>[2,25]</sup> and in patients with nasopharyngeal carcinoma<sup>[27]</sup>, the diagnostic use of these antibodies is still being debated. In general, a search for EA (D) IgG alone does not identify the stage of disease<sup>[43,109]</sup>, but combining these antibodies with other parameters can be useful in laboratory diagnoses<sup>[110]</sup> because it has been found that only 12% of the patients with isolated VCA IgG and past infection are EA (D) IgG positive, as against 90% of subjects with isolated VCA IgG and acute infection<sup>[111]</sup>.

### **Simultaneous presence of EBNA-1 IgG, VCA IgG and VCA IgM**

VCA IgM may persist for several months after an acute infection<sup>[55]</sup>, and may also reappear during EBV reactivation<sup>[33,112]</sup>. Consequently, EBNA-1 IgG, and VCA IgG and IgM may be simultaneously present in patients with primary EBV infection if VCA IgM persist and EBNA-1 IgG have already been produced (a phase that has been variously defined as “recent infection”, “primary infection, transient phase or convalescence”, “past infection, IgM persisting”), or in those with reactivation and the simultaneous presence of VCA IgM and EBNA-1 IgG<sup>[42]</sup>. Reactivation is still relatively rare and often short in immunocompetent subjects, and is generally considered of no clinical relevance<sup>[113,114]</sup>; however, it can cause serious complications in immunocompromised patients.

This serological pattern is uncommon (approximately 5% in normal routine laboratory practice)<sup>[107]</sup>, and further diagnostic tests are needed to distinguish transient infection and reactivation (Table 6). First of all, it is important to verify the specificity of VCA IgM because there may be false positive results<sup>[115-117]</sup> during the course of infection with other pathogens, such as HCMV, parvovirus B19, *Toxoplasma gondii*, hepatitis A or HIV<sup>[118-124]</sup>. It has been shown that primary HCMV infection often causes a further antibody response in anti-EBV IgM<sup>[125]</sup>, and cross-reactive antibody responses against conserved epitopes are well known among herpes viruses, such as the glycine-alanine epitope shared by EBV and HCMV<sup>[119]</sup>. In some studies, up to about 50% of VCA IgM in patients with past infection may be due to non-specific reactivity<sup>[67]</sup> and a positive result should be confirmed (e.g. by immunoblotting for EBV IgM)<sup>[124]</sup>. It is also possible to test for cross-reactivity or polyclonal IgM, especially in the case of HCMV and parvovirus B19<sup>[98]</sup>, and a search for HCMV or parvovirus B19 DNA can also help in

some cases<sup>[98]</sup>. Moreover, as false positive reactions may also result from the presence of autoantibodies or rheumatoid factor, it can be useful to look for these interfering factors. However, it needs to be pointed out that cold agglutinins, rheumatoid factor and autoantibodies can be found for a short period during the course of EBV mononucleosis because of the polyclonal activation of B-cells<sup>[126]</sup>, therefore, the presence of rheumatoid factor does not automatically mean a falsely positive VCA IgM result. Finally, EBNA-1 IgG positivity in patients with primary infection may also result from aspecific reactivity, which can be detected by immunoblotting for IgG antibodies<sup>[124]</sup>. Furthermore, the recently developed EIA for antibodies to EBNA-1 IgG based on recombinant or synthetic peptides may be more sensitive than its predecessors, and allow their identification early in the course of primary EBV infection<sup>[124]</sup>.

Once the specificity of the results obtained has been established, additional diagnostic approaches are necessary (Table 6). In addition to repeating the test after a reasonable period of time in order to detect any changes in the antibody profile, VCA IgG avidity has proved to be particularly useful because low levels of avidity have been found in the course of recent infection, and high levels in the course of past infection or reactivation<sup>[62-64]</sup>. Some studies have found that fewer than half of immunocompetent patients with this profile and a low degree of avidity have primary infection, and about 20% or less a reactivation that is probably clinically insignificant<sup>[112,124]</sup>. In order to fully understand the IgG avidity test and evaluate the results in relation to antibody maturation time, it is important to know how long after the onset of symptoms the blood sample was taken.

The use of heterophile antibodies is controversial because some studies have shown that they are very sensitive (94% of infected patients with transient infection)<sup>[124]</sup>, whereas others have found very few cases of simultaneous positivity for EBNA-1 IgG, VCA IgG, VCA IgM and heterophile antibodies<sup>[109]</sup>.

The use of molecular biology seems to be a rather delicate question. Patients with latent infection have an almost constant number of circulating infected B cells in peripheral blood and, in the case of reactivation, these differentiate into plasma cells, leading to the start of the replicative cycle and increased EBV DNA levels in PBMCs and serum/plasma. It has been reported that a search for EBV DNA in PBMCs and serum/plasma is important for an immediate diagnosis of reactivation but, although this is true in the patients who are followed up over time in order to detect any changes in viral load, the finding of EBV DNA in a single sample should not necessarily be seen as a sign of reactivation<sup>[95]</sup>. Consequently, as EBV DNA is present in cases of reactivation or primary infection, it is unlikely that testing one sample will be able to distinguish the two situations<sup>[75,95]</sup>.

Persistent or reactivated EBV infection is characterized by high titers of EA (D) IgG, especially in immunocompromised patients<sup>[26,29]</sup>. After reactivation, the levels

of these antibodies increase with VCA IgG, whereas the levels of EBNA-1 IgG decrease. An increase in the titer of EA (D) IgG can therefore be considered a useful marker of reactivation. However, as EA (D) IgG can also be found in 85% of primary infections and 20%-30% of past infections, and simultaneous VCA IgM, VCA IgG, EBNA-1 IgG and EA (D) IgG positivity has been seen in both the transient phase and reactivation<sup>[76]</sup>, the detection of anti-EA (D) IgG seems to be useful only if serial sampling is possible. Other antibodies have been found in cases of reactivation. It has been suggested that EBNA IgM may be useful in identifying reactivation<sup>[114]</sup>. The combination of negative EBNA IgG and positive EBNA IgM, in addition to a high degree of avidity for VCA IgG within 3 mo of an acute infection, should reliably indicate reactivation rather than primary EBV infection<sup>[67]</sup>.

It has also been suggested that VCA IgA may be a marker of reactivation. These antibodies reach a peak level 3-4 wk after primary infection and decline slowly, but may last indefinitely. Different studies have found them in 35%-74% of acute cases<sup>[34,127,128]</sup>, but they were also seen in 10% of healthy subjects. High levels were also found in patients with immunodeficiencies, recurrent parotitis, multiple sclerosis or nasopharyngeal cancer, as well as in pregnant women and elderly subjects<sup>[26,129-132]</sup>. Consequently, a search for VCA IgA is considered useful only in the diagnosis and management of patients with nasopharyngeal carcinoma<sup>[133-136]</sup>.

EA (R) IgG, EA IgM, EA IgA, VCA IgG-3 and the EBNA-1/EBNA-2 ratio have also been used as markers of reactivation in some studies<sup>[18,95,114,137-139]</sup> but, as many of these antibodies are also present in primary infections, and some even in past infections, the usefulness of a single sample in patients with the simultaneous presence of EBNA-1 IgG, VCA IgG and IgM has yet to be evaluated.

Finally, the use of different cut-off values proposed by the most recent CLIAs for determining VCA IgG, VCA IgM and IgG EBNA-1 in parallel seems to be a promising approach as it should make it possible to distinguish the different stages of EBV infection, especially the difference between transient infection and past infection or reactivation<sup>[55,56]</sup>.

### Isolated EBNA-1 IgG

VCA IgM usually appears at the same time as VCA IgG antibodies, but they disappear completely within a few weeks<sup>[32-37]</sup>, whereas patients are VCA IgG positive for the rest of their lives<sup>[32]</sup>. EBNA-1 IgG cannot usually be detected during the first 3-4 wk after the onset of clinical symptoms<sup>[32,140]</sup> and as VCA IgG antibodies persist but EBNA-1 IgG antibodies may disappear, especially in the case of immunosuppression<sup>[23,103,106]</sup>, a pattern of isolated VCA IgG without VCA IgM or EBNA-1 IgG has been widely documented as mentioned above, whereas the presence of EBNA-1 IgG without VCA IgG is generally considered implausible<sup>[43]</sup>. However, some immunoblotting studies have found that 2% of subjects who have



experienced previous infections may be negative for VCA IgG (anti-p23 or anti-p18)<sup>[23]</sup> and, although rare, this pattern can be found in routine laboratory tests and may represent about 1.7% of all EBNA-1 IgG-positive samples<sup>[39]</sup>. An isolated EBNA-1 IgG pattern therefore gives rise to some interpretative doubts: it is possible to envisage aspecific EBNA-1 IgG, a lack of VCA IgG production or their loss over time. At clinical level, the problem is to discover whether the patient has experienced a past infection or is still susceptible. As the doubt is to distinguish past infection and non-specificity, it is useless to search for heterophile antibodies or EBV DNA. The presence of EA (D) IgG may help indicate a past infection as approximately 20%-30% of healthy subjects with a history of EBV infection have EA (D) IgG<sup>[2,23,25]</sup>, but nothing can be said in the case of negativity. However, immunoblotting for IgG can solve the problem as it can confirm the real presence of EBNA-1 IgG. One immunoblotting study found that such patients were not only all anti-p72 (EBNA-1) positive, but also anti-p23 (VCA) positive, which was not detected by the screening tests using p18 antigen alone to detect VCA IgG<sup>[140]</sup>. It therefore seems that EBNA-1 IgG does not exist without the presence of at least one VCA IgG, such as anti-p23.

## CONCLUSION

A number of tests have recently been developed that may help clarify doubtful results in the serological diagnosis of EBV infection, and it is now possible to reach a conclusion without having to wait for a second sample taken after a certain lapse of time. What tests should be used after screening depends on various factors in addition to their scientific and technical suitability; these include organizational and economic questions (such as differences in costs and the reimbursements foreseen by the National Health Service or insurance companies), as well as the availability of space and adequately trained personnel. Furthermore, the number of routine tests (the number of samples with inconclusive results) affects the decision to undertake further more or less expensive laboratory tests or to send the sample (or the patient) to a reference laboratory. Finally, the type of patient may also be decisive. If the laboratory has to deal not only with samples from immunocompetent patients, but also with samples from immunosuppressed patients and/or patients with other EBV-related diseases, the choice of methods should privilege those suitable for all patients.

In conclusion, considerable progress has been made in the serological diagnosis of EBV infection and, using appropriate algorithms and methodologies, it is possible to solve all of the problems that may arise during the course of routine laboratory practice.

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