Diploma thesis

Evaluation of a new EBV DNA quantitative prototype test

submitted by

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Graz, August 31, 2021

I declare that I have written this diploma thesis independently, that I have not used other than the sources/ resources cited, and that I have explicitly marked all material, which has been quoted either literally or by content from the sources used.

Graz, August 31, 2021 Anna Achathaler eh.

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Abstract

Background: After infection, the Epstein-Bar virus (EBV) maintains lifelong but the healthy immune system keeps it in check. In immunocompromised patients, EBV may reactivate and lead to chronic disease such as post-transplant lymphoproliferative disorder (PTLD). Detection and accurate quantitation of EBV DNA is of paramount importance to recognize reactivation as early as possible in order to initiate a preemptive strategy.

Objectives: The aim of this study was to compare results obtained by a prototype test for detection and quantitation of EBV DNA on the cobas® 6800/8800 systems.

Materials and Methods: The performance of the cobas® EBV prototype test was evaluated at the Medical University of Graz, Austria. The cobas® EBV prototype test was compared to a CE-IVD labeled EBV test (bioMerieux R-GENE®) after extraction with either the eMAG® or the MagNA Pure 24 platform. For determination of analytical performance, an EBV EDTA plasma panel based on the 1st WHO International Standard for EBV was used. EBV DNA concentrations ranged from 1.0E+05 to 1.0E+02 International Units IU/mL. For determination of clinical performance, 30 plasma samples positive for EBV DNA were diluted in EBV negative human plasma and dilutions were tested.

Results: When the analytical performance of the cobas® EBV prototype test was studied with dilutions of the WHO panel, concentrations obtained with the cobas® EBV prototype test were found to be very close to those expected (-0.04 to 0.06 $\log_{10}$ IU/mL). When clinical samples were tested, the mean difference observed with the cobas® EBV prototype test was found to be 0.07 $\log_{10}$ IU/mL. The mean difference between the cobas® EBV prototype test and R-GENE® was -0.23 $\log_{10}$ IU/mL, respectively.

Conclusions: The cobas® EBV prototype test showed high agreement when tested with the 1st WHO International Standard for EBV. When clinical samples were tested with the cobas® EBV prototype test and compared to alternative test systems, an excellent correlation was observed.

Kurzfassung

Hintergrund: Das Epstein-Barr Virus gehört zur Gruppe der Herpes-Viren. Die meisten Menschen sind Träger dieses Virus. Mit Hilfe eines intakten Immunsystems kann das Virus unter Kontrolle gehalten werden. Kommt es allerdings zu einer Suppression des Immunsystems, kann es zu einer Reaktivierung kommen und in weiterer Folge zu einer post-transplant lymphoproliferative disorder (PTLD). Aus diesem Grund ist die exakte Messung des EBV DNA-Spiegels von größter Bedeutung. Nur dadurch wird man frühzeitig auf eine PTLD aufmerksam und kann eine präemptive Therapie einleiten.

Materialien und Methoden: In dieser Studie wurden zur Bestimmung der Plasma EBV-Viruslast der cobas® EBV-Prototyptest am cobas® 6800/8800 System und der bioMerieux R-GENE® Test nach Extraktion entweder auf der eMAG® oder auf der MagNA Pure 24-Plattform verglichen. Um die analytische Leistungsfähigkeit zu bestimmen, wurde der 1. Internationale WHO Standard für EBV verwendet. Die verwendeten EBV DNA-Konzentrationen lagen zwischen 1.0E+05 und 1.0E+02 International Units (IU)/mL. Um die klinische Leistungsfähigkeit zu bestimmen, wurden 30 EBV-DNA-positive Plasmaproben untersucht.

Ergebnisse: Die Untersuchung der analytischen Leistungsfähigkeit des cobas® EBV-Prototyptests zeigt, dass die bestimmten Konzentrationen sehr nahe an den erwarteten Werten lagen: -0.04 bis 0.06 $\log_{10}$ IU/mL. Die Auswertung der klinischen Proben ergabt mit dem cobas® EBV-Prototyptest einen mittleren Unterschied von -0.07 $\log_{10}$ IU/mL. Der mittlere Unterschied zwischen dem cobas® EBV-Prototyptest und dem R-Gen® betrug -0.23 $\log_{10}$ IU/mL.

Fazit: Der cobas® EBV-Prototyptest zeigte im Vergleich mit den Proben des 1. Internationalen WHO Standard eine nur minimale Abweichung. Bei klinischen Proben, die mit dem cobas® EBV-Prototyptest getestet wurden, wurde eine gute Korrelation mit dem R-GENE® beobachtet.

1. Introduction

The history of EBV started in central Africa in 1958. The surgeon Denis Burkitt described a certain type of cancer, which occurred in young children. He noticed that the so-called Burkitt lymphoma appeared in a specific geographic area in central Africa with high temperatures and rainy weather. He first thought that an insect-born virus would cause the cancer. Nevertheless, a few years later he met Michael Anthony Epstein who had huge interest to detect the reason for this cancer. In 1965, Epstein and his team, Yvonne Barr and Burt Achong, detected a virus in the tissue of a patient with Burkitt lymphoma, which was later named after its discoverers [1], [2].

1.1 The *herpesviridae* family

There are eight different herpes viruses, which can infect humans and lead to serval diseases. All of them are organized in three subfamilies, so called α, β, and γ herpes virus. Herpes simplex virus types 1 and 2 (HSV 1, HSV 2), and varicella-zoster virus (VZV) belong to the alpha herpes virus group. These cause herpes labialis, genitalis, and chickenpox. Beta herpes virus group include roseolovirus (HHV 6, HHV 7) and cytomegalovirus (CMV) which are associated with roseola infantum and infectious mononucleosis. Moreover, Kaposi's sarcoma-associated virus (KSHV) and Epstein-Barr virus (EBV) belong to gamma herpes virus group and cause serval types of cancer and autoimmune diseases [3].

1.2 The Epstein-Barr virus

The Epstein-Barr virus (EBV) also called human gamma herpesvirus 4 is an enveloped DNA virus. The EBV genome consists of a linear double stranded DNA genome, which is encased from an icosahedral capsid. The virus infects B cells but also epithelial cells [3].

The virus has a toroid-shaped protein core, which is coated with DNA, a nucleocapsid with 162 capsomers and an envelope with virus-encoded glycoprotein spikes. Between the envelope and nucleocapsid is a protein tegument [4].

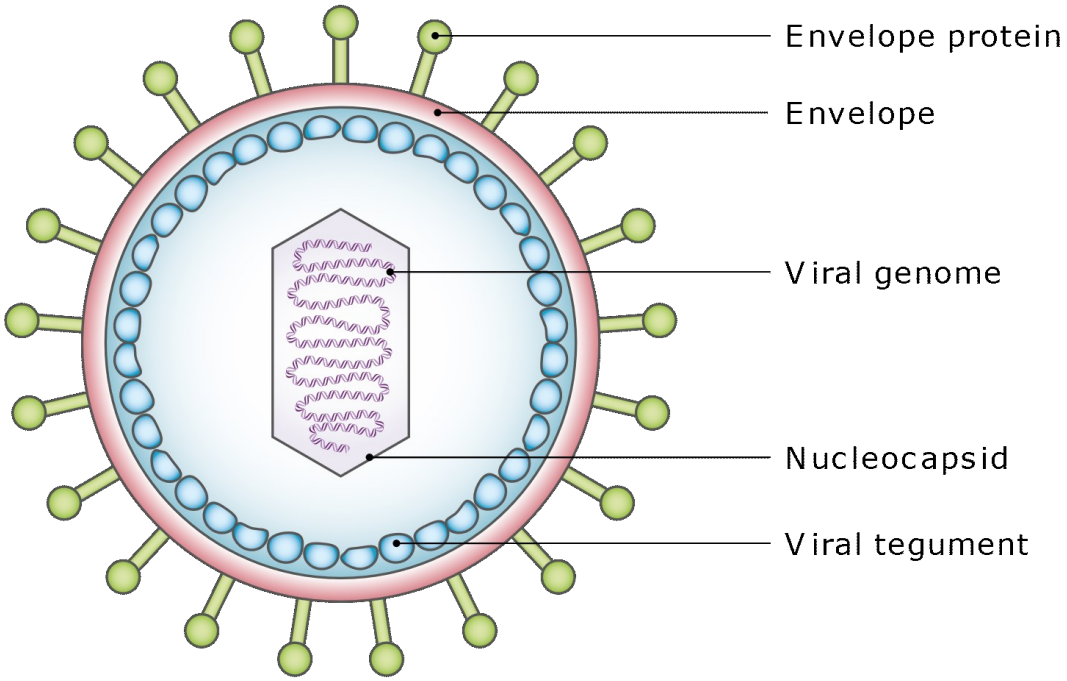

Fig. 1. Simplified structure of EBV.

(https://en.wikipedia.org/wiki/Epstein%E2%80%93Barr_virus#/media/File:Viral_Tegument.svg)

1.3 Epidemiology

EBV is highly prevalent all over the world. It is estimated that worldwide nearly 90% of people carry the EBV. After primary infection, the virus persists lifelong in a latent stadium in B lymphocytes [1].

Usually, primary infection takes place during childhood or early adolescence with the majority lacking symptoms. In case of symptoms, patients present swollen lymph nodes, sore throat, fever, and severe fatigue. Even though the symptoms usually disappear within 2 to 4 weeks, about 10% of the patients suffer from fatigue for 6 months or longer. Serious complications such as liver, neurological, or hematological diseases are rarely found. A longer-lasting immune dysfunction can be observed more frequently [5].

In healthy adults, the virus is usually suppressed by the immune system. If the immune system is weakened because of medication, illness, or infection (especially HIV infection), the virus may reactivate [5]. Several diseases associated with impaired immune function and EBV reactivation have been described including post-transplant lymphoproliferative disease (PTLD), Burkitt lymphoma, and nasopharyngeal carcinoma [6]. PTLD is typically observed in bone marrow and solid organs transplant recipients and found worldwide. While EBV-positive Burkitt lymphoma is a frequent childhood tumor in equatorial Africa and New Guinea, EBV-associated nasopharyngeal carcinoma is mainly observed in Southern China [5].

1.4 Transmission

There are several ways to transmit the virus. The first route is through deep kissing which is very popular among young adults and adolescents. Usually, infected people present without any symptoms and so they act as if they were healthy. This leads to more infections and makes it to the most common route of transmission. The second way of transmission is through sexual intercourse. EBV may also be transmitted through blood transfusions. It is estimated that the risk of getting EBV via blood products is low, but an accurate number is not known, because most patients remain asymptomatic. The fourth transmission route is via allograft transplantation. After hematopoietic cell and solid organ transplantation there is a documented transmission from donor to recipient. It is not known how babies are infected by EBV. Finally, EBV might be transmitted through close contact with household members or caregivers. It is assumed that children take up the virus through contact with oral secretions of “carriers” such as siblings, parents, or caregivers. It is expected that sharing cutlery, toothbrushes, or drinking glasses might transmit the virus but this is not proven yet [7].

1.5 Genome and Genotypes

1.5.1 Genome

The genome of EBV is a linear, double-stranded, about 172 kb long DNA molecule, which encodes more than 85 genes. According to a map of BamHI-restrictions fragments the nomenclature of EBV open-reading frames (ORF) was accomplished. The fragments were put in order, depending on their size, descending from A to Z. Furthermore, the EBV ORFs are classified into two subgroups: lytic and latent genes [8].

The EBV genome encodes serval proteins. Most of them are involved in the metabolism of the nucleotides to support the replication and build the different compartments of the viral structure. There are some latent genes, which are a not translated during the lytic phase. In this phase only EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C, EBNA-LP and three membrane proteins are expressed. One of them is called LMP-1, which has oncogenic properties [9].

1.5.2 Genotypes

There are two genotypes of EBV: Type 1 and Type 2 or Type A and Type B. Based on the difference in the EBNA-2 gene these two groups are distinguished. The other EBV gens are nearly similar. They only differ in under 5 percent of their sequences. Conspicuously, the two types differ in transformation abilities. Furthermore, EBV Type 1 and Type 2 can be subtyped in several EBV strains. These strains do have a huge genetic variability. Interestingly, multiple EBV variants can be found in one patient.

The most investigated EBV strain is the LMP-1 oncogene sequence. It has a great polymorphism and so it is divided in seven main groups. Some activities of LMP-1 include production of cytokines, cell surface markers, suppression of cell senescence and DNA synthesis [8], [9].

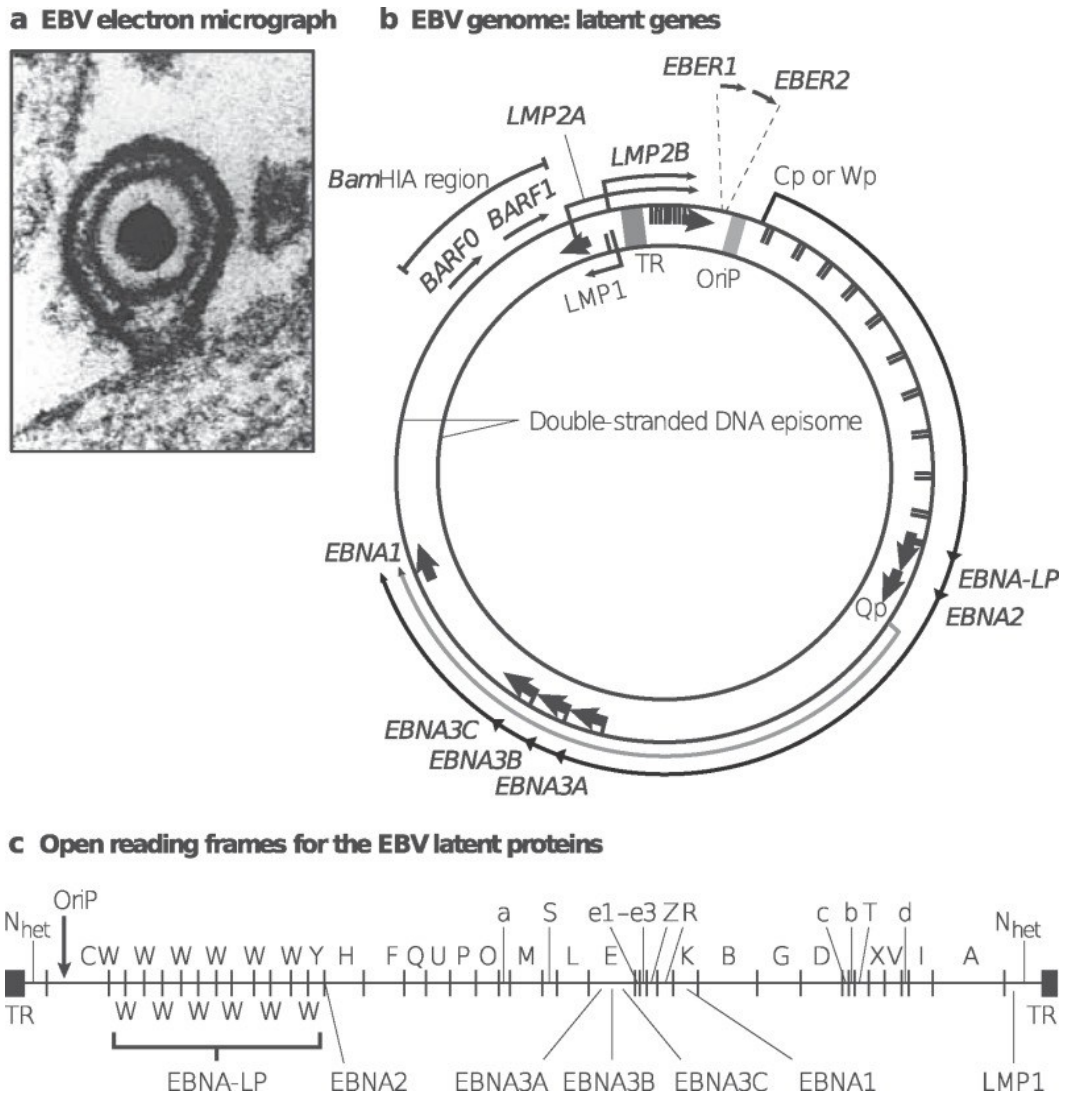

Fig. 2. The EBV Genom.

(Biological Agents.IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, No. 100B.IARC Working Group on the Evaluation of Carcinogenic Risk to Humans.Lyon (FR): International Agency for Research on Cancer; 2012.)

1.5.3 EBV life cycle

Usually, the EBV is transmitted via saliva and after entering epithelial cells of the tonsils, the lytic phase of infection begins. This phase includes virus replication. After infection, the naive B lymphocytes turn into activated lymphoblasts and enter the lymph node. In this phase, EBV nuclear antigens such as EBNA-1 and EBNA-2 and latent membrane proteins were expressed. It is possible that infected memory B lymphocytes migrate back to the tonsils and infect other B lymphocytes also. During latency phase no viral proteins are expressed and so the virus can hide from the immune system. The EBV remains in the resting memory B lymphocytes, but under certain circumstances it can get reactivated. Causes for reactivations are inflammation, drugs, other infections, immunodeficiency, and immunosuppression. This leads to a new EBV cycle, which includes replication and infecting new cells [8], [9].

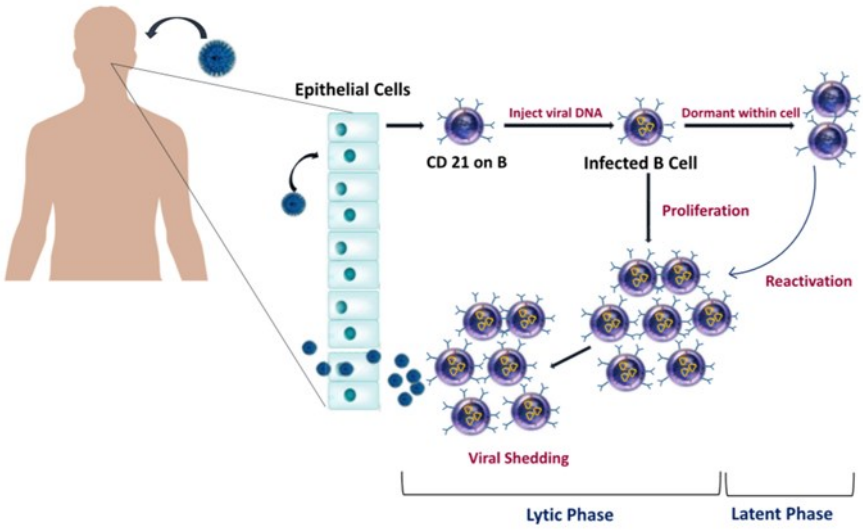

Fig. 3. EBV life cycle.

(Maria K. Smatti et al., "Epstein–Barr virus epidemiology, Serology, and Genetic variability of LMP-1 Oncogene Among Healthy Population: An Update", review, frontiers in oncology, June 2018, Volume 8, article 211, S. 4)

1.6 Infection

1.6.1 Symptomatic infection

The primary infection with EBV is called infectious mononucleosis, Pfeiffer's glandular fever, or kissing disease. After the incubation period, which varies from one to seven weeks the disease manifests primarily in the form of tonsillitis and sore throat. Furthermore, patients present with swollen lymph nodes, fever, and fatigue, which can last over several weeks. The tonsils usually look enlarged with white-grayish confluent deposits. Sometimes, organs are co-affected, presenting as splenomegaly and/or hepatomegaly. Rarely, other organs such as heart, kidneys, and central nervous system may be involved. Other rare symptoms include petechial enanthema on the hard palate and fine-stained rash on the trunk.

Symptom	Number of subjects (percent)	Median duration (days)[a]
Sore throat	59 (98%)	7.5
Swollen or tender cervical lymphadenopathy	53 (88%)	15.0
Fatigue	47 (78%)	15.5
Decreased appetite	39 (65%)	10.0
Headache	38 (63%)	8.0
Felt febrile	32 (53%)	5.5
Body aches (myalgia)	30 (50%)	8.0
Upper respiratory symptoms (cough, runny nose, nasal stuffiness)	29 (48%)	8.0
Abdominal pain	9 (15%)	16.0

[a] Of symptomatic subjects.

Fig. 4. Symptoms of infectious mononucleosis in 60 undergraduate students studied prospectively.

(Samantha K. Dunmire, Priya S. Verghese, Henry H. Balfour Jr, "Primary Epstein-Barr virus infection", review, Journal of Clinical Virology, 102 (2018) 84–92)

Figure 4 shows the data of three prospective studies in which college students with infectious mononucleosis were observed. They presented with different symptoms and the most common ones were sore throat and swollen cervical lymph nodes. Abdominal pain, for instance, occurred in only 15 percent of all symptomatic students. That study showed that the period of the acute illness is between 8 and 18 days. Nevertheless, most of the patients with primary EBV infection do not present any symptoms [7].

1.6.2 Asymptomatic infection

The asymptomatic infection is defined as infection without any symptoms. Nevertheless, patient carry the virus and may transmit it [7]. Younger people under the age of 10 years typically present asymptomatic, whereas adolescent and adults show symptoms mentioned above [10].

1.6.3 Complications

Acute illness

During the acute phase, severe complications are not common because most patients have an intact immune defense. It is estimated that serious complications such as streptococcal pharyngitis, hemolytic anemia, thrombocytopenia, and meningoencephalitis occur in less than one percent. Despite that, the most dangerous complication is splenic rupture [7].

Chronic infectious mononucleosis

Primary EBV infection may result in chronic disease. The patient’s immune system may not be able to control the virus appropriately and persistent or recurring symptoms may be observed [7].

Posttransplant lymphoproliferative disorder (PTLD)

In solid organ recipients, PTLD is a potentially fatal complication. There are two different ways how patients can get PTLD. Firstly, after every transplantation, patients must take drugs, which suppress their immune system. If the patient has already a latent infection, it can lead to a reactivation of the virus because of the immunosuppressive drugs. Secondly, when a seronegative patient gets an EBV seropositive donor organ, EBV infected B cells can grow uninhibited, and patients develop PTLD. The number of infected B cells can be 50 time higher in the immunosuppressed than in the healthy population. Because of immunosuppression, the number of cytotoxic EBV specific T cells decreases, the lymphoproliferative blasts cannot be controlled any more, and grow unimpeded [10].

1.7 Diagnosis

Clinical presentation of EBV is equal to other acute viral infections such as CMV or hepatitis and therefore, it is important to have reliable laboratory tests for making accurate differential diagnosis. The two main methods for testing EBV are serological testing and molecular assays. The choice which diagnostic tool is used depends on the immune condition of the patient as well as the urgency of the therapy [9].

1.7.1 Serological testing

Serological testing means to detect antibodies in the serum of the patient. Even though this method shows a high variability, it is the most common used one. With the serology, testing of EBV it is possible to identify the infection status of the patient. There are three important parameters to differ the disease status in immunocompetent patients: VCA-IgG, VCA-IgM, and EBNA-1 IgG. VCA is a complex that is synthesized in the lytic phase of replication and humoral response is found early at the onset of symptoms.

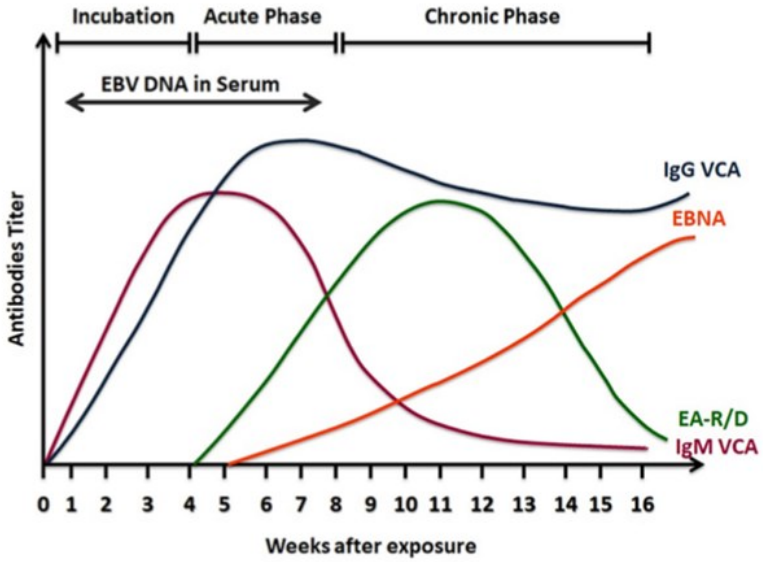

Fig. 5. Serological parameters during EBV infection.

(Maria K. Smatti et al., "Epstein–Barr virus epidemiology, Serology, and Genetic variability of LMP-1 Oncogene Among Healthy Population: An Update", review, frontiers in oncology, June 2018, Volume 8, article 211, S. 7)

Figure 5 shows a pattern of serological response to EBV during an infection. VCA-IgM immediately produced and is used to detect the active phase of primary infection. After convalescence, the antibody titer declines. At the same time VCA-IgG is increasing too, but as the graphic exhibits the titer remains positive lifelong. For this reason, VCA-IgG is a good single test to detect a previous EBV infection. The EBV nuclear antigen (EBNA) consists of six proteins and one of these can be detected in all EBV infected cells. EBNA antibodies occur 3 to 6 months after the first symptoms and remain at a detectable level for life. Nevertheless, VCA-IgG a more accurately than EBNA because EBNA-IgG are not developed in every patient. A disadvantage of detecting EBNA-IgM and VCA-IgM is, that there are often cross-reactivity with antigenically related infections like CMV or Parvovirus B19. Early antigens (EA) are a complex of nonstructural proteins and it increase in the acute phase and later declines to an undetectable level [8, 9, 11].

1.7.2 Molecular assays

Real-time PCR (qRT-PCR), southern blotting and dot blotting are methods to detect EBV DNA out of blood samples and quantify the viral load. The qRT-PCR is a sensitive method for diagnose the acute infection and monitor a silent reactivation. Monitoring is especially important for immunocompromised patients. All over the laboratories, these tests do not have any standardization and differ in sensitivity and specificity. Furthermore, there is also a variation in unit of measurement. The EBV DNA can be detected in peripheral blood mononuclear cells (PMNC), serum or unfractionated blood. In healthy people, the EBV DNA is below the limit of detection in serum, but very low concentrations can be proven in PMNC. The virus load is a prognostic marker in EBV associated diseases because the amount of virus correlates with the severity of EBV infection. Molecular detection is very important for immunocompromised patients, additionally when serology testing is unclear or confusing. However, a combination of serology and molecular testing guarantee an early and accurate detection of the EBV [9, 11].

1.8 Prevention

As EBV has a high prevalence in population worldwide, it is nearly impossible to avoid exposure. Some measures to reduce transmission would be frequent handwashing and not to share drinking glasses, eating utensils, or toothbrushes. In general, a good hygiene is useful to reduce EBV infections [7].

To protect solid organ and stem cells recipients from primary EBV infection, it is wise to select EBV-negative donors. Although, this can be difficult because nearly 90 percent of adults have positive antibodies [7, 12]. After a transplantation, the patient should get an antiviral prophylaxis for 3 to 6 months. Unfortunately, studies showed that these drugs only reduce the severity of CMV posttransplant disease but not of EBV-related posttransplant disease. Many different prophylactic EBV vaccinations have been tested yet but without a satisfactory outcome [7].

1.9 Therapy

There is no causal therapy for primary EBV infection. The only way to deal with the disease is symptomatic therapy. This means physical rest, administration of liquids, analgesia, and antipyresis if needed. It has been reported that patients who take valacyclovir show less symptoms and a milder course of disease [7].

Patients with PTLD have an abnormal proliferation of immune cells and need special treatment immediately. The most important thing is to reduce the immunosuppression therapy. Further treatments include the administration of rituximab, chemotherapy, surgery, or radiation. Antiviral drugs in PTLD do not have a proven benefit [7, 13].

2. Objectives

(1) To evaluate the analytical performance of the newly developed cobas® EBV prototype test for the cobas® 6800/8800 systems and calculate the correction factors allowing comparison with alternative test systems, the EBV R-GENE® in combination with the eMAG® nucleic acid extraction platform and in combination with the MagNA Pure 24 platform.

(2) To compare results obtained from clinical specimens with the new cobas® EBV prototype test and the EBV R-GENE® in combination with the eMAG® nucleic acid extraction platform.

(3) To compare turn-around time and hands-on time of the three different analytical workflows.

3. Materials and Methods

3.1 Study design

3.1.1 Analytical performance

At the Medical University of Graz, Molecular Diagnostics Laboratory, three different molecular assays, the cobas® EBV prototype test for the cobas® 6800/8800 systems, the EBV R-GENE® in combination with the eMAG® platform, and the EBV R-GENE® in combination with the MagNA Pure 24 platform were evaluated. Table 1 shows the study design of analytical performance testing and comparison studies. An EBV EDTA plasma dilution series with concentration levels from 1.0E+05 to 1.0E+02 International Units per milliliter (IU/mL) was prepared using the 1st WHO International Standard for EBV which is defined as 5.0E+06 IU/mL of a whole virus preparation [14]. Four replicates per panel member were tested. Imprecision was calculated as delta observed minus expected [log IU/mL] for panel members. Correlation between tests was also evaluated, with the WHO panel being further used to calculate a correction factor to IU/mL for the EBV R-GENE® assays that report results in copies per milliliter (cop/mL). Samples with results of Target Not Detected (TND), under lower limit of quantitation (≤LLOQ), or above upper limit of quantitation (≥ULOQ) were excluded from analysis.

Table 1.
Study design for evaluation of analytical performance.

Molecular assays	cobas® EBV prototype test for the cobas® 6800/8800 systems	EBV R-GENE® (on the eMAG® platform)	EBV R-GENE® (on the MagNA Pure 24 platform)
Comparison testing	cobas® vs. R-GENE ®(eMAG®)		cobas® vs. R-GENE ®(MP24)

3.1.2 Clinical performance

Clinical performance of the cobas® EBV prototype test was evaluated with 30 de-identified leftover EBV positive EDTA plasma samples. Results were compared to those obtained with the EBV R-GENE® in combination with the eMAG® platform.

3.1.3 Lab flow analysis

For the lab flow analysis, the turn-around time including hands-on time of the 3 different analytical workflows were estimated. The turn-around times required for the three molecular assays were compared.

3.2 Methods

3.2.1 Extraction of nucleic acids

Nucleic acid extraction was performed on the following platforms: cobas® 6800/8800 systems (Fig.6), eMAG® (Fig.7), and MagNA Pure 24 (Fig.8). The EDTA plasma input volume was 350 µL for the cobas® 6800/8800 systems and 200 µL for the eMAG® and the MP24 (Table 2). Nucleic acid extraction on these extraction platforms is based on the magnetic silica particle technology. In a single run, up to 94 samples can be extracted on the cobas® 6800/8800 system, up to 48 samples on the eMAG®, and up to 24 samples on the MP24.

Table 2.

Extraction platforms and input volume.

Extraction platform	Material	Input volume (µL)
cobas® 6800/8800	EDTA plasma	350*
eMAG®	EDTA plasma	200
MP24	EDTA plasma	200

*including dead volume of 150 µL

3.2.2 Amplification and detection

Amplification and detection of nucleic acid extracts was performed on the cobas® 6800/8800 system and on the Light Cycler® 480II (Roche Molecular Diagnostics, Rotkreuz, Switzerland) instrument (Fig.9).

The cobas® EBV prototype test has been designed for the fully automated cobas® 6800/8800 systems. This system includes automated nucleic acid extraction (sample preparation) and nucleic acid amplification and detection. With the cobas® EBV prototype test, it is possible to quantify the EBV DNA load. The viral load is quantified against a quantitation standard (QS), which also serves as an internal control and is added to each patient sample and external control to monitor the entire sample preparation and amplification process.

The EBV R-GENE® was performed on the Light Cycler 480II instrument. Samples had been extracted either on the eMAG® or the MP24 platforms. PCR setup and the amplification protocol were done according to the manufacturer's package insert. Test results were analyzed in Second Derivative Max mode.

Fig. 6. The cobas® 6800/8800 system (picture taken by Anna Achathaler, March 17, 2020).

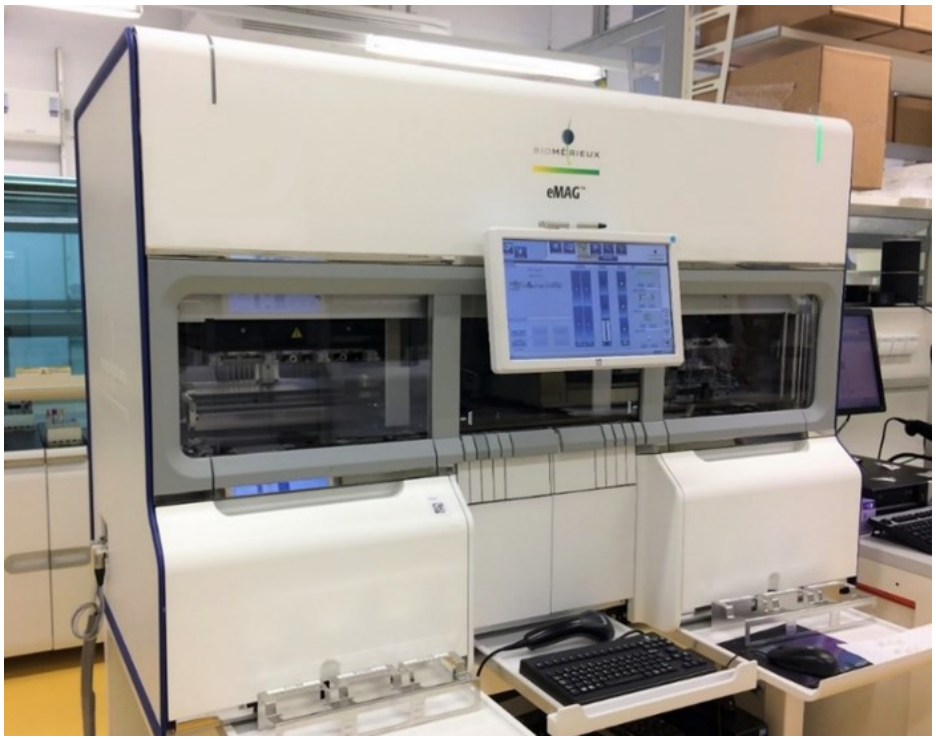

Fig. 7. The eMAG® nucleid acid extraction platform (picture taken by Anna Achathaler March 17, 2020).

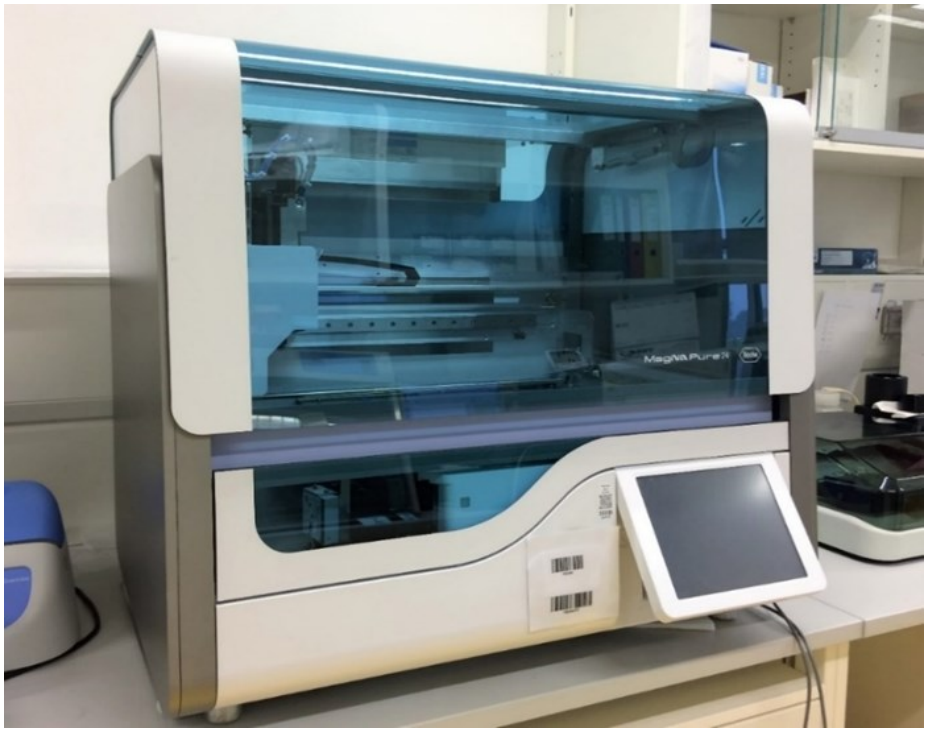

Fig. 8. The MagNA Pure 24 platform. (picture taken by Anna Achathaler March 17, 2020).

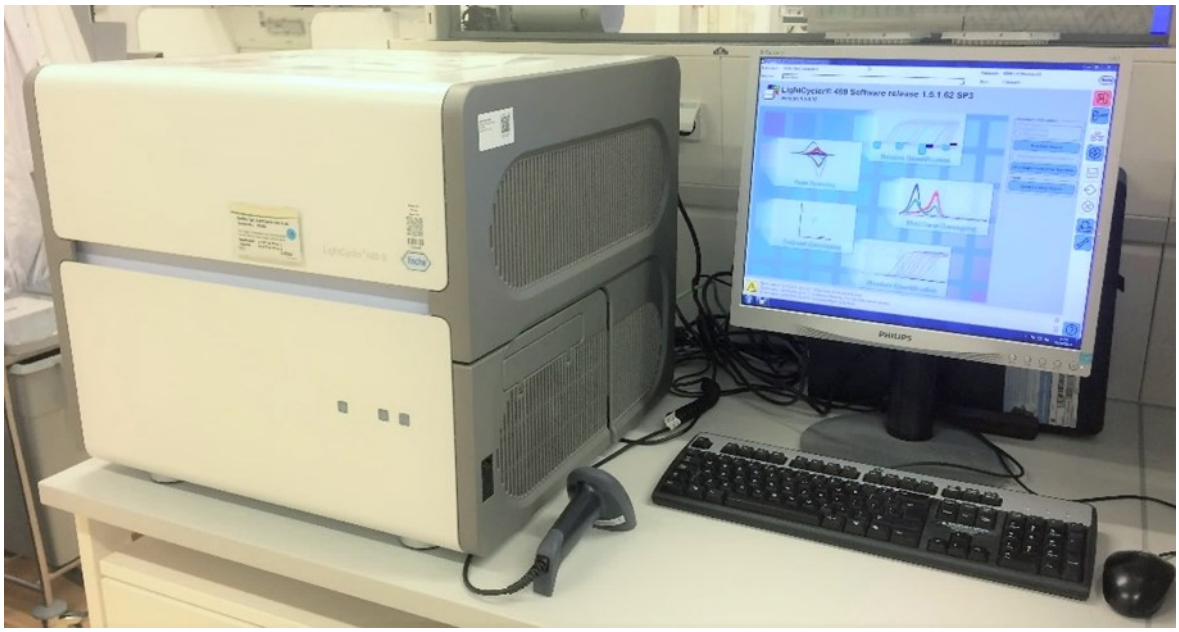

Fig. 9. The Light Cycler® 480 II instrument. (picture taken by Anna Achathaler March 17, 2020).

3.2.3 Reporting results

With the cobas® EBV prototype test, the EBV DNA viral load result is reported as a numerical number in IU/mL if within the analytical measuring range (3.5E+01 IU/mL – 1.0E+08 IE/mL). Otherwise, results are reported as “target not detected” (TND), as “EBV DNA detected but below the lower limit of quantitation” (<LLoQ), or as “EBV DNA detected but above the upper limit of quantitation” (>ULoQ).

With the EBV R-GENE®, the EBV DNA viral load result is reported as a numerical number in cop/mL. For comparison purposes, data must be given in identical units. Because results obtained by the cobas® EBV prototype test are reported in IU/mL while results reported by the EBV R-GENE® are reported in cop/mL a conversion factor must be calculated. This was done using a dilution series of the 1st WHO International Standard for EBV.

4. Results

4.1 Analytical performance

Obtained versus expected data for the cobas® EBV prototype test are shown in Table 3 and Figure 10. For each concentration, four samples were tested. The mean differences between concentrations obtained and those expected were 0.06 or less. The highest standard deviation was found for the concentration of 1.0E+02 IU/mL.

Table 3.

Analytical performance of the cobas® EBV prototype test when using dilutions of the 1st WHO International Standard for EBV.

Dilution (log_{10} IU/mL)	N	Mean titer (log_{10} IU/mL) obtained	Standard deviation	Mean difference (obtained vs. expected)
5.00	4	4.99	0.04	-0.01
4.00	4	3.96	0.02	-0.04
3.00	4	2.99	0.03	-0.01
2.00	4	2.06	0.12	0.06

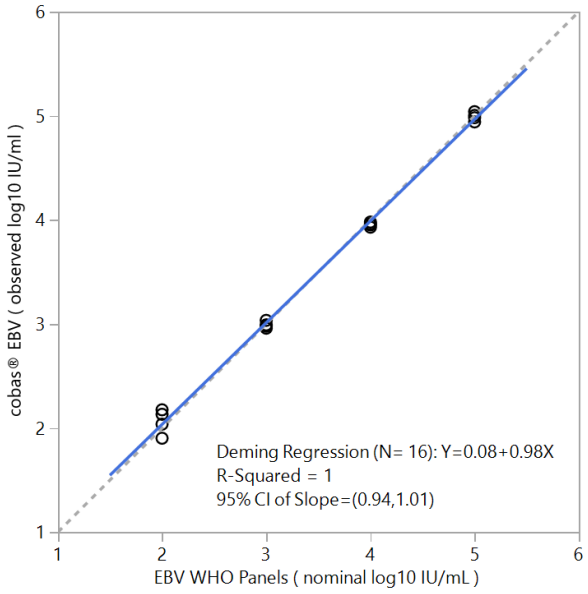

Fig. 10. EBV DNA concentrations obtained by the cobas® EBV prototyp test compared to the 1st International WHO Standard.

4.2 Calculation of conversion factors

To make results obtained by the cobas® EBV prototype test comparable to those obtained by the R-GENE® EBV, the conversion factor for the alternative test system was calculated. In this study, a dilution series based on the 1st WHO International Standard for EBV was used. Figure 11 and 12 show results obtained with the R-GENE® EBV in combination with extraction on the eMAG® or on the MP24 platform compared to those expected.

When using the R-GENE® EBV in combination with the eMAG® platform, the conversion factor was calculated as 1.25.

The formula for calculation when using the eMAG® platform is:

R-GENE® EBV in cop/mL x 1.25 = concentration in IU/mL

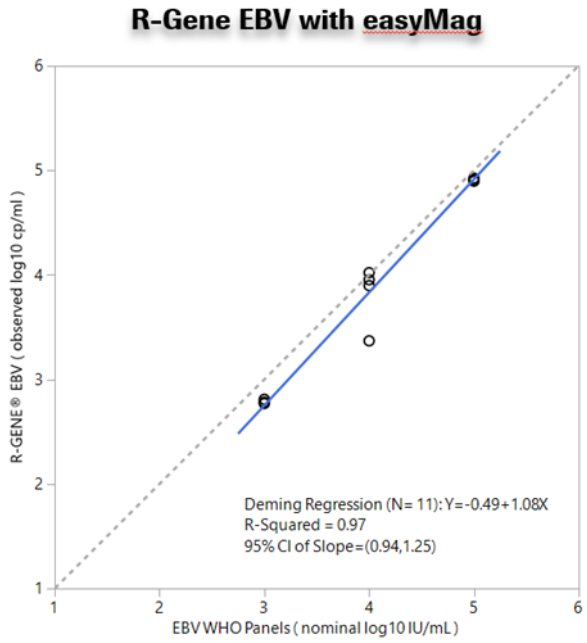

Fig. 11. Titers in log_{10} IU/mL for the EBV R-GENE® in combination with the eMAG® platform compared to 1st International WHO Standard.

When using the R-GENE® EBV in combination with the MP24 platform, the conversion factor was calculated as 0.82.

The formula for calculation when using the MP24 platform is:

R-GENE® EBV in cop/mL x 0.82 = concentration in IU/mL

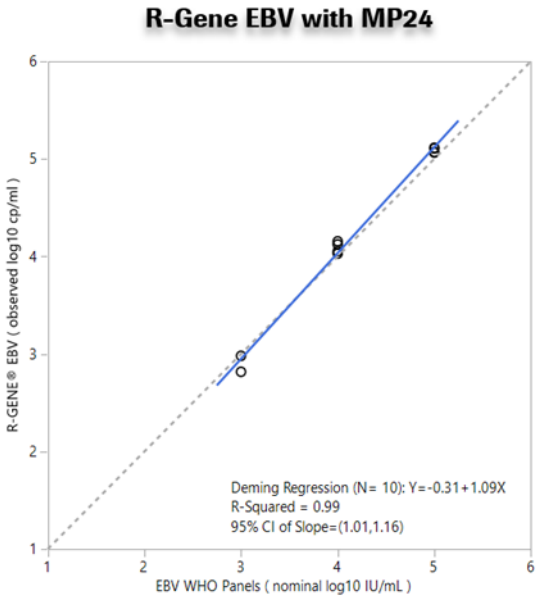

Fig. 12. Titers in $_{log}$ cp/mL for EBV R-GENE® on the MP24 platform compared to 1st WHO International Standard.

4.3 Clinical performance

To compare the clinical performance of the cobas® EBV prototype test with that of the R-GENE® test on the eMAG® platform, 16 de-identified leftover EBV positive EDTA plasma samples quantifiable with both tests were used. The cobas® EBV prototype test showed a mean 0.22 log_{10} unit lower measurement (Fig. 13). None of the results differed more than ±0.5 log_{10}. For all 16 positive samples, a correlation coefficient (R^2) of 0.96 was obtained. The ICs included in both test systems were detected within the expected range in all samples throughout this study.

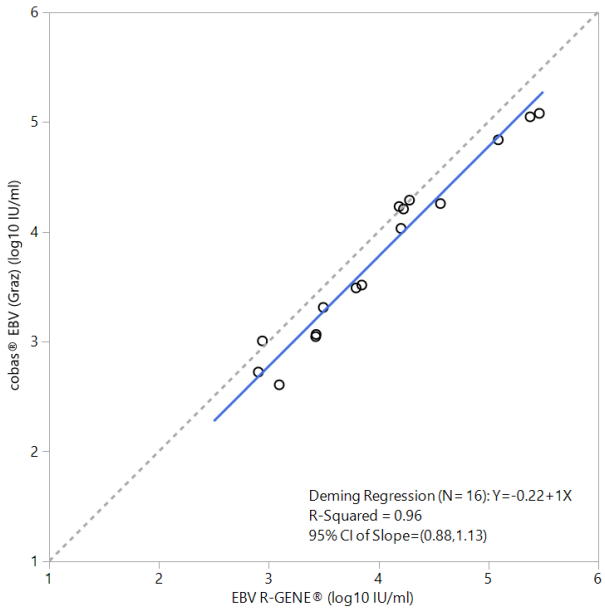

Fig. 13. Titers in log_{10} IU/mL for R-GENE® on eMAG® compared to cobas® EBV prototype test.

4.4 Lab flow analysis

For the lab flow analysis, the turn-around time including hands-on time for the three different test systems were estimated and compared. Table 4 shows the lab flow analysis for 24 samples. When samples were run on the cobas® 6800/8800 system, hands-on time was 15 minutes, for the alternative test systems, hands-on time was 20 minutes. The time required for nucleic acid extraction was 75 minutes on the eMAG® platform and 80 minutes on the MagNA Pure 24 platform. Overall, the turn-around time was 195 minutes for the cobas® 6800/8800 system, 175 minutes for the eMAG® +LC® and 180 minutes for MagNA Pure 24+LC®.

Table 4.

Lab flow analysis (for 24 samples).

	cobas® 6800/8800	eMAG® +LC®	MagNA Pure 24+LC®
Hands -on time (min)	15	20	20
Time for extraction (min)	180	75	80
Time for amplification (min)		80	80
Turn-around time (min)	195	175	180

5. Discussion

In the immunosuppressed patient, detection, and quantitation of EBV DNA is state of the art and has replaced serological testing. Standardized detection of EBV DNA is important. Especially for patients after organ or stem cell transplantation, it is necessary to detect an increase in virus load as early as possible. An increase of 0.5$\log_{10}$ is considered as significant change. In excess of this value, pre-emptive measures such as reduction of immunosuppression and/or antiviral therapy can be taken.

Standardization of diagnostic test systems for infectious disease markers is of paramount importance to facilitate clinical guidelines used for the diagnosis, treatment, and monitoring of patients (LIT). The major goal of standardization of nucleic acid-based diagnostic tests is to ensure that the sample numeric result will be obtained for a sample irrespective of the assay method used. One of the major issues with standardization is the availability of standardized reference materials. To overcome this problem, the 1st WHO International Standard for EBV DNA was introduced, with titer assignment in IU/mL. In this study, the new fully automated cobas® EBV prototype test was compared to alternative test systems for detection and quantitation of EBV DNA. The new cobas® EBV prototype test reports EBV DNA concentrations in IU/mL, the alternative tests in cop/mL. To make concentrations comparable, conversion factors were calculated [15].

When accuracy was tested, the cobas® EBV prototype test demonstrated a high agreement with the 1st WHO International Standard panel. When clinical performance was tested, conversion factors calculated were applied. EBV concentrations obtained by the cobas® EBV prototype test were found to be similar to those obtained by the alternative test systems. The cobas® EBV prototype test showed slighty lower EBV concentrations.but none of the results differed more than 0.5 $\log_{10}$.

The turn-around time including hands-on time for the cobas® EBV prototype test was found to be shorter compared to that of the EBV R-GENE®. The major advantage of this test is that it is performed on the fully automated cobas® 6800/8800 system. By using this system, time can be saved as well as human error avoided.

Limitations of this study include that only a low number of samples was used for testing clinical performance and that due to the lack of further samples dilutions had to be done.

In conclusion, the cobas® EBV prototype test proved to be a good tool for accurate estimation of plasma EBV DNA concentrations in the routine diagnostic laboratory. Values obtained are stated in IU/mL facilitating interpretation of clinical guidelines used for the diagnosis, treatment, and monitoring of patients.

6. References

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7. List of figures

Fig. 9. The Light Cycler® 480 II instrument. (picture taken by Anna Achathaler March 17, 2020)

Fig. 10. EBV DNA concentrations obtained by the cobas® EBV prototyp test compared to the 1st International WHO Standard.

Fig. 11. Titers in $\log_{10}$ IU/mL for the EBV R-GENE® in combination with the eMAG® platform compared to 1st International WHO Standard.

Fig. 12. Titers in $\log_{10}$ cp/mL for EBV R-GENE® on the MP24 platform compared to 1st WHO International Standard.

Fig. 13. Titers in $\log_{10}$ cp/mL for EBV R-GENE® on the MP24 platform compared to 1st WHO International Standard.