Acta virologica **57**: 283 – 291, 2013 doi:10.4149/av_2013_03_283

# How accurate are diagnostic tools for Epstein-Barr virus (EBV) to establish causal association of an uncommon clinical condition with EBV?

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*Received December 12, 2012; accepted June 24, 2013*

**Summary.** – Epstein-Barr virus (EBV) infection has been implicated as a possible cause of a wide range of clinical conditions in children and young adults. In uncommon clinical conditions, where clinical experience is missing, it is important to evaluate both the biological plausibility and the virological basis that substantiates their causal association with EBV. By reviewing the diagnostic procedures performed in the diagnosis of EBV infection in case reports concerning uncommon clinical conditions causally related to EBV infection in children and young adults, the aim of the present study was to discuss the limitations of the diagnostic procedure used to establish EBV diagnosis, which may cause false-positive results and compromise the reliability of such a diagnosis. We should be aware not only of the nuances of serological tests and virus detection tests for latent viruses such as EBV, but also of the risk of using them alone or in combination with molecular methods as the sole mean for establishing a causal relation between EBV infection and an uncommon clinical condition. Accurate laboratory tests for EBV detection, strict criteria for EBV infection diagnosis, and a cumulative clinical experience coupled with biological plausibility and experimental data are needed to avoid a possible coincidental association between several clinical manifestations, mainly uncommon clinical conditions, and EBV infection.

**Keywords:** Epstein-Barr virus; diagnostics; uncommon condition

**Contents:**



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**Abbreviations:** Abs = antibodies; EA-D = early antigen-diffuse; EBV = Epstein-Barr virus; EBER = EBV-encoded RNA; EBNA = EBV nuclear antigen; HAbs = heterophile Abs; IM = infectious mononucleosis; LMP = latent membrane protein; VCA = virus capsid

## 1. Introduction

Epstein-Barr virus (EBV) or human herpesvirus 4 is ubiquitous and about 90% of adults throughout the world have antibodies against it (De Paschale and Clerici, 2012; Rickinson and Kieff, 2001). Nearly one-half to two-thirds of primary EBV infections result in infectious mononucleosis syndrome (IM) (Jenson, 2000). IM syndrome is characterized by systemic somatic complaints, such as prominent fever, tonsilopharyngitis, lymphadenopathy, hepatosplenom-

egaly, fatigue and malaise. IM usually resolves over a period of weeks or months without sequel, although it may be occasionally complicated with a wide variety of neurological, hematological, hepatic, respiratory, and psychological complications (Jenson, 2000).

In developing countries and in socio-economically disadvantaged population in industrialized countries, a primary EBV infection usually occurs during infancy and early childhood. In this age group, patients are asymptomatic or with only mild symptoms. In more affluent population, in industrialized countries, EBV infection is also more common during early childhood, but about one-third of EBV cases occur during adolescence and early adulthood (Jenson, 2000).

Since the EBV′s discovery in 1964, EBV infection has been implicated as a possible cause in a wide range of uncommon clinical conditions (Jenson, 2000; Okano *et al.*, 1988, 1991). However, whether this causal association is strong or not remains unconvincing in many instances. This is because in many instances the current clinical experience and experimental data are insufficient to support this causal association. It is well known that an inference of causality tends to be strengthened by consistency with acquired cumulative clinical experience or data from experimental studies and other sources demonstrating plausible biological mechanisms.

To accept a causal association of severe uncommon clinical condition with EBV, especially in children, it is necessary to consider the limitations of the diagnostic methods, commonly used today. Otherwise, false-positive results may be followed by an inappropriate treatment with unpredictable consequences in patient's health. For this reason the question whether the diagnostic tools for EBV are accurate enough to establish a causal association of uncommon clinical conditions with EBV or not, is tenable.

The aim of this review was to analyze the risk of incorrect diagnostics of EBV-associated uncommon conditions. For this purpose we reviewed the English language literature via Pubmed database for case reports published in the last 15 years, concerning uncommon clinical conditions causally related to EBV in children and young adults (3–22 years). After that we reviewed the diagnostic procedures, followed by authors, for the diagnosis of EBV. Finally, we were focused on the possible pitfalls of the whole diagnostic procedure,

**Table 1. Diagnostic methods for EBV**

| Age/Sex | Clinical condition associated with EBV | Diagnostic methods | Consideration of other infections | Diagnostic methods on follow-up stage | References |
|---|---|---|---|---|---|
| 12y/f | Aspecific membranous laryngitis | Serological | Yes | – | Di Girolamo *et al.*, 1996 |
| 3y/m | Chronic bullous disease | Serological | No | Serological | Baldari *et al.*, 1996 |
| 12y/f | Conjunctival tumor | Serological, *in situ* hybridization | No | – | Hundsdoerfer *et al.*, 2000 |
| 13y/m | Intestinal pseudo-obstruction and acute pandysautonomia | Serological, PCR | Yes | Serological, *in situ* hybridization | Besnard *et al.*, 2000 |
| 6y, 13y | Henoch schönlein purpura | Serological | No | – | Grech and Vella, 2002 |
| 22y/f | Systemic lupus erythematosus | Serological, *in situ* hybridization | No | Serological | Verdolini *et al.*, 2002 |
| 6y/f | Gianotti- Crosti syndrome | Serological, immunohistological | Yes | – | Terasaki *et al.*, 2003 |
| 14y/m | Cervical ankylosis | Not mentioned | No | – | Haidar *et al.*, 2005 |
| 11y/f | Glomerulonephritis | Serological, *in situ* hybridization | Yes | Serological, PCR | Kano *et al.*, 2005 |
| 6y/m | Acquired chiari I malformations | Serological | Yes | – | Shokouhi and Naghili., 2005 |
| 3y-f | Atypical subacute thyroiditis | Serological, PCR | No | Serological | Volta *et al*, 2005 |
| 12y/f, 18y/f | Genital ulcers | Serological, PCR | Yes | Serological | Halvorsen *et al.*, 2006 |
| 3y/m | C1q nephropathy | Serological | Yes | Serological | Lim *et al.*, 2007 |
| 6y/m | Polyglandular syndrome type II | Serological | Yes | – | Roa *et al.*, 2008 |
| 5y/m, 4y/f | Acute acalculous cholecystitis | Serological, PCR | Yes | Serological | Attilakos *et al.*, 2009 |
| 18y/f | Lemierre′s syndrome | Serological | No | – | Garimorth *et al.*, 2008 |
| 7y/m | Frosted branch angiitis | Serological, PCR | Yes | Serological | Farrando *et al.*, 2008 |
| 15y/m | Erythema multiforme-like lesions | Serological | Yes | – | Zawar *et al.*, 2009 |
| 22y/f | Kikuchi′s disease | Serological | Yes | – | Bhargava and Matthew, 2009 |
| 20y/f, 19y/f, 19y/f | Graves′ disease | Serological | Yes | – | Akahori *et al.*, 2010 |
| 17y/f | Gastritis | Serological, *in situ* hybridization | Yes | *in situ* hybridization | Hisamatsu *et al.*, 2010 |
| 9y/f, 4y/m | Acute dacryocystitis | Serological | No | Serological | Ghauri *et al.*, 2011 |

which could lead to false-positive results and therefore compromise the causal association.

Our primary goal was to turn clinicians' interest to the pitfalls that may be present during the diagnosis of EBV infection with the available diagnostic tools that can lead to false-positive results. It is crucial that these pitfalls are taken into account, when a dilemma about a possible causal association of uncommon clinical condition with EBV is raised in their daily clinical practice. It is noteworthy that we were specifically focused on uncommon clinical conditions which rarely appear in clinical practice and there is not enough clinical experience for the causal association of this condition with EBV. On the other hand, cases with common or rare clinical manifestations of EBV were excluded from our study, when the current cumulative clinical experience or the experimental data demonstrating biological plausibility have already supported this causal association (Jenson, 2000).

Based on these criteria, our search revealed twenty-two uncommon clinical conditions causally related to EBV, that were described in 28 patients with mean age of 11.5 years (3–22 years) (Table 1) (Di Girolamo *et al.*, 1996; Baldari *et al.*, 1996; Hundsdoerfer *et al.* 2000; Besnard *et al.*, 2000; Grech and Vella, 2002; Verdolini *et al.*, 2002; Terasaki *et al.*, 2003; Haidar *et al.*, 2005; Kano *et al.*, 2005; Shokouhi and Naghili, 2005; Volta *et al.*, 2005; Halvorsen *et al.*, 2006; Lim *et al.*, 2007; Roa *et al.*, 2008; Attilakos *et al.*, 2009; Garimorth *et al.*, 2008; Farrando *et al.*, 2008; Zawar *et al.*, 2009; Bhargava and Matthew, 2009; Akahori *et al.*, 2010; Hisamatsu *et al.*, 2010; Ghauri *et al.*, 2011). We were specifically focused on the diagnostic procedures used for the diagnosis of EBV at present as well as on follow-up stage. Only atypical symptoms of IM syndrome were taken into account. The whole diagnostic procedure of all reviewed cases is summarized in two tables (Table 1, 2).

## 2. Diagnostic tools

The sophistication of the diagnostic testing greatly improved since 1960s and 1970s, as many uncommon clinical conditions have been reported. Today, diagnosis of EBV infection is commonly based on clinical symptoms and a combined use of serological and molecular methods.

**Table 2. Serological methods for EBV diagnosis**

| Clinical condition associated with EBV | Serological methods | | | | | Serological methods at follow-up stage |
|---|---|---|---|---|---|---|
| | HAbs | IgM-VCA Abs | IgG-VCA Abs | IgG-EBNA Abs | IgG-EA-D Abs | |
| Aspecific membrabous laryngitis | + | – | – | – | – | - |
| Chronic bullous disease | – | + | + | + | – | IgG-VCA Abs |
| Conjunctival tumor | Not mentioned | | | | | - |
| Intestinal pseudo-obstruction and acute pandysautonomia | – | – | + | + | – | IgG-EA-D Abs |
| Henoch schönlein purpura | – | + | – | – | – | - |
| Systemic lupus erythematosus | + | + | + | + | – | IgG-VCA Abs |
| Gianotti- Crosti syndrome | – | + | + | + | + | - |
| Cervical ankylosis | Not mentioned | | | | | - |
| Glomerulonephritis | – | + | + | + | – | IgG-VCA Abs |
| Acquired chiari I malformations | + | – | – | – | – | - |
| Atypical subacute thyroiditis | – | + | + | – | – | IgM-VCA Abs, IgG-VCA Abs |
| Genital ulcers | + | + | + | + | – | IgG-VCA Abs IgG-EBNA Abs |
| C1q nephropathy | – | + | + | + | + | IgM-VCA Abs, IgG-VCA Abs, IgG-EBNA Abs, IgG-EA-D Abs |
| Polyglandular syndrome type II | – | + | – | – | – | - |
| Acute acalculous cholecystitis | – | + | + | + | + | IgG-VCA Abs, IgG-EBNA Abs |
| Lemierre´s syndrome | + | – | – | – | – | - |
| Frosted branch angiitis | + | + | – | – | – | IgM-VCA Abs, IgG-VCA Abs |
| Erythema multiforme-like lesions | + | – | – | – | – | - |
| Kikuchi´s disease | + | + | – | – | – | - |
| Graves´ disease | – | + | + | + | – | - |
| Gastritis | – | + | + | + | + | - |
| Acute dacryocystitis | + | + | + | – | – | IgM-VCA Abs, IgG-VCA Abs |

### *2.1 Serological methods*

There are long lasting and easily detectable levels of specific and non-specific EBV antibodies, including the following: non specific IgM heterophile antibodies (HAbs), IgM and IgG antibodies to the viral capsid antigen (IgM-VCA Abs, IgG-VCA Abs), IgG antibodies to the EBV early antigen-diffuse (IgG-EA-D Abs) and IgG antibodies to the EBV nuclear antigen (IgG-EBNA Abs) (Klutts *et al.*, 2009). Moderate-to-high levels of HAbs are present during the first month of illness and decrease rapidly after the 4$^{th}$ week. IgM-VCA Abs appear early during an infection and disappear within 4 to 6 weeks. IgG-VCA Abs appear in the acute phase, culminate at 2$^{nd}$ to 4$^{th}$ week after onset, decline slightly, and then persist forever. IgG-EA-D Abs appear in the acute phase and generally fall to undetectable levels after 3 to 6 months. Finally, IgG-EBNA Abs are absent in the acute phase, but they slowly appear in 2$^{nd}$ to 4$^{th}$ month after onset and persist forever (Fig. 1).

### *2.2 Molecular methods*

#### *2.2.1 Qualitative and quantitative PCRs*

It was suggested that PCR for EBV DNA in serum is a useful addition to the available test-panel, particularly when it is used as a confirmatory test in conjunction with serological tests (Chan *et al.*, 2001). More recent studies indicate that real-time quantitative PCR is a very sensitive, as well as useful method to define infection status, especially in immunocompromised patients and in those who are at risk to develop EBV-related disorders (De Paschale and Clerici, 2012). The most ideal samples for the PCR method were suggested to be the whole blood, tissue sections or cells from swabs (Siennicka and Trzcińska, 2007).

#### *2.2.2 Histochemical assays*

Histochemical assays are widely used to localize EBV nucleic acid or protein in malignant cells. These tumor cells express a limited spectrum of viral proteins, including

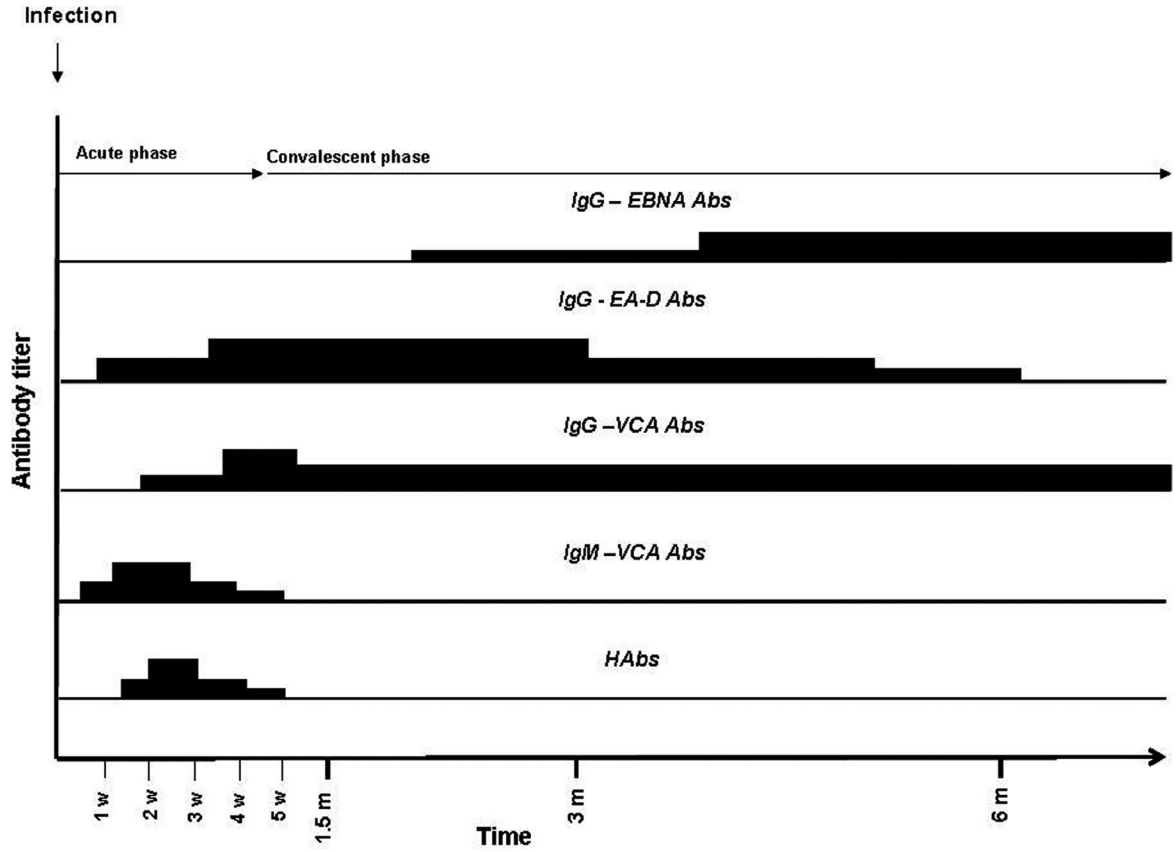


**Fig. 1**

**Time course of induction of various EBV antibodies**

w = week; m = month.

EBNA-1 and the latent membrane proteins (LMP1, LMP2a, and LMP2b). In addition, EBV-encoded RNA (EBER1 and EBER2) is expressed abundantly, although EBER transcripts are non-polyadenylated and remain untranslated (Gulley *et al.*, 2002). There are several methods to detect EBV in tissue samples: *in situ* hybridization with probes directed against EBER1 and EBER2, Southern blot analysis and hybridization with radioactive probes, immunohistochemical staining for EBV LMP1, LMP2a, and LMP2b or EBNA, and detection of EBV-specific DNA sequences with PCR analysis (Frías *et al.*, 2000). *In situ* hybridization has been considered as the gold standard for detecting EBV in tissue samples because it is sensitive, specific and fast (Leblond *et al.*, 1998). Furthermore, EBER *in situ* hybridization has been recommended as the best test for the detection and localization of latent EBV in tissue samples (Gulley *et al.*, 2002).

## 3. False-positive results of diagnostic procedures

### *3.1 Serological methods*

Serological tests (21 out of 22 cases), remain the most commonly used diagnostic tool for the diagnosis of EBV infection (Table 1). Although the assignment of certain serological patterns is a point of disagreement, it was suggested that, if all five antibodies (HAbs, IgM-VCA Abs, IgG-VCA Abs, IgG-EA-D Abs, and IgG-EBNA Abs) are determined, there are 32 possible serological patterns that can be generated (Klutts *et al.*, 2009). Therefore, there is a high possibility of incorrect result interpretation (Jenson, 2000). Thus, the interpretation of EBV serological patterns so far remains a challenge for the physicians (Hess, 2004).

#### *3.1.1 IgM-VCA antibodies*

Detection of specific IgM antibodies against EBV (15 out of 22 cases) is the method of first choice (Table 2) (de Ory *et al.*, 2011). Nevertheless, it is now accepted that there is a high degree of variability in IgM-VCA Abs serological response against EBV infection (Vilibic-Cavlek *et al.*, 2011). This variation may be caused by many reasons: an infection with heterologous virus and a subsequent development of cross-reactive antibodies, a selective stimulation of memory B cells by related antigens, autoantibodies, other serum factors, an anamnestic reaction to other recent infections (Matheson *et al.*, 1990), a polyclonal B cell stimulation (Jenson, 2000) and a variety of immunosuppressive conditions (Henle and Henile, 1980). Furthermore, in a certain percentage of patients a persistent IgM-VCA Abs response could be observed (Bauer, 2009). It is therefore impossible to derive a reliable indication for an acute EBV infection from a positive IgM-VCA Abs results (Bauer, 2009).

#### *3.1.2 IgG-EBNA antibodies*

In ten cases IgG-EBNA Abs were determined in combination with other serological markers (Table 2). Despite the fact that this parallel IgG-EBNA Abs determination could bring clarity in some cases, the result remained inconclusive in other cases (Bauer, 2009). This effect can be explained by the fact that the primary infection in children or immunocompromised patients could cause delayed appearance of IgG-EBNA Abs (Chan *et al.*, 2001). In addition, about 5% of the patients do not produce IgG-EBNA Abs after EBV infection (Bauer, 1995; Kampmann *et al.*, 1993) or their levels remain below the detection´s limit (Kampmann *et al.*, 1993; Lamy *et al.*, 1982). On the other hand, even when they are produced, they may be lost over the time particularly, but not exclusively, in immunocompromised patients (De Paschale and Clerici, 2012; Bauer, 1995, 2001; Vetter *et al.*, 1994). Therefore, in all patients with negative IgG-EBNA Abs and positive IgG-VCA Abs there is no clear differentiation among an acute EBV infection, a lack of IgG-EBNA Abs formation despite a past infection and a secondary loss of IgG-EBNA Abs (Bauer, 2009). In conclusion, it was found that test for IgM-VCA Abs or IgG-VCA Abs solely is not reliable for active EBV infection diagnosis, when IgG-EBNA Abs are absent (Chan *et al.*, 2001).

#### *3.1.3 IgG-EA-D antibodies*

It should be noted that IgG-EA-D Abs determination could elucidate some cases but it is common practice for physicians to order only the IgG-VCA Abs, IgG-VCA Abs or HAbs, so as to determine the EBV disease stage or patient's serological status (Klutts *et al.*, 2009). Similarly, only five of the previously mentioned cases used this serological marker in combination with the other serological markers for the diagnosis of EBV infection (Table 2). In addition, 20%-30% of healthy people with a history of EBV infection have IgG-EA-D Abs, for years, compromising its importance in acute EBV infection diagnosis (De Paschale and Clerici, 2012).

#### *3.1.4 Heterophile agglutination test*

The use of HAbs is controversial because although some studies show that they are very sensitive (94% of infected patients with transient infection) (Jensen and Vestergaard, 1997), others find only a few cases with simultaneous positive IgG-EBNA Abs, IgG-VCA Abs, IgM-VCA Abs, and HAbs (Klutts *et al.*, 2009). Although the presence of HAbs has a high specificity for EBV infection (Fisher and Bhalara, 2004), similarly to other serological markers, false-positive results have occasionally been reported. Leukemia, rubella virus, malaria, systemic lupus erythematosus, pancreatic carcinoma, viral hepatitis and HIV infection are some agents which can cause false-positive HAbs results (Schumacher

*et al.*, 1979; Hendry and Longmore, 1982; van Essen *et al.*, 1988).

*3.1.5 Second serum sample test*

A second serum sample, taken after a certain lapse of time, can elucidate any inconclusive result, raised from the initial serological tests. This occurs in patients, who are checked frequently over the time, in order to detect changes in specific antibody titres. A second serological test was performed in only ten previously mentioned cases, but in six of them (Besnard *et al.*, 2000; Verdolini *et al.*, 2002; Kano *et al.*, 2005; Volta *et al.*, 2005; Halvorsen *et al.*, 2006; Attilakos *et al.*, 2009) the serological tests were performed 4–24 months after the initial serological test (Table 2). It is obvious that some patients might have had a mild or an asymptomatic EBV infection during these months, so that the changes in the antibody titres were independent from the initial presentation.

### *3.2 Molecular methods*

A number of different methods, techniques and protocols have been used to determine the presence of EBV DNA and to measure viral load (De Paschale and Clerici, 2012). Dot blot analysis, Southern blot analysis, PCR and in situ hybridization have been applied to various materials. Their differences in sensitivity and specificity, have led to results that need to be carefully considered (Jenson, 2004), because they varied from laboratory to laboratory (Macsween and Crawford, 2003; Preiksaitis *et al.*, 2009).

*3.2.1 Qualitative and quantitative PCRs*

Similarly to six previous cases (Table 1), infections caused by EBV can also be diagnosed with molecular techniques, such as PCR. EBV DNA is present in a small fraction of lymphoid cells and healthy virus carriers harbor 1 to 50 EBV genomes per $10^6$ mononuclear cells, with B lymphocytes representing the major cellular reservoir. Therefore, qualitative PCR assays are unable to distinguish an active from a latent infection. Consequently, clinical interpretation of positive results is difficult (Ruiz *et al.*, 2005). Although real-time quantitative RNA assays are performed with high sensitivity and specificity, there is still no consensus regarding the appropriate material, the units of measurement, or the quantitative levels requiring intervention or predicting prognosis (De Paschale and Clerici, 2012). Furthermore, there is a debate about the appropriate material that should be used for EBV DNA detection (whole blood, peripheral blood mononuclear cells, plasma or serum) (Kimura *et al.*, 2008; Henle and Henle, 1966). The whole blood may be incorrectly stored, so that it leaves the intracellular compartments and gives rise to false-positive results in plasma or serum (De Paschale and Clerici, 2012; Ruiz *et al.*, 2005). We should also keep in mind that there may be an individual variation as a result to individual differences in kinetics of viral load. This fact may cause an increase of viral load after an initial decline, while in some cases it may take as long as a year or more before it reaches stably low levels (De Paschale and Clerici, 2012). All the above mentioned conditions may lead to false-positive results.

*3.2.2 In situ hybridization*

Similarly to six previous cases, *in situ* hybridization (especially EBER *in situ* hybridization) is widely used to define cases of EBV-related diseases (Table 1). Several pitfalls in technique and interpretation have been described. For example, false-positive EBER interpretations are attributable to a confusion among a latent infection of background lymphocytes, nonspecific staining or cross reactivity with mucin, yeast, or plant materials. Furthermore LMP1 immunostaining results should be cautiously interpreted, since false-positive staining was reported in poorly fixed tissues, nervous system cells, and some uninfected hematopoietic elements, including eosinophils and plasma cells (Gulley *et al.*, 2002).

## 4. Diseases not associated with EBV

It is possible that the clinical features of various infectious and noninfectious diseases overlap EBV infection symptoms (Chan *et al.*, 2001). It is well known that other acute bacterial or viral syndromes (especially due to heterologous viruses), including human cytomegalovirus, adenovirus, herpes simplex virus, parvovirus B19, hepatitis viruses, HIV, *mycoplasma*, *Toxoplasma gondii* and possibly rubella virus, can also lead to similar clinical syndromes (IM-like syndromes) (Klutts *et al.*, 2009). In eight cases (Table 1) there was no consideration for other infectious agents, especially heterologous viruses, as possible IM syndrome causes. Consequently, some of these patients might not be included because of a true EBV infection, but because of an infection from a heterologous virus, with subsequent development of cross-reactive antibodies to EBV.

Only in a few cases the patients had typical symptoms indicating IM syndrome (Baldari *et al.*, 1996; Grech and Vella, 2002; Halvorsen *et al.*, 2006; Akahori *et al.*, 2010) (Table 1). On the contrary, in the majority (18 cases), the patients had atypical symptoms or symptoms indicating only possible IM syndrome. It is true that an acute infection from EBV can vary widely regarding the severity of illness, ranging from an asymptomatic infection to a serious, life-threatening version of IM syndrome. It is also possible though, that these patients acquired EBV infection in the recent past, with a coincidental persistence of serological markers (Bauer, 2009; Fisher and Bhalara, 2004).

Furthermore, reliability of some cases might be reduced because their clinical conditions were reported before the EBV identification as causative agent. For this reason they lack any specific virological confirmation (Hundsdoerfer *et al.* 2000; Grech and Vella, 2002; Kano *et al.*, 2005; Shokouhi and Naghili, 2005; Halvorsen *et al.*, 2006; Garimorth *et al.*, 2008; Ghauri *et al.*, 2011) (Table 1).

Finally, there are only a few detailed cases reported for these uncommon clinical conditions. Small number of cases that have been studied does not offer enough evidence to confirm a true causal relation between EBV and the uncommon clinical conditions. This rarity might reflect only a coincidental association with EBV infection.

## 5. Conclusions

There must be an awareness of the nuances of available serological tests for latent viruses, such as EBV. We should also be cautious about using them alone or in combination with molecular methods as the sole mean to establish a casual association of an uncommon clinical condition with EBV. For this reason accurate laboratory tests for EBV detection and also strict criteria for an EBV infection's diagnosis are needed, in order to avoid a possible coincidental association of uncommon clinical conditions with EBV. Until then, it is crucial to realize that the essential components for such a true casual association are the cumulative clinical experience or the experimental data that demonstrates plausible biological mechanisms.

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