Diploma Thesis

Comparison of automated systems for detection of viruses relevant for immunosuppressed patients

Submitted by

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Declaration of Originality

Herewith I, Lisa Blazek, declare that I have written the present diploma thesis fully on my own and without any assistance from third parties. Furthermore, I confirm that no sources have been used in the preparation of the thesis other than those indicated in the thesis itself.

Graz, December 2014 Lisa Blazek

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I am very thankful to my parents who made it possible for me to study medicine and to my sisters who supported and believed in me the entire time.

Abstract

Background: Today, real-time PCR-based techniques are the method of choice for detection of viruses relevant for immunosuppressed patients. Recently, novel platforms have been introduced including automated PCR setup.

Objectives: To evaluate the accuracy, clinical performance, and workflow of the new Sentosa™ SA assays for detection of human cytomegalovirus (CMV), Epstein-Barr virus (EBV), herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), and varizella-zoster virus (VZV). To compare the clinical performance and the times-to-results to the corresponding R-gene® kits employed in the routine diagnostic laboratory.

Materials and methods: The accuracy of the Sentosa™ SA CMV and EBV assays was determined using proficiency panels provided by an international external quality assessment/proficiency testing organization (QCMD). For evaluation of the clinical performance, 106 EDTA whole blood specimens that remained following routine clinical testing were tested and results compared.

Results: When the accuracy of the Sentosa™ SA CMV and EBV assays was determined, the difference between results was found to be 0.1 $\log_{10}$ or less in the majority of the panel members. When the clinical performance was compared, concordant results were observed in 76% for CMV, 63% for EBV, 91% for HSV-1, 97% for HSV-2, and 97% for VZV. The mean difference in values obtained with the Sentosa™ SA CMV PCR Test compared to those obtained with the CMV R-gene® kit was 0.31 $\log_{10}$ unit. The corresponding value for EBV assays was 0.62 $\log_{10}$ unit. Times-to-results for 16 samples were 270 min for the Sentosa™ SA assays and 230 min for the R-gene® kits.

Conclusions: The Sentosa™ SA assay showed excellent accuracy. Comparison of the clinical performance revealed concordant results in the majority of samples. However, the variability in viral load results is remarkably high. It is thus still mandatory to monitor a single patient with the identical assay.

Kurzfassung

Hintergrund: Heutzutage ist die real-time PCR die Methode der Wahl zur Diagnostik von Viren welche im Besonderen für immunsupprimierte Patienten relevant sind. Vor kurzem wurden neue Plattformen mit automatisiertem PCR Setup eingeführt.

Ziele: Beurteilung der Richtigkeit, der Leistungsfähigkeit und des Arbeitsablaufs des neuen Sentosa™ SA Tests zur Diagnostik des Cytomegalievirus (CMV), Epstein-Barr-Virus (EBV), Herpes-simplex-Virus Typ 1 (HSV-1), Herpes-simplex-Virus Typ 2 (HSV-2) und Varizella-Zoster-Virus (VZV). Vergleich der Leistungsfähigkeit und der Arbeitszeiten mit den dementsprechenden R-gene® Test welche in der Routine-Diagnostik verwendet werden.

Methoden: Die Richtigkeit der neuen Sentosa™ SA CMV and EBV Tests wurde mit Hilfe von Ringversuchproben, zur Verfügung gestellt von der „international external quality assessment/proficiency testing organization“ (QCMD) bestimmt. Zur Beurteilung der Leistungsfähigkeit wurden 106 EDTA Vollblut-Proben mit beiden Testsystemen getestet und miteinander verglichen.

Ergebnisse: Bei der Überprüfung der Richtigkeit des Sentosa™ SA CMV and EBV Tests, wurde beim Großteil der Ringversuchproben ein Unterschied zwischen den Ergebnissen von 0.1 $\log_{10}$ oder weniger festgestellt. Beim Vergleich der Leistungsfähigkeit wurden in 76% bei CMV, 63% bei EBV, 91% bei HSV-1, 97% bei HSV-2 und 97% bei VZV übereinstimmende Ergebnisse beobachtet. Beim Vergleich der erzielten Werte vom Sentosa™ SA CMV PCR Test mit dem Test CMV R-gene® Test lag die mittlere Differenz bei 0.31 $\log_{10}$ unit. Die dementsprechenden Werte des EBV Tests lagen bei 0.62 $\log_{10}$ unit. Die Gesamtanalysezeiten lagen für 16 Proben bei 270 min für den Sentosa™ SA Test und bei 230 min für den R-gene® Test.

Schlussfolgerung: Der Sentosa™ SA Test zeigte eine ausgezeichnete Richtigkeit. Beim Vergleich der Leistungsfähigkeit zeigten sich übereinstimmende Ergebnisse beim Großteil der Proben. Allerdings waren die Schwankungen der Viruslast ungewöhnlich hoch. Es ist daher immer noch obligat jeden Patienten mit dem gleichen Test zu kontrollieren.

TABLE OF CONTENTS

1 INTRODUCTION

1.1 Herpesviridae

The family of the human pathogenic Herpesviridae currently counts eight members, including the cytomegalovirus (CMV), Epstein-Barr virus (EBV), herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), varicella zoster virus (VZV), human herpesvirus 6 (HHV-6), human herpesvirus 7 (HHV-7), and human herpesvirus 8 (HHV-8). These viruses are classified into three subfamilies depending on their genetic and biological similarities: alpha-, beta- and gammaherpesviruses. Nearly hundred percent of the adult population is infected with at least one of these viruses, which accounts for 35–40 billion infections worldwide making herpesviruses the most common pathogens known to exist. The primary infection most commonly emerges in the first two decades of life. Compared to most other viruses, herpesviruses are known for their ability to establish a lifelong subclinical infection. Primary acute infection is followed by a prolonged period called latency without evidence of disease. Reactivation usually occurs with periods of stress or immunosuppression (1).

All human herpesviruses share a common structure. Typically the virions are 100-200 nm large, enveloped and contain double-stranded, linear DNA genomes encoding 70-200 genes. These genomes are packed in an icosahedral protein cage called the nucleocapsid, enveloped by a proteinaceous matrix (the tegument). These components are wrapped in a lipid bilayer membrane called the envelope (2).

In the immunosuppressed patient, infectious complications are a major cause of mortality and morbidity. Several herpesviruses including CMV, EBV, HSV, and VZV have been recognized as significant pathogens in the immunocompromised patient (3).

1.2 Cytomegalovirus

The human cytomegalovirus (CMV) is a ubiquitous betaherpesvirus with a genome of approximately 235 kb containing about 250 open reading frames (ORF's) making it the largest discovered herpesvirus (4). Mature virions vary in diameter from 200 to 300 nm (5).

The lipid bilayer envelope comprehends at least 20 virus-encoded glycoproteins that are participating in cell attachment and penetration (Fig.1).

Viral glycoproteins include glycoprotein B (gB), gH, gL, gM, gN, and gO. Of these, glycoprotein B is the most important glycoprotein that plays a critical role in the CMV entry process (6).

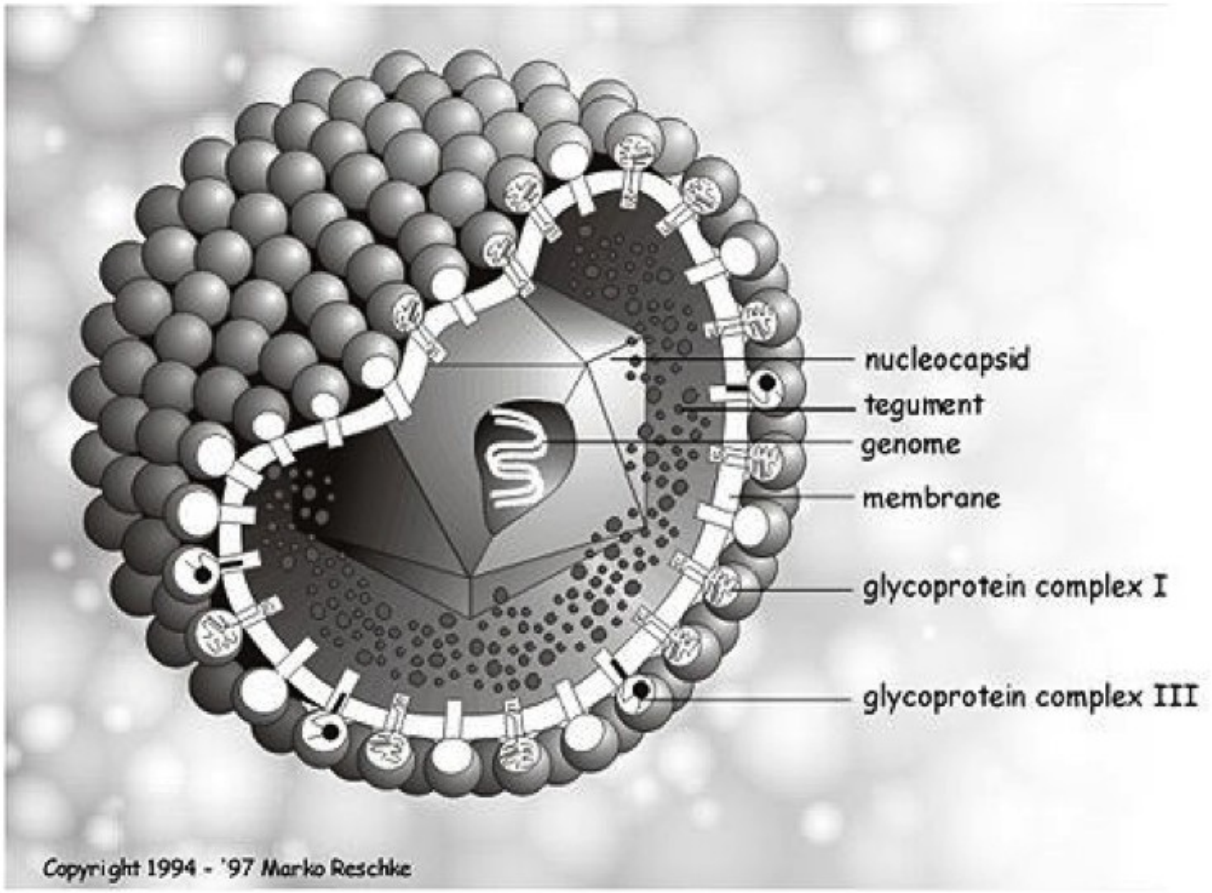

Fig.1. The human cytomegalovirus.

The CMV genome contains two regions of unique sequences, the unique long sequence (UL), and the unique short sequence (US), confined by two sets of inverted repeats, TRL/IRL and IRS/TRS (7) (Fig.2).

The different segments of the UL encodes the major tegument proteins including the UL82 segment encoding the virion transactivator pp71 (upper matrix protein), the UL83 segment encoding the lower matrix phosphoprotein (pp65), the UL32 segment encoding the herpesvirus core virion maturation protein pp150 (large matrix phosphoprotein), the UL48 segment encoding the largest tegument protein, and the UL99 segment encoding the pp28 (5).

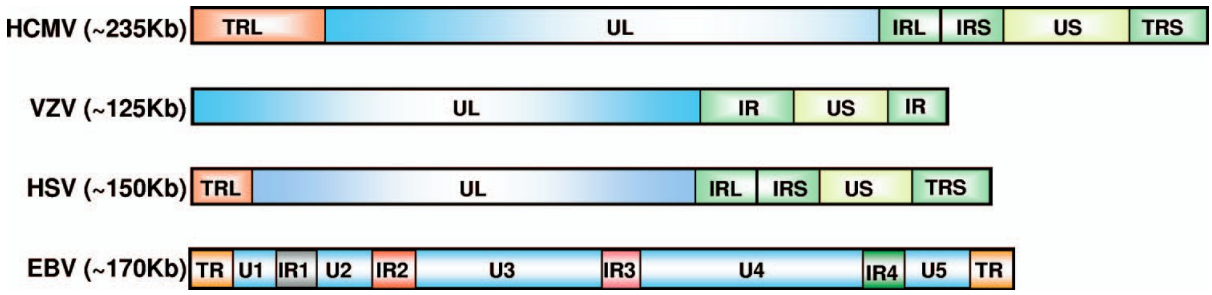

Fig.2. Comparative schematic genome organizations of human herpesviruses. TRL, terminal repeat long; UL, unique long; US, unique short; IRL, internal repeat long; IRS, internal repeat short; TRS, terminal repeat short; IR, internal repeat.

1.2.1 Viral Replication and Pathogenesis

The CMV is transmitted through direct exposure to infected bodily fluids including saliva, semen, stool, tears, urine, breast milk, placental transfer, blood transfusion, solid-organ transplantation (SOT), and hematopoietic stem cell transplantation (SCT) (8).

Following primary CMV infection in an immunocompetent host, which is usually asymptomatic, the virus establishes lifelong latency within the host and reactivates periodically (5).

Compared to other herpesviruses such as HSV and VZV, which remain latent in highly restricted areas of the body, latent CMV can be found in multiple body sites,

although it does not cause disease in all of them. CMV genomic viral DNA has been found in monocytes/macrophages, neutrophils, lymphocytes, and endothelial cells; however, the exact site of latency has not been determined definitively (8).

CMV enters human cells via two possible pathways, either through direct fusion or through the endocytic pathway. The virus integrates to the cell via interactions between viral glycoproteins (gB and gH) and a specific surface receptor(s) (platelet-derived growth factor). To complete the entry process, the viral envelope and the cellular membrane fuse to release nucleocapsids into the cytoplasm. These nucleocapsids are migrated into the nucleus, where viral DNA is delivered. This initiates the expression of viral immediate early genes (IE-1/IE-2) (5).

These genes initiate the production of proteins that are responsible for replicating the double-stranded viral genomic DNA and stimulate the expression of viral late genes.

The viral late proteins are particularly structural components of the virion that assist in the assembly and escape of newly formed virions (9).

Viral replication and maturation succeed the stimulation and parallel congestion of viral synthesis function. This process includes the encapsulation of replicated viral DNA as capsids, which are then transported from the nucleus to the cytoplasm. Secondary envelopment appears in the cytoplasm at the endoplasmic reticulum (ER)-Golgi intermediate compartment. This is followed by a complex two-stage final envelopment and egress process. Finally, the virion is ejected by exocytosis at the plasma membrane (5).

The CMV is controlled by both the innate and the adaptive immune system (Fig. 4).

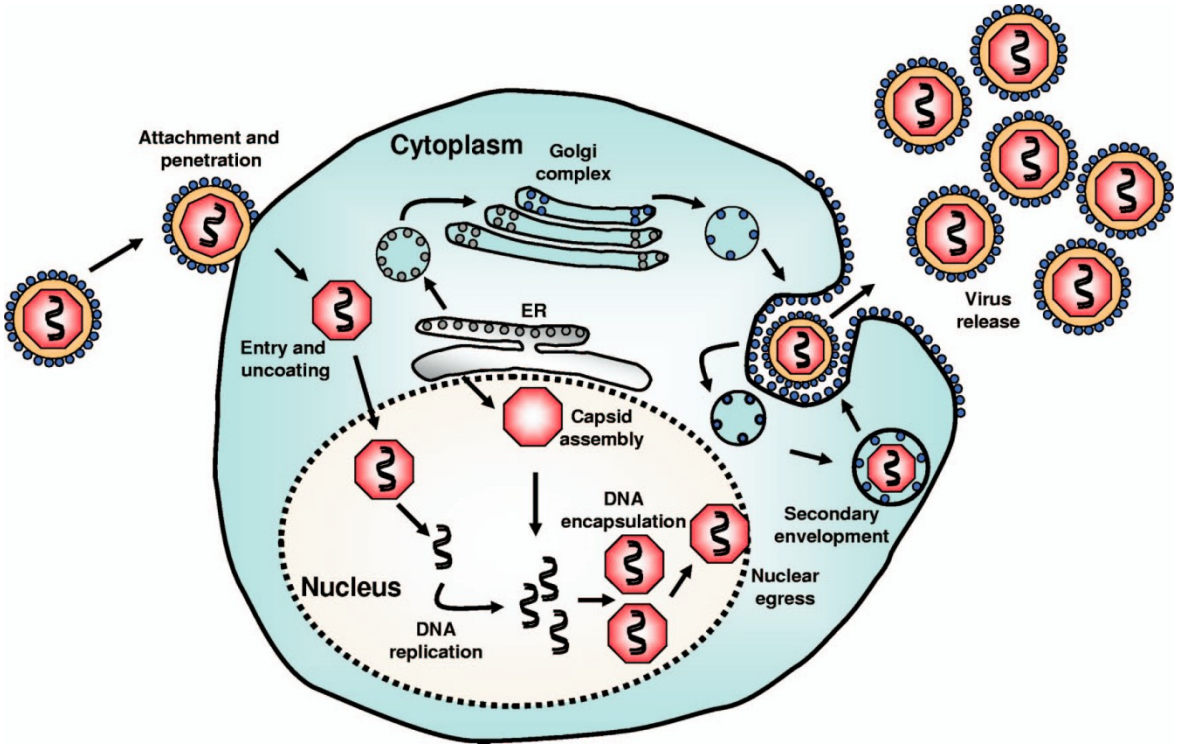

Fig. 3. Life cycle of CMV in a human cell.

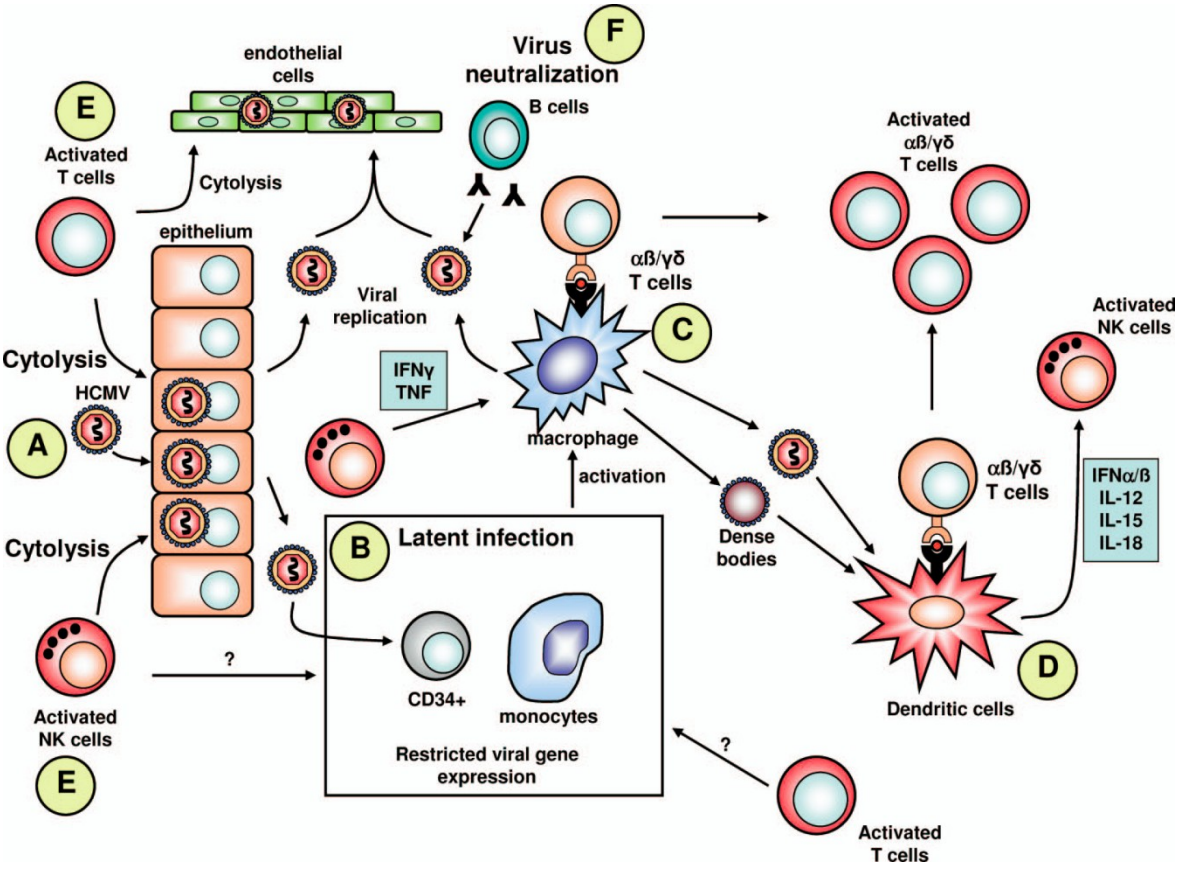

Fig. 4. CMV and immunity. Primary infection with CMV in immunocompetent individuals usually initiates with replication in mucosal epithelium (A), after which the virus distributes to monocytic cells of myeloid lineage including monocytes and CD34+ cells, where it establishes latent infection (B). Constricted viral gene expression is noticed in these latently infected cells, thus limiting their immune recognition by effector cells. Productive infection can be initiated by the separation of these virus-infected monocytes into macrophages (C). Professional antigen-presenting dendritic cells (DCs) can process virus particles or virus-associated dense bodies. These DCs activated through toll-like receptors (TLRs) can stimulate antigen-specific T cells and secrete a variety of cytokines/chemokines, which activate the innate arm of the immune system (e.g., NK cells) (D). Antigen-specific T cells can also be directly stimulated by virus-infected macrophages (C). Activated T cells (CD8+, CD4+, and/or γδ T cells) and NK cells can directly degrade virus-infected cells by cytolysis or block virus replication through the secretion of cytokines such as IFNγ-and/or TNF (E). Another important arm of adaptive immunity involves B cells activated by the professional antigen-presenting cells. They can control extracellular virus through antibody-mediated neutralization (F).

1.2.2 Clinical Manifestations

Primary CMV infection in immunocompetent adults is usually asymptomatic but in the fetus, neonate, and in immunocompromised patients, CMV infection may cause severe disease. Rarely, a mononucleosis syndrome with fever, myalgia, lymphadenopathy, and hepatomegaly may develop, which is difficult to distinguishable from clinical presentation caused by primary EBV infection.

Congenital CMV infection leads to high morbidity and mortality in newborns. Primary CMV infection in a seronegative mother during the first trimester of pregnancy implicates the highest risk and severity of CMV disease. The risk of primary infection during pregnancy is 1 to 4%, which carries a 30 to 40% risk of congenital infection and approximately 10 to 15% of congenitally infected babies are symptomatic at birth. Symptoms include intrauterine growth retardation, hepatitis with jaundice and hepatosplenomegaly, thrombocytopenia with petechiae, pneumonitis, and severe central nervous system damage with microcephaly, intracerebral calcifications, and chorioretinitis. Furthermore, congenital CMV infection is the leading infectious cause of deafness. CMV reactivation in pregnancy carries a lower risk of congenital infection because of the preexisting maternal CMV antibodies and their protective role against intrauterine transmission. CMV infection in immunocompromised individuals like human immunodeficiency virus (HIV)-infected individuals and transplant patients on immunosuppressive medication is a major problem (5).

In SOT recipients, CMV reactivation is the most common complication after transplantation and has a significant impact on morbidity, mortality and graft survival. Optimal prevention, diagnosis, and treatment of active CMV infection significantly impact and improve transplant outcomes (10). Clinical manifestations of CMV infection in SOT recipients range from febrile illness with leukopenia and malaise to pneumonitis, enterocolitis, esophagitis, gastritis, hepatitis, retinitis, and other tissue-invasive disease such as nephritis, cystitis, myocarditis, pancreatitis (5).

CMV is a serious opportunistic infection in HIV-infected individuals and approximately 40% of HIV-infected patients suffered from CMV disease before the introduction of highly active antiretroviral therapy. Accounting for 85% of all cases, retinitis is the most common manifestation of CMV disease in HIV-infected patients

and is characterized by hemorrhagic retinal necrosis. Other manifestations of CMV-associated disease include gastrointestinal disease, encephalitis, and pneumonia (11).

1.2.3 Molecular Diagnostics

In contrast to the immunocompetent, CMV disease may be lethal in the immunocompromised. To avoid a lethal outcome, rapid and accurate diagnosis is of paramount importance to start treatment at the earliest stage (8, 12). CMV infection can be diagnosed by serology, antigenemia testing, and nucleic acid testing (NAT). For NAT, either ethylenediaminetetraacetate (EDTA) whole blood or plasma are used. Current guidelines do not recommend a specific kind of sample, although EDTA whole blood may be superior to plasma (12). Other sample materials useful for detection of CMV DNA in the immunocompromised patient include cerebrospinal fluid, throat washings, bronchoalveolar lavages, stool, urine, aqueous humor, and bone marrow (3). The level of CMV DNA has been reported to be an important prognostic marker for the ongoing disease (13).

NAT consists of CMV DNA extraction followed by amplification of nucleic acid extracts. Nucleic acid amplification is usually performed by real-time polymerase chain reaction (qPCR), which is nowadays the method of choice for detection and quantification of CMV DNA (3).

Molecular assays based on qPCR show greater sensitivity and reduced hands-on time and have largely replaced pp65 antigenemia testing in routine diagnostic laboratory (14). Further advantages include increased sensitivity for early detection of CMV infection or reactivation, applicability in patients with neutropenia, wide range of linearity (up to 8 $\log_{10}$), the ability to process a large number of specimens, and the potential for increased accuracy of results through precision instrumentation (13).

For the detection of CMV DNA, the choice of the target region of the CMV genome requires special attention. More than ten different target regions and at least three different units of result reports have been described. The glycoprotein B gene (UL55) has been used as the target region in diverse laboratory-developed

molecular assays. The majority of presently frequently used commercial assays in Europe for the detection of CMV DNA employ the qPCR method (Table1) (13).

Table 1
Comparison of currently frequently used commercially available assays for the quantification of CMV DNA.

Characteristics	**Manufacturer and details**				
	Abbott	**bioMerieux/Argene**	**Nanogen/ELITechGroup**	**Qiagen**	**Roche**
Kit name	RealTime CMV	CMV R-gene	CMV ELITe MGB Kit	artus CMV QS-RGQ Kit	COBAS AmpliPrep/ COBAS TaqMan CMV Test
Target sequence	UL34 (early protein) and pUL80.5 (scaffolding protein)	UL83 (lower matrix phosphoprotein 65)	UL123 (major immediate early protein)	UL123 (major immediate early protein)	UL54 (DNA polymerase)
Amplification method	qPCR	qPCR	qPCR	qPCR	qPCR
Detection method	Fluorescence	Fluorescence	Fluorescence	Fluorescence	Fluorescence
Internal control	Heterologous	Heterologous	Heterologous	Heterologous	Homologous
Standards	Two EQS	Four EQS	Four EQS	Four EQS	One IQS
Analytic measuring range	$3.1 \times 10^1 – 1.6 \times 10^8$ IU/ml (plasma); $6.2 \times 10^1 – 1.6 \times 10^8$ IU/ml (EDTA whole blood)	$5.0 \times 10^2 – 1.0 \times 10^7$ copies/ml	$3.2 \times 10^2 – 2.5 \times 10^7$ genome equivalents/ml (EDTA whole blood)	$7.9 \times 10^1 – 1.0 \times 10^8$ copies/ml (plasma); $1.0 \times 10^3 – 5.0 \times 10^7$ copies/ml (EDTA whole blood)	$1.5 \times 10^2 – 1.0 \times 10^7$ copies/ml (plasma)

EQS: external quantification standards; IQS: internal quantification standard

1.3 Epstein-Barr virus

The Epstein-Barr virus (EBV) is a ubiquitous gammaherpesvirus with a genome of approximately 184 kb that encodes more than 85 genes (15, 16). Mature virions vary in diameter from 120 to 180 nm. Two major types of EBV are known to infect humans: EBV-1 and EBV-2. These two subtypes have different EBV nuclear antigens (EBNA-2, EBNA-3a, EBNA-3b, and EBNA-3c) and they differ in geographic distributions (17). Type 1 is dominant in most populations, whereas types 1 and 2 are nearly equally prevalent in New Guinea and equatorial Africa (16).

1.3.1 Viral Replication and Pathogenesis

EBV establishes lifelong latent infection in the resting memory B-cell compartment and epithelial cells following primary infection (18). It is estimated that more than 90% of the world's population are persistently infected with EBV, which can be transmitted through contact with oropharyngeal and genital tract secretions, through blood transfusions, and through organ and tissue transplantation (15).

Primary infection is thought to occur at the oropharyngeal epithelium. EBV attaches to B-cells through binding of the gp 350/220 viral membrane glycoproteins to the CD21 molecule on these lymphocytes (16). Then gp42 interacts with B-cell HLA class II molecules and initiates fusion with the host membrane. In epithelial cells with lack CD21, the EBV BMRF-2 protein and the EBV gH/gL envelope protein trigger fusion via interaction with the β1 and the αvβ6/8 integrins. Due to endocytosis of the virus into vesicles and fusion of the virus with the vesicle membrane the viral nucleocapsid release into the cytoplasm. Viral entry results in transport of the viral genome into the B-cell nucleus, where it is replicated by DNA polymerases. Viral gene products induce infected B-cells to activate their growth program and trigger proliferation of blasting B-cells. Concurrent priming of naïve T cells by antigen-presenting blasting B-cells occurs. Usually, these blasting B-cells are destroyed by cytotoxic T lymphocytes. Previously activated memory B-cells, which are released into the peripheral

circulation, may continue to undergo lytic replication or, if EBV shuts down most of its protein encoding genes, latency occurs. Later on, resting B-cells may be activated, resulting in viral reactivation and shedding (Fig.5) (17).

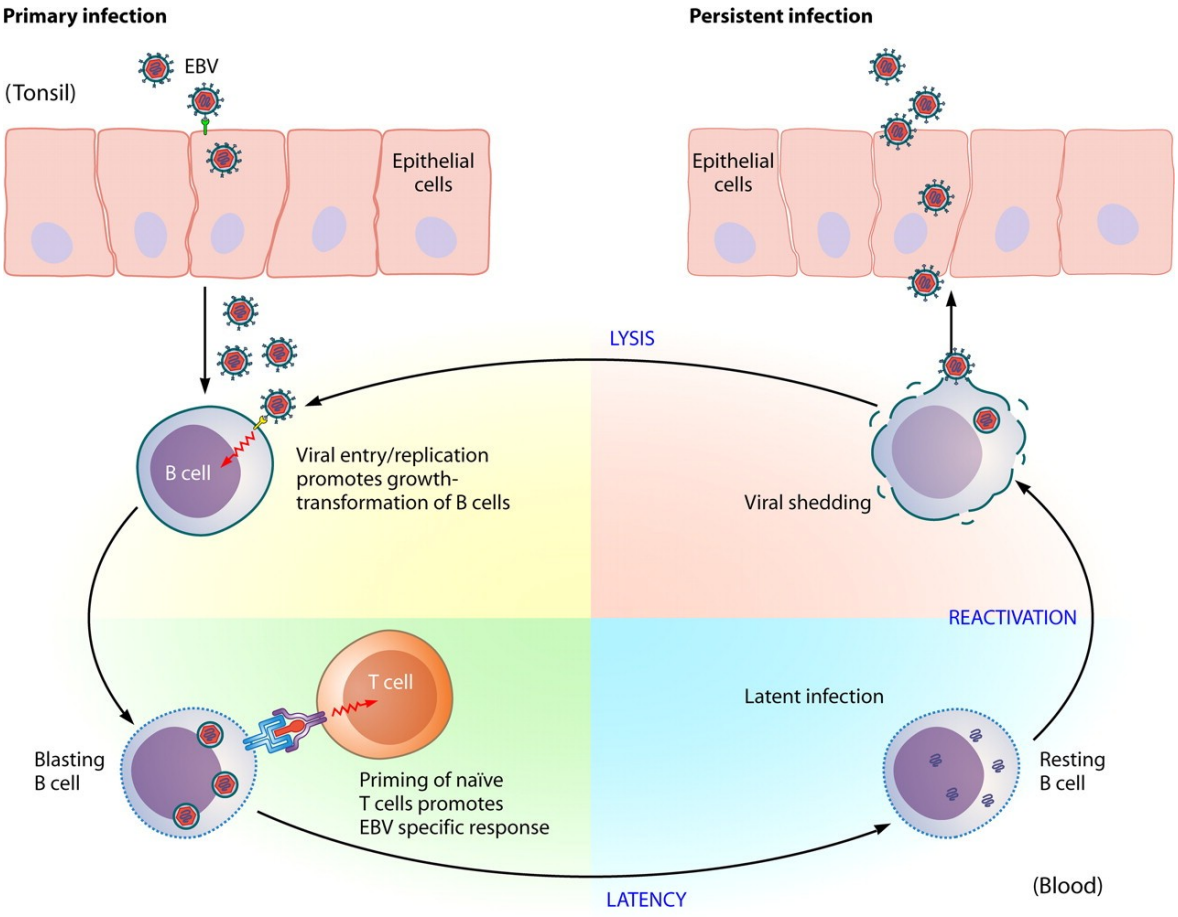

Fig. 5. EBV infection in healthy carriers.

1.3.2 Clinical Manifestations

Primary EBV infection usually occurs during childhood and adolescence. Primary infection is often asymptomatic or results in acute illness that is often not recognized as EBV infection. However, in adolescents and adults, primary infection regularly presents as infectious mononucleosis (IM).

After an incubation period of approximately 6 weeks, IM mostly begins with vague malaise, followed a few days later by fever, sore throat, swollen posterior cervical lymph nodes, and fatigue. The duration of IM is about 16 days, although it

may take much longer for the patient to feel entirely well. Some patients experience a sudden influenza-like onset, including fever, chills, body aches, and sore throat. Splenomegaly occurs in 33% of cases and hepatomegaly is observed in 25% of cases. A small minority of patients spontaneously presents a rash. Many complications have been associated with IM, including airway obstruction, hepatitis, meningoencephalitis, myocarditis, hemolytic anemia, thrombocytopenia, and splenic rupture. However, almost all of them are very rare with a frequency by less than 1% of patients.

The chronic active EBV (CAEBV), which occurs very rarely, is due to an inappropriate control of viral replication. The disease is characterized by chronic infectious mononucleosis-like symptoms with illness lasting more than 6 months. The accurate diagnosis is very important, due to the fact that there is a high morbidity and mortality rate for patients with CAEBV infection (17).

EBV as a potent growth-transforming factor has been implicated in the pathogenesis of various human cancers including Burkitt's lymphoma, Hodgkin's disease, non-Hodgkin's lymphoma, gastric carcinoma, and nasopharyngeal carcinoma. Furthermore, EBV is associated with autoimmune diseases such as systemic lupus erythematosus and multiple sclerosis (16, 18, 19).

EBV infection in the immunocompromised patient may result in the development of various lymphoproliferative disorders (LPDs), including the posttransplant lymphoproliferative disorder (PTLD), the X-Linked LPD, and AIDS-related LPD (16). Primary infection or reactivation of latent EBV infection in transplant recipients occurs due to the iatrogenic suppression of T cell functions, which may result in a fatal disorder of uncontrolled B-cell proliferation, plasmacytic hyperplasia, and lymphoma (15, 16, 17). The X-Linked LPD is a rare inherited disorder characterized by fatal or fulminant outcome, B-cell lymphomas, and dysgammaglobulinemia. The AIDS-related LPDs are a heterogeneous group of diseases including both central nervous system and systemic lymphomas, which virtually always contain EBV (16).

1.3.3 Molecular Diagnostics

In the immunocompromised patient, quantification of EBV DNA provides information for initiating treatment and is essential for monitoring response to

therapy. Information on viral load may reduce the incidence and level of EBV reactivation in transplant recipients, as well as the potentially following development of PTLD and lymphomas. A correlation between the EBV DNA level and the likelihood of development of PTLD exists, as high levels of EBV DNA in whole blood or plasma are observed in almost all patients with PTLD (3). For detecting and quantifying EBV DNA in body fluids, qPCR is the method of choice and very useful for defining infection status, especially in immunocompromised patients (17, 20). There is much debate concerning the optimal material for detection and quantification of EBV DNA. EDTA whole blood and plasma appear to be most informative in transplant recipients (3). In patients with EBV disease of the central nervous system, detection of EBV DNA in cerebrospinal fluid is considered the standard method.

Similar to the detection of CMV, the choice of the target region requires special attention, as EBV sequence variation is a recognized phenomenon. Laboratory-developed and commercially available assays for the quantification of EBV DNA based on qPCR have been established recently. Several gene targets have been selected for the quantification of EBV DNA including genes coding for DNA polymerase, thymidine kinase, Epstein-Barr nucleic antigen type 1, glycoprotein B, ZEBRA protein, and a nonglycosylated membrane protein. A summary of presently frequently used commercial assays in Europe is provided in Table 2 (3).

Table 2
Comparison of currently frequently used commercially available assays for the quantification of EBV DNA.

Characteristics	**Manufacturer and details**				
	bioMerieux/Argene	**diagenode**	**Nanogen/ELITechGroup**	**Qiagen**	**Roche**
Kit name	EBV R-gene	EBV Realtime PCR	EBV ELITe MGB Kit	artus EBV QS-RGQ Kit	LightCycler EBV Quant Kit
Target sequence	BXLF1 (thymidine kinase)	BNRF1 (nonglycosilated membrane protein)	EBNA-1 (Epstein-Barr nucleic antigen 1)	EBNA-1 (Epstein-Barr nucleic antigen 1)	LMP2 (latent membrane protein 2)
Amplification method	qPCR	qPCR	qPCR	qPCR	qPCR
Detection method	Fluorescence	Fluorescence	Fluorescence	Fluorescence	Fluorescence
Internal control	Heterologous	Heterologous	Heterologous	Heterologous	Heterologous
Standards	Four EQS	Four EQS	Four EQS	Four EQS	Four EQS
Analytic measuring range	5.0×10^2- 1.0×10^7 copies/ml	8.2×10^2 – 1.0×10^7 copies/ml	2.5×10^2 – 2.5×10^7 genome equivalents/ ml (cellular samples); 1.3×10^2 – 1.3×10^7 genome equivalents/ml (noncellular samples)	3.2×10^2 – 1.0×10^7 copies/ml (plasma)	1.0×10^3 – 2.0×10^7 copies/ml (plasma); 2.0×10^3 – 2.0×10^7 copies/ml (EDTA whole blood)

EQS: external quantification standards.

1.4 Herpes simplex virus

Herpes simplex viruses (HSVs) are ubiquitous alphaherpesviruses. Two subtypes of HSV are known, HSV-1 and HSV-2 (21). HSV-1 and HSV-2 are large enveloped double-stranded DNA viruses with a genome of approximately 152 kb that encode more than 80 polypeptides (22, 23).

1.4.1 Viral Replication and Pathogenesis

HSV-1 and HSV-2 are the most prevalent human viral infections worldwide. Following primary infection they establish lifelong latent infection in sensory neurons (22, 23). Reactivation occurs periodically and is associated with numerous diseases (22).

HSV infects a variety of host cells, including lymphocytes, epithelial cells, fibroblasts, and neurons. HSV can enter the cell by direct fusion with the plasma membrane or via pH-dependent or -independent endocytosis, depending on the target cell. The entry-fusion system involves a multiprotein complex of virion glycoproteins, gB, gC, gD, and gH/gL, and three classes of HSV entry receptors including herpesvirus entry mediator, nectin-1 and -2, and 3-O-sulfated heparan sulfate (21, 24, 25). Following entry into the cell, the HSV envelope is lost and the de-enveloped particles travel to the nucleus where the viral genes are expressed in a tightly regulated temporal sequence and consist of immediate early (IE), early (E), and late (L) gene functions. Proteins encoded by early and then late genes include enzymes necessary for viral DNA replication and structural proteins that are assembled into new viral particles into the nucleus. Envelopment is acquired by budding through the nuclear membrane with further processing in the Golgi apparatus. The virus replication cycle leads to rapid cell death and release of new viral particles during cell lysis. The virus may enter the latent state without detectable expression of IE, E or L gene products or initiate lytic replication (Fig.6) (26).

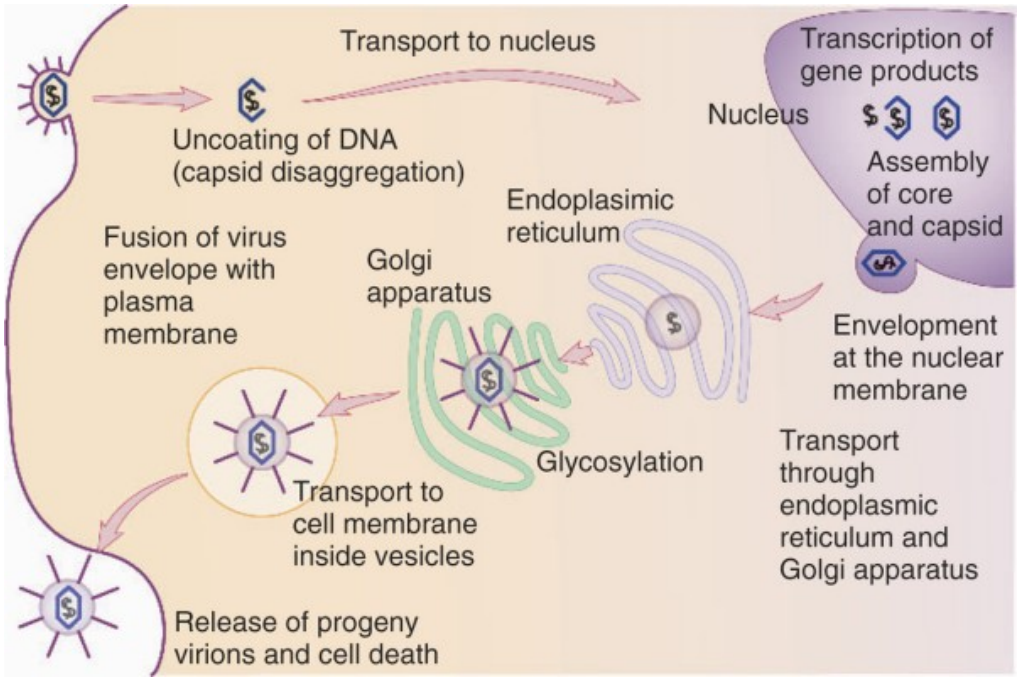

Fig. 6. Schematic diagram of replication of herpes simplex virus.

1.4.2 Clinical Manifestations

HSV-1 and HSV-2 are neurotropic viruses and primarily infect oral and epithelial cells. They can be transmitted through casual or sexual contact, but HSV-2 is mostly transmitted sexually (21). After primary infection, the virus can travel along axons to the trigeminal ganglia for HSV-1 and the lumbosacral dorsal root ganglia for HSV-2, where it establishes a lifelong latency. Reactivation can occur spontaneously or be induced by various stimuli, including UV light, fever, and trauma. Recurrent infection is usually restricted to epidermal lesions around the mouth (herpes labialis) and genitals (herpes genitalis) (23, 27, 28). The blisters contain viral particles and probably represent a main strategy for viral transmission. HSV-1 commonly infects the eye and produces oropharyngeal infections and keratitoconjunctivitis (a leading cause of infectious blindness), whereas HSV-2 commonly produces genital infections. In very rare cases, HSV-1 enters the central nervous system (CNS) due to its affinity for neurons and epithelial cells and may produce life-threatening encephalitis or meningitis. Herpes

encephalitis, predominantly caused by HSV-2, is most frequent in neonates infected during birth and is associated with high mortality and morbidity (2, 13, 28). Immunocompromised adults, including patients with AIDS, cancer and transplant patients can suffer disseminated infections that become resistant to acyclovir (28).

1.4.3 Molecular Diagnostics

Nowadays, effective therapeutic management of HSV infection is possible; however, especially for CNS disease, antiviral drugs must be applied at a very early stage. Therefore, rapid laboratory diagnosis is required for degrading the lethality as well as the sequelae of HSV infection. Several specimen types for the detection of HSV DNA exist. For CNS disease, the detection of HSV DNA in cerebrospinal fluid specimens has been approved as the gold standard. For dermal or genital HSV infection, the detection of HSV DNA in swabs is the standard method. Further specimen types for detection of HSV DNA include aqueous humor in case of retinitis and/or conjunctivitis and EDTA whole blood in case of generalized HSV infection. Laboratory-developed assays for the qualitative detection/quantification of HSV DNA based on qPCR have been established recently. Various gene targets have been selected including genes coding for glycoproteins B, C, D, and G, thymidine kinase, DNA polymerase, and DNA binding protein. For diagnosis of HSV infections molecular assays based on qPCR have shown increased detection rates when compared to cell culture methods. A summary of presently frequently used commercial assays in Europe is provided in Table 3 (13).

Table 3
Comparison of currently frequently used commercially available assays for the qualitative detection/quantification of HSV DNA.

Characteristics	**Manufacturer and details**				
	Argene	**Cepheid**	**Nanogen/ELITechGroup**	**Qiagen**	**Roche**
Kit name	HSV1 HSV2 VZV R-gene	affigene HSV 1/2 tracer	HSV 1, HSV 2 ELITe MGB Kit	artus HSV-1/2 PCR Kit	LightCycler HSV 1/2 Qual Kit
Target sequence	US7 (glycoprotein I, HSV-1); US2 (hypothetical protein, HSV-2)	US6 (glycoprotein D, HSV-1); US4 (glycoprotein G, HSV-2)	US 6 (glycoprotein D, HSV-1) and US4 (glycoprotein G, HSV-2)	UL27 (glycoprotein B)	UL30 (DNA polymerase)
Amplification method	qPCR	qPCR	qPCR	qPCR	qPCR
Detection method	Fluorescence	Fluorescence	Fluorescence	Fluorescence	Fluorescence
Internal control	Heterologous	Heterologous	Heterologous	Heterologous	Heterologous
Standards	Four EQS	–[a]	Four EQS	Four EQS	–[a]
Analytic measuring range	$2.8 \times 10^2 – 1.5 \times 10^5$ copies/ml (HSV-1); $7.0 \times 10^1 – 1.0 \times 10^6$ copies/ml (HSV-2)	–[b]	$1.25 \times 10^2 – 1.25 \times 10^6$ copies/ml	NA	–[c]

[a]Qualitative test.
[b]Limit of detection $1.3 \times 10^2 – 1.8 \times 10^2$ copies/ml (depending on the sample preparation protocol employed).
[c]Limit of detection 4×10^2 copies/ml.
EQS: external quantification standards; NA: data not available.

1.5 Varicella Zoster Virus

The Varicella zoster virus (VZV) is a human neurotropic alphaherpesvirus with a genome of approximately 125 kb that encodes at least 71 unique ORF's and related promotor sequences (29, 30). Mature virions vary in diameter from 80 to 120 nm. Similar to all herpesviruses, VZV has a lipid-rich envelope, which comprehends viral glycoproteins (30).

1.5.1 Viral Replication and Pathogenesis

VZV is highly contagious and is restricted to humans. VZV infection is typically acquired through inhalation of aerosolized virus. After exposure, VZV infects Langerhans and plasmacytoid dendritic cells of the upper respiratory mucosa and nasopharyngeal region. Subsequently these antigen-presenting cells transfer the virus to CD4 T-cells, which, in turn, transmit the virus to dermal endothelial cells (29). VZV establishes latency in sensory ganglia following transport to neuronal nuclei along neuronal axons or by viremia. Reactivation from latency permits a second phase of replication to occur in skin, which typically causes lesions in the dermatome that is innervated by the affected sensory ganglion (Fig. 7a) (30).

The viral life cycle begins with VZV entry that is supposed to involve either direct fusion of viral particles with the plasma membrane or endocytosis. Viral envelope proteins are forecasted to interact with cell surface molecules, such as mannose-6-phosphate receptor or myelin-associated glycoprotein. VZV glycoprotein B (gB), gH, and gL form the core fusion complex but other envelope glycoproteins probably play a part as accessory proteins. Following entry, the virions undergo uncoating followed by release of tegument proteins and transportion to the nucleus. Similar to the events that have been described for HSV-1 replication, immediate-early genes are expressed followed by early and late genes. Nucleocapsids are assembled and package newly synthesized genomic DNA, move to the inner nuclear membrane, and bud across the nuclear membrane. Capsids enter the cytoplasm, and virion glycoproteins mature in the trans-Golgi region and tegument proteins assemble in vesicles. Capsids undergo

secondary envelopment and are transported to cell surfaces, where newly assembled virus particles are released (Fig. 7b) (30).

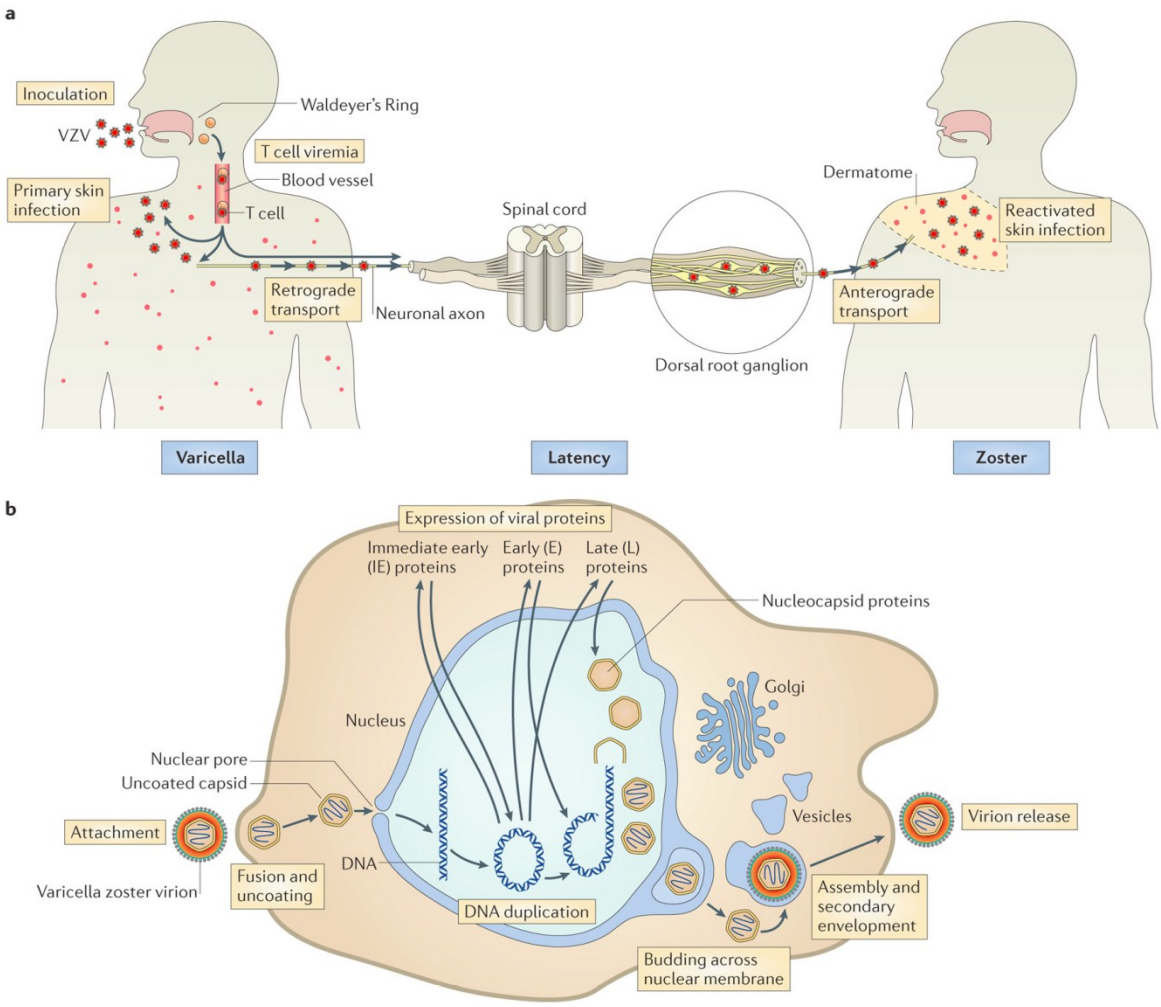

Fig. 7. VZV life cycle and replication. Model of the VZV life cycle (a). Model of the VZV replication (b).

1.5.2 Clinical Manifestations

Primary VZV infection normally results in varicella (chickenpox), wherein the virus replicates in multiple organs, especially the skin. Subsequently, VZV remains latent in neurons of cranial nerve ganglia, dorsal root ganglia, and autonomic ganglia along the entire neuraxis (31). After an incubation period of 10-21 days, varicella starts with a vesicular, itchy rash that primarily affects the trunk and the

head and is typically accompanied by malaise, such as low-grade fever, nausea, aching muscles, and headache (2, 30).

With VZV reactivation resulting in herpes zoster (shingles), the clinical picture is somewhat different. New virions are assembled and transported anterograde to infect dermal cells, which is characterized by a painful, vesicular rash in the dermatome that is innervated by the affected ganglion (30, 31). Rash and pain usually develop within a few days, although pain can precede rash and often becomes chronic (postherpetic neuralgia). After reactivation, the virus may also travel retrograde and produce meningoencephalitis, cerebellitis, zoster paresis, cranial nerve palsies, vasculopathy, myelopathy, and various inflammatory ocular disorders (29, 31).

Zoster most frequently occurs with advancing age as cell-mediated immunity to VZV declines or in immunocompromised patients including organ transplant recipients and patients with cancer or AIDS (29).

1.5.3 Molecular Diagnostics

The clinical presentation of VZV dermal disease can be confused with that produced by HSV therefore laboratory diagnosis is of paramount importance for distinguishing HSV from VZV infections especially in the immunocompromised patient. Quantification of VZV DNA may provide information for initiating treatment and monitoring response to therapy. In transplant recipients, information on quantitative viral load may guide a preemptive strategy to reduce the incidence and level of VZV reactivation.

Detection of VZV DNA in swabs is the standard method for suspected dermal infection. In immunocompromised patients, the most significant results may be achieved by quantification of VZV DNA in EDTA whole blood including both the cellular and the cell-free compartments from serial or sequential specimens obtained from the same patient. The standard method for CNS disease is the detection of VZV DNA in cerebrospinal fluid specimens. For respiratory VZV infection, detection of VZV DNA in bronchoalveolar lavages is significant.

For the detection and quantification of VZV DNA several laboratory-developed and commercially available assays based on qPCR have been established. Several gene targets have been selected including genes coding for the DNA

polymerase, the glycoprotein B, the DNA binding protein, and the immediate-early transactivator. A summary of presently frequently used commercial assays in Europe is provided in Table 4 (13).

Table 4
Comparison of currently frequently used commercially available assays for the qualitative detection/quantification of VZV DNA.

Characteristics	**Manufacturer and details**				
	bioMerieux/Argene	**Cepheid**	**Nanogen/ELITechGroup**	**Qiagen**	**Roche**
Kit name	HSV1 HSV2 VZV R-gene	Affigene VZV tracer	VZV ELITe MGB Kit	artus VZV QS-RGQ Kit	LightCycler VZV Qual Kit
Target sequence	ORF17 (tegument host shut-off protein)	ORF62 (immediate early protein)	ORF29 (major DNA binding protein)	ORF38 (virion protein)	ORF29 (major DNA binding protein)
Amplification method	qPCR	qPCR	qPCR	qPCR	qPCR
Detection method	Fluorescence	Fluorescence	Fluorescence	Fluorescence	Fluorescence
Internal control	Heterologous	Heterologous	Heterologous	Heterologous	Heterologous
Standards	Four EQS	–[a]	Four EQS	Four EQS	–[a]
Analytic measuring range	5.0×10^2 – 1.0×10^7 copies/ml	–[b]	2.5×10^2 – 2.5×10^7 genome equivalents/ml (cellular samples); 1.0×10^2-1.2×10^7 (noncellular samples)	5.0×10^2 – 1.0×10^8 copies/ml	–[c]

[a]Qualitative assay.
[b]Limit of detection 7.2×10^1 – 3.3×10^2 copies/ml (depending on the sample preparation protocol employed).
[c]Limit of detection 1.5×10^2 copies/ml (CSF), 3.8×10^2 copies/ml (swab).
EQS: external quantification standards.

2 OBJECTIVES

The aim of this study was to evaluate the accuracy, clinical performance, and workflow of the new Sentosa™ SA assays (Vela Diagnostics, Singapore) for detection of CMV, EBV, HSV-1, HSV-2, and VZV. For accuracy testing, a secondary reference material was used. The clinical performance was evaluated with 106 EDTA whole blood specimens. Both the clinical performance and the time-to-result including the automated and the manual times were compared to the current assays employed in the routine diagnostic laboratory (standard assays).

3 STUDY DESIGN

3.1 Molecular methods

Characteristics of the molecular assays used in this study are summarized in Table 5. All experiments were done in an International Standard Organization (ISO 9001:2008)-certified laboratory; the Molecular Diagnostics Laboratory, IHME, Medical University of Graz. All kits were performed according to the manufacturer's package insert instructions.

Table 5
Characteristics of the molecular assays used in this study.

Kit name	**Sentosa™ SA CMV PCR Test**	**CMV R-gene®**	**Sentosa™ SA EBV PCR Test**	**EBV R-gene®**	**Sentosa™ SA HSV PCR Test**	**HSV R-gene®**	**Sentosa™ SA VZV PCR Test**	**VZV R-gene®**
Target sequence (gene)	UL54	UL83	EBNA1	BXLF1	UL30	US7 (HSV-1); US2 (HSV-2)	ORF31	ORF17
Amplification method	Real-time PCR							
Detection method	Fluorescence							
Internal control	Heterologous							
Standards	Four EQS							
Limit of detection (whole blood)	1.1×10^2 IU/ml	4.5×10^2 cop/ml	2.1×10^2 IU/ml	1.8×10^2 cop/ml	HSV-1: 1.5×10^2 cop/ml; HSV-2: 0.8×10^2 cop/ml	HSV-1: 2.8×10^2 cop/ml; HSV-2: 0.7×10^2 cop/ml	1.7×10^2 cop/ml	2.5×10^2 cop/ml
Analytic measuring range	1.1×10^2 - 1.0×10^7 IU/ml	5.0×10^2 - 1.0×10^7 cop/ml	2.1×10^2 - 1.0×10^7 IU/ml	8.2×10^2 - 1.0×10^7 cop/ml	HSV-1: 6.3×10^2 - 1.0×10^7 cop/ml; HSV-2: 0.9×10^2 - 1.5×10^7 cop/ml	HSV-1: 2.8×10^2 - 1.5×10^6 cop/ml; HSV-2: 0.7×10^2 - 1.0×10^6 cop/ml	1.7×10^2 - 1.0×10^8 cop/ml	5.0×10^2 - 1.0×10^7 cop/ml

3.2 Assays

3.2.1 The Sentosa™ SA assays

The Sentosa™ SA CMV PCR Test

The Sentosa™ SA CMV Quantitative PCR Test (Vela) is an in vitro qPCR-based test intended for the detection and quantification of CMV DNA in human EDTA plasma, serum, and cerebrospinal fluid (CSF). This assay includes the Sentosa™ SX Virus Total Nucleic Acid Kit v2.0 (Vela) and is performed on the Sentosa™ SX101 instrument (Vela) including the Rotor-Gene® Q MDx 5plex HRM instrument (Qiagen, Hilden, Germany) (Fig. 8).

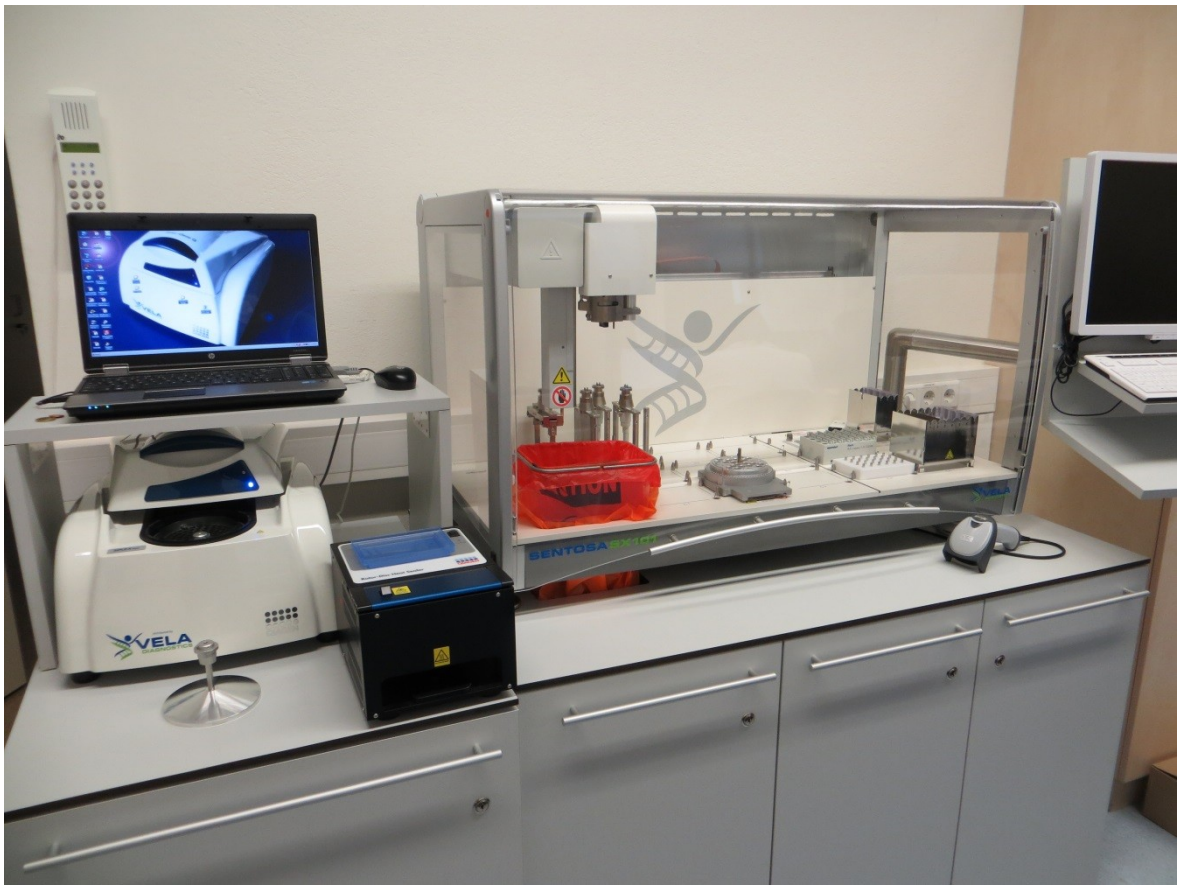

Fig.8. The Sentosa™ SX101 instrument (Vela) including the Rotor-Gene® Q MDx 5plex HRM instrument (Qiagen).

The Sentosa™ SA CMV Quantitative PCR Test is intended for use in detection of CMV DNA from symptomatic patients as an aid in the diagnosis of CMV

infection as well as an aid in the management of transplant patients who are undergoing anti-CMV therapy. According to the manufacturer's package insert, the results from the Sentosa™ SA CMV Quantitative PCR Test must be interpreted within the context of all relevant clinical and laboratory findings.

The Sentosa™ SA CMV PCR Test is intended for detection of CMV DNA. The concentration of CMV DNA may be automatically calculated using a stored CMV standard curve, which is generated with Sentosa™ SA CMV Quantitation Standards on the instrument.

Principle: Pathogen detection by qPCR is based on the amplification of specific regions of the viral genome. The amplified product is detected via fluorescent dyes linked to oligonucleotide probes that bind specifically to the target sequences.

Nucleic acid extraction and PCR set-up: The procedure is automated using the Sentosa™ SX101 platform.

qPCR (amplification and detection): For qPCR and detection of amplification products, the Rotor-Gene® Q MDx 5plex HRM instrument was used.

The Sentosa™ SA EBV PCR Test

The Sentosa™ SA EBV Quantitative PCR Test (Vela) is an in vitro qPCR-based test intended for the detection and quantification of EBV DNA in human EDTA plasma, serum, and CSF. This assay includes the Sentosa™ Virus Total Nucleic Acid Kit v2.0 and is performed on the Sentosa™ SX101 instrument (Vela) including the Rotor-Gene® Q MDx 5plex HRM instrument (Qiagen, Hilden, Germany) (Fig. 8).

The Sentosa™ SA EBV Quantitative PCR Test is intended for use in the quantification of EBV DNA load in transplant or immunosuppressed patients who have been diagnosed with EBV infection. According to the manufacturer's package insert, the results from the Sentosa™ SA EBV Quantitative PCR Test must be interpreted within the context of all relevant clinical and laboratory findings.

The Sentosa™ SA EBV PCR Test is intended for detection of EBV DNA. The concentration of EBV DNA may be automatically calculated using a stored EBV standard curve, which is generated with Sentosa™ SA EBV Quantification Standards on the instrument.

Principle: Pathogen detection by qPCR is based on the amplification of specific regions of the viral genome. The amplified product is detected via fluorescent dyes linked to oligonucleotide probes that bind specifically to the target sequences.

Nucleic acid extraction and PCR set-up: The procedure is automated using the Sentosa™ SX101 platform.

qPCR (amplification and detection): For qPCR and detection of amplification products, the Rotor-Gene® Q MDx 5plex HRM instrument was used.

The Sentosa™ SA HSV1/2 PCR Test

The Sentosa™ SA HSV1/2 Quantitative PCR Test (Vela) is a qPCR-based in vitro diagnostic test intended for the detection and quantification of HSV1/2 viral load in swab in universal transport medium (UTM), EDTA plasma, serum, and CSF of transplant or immunocompromised patients who have been diagnosed with HSV1/2 infection.

This assay includes the Sentosa™ Virus Total Nucleic Acid Kit v2.0 and is performed on the Sentosa™ SX101 instrument (Vela) including the Rotor-Gene® Q MDx 5plex HRM instrument (Qiagen, Hilden, Germany) (Fig. 8).

The concentration of HSV1/2 DNA may be automatically calculated using a stored HSV1/2 standard curve, which is generated with Sentosa™ SA HSV1/2 Quantitation Standards on the instrument.

Principle: Pathogen detection by qPCR is based on the amplification of specific regions of the viral genome. The amplified product is detected via fluorescent dyes linked to oligonucleotide probes that bind specifically to the target sequences.

Nucleic acid extraction and PCR set-up: The procedure is automated using the Sentosa™ SX101 platform.

qPCR (amplification and detection): For qPCR and detection of amplification products, the Rotor-Gene® Q MDx 5plex HRM instrument was used.

The Sentosa™ SA VZV PCR Test

The Sentosa™ SA VZV Quantitative PCR Test (Vela) is an in vitro qPCR-based test intended for the detection and quantification of VZV DNA in human EDTA plasma, serum, and CSF. This assay includes the Sentosa™ SX Virus Total

Nucleic Acid Kit v2.0 (Vela) and is performed on the Sentosa™ SX101 instrument (Vela) including the Rotor-Gene® Q MDx 5plex HRM instrument (Qiagen, Hilden, Germany) (Fig. 8).

The Sentosa™ SA VZV Quantitative PCR Test is intended for quantification of VZV DNA load in transplant or immunosuppressed patients who have been diagnosed with VZV infection. According to the manufacturer's package insert, the results from the Sentosa™ SA VZV Quantitative PCR Test must be interpreted within the context of all relevant clinical and laboratory findings.

The Sentosa™ SA VZV PCR Test is intended for detection of VZV DNA. The concentration of VZV DNA may be automatically calculated using a stored VZV standard curve, which is generated with Sentosa™ SA VZV Quantitation Standards on the instrument.

Principle: Pathogen detection by qPCR is based on the amplification of specific regions of the viral genome. The amplified product is detected via fluorescent dyes linked to oligonucleotide probes that bind specifically to the target sequences.

Nucleic acid extraction and PCR set-up: The procedure is automated using the Sentosa™ SX101 platform.

qPCR (amplification and detection): For qPCR and detection of amplification products, the Rotor-Gene® Q MDx 5plex HRM instrument was used.

3.2.2 The routinely used assays

The CMV R-gene® kit

The CMV R-gene® kit (bioMérieux, Marcy l'Etoile, France) enables the quantification of CMV genome. After CMV DNA isolation, the quantification is performed using the CMV R-gene® qPCR assay. Several types of specimen and numerous DNA purification systems (automatic and manual) have been validated with the kit. Extracted DNA is then amplified and detected by qPCR on the common available platforms. Results are validated with various controls, including an extraction control, all provided with the kit.

Sample type: CMV R-gene® is used to detect and measure the viral load on CMV virus in whole blood, CSF, plasma, serum, bronchoalveolar lavage (BAL)

fluid, urine, biopsies, and amniotic fluid subject to the conditions of a specific protocol.

DNA purification and automated qPCR setup: In this study, DNA was extracted on the NucliSENS® easy MAG® (bioMerieux) (Fig. 9). The qPCR setup was performed on the easySTREAM™ platform (bioMerieux) (Fig. 10).

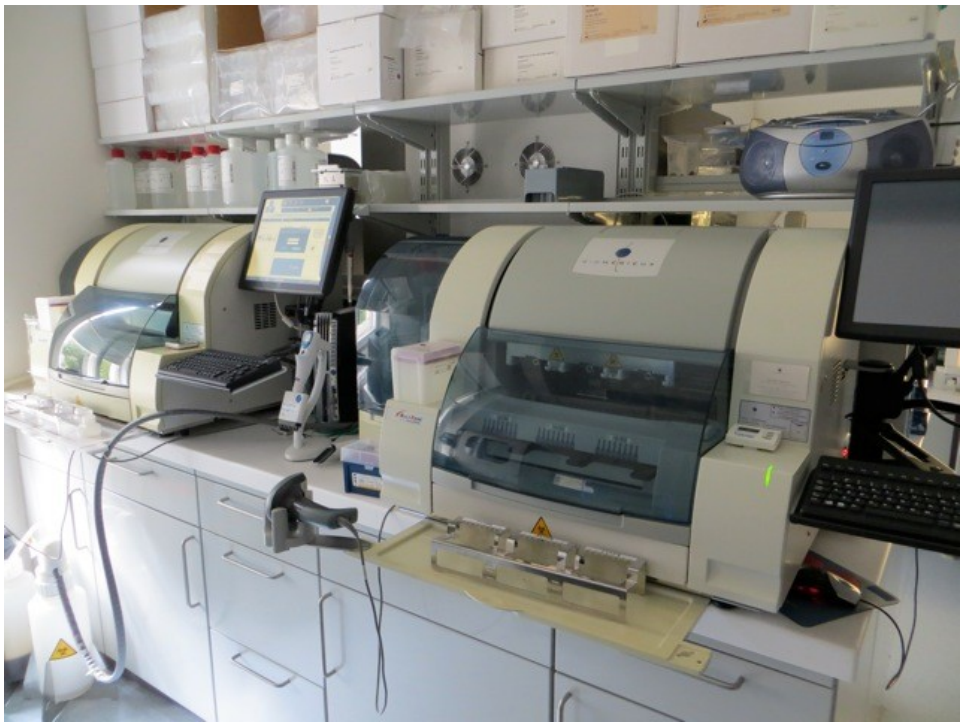

Fig. 9. The NucliSENS® easy MAG® (bioMerieux).

Fig. 10. The easySTREAM™ platform (bioMerieux).

Real time amplification and quantification: Amplification is performed using the 5' nuclease TaqMan® technology also called hydrolysis probes. The ready-to-use amplification mixture includes: primers, dNTPs, amplification buffer, Taq Polymerase, specific CMV primers and probe as well as the primers and probe specific for the internal control which also must be subjected to the entire extraction procedure (including lysis). In this study, amplification and detection was performed on the LightCycler® 480II platform (Roche Diagnostics Ltd., Rotkreuz, Switzerland (Fig. 11).

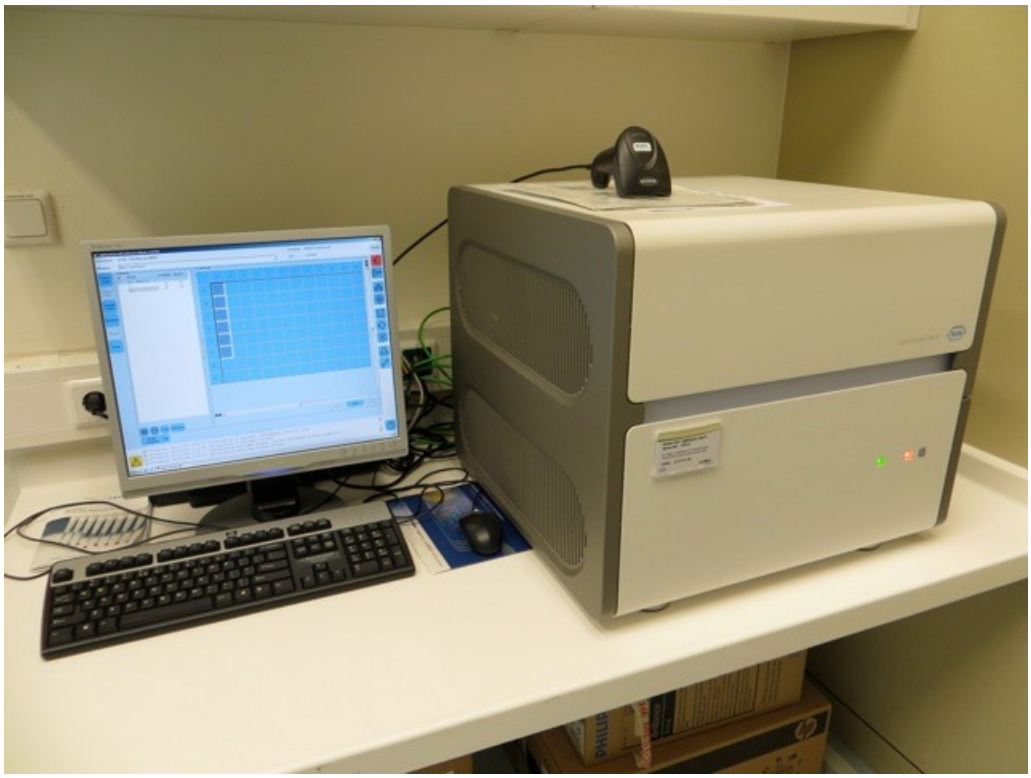

Fig. 11. The LightCycler® 480II platform (Roche).

The EBV R-gene® kit

The EBV R-gene® kit (bioMérieux) enables the quantification of EBV genome. After EBV DNA isolation, the quantification is performed using the EBV R-gene® qPCR assay. Several types of specimen and numerous DNA purification systems (automatic and manual) have been validated with the kit. Extracted DNA is then amplified and detected by qPCR on the common available platforms. Results are

validated with various controls, including an extraction control, all provided with the kit.

EBV R-gene® kit measures the viral load in whole blood and various other samples. The viral load represents the number of virions or the number of EBV-infected B lymphocytes in the replication phase. The viral load can be measured using a quantification range supplied with the kit. Viral DNA must be extracted prior to the qPCR amplification.

Sample type: EBV R-gene® measures the viral load of EBV in whole blood specimens, CSF, plasma, BAL fluid, and biopsies using the extraction systems shown in section “real time amplification and quantification”.

DNA purification and automated qPCR setup: In this study, DNA was extracted on the NucliSENS® easy MAG® (bioMerieux) (Fig. 9). The qPCR setup was performed on the easySTREAM™ platform (bioMerieux) (Fig. 10).

Real time amplification and quantification: Amplification is performed using the 5’ nuclease TaqMan®R-gene® kit technology also called hydrolysis probes. The ready-to-use amplification mixture includes: primers, dNTPs, amplification buffer, Taq Polymerase, specific EBV primers and probe as well as the primers and probe specific for the internal control which also must be subjected to the entire extraction procedure (including lysis).

In this study, amplification and detection was performed on the LightCycler® 480II platform (Roche Diagnostics Ltd., Rotkreuz, Switzerland (Fig. 11).

Extracted samples are amplified and quantified at the same time. The amplified gene is the BXLF1 gene coding for thymidine kinase. A range of 4 quantification standards is provided with the EBV R-gene® kit.

The HSV-1, HSV-2, and VZV R-gene® kit

The HSV-1, HSV-2, and VZV R-gene® kit (bioMérieux) enables the quantification of HSV-1, HSV-2, and VZV viruses in qPCR after viral DNA extraction. Several types of specimen and numerous DNA purification systems (automatic and manual) have been validated with the kit. Extracted DNA is then amplified and detected by qPCR on the common available platforms. Results are validated with various controls, including an extraction control, all provided with the kit.

The HSV-1, HSV-2, and VZV R-gene® kit is used to detect and/or measure the viral load of HSV-1, HSV-2, and VZV in CSF and various other samples. Combined with other methods of biological investigation (medical imaging, biochemical and immunological analysis, etc.), the results obtained with the HSV-1, HSV-2, and VZV R-gene® kit enable one to diagnose the first infections or reactivations of these viruses, and consequently to follow the progress of and to improve the therapeutic efficacy.

Sample type: The HSV-1, HSV-2, and VZV R-gene® assays are used to detect and measure the viral load of HSV-1, HSV-2, and VZV in CSF, gynecological smears, ENT and ophthalmological samples, skin and mucosal smears, and bronchoalveolar lavage (BAL) fluid. The viral load is measured using a quantification sequence common to the three viruses, provided in the kit.

DNA purification and automated qPCR setup: In this study, DNA was extracted on the NucliSENS® easy MAG® (bioMerieux) (Fig.9). The qPCR setup was performed on the easySTREAM™ platform (bioMerieux) (Fig.10).

Real time amplification and quantification: The principle of the real-time amplification utilizes the 5' nuclease technology TaqMan®. Amplification premix is provided ready-to-use with dNTP, amplification buffer Taq polymerase, probes specific to each virus and primers and probes for an internal control, which goes through the extraction step (before lysis).

In this study, amplification and detection was performed on the LightCycler® 480II platform (Roche Diagnostics Ltd., Rotkreuz, Switzerland (Fig.11).

Extracted samples are amplified and quantified at the same time.

A range of 4 quantification standards is provided with the HSV-1, HSV-2 and VZV R-gene® kit.

Quality control

To exclude false-negative results due to interference from inhibitors, a heterologous internal control is incorporated in each of the molecular assays evaluated. The internal control is added to each of the samples before the start of the nucleic acid extraction procedure.

3.3 Accuracy testing

Accuracy of the new Sentosa™ SA CMV PCR and EBV PCR assays was determined using the appropriate proficiency panels provided by an international external quality assessment/proficiency testing organization (Quality Control for Molecular Diagnostics, QCMD; www.qcmd.org): the QCMD 2014 Cytomegalovirus Whole Blood EQA Programme (including 8 members) and the QCMD 2014 Epstein-Barr Virus Whole Blood EQA Programme (including 8 members). For all members of the panels, whole blood was used as matrix.

3.4 Standardization of quantification results with the CMV R-gene® and the EBV R-gene® kits

While results obtained with the Sentosa™ SA kits are reported in International Units/milliliter (IU/ml), results obtained with the CMV R-gene® and the EBV R-gene® kits are reported in copies/milliliter (cop/ml). In order to make results comparable, those reported in cop/ml must be converted into IU/ml. For establishing specific conversion factors for results obtained with the CMV R-gene® and the EBV R-gene® kits, the first international WHO standards for CMV and EBV were used. These standards were developed and have become available at the National Institute for Biological Standards and Control (NIBSC; NIBSC code: 09/162 and NIBSC code: 09/260) in 2010/2011 (32, 33).

The conversion factors were calculated according to results obtained by specially designed protocols. Aliquots of the first international WHO standards for CMV and EBV were tested with the CMV R-gene® and the EBV R-gene® kits. Three quantifications of each aliquot were performed on 4 consecutive days.
The specific conversion factor (CF) is calculated as follows:

CF = value of International Standard (IU/ml) / mean value of Argene quantifications (cop/ml)

The CF can only be validated if the deviation of the 12 values individually measured is less than 0.5 log units compared to the mean value. The specific

conversion factors allow the conversion of results obtained with the CMV R-gene® and the EBV R-gene® kits into IU/ml.

3.5 Clinical specimens

For evaluation of the clinical performance, 106 EDTA whole blood specimens that remained following routine clinical testing were tested. Specimens had been collected from 106 patients presenting at different departments of the University Hospital Graz. Specimens had been derived from patients (42 females, 64 males; mean age, 47.3 years; age range, 5 to 82 years) after bone marrow (n=77) or solid organ (n=29) transplantation. Immediately after collection, samples were transferred to the molecular diagnostics laboratory and either processed within 2 hours after collection or frozen at ms70° C until procession. Due to the capacity limitation of the Sentosa™ SX101 instrument, 16 samples were processed in parallel within each experiment.

3.6 Interpretation of results

All experiments were done with single kit lots of the molecular assays. Runs were considered valid and patient results reported if all conditions outlined in the manufacturer´s package insert instructions occurred. Valid results were reported quantitatively (IU/ml), as “positive” if the value obtained was between the limit of quantification (LOQ) and the limit of detection (LOD), as “positive <LOD” if the value obtained was under the LOD, or as “not detectable”.

4 RESULTS

4.1 Accuracy testing

When accuracy was tested with whole blood QCMD proficiency panels, all results were found to be concordant with the expected results. All members of the QCMD 2014 Cytomegalovirus Whole Blood EQA Programme yielded results within ±0.5 log_{10} unit of the expected panel results (Table 6). In the sample without CMV DNA, no target was detected. Of 8 members of the QCMD 2014 Epstein-Barr Virus Whole Blood EQA Programme, 6 yielded results within ±0.5 log_{10} unit of the expected panel results (Table 7). One result showed a difference of -0.72 log_{10} unit when compared to the expected result. In the sample without EBV DNA, no target was detected.

Table 6
Results obtained by the Sentosa™ SA CMV PCR Test in comparison with those obtained by consensus values of the QCMD 2014 Cytomegalovirus Whole Blood EQA Programme calculated from all of the data returned by participants.

Panel member ID	Result (IU/ml)		Difference between results (log_{10})
	Sentosa™ SA CMV PCR Test	Consensus value	
1	7.4×10^2	7.3×10^2	<0.1
2	4.8×10^2	4.9×10^2	<0.1
3	3.8×10^2	3.7×10^2	<0.1
4	5.5×10^2	7.5×10^2	0.1
5	5.4×10^3	7.3×10^3	0.1
6	0	0	TND
7	2.9×10^4	3.8×10^4	0.1
8	7.6×10^1	2.0×10^2	0.4

TND, target not detected.

Table 7
Results obtained by the Sentosa™ SA EBV PCR Test in comparison with those obtained by consensus values of the QCMD 2014 Epstein-Barr Virus Whole Blood EQA Programme calculated from all of the data returned by participants.

Panel member ID	Result (IU/ml)		Difference between results (log_{10})
	Sentosa™ SA EBV PCR Test	Consensus value	
1	1.5×10^3	2.0×10^3	0.2
2	2.8×10^2	6.4×10^2	0.4
3	5.4×10^3	5.8×10^3	<0.1
4	5.2×10^3	6.5×10^3	<0.1
5	3.2×10^2	1.7×10^3	0.7
6	1.5×10^4	1.7×10^4	<0.1
7	0	0	TND
8	4.4×10^4	7.1×10^4	0.2

TND, target not detected.

4.2 Results of standardization

In order to make results obtained with the Sentosa™ SA CMV and EBV assays on the one hand and the appropriate R-gene® kits on the other comparable, conversion factors for results were calculated according to the specifically designed protocol. Conversion factors were found to be 0.36 for the CMV R-gene® kit and 0.48 for the EBV R-gene® kit (Table 8).

Table 8
Standardization of the CMV and the EBV R-gene® kits according to the first international WHO standards for CMV and EBV.

Limits	Results obtained by the CMV R-gene® in		Results obtained by the EBV R-gene® in	
	cop/ml	IU/ml	cop/ml	IU/ml
Limit of detection	4.5×10^2	1.6×10^2	1.8×10^2	0.9×10^2
Limit of quantification	5.0×10^2 - 1.0×10^7	1.8×10^2 - 3.6×10^6	8.2×10^2 - 1.0×10^7	3.4×10^2 - 4.8×10^6

4.3 Clinical performance

Validity of results

The internal controls included in each of the molecular assays employed were detected within the range expected in all samples throughout the whole study. In addition, external run controls gave results as expected.

Comparison of results obtained by CMV assays

When the results obtained by the Sentosa™ SA CMV PCR Test were compared to those obtained by the CMV R-gene® kit, 81 of 106 (76.4%) results were found to be concordant. Twenty-four results were found to be within the analytical measuring range, 3 tested positive under LOD with both of the assays, and in 54 samples, CMV DNA was not detectable (Table 9).

Table 9
Comparison of results obtained by the Sentosa™ SA CMV PCR Test and the CMV R-gene® kit.

	The Sentosa™ SA CMV PCR Test				
CMV R-gene®		Within analytical measuring range	Between LOQ and LOD	Positive under LOD	TND
	Within analytical measuring range	24	0	0	0
	Between LOQ and LOD	3	0	0	0
	Positive under LOD	10	0	3	3
	TND	5	0	4	54

Twenty-five of 106 (23.6%) results were found to be discordant. Ten of the results were found to be within the analytical measuring range with the Sentosa™ SA CMV PCR Test (range, 143 – 1857 IU/ml) but tested positive under LOD with the CMV R-gene® kit. Another 5 of the results were found to be within the analytical measuring range with the Sentosa assay (range, 125 – 570 IU/ml) but CMV DNA was not detectable with the R-gene® kit. Four of the results were found to be positive under LOD with the Sentosa assay but CMV DNA was not detectable with the R-gene® kit and another 3 results vice versa (positive under LOD with the R-gene® kit and not detectable with the Sentosa assay). Three samples were found to be within the analytical measuring range with the Sentosa assay (136 IU/ml, 1014 IU/ml, and 2517 IU/ml) but were found to be positive (between LOQ and LOD) with the R-gene® kit.

When the results obtained for the 24 samples with viral concentrations within the analytical measuring range by both of the test systems were compared, the Sentosa™ SA CMV PCR Test revealed a 0.32 $\log_{10}$ units higher measurement (Fig. 12). Of 24 samples compared, 19 measured higher by this test system. Six of 24 samples showed values within the SD range. For all 24 samples, a correlation coefficient (R^2) of 0.37 was obtained (Fig. 13).

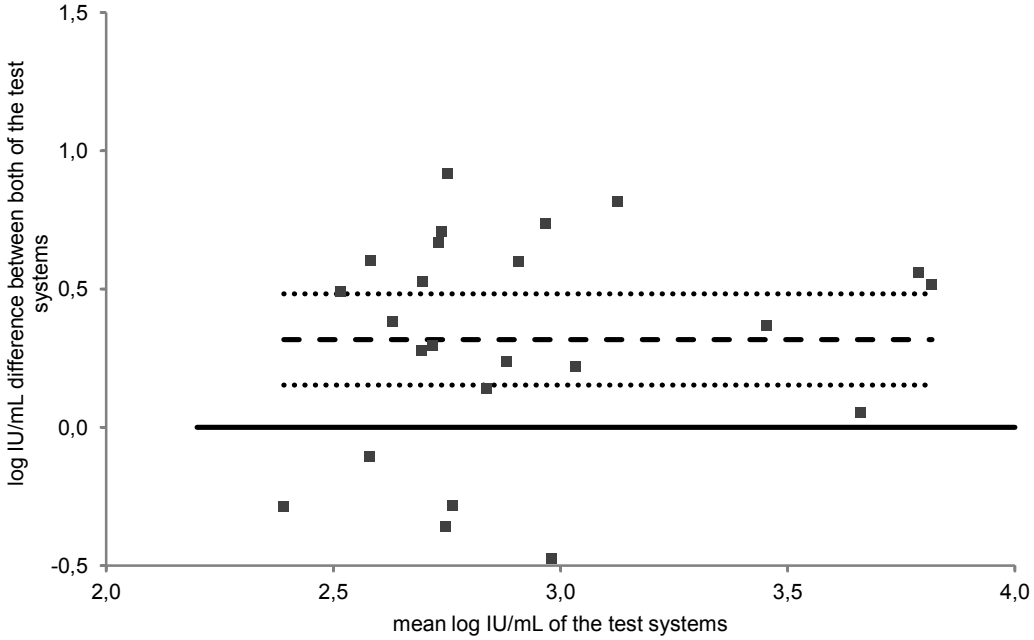

Fig. 12. Agreement between 24 results (IU/ml) obtained with the Sentosa™ SA CMV PCR Test and the CMV R-gene® kit. Bland-Altman plot; the *x* axis shows the mean values for each sample obtained by both of the assays; the *y* axis shows the difference between the values obtained with the 2 assays. The *solid line* represents the zero bias, the *dotted line* represents the mean difference between values, and the *dashed lines* represent the mean difference plus or minus 1.96 SD (95% limits of agreement).

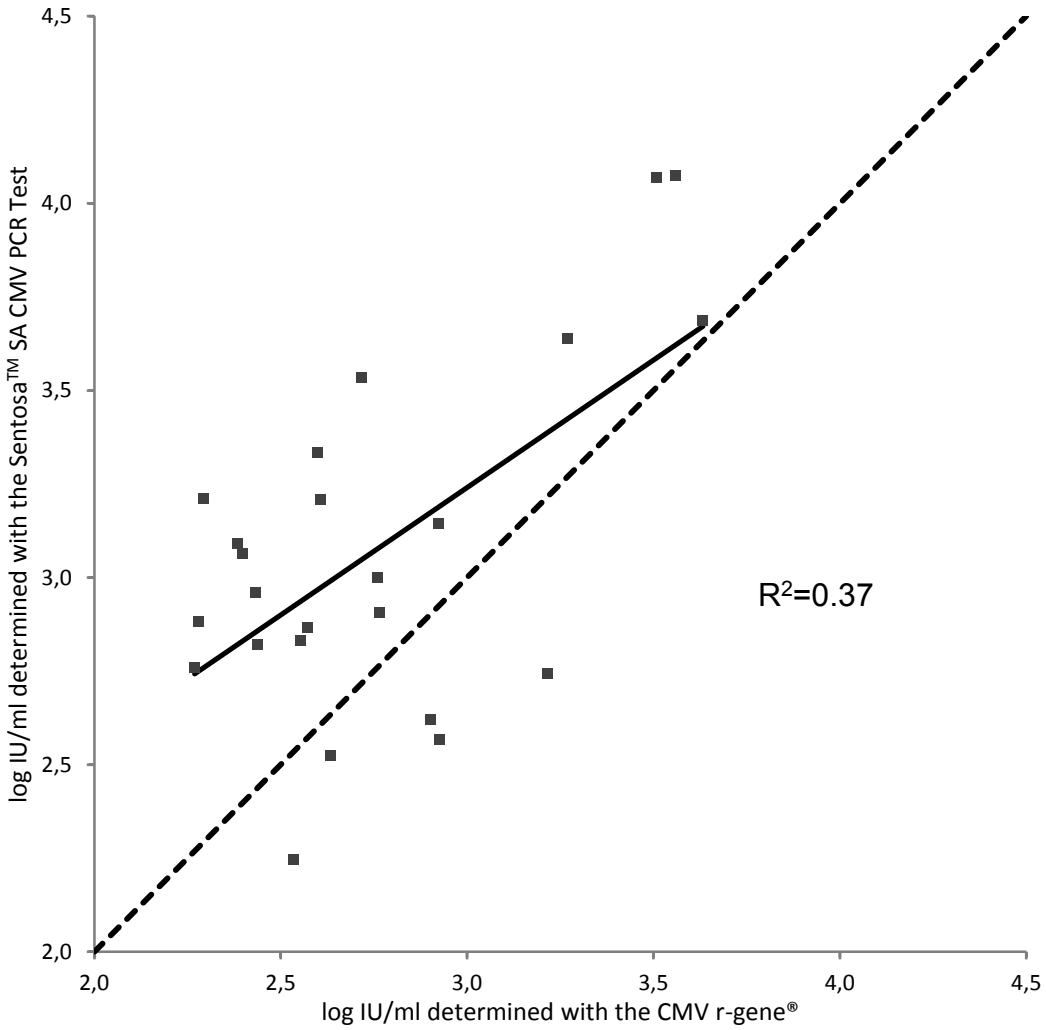

Fig. 13. Concordance of the results (IU/ml) obtained with the Sentosa™ SA EBV PCR Test (referred to as the new assay) and the EBV R-gene® kit (referred to as the routinely used assay). *Solid line*, regression curve; *dashed line*, identity line.

Comparison of results obtained by EBV assays

When the results obtained by the Sentosa™ SA EBV PCR Test were compared to those obtained by the EBV R-gene® kit, 67 of 106 (63.2%) results were found to be concordant. Sixteen results were found to be within the analytical measuring range, 5 tested positive under LOD with both of the assays, and in 46 samples, EBV was not detectable (Table 10).

Table 10
Comparison of results obtained by the Sentosa™ SA EBV PCR Test and the EBV R-gene® kit.

	Sentosa™ SA EBV PCR Test				
EBV R-gene®		Within analytical measuring range	Between LOQ and LOD	Positive under LOD	TND
	Within analytical measuring range	16	0	3	0
	Between LOQ and LOD	7	0	4	0
	Positive under LOD	4	0	5	5
	TND	4	0	12	46

Thirty-nine of 106 (36.8%) results were found to be discordant. Four of the results were found to be within the analytical measuring range with the Sentosa™ SA EBV PCR Test (range, 251 – 1629 IU/ml) but tested positive under LOD with the EBV R-gene® kit and another three results were found to be within the analytical measuring range with the R-gene® kit but tested positive under LOD with the Sentosa assay (range, 255 – 2189 IU/ml). Another 4 of the results were found to be within the analytical measuring range with the Sentosa assay (range, 215 – 849 IU/ml) but EBV DNA was not detectable with the R-gene® kit. Twelve of the results were found to be positive under LOD with the Sentosa assay but EBV DNA was not detectable with the R-gene® kit and another 5 results vice versa (positive under LOD with the R-gene® kit and not detectable with the Sentosa assay). Seven samples were found to be within the analytical measuring range with the

Sentosa assay (236 IU/ml, 456 IU/ml, 480 IU/ml, 633 IU/ml, 852 IU/ml, 870 IU/ml, and 1314 IU/ml) but were found to be positive (between LOQ and LOD) with the R-gene® kit. Four samples were found to be positive under LOD with the Sentosa assay but were found to be positive (between LOQ and LOD) with the R-gene® kit.

When the results obtained for the 16 samples with viral concentrations within the analytical measuring range by both of the test systems were compared, the Sentosa™ SA EBV PCR Test revealed a 0.62 $\log_{10}$ units higher measurement (Fig. 14). All samples measured higher by this test system. Of 16 samples, 6 showed values within the SD range. For all 16 samples, a correlation coefficient (R^2) of 0.61 was obtained (Fig. 15).

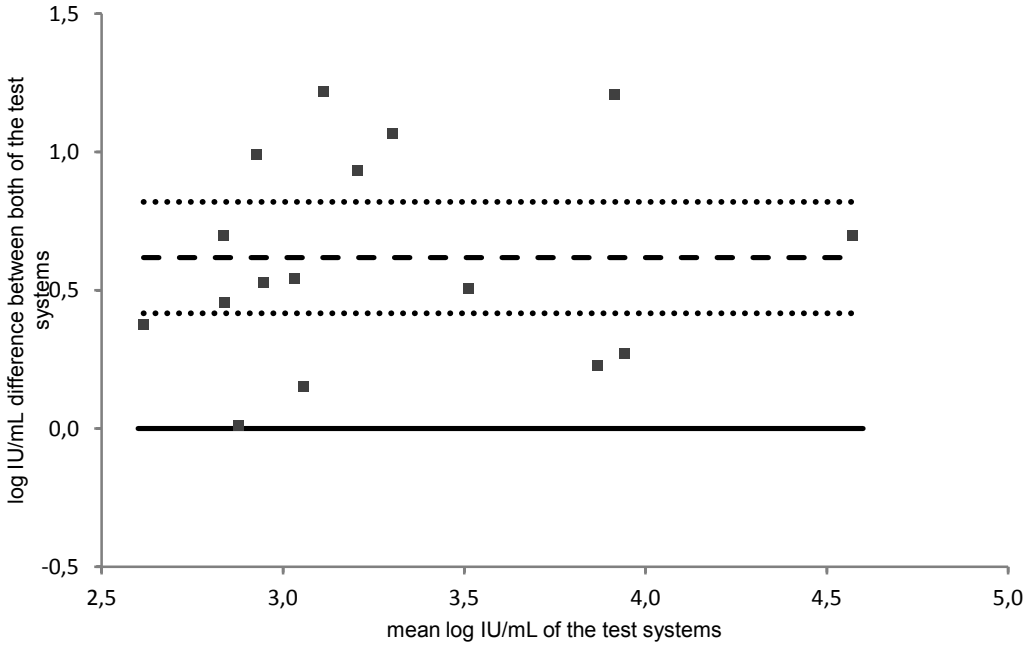

Fig. 14. Agreement between 16 results (IU/ml) obtained with the Sentosa™ SA EBV PCR Test and the EBV R-gene® kit. Bland-Altman plot; the *x* axis shows the mean values for each sample obtained by both of the assays; the *y* axis shows the difference between the values obtained with the 2 assays. The *solid line* represents the zero bias, the *dotted line* represents the mean difference between values, and the *dashed lines* represent the mean difference plus or minus 1.96 SD (95% limits of agreement).

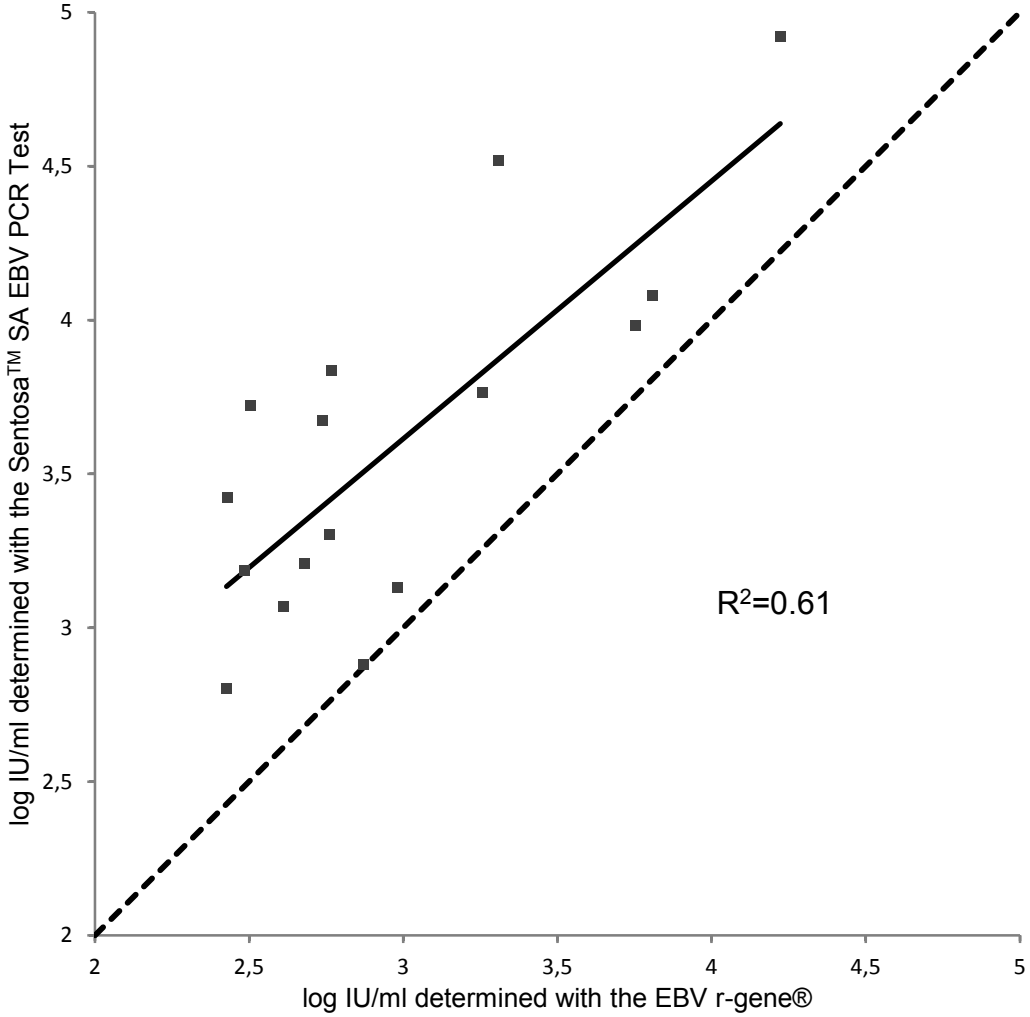

Fig. 15. Concordance of the results (IU/ml) obtained with the Sentosa™ SA EBV PCR Test (referred to as the new assay) and the EBV R-gene® kit (referred to as the routinely used assay). *Solid line*, regression curve; *dashed line*, identity line.

Comparison of results obtained by HSV assays; HSV-1

When the results obtained by the Sentosa™ SA HSV PCR Test were compared to those obtained by the HSV R-gene® kit, 96 of 106 (90.6%) results were found to be concordant. In all of those 96 samples, HSV-1 DNA was not detectable (Table 11).

Table 11
Comparison of results obtained for HSV-1 by the Sentosa™ SA HSV PCR Test and the HSV R-gene® kit.

	The Sentosa™ SA HSV PCR Test				
HSV I R-gene®		Within analytical measuring range	Between LOQ and LOD	Positive under LOD	TND
	Within analytical measuring range	0	0	0	0
	Between LOQ and LOD	0	0	0	0
	Positive under LOD	5	4	0	0
	TND	0	0	1	96

Ten of 106 (9.4%) results were found to be discordant. Five of the results were found to be within the analytical measuring range with the Sentosa™ SA HSV PCR Test (range, 642 – 1971 cop/ml) but tested positive under LOD with the HSV R-gene® kit. Four samples were found to be positive under LOD with the R-gene® kit but were found to be positive (between LOQ and LOD) with the Sentosa assay. One result was found to be positive under LOD with the Sentosa assay but was not detectable with the R-gene® kit.

Comparison of results obtained by HSV assays; HSV-2

When the results obtained by the Sentosa™ SA HSV PCR Test were compared to those obtained by the HSV R-gene® kit, 103 of 106 (97.2%) results were found to be concordant. Six results were found to be within the analytical measuring range; in 97 samples, HSV-2 DNA was not detectable (Table 12).

Table 12
Comparison of results obtained for HSV-2 by the Sentosa™ SA HSV PCR Test and the HSV R-gene® kit.

	The Sentosa™ SA HSV PCR Test				
HSV II R-gene®		Within analytical measuring range	Between LOQ and LOD	Positive under LOD	TND
	Within analytical measuring range	6	0	0	0
	Between LOQ and LOD	0	0	0	0
	Positive under LOD	2	0	0	1
	TND	0	0	0	97

Three of 106 (2.8%) results were found to be discordant. Two of the results were found to be within the analytical measuring range with the Sentosa™ SA HSV PCR Test (range, 616 – 704 cop/ml) but tested positive under LOD with the HSV R-gene® kit. One result was found to be positive under LOD with the R-gene® kit but was not detectable with the Sentosa assay.

Comparison of results obtained by VZV assays

When the results obtained by the Sentosa™ SA VZV PCR Test were compared to those obtained by the VZV R-gene® kit, 103 of 106 (97.2%) results were found to be concordant. Six results were found to be within the analytical measuring range; in 97 samples, VZV DNA was not detectable (Table 13).

Table 13
Comparison of results obtained by the Sentosa™ SA VZV PCR Test and the VZV R-gene® kit.

	The Sentosa™ SA VZV PCR Test				
VZV R-gene®		Within analytical measuring range	Between LOQ and LOD	Positive under LOD	TND
	Within analytical measuring range	6	0	0	0
	Between LOQ and LOD	0	0	0	0
	Positive under LOD	1	0	0	1
	TND	0	0	1	97

Three of 106 (2.8%) results were found to be discordant. One result was found to be within the analytical measuring range with the Sentosa™ SA VZV PCR Test (879 cop/ml) but tested positive under LOD with the VZV R-gene® kit. Another one of the results was found to be positive under LOD with the Sentosa assay but was not detectable with the R-gene® kit and another 1 result vice versa (positive under LOD with the R-gene® kit and not detectable with the Sentosa assay).

Technical considerations

Both test systems were easy to handle and reagents conveniently packaged. Times required for sample preparation and PCR setup were found to be similar for both the Sentosa™ SA PCR assays and the R-gene® kits. Times-to-results for 16 samples were 270 min for the new assays and 230 min for the kits used routinely. This included a hands-on time of 30 min for each of the molecular assays evaluated (Table 14).

Table 14
Comparison of workflows and times required for assays used in this study.

Steps	**Sentosa™ SA PCR assay**	**Time/16 samples (min)**	**R-gene® kits**	**Time/16 samples (min)**
Sample preparation	Sentosa SX 101 (including PCR setup)	150	NucliSens easyMag (generic protocol)	65
PCR setup			easySTREAM	75
Amplification/ Detection	Rotor-Gene Q	120	Lightcycler 480 II	90
Total		**270**		**230**

5. DISCUSSION

Infectious complications are a major cause of mortality and morbidity in immunocompromised patients such as bone marrow and solid organ transplant recipients. Several viruses including the herpesviruses CMV, EBV, HSV, and VZV have been recognized as significant pathogens in transplant recipients. Detection and quantification of herpesvirus DNA has been state-of-the-art in those patients. The Sentosa™ SA PCR assays for detection and quantification of CMV, EBV, HSV-1, HSV-2, and VZV have recently been brought on the market. In the present study, these assays were evaluated in a routine clinical setting and compared to the routinely used assays.

When the accuracy of the Sentosa™ SA CMV PCR and the Sentosa™ SA EBV PCR assays was evaluated with panels of secondary reference material, all results were found to be concordant with the results expected. Proficiency panels of the CMV and EBV QCMD programs included members with different viral loads as well as negative members. In this study, the accuracy of the Sentosa™ SA PCR assays was found to be superior to that obtained from other assays published recently (34, 35).

Standardization of diagnostic tests for infectious disease markers is inevitable to facilitate clinical guidelines used in the diagnosis, treatment, and monitoring of patients (36). Lack of standardization not only makes comparison of results from different assays difficult but also contributes to the difficulty in interpretation of results. To overcome these problems, the first international WHO standards for CMV and EBV were developed and have become available at the National Institute for Biological Standards and Control (NIBSC; NIBSC code: 09/162 for CMV and NIBSC code: 09/260 for EBV) in 2010/2011 (32, 33). Assay validation with this new standard allows reporting the CMV and EBV concentration measured in IU's instead of the different units used so far. Despite this possibility, recent proficiency panel results obtained in Europe (QCMD) and North America (College of American pathologists) revealed that the vast majority of assays for detection and quantification of EBV DNA still report results in units different from IU.

When the clinical performance of the new assay was evaluated, concordant results were found in the majority of samples for HSV-1 (91%), HSV-2 (97%), and

VZV (97%). However, the percentage of concordant results was relatively low for CMV (76%) and EBV (63%). A relatively high number of discrepant results were observed in the present study. As expected, the majority of discrepant results were found in samples with viral loads close to the LODs of the assays compared.

The mean difference in values obtained with the Sentosa™ SA CMV PCR Test compared to those obtained with the CMV R-gene® kit was 0.31 $\log_{10}$ unit. The corresponding value for EBV assays was 0.62 $\log_{10}$ unit. For EDTA whole blood samples with lower viral concentrations, an increased variation was observed. These results are in agreement with those reported recently showing significant interlaboratory variations of CMV and EBV DNA quantification (37, 38, 39). For instance, the variability of CMV viral load testing across 33 laboratories demonstrated an inconsistency of up to 3 $\log_{10}$ in viral load values for individual samples (38). Variation in nucleic acid extraction and assay design has been blamed for the wide variation of results but this may not be applicable for the present study. However, high viral copy variation may be caused by different gene targets as reported recently (40, 41, 42). Indeed, each assay employed in the present study uses different amplification target genes. Anyway, results obtained indicate that results obtained from different assays still cannot be compared making it mandatory to monitor a single patient with the identical assay.

The total time required for the Sentosa™ SA PCR assays per 16 samples was found to be 40 min longer in comparison to that for the assays used routinely. The times required for manual steps were found to be similar. The throughput of the Sentosa™ system is limited to 16 samples to be analyzed in parallel on the SX101 instrument.

In conclusion, the Sentosa™ SA assay showed excellent accuracy. Comparison of the clinical performance revealed concordant results in the majority of samples. However, the variability in viral load results is remarkably high. It is thus still mandatory to monitor a single patient with the identical assay.

References

1. Douglas W, White R, Beard S, Barton ES. Immune modulation during latent herpesvirus infection. Immunol Rev 2012; 245(1): 189–208.

2. Grinde B. Herpesviruses: latency and reactivation, viral strategies and host response. J Oral Microbiol 2013(ePub).

3. Ciotti M and Kessler HH. Pathogens relevant in transplantation medicine. In: Kessler HH (Ed.). Molecular diagnostics of infectious diseases, 3rd edition. Berlin, Boston: Walter de Gruyter 2014; 125-143.

4. Kosugi I. Cytomegalovirus (CMV). The Japanese Society for Virology 2010; 60(2): 209-220.

5. Crough T and Khanna R. Immunobiology of human cytomegalovirus: from bench to bedside. Clin Microbiol Rev 2009; 22(1): 76–98.

6. Melnik LI, Garry RF, Morris CA. Peptide inhibition of human cytomegalovirus infection. Virol J 2011; 8:76(ePub).

7. Kotenko SV, Saccani S, Izotova LS, Mirochnitchenko OV, Pestka S. Human cytomegalovirus harbors its own unique IL-10 homolog (cmvIL-10). PNAS USA 2000; 97(4): 1695–1700.

8. Sia IG and Patel R. New strategies for prevention and therapy of cytomegalovirus infection and disease in solid-organ transplant recipients. Clin Microbiol Rev 2000; 13(1): 83–121.

9. Tomtishen JP III. Human cytomegalovirus tegument proteins (pp65, pp71, pp150, pp28). Virol J 2012; 9: 22(ePub).

10. Kotton CN. CMV: Prevention, diagnosis and therapy. Am J Transplant 2013; 13: 24–40.

11. Steininger C, Puchhammer-Stockl E, Popow-Kraupp T. Cytomegalovirus disease in the era of highly active antiretroviral therapy (HAART). J Clin Virol 2006; 37: 1–9.

12. Michelin BDA, Hadzisejdic I, Bozic M, Grahovac M, Hess M, Grahovac B, Marth E, Kessler HH. Detection of cytomegalovirus (CMV) DNA in EDTA whole-blood samples: evaluation of the quantitative artus CMV LightCycler PCR Kit in conjunction with automated sample preparation. J Clin Microbiol 2008; 46(4): 1241–1245.

13. Kessler HH and Heyszel S. Molecular detection of herpesviruses; In: Stephenson, JR, Warnes, A (Eds.). Diagnostic Virology Protocols. New York, Dordrecht, Heidelberg, London: Springer 2011; 101 - 121.

14. Schnepf N, Scieux C, Resche-Riggon M, Feghoul L, Xhaard A, Gallien S, Molina JM, Socié G, Viglietti D, Simon F, Mazeron MC, Legoff J. Fully automated quantification of cytomegalovirus (CMV) in whole blood with the new sensitive Abbott RealTime CMV Assay in the era of the CMV international standard. J Clin Microbiol 2013; 51(7): 2096–2102.

15. Gequelin LC, Riediger IN, Nakatani SM, Biondo AW, Bonfim CM. Epstein-Barr virus: general factors, virus-related diseases and measurement of viral load after transplant. Rev Bras Hematol Hemoter 2011; 33(5): 383-8.

16. Thompson MP and Kurzrock R. Epstein-Barr virus and cancer. Clin Cancer Res 2004; 10: 803-821.

17. Odumade OA, Hogquist KA, Henry H, Balfour Jr. Progress and problems in understanding and managing primary Epstein-Barr virus infections. Clin Microbiol Rev 2011; 24(1): 193–209.

18. Siennicka J, Trzcinska A, Czescik A, Dunal-Szczepaniak M, Lagosz B. The influence of toll-like receptor stimulation on expression of EBV lytic genes. Pol J Microbiol 2013; 62(3): 237–242.

19. Dunmire SK, Odumade OA, Porter JL, Reyes-Genere J, Schmeling DO, Bilgic H, Fan D, Baechler EC, Balfour HH Jr, Hogquist KA. Primary EBV infection induces an expression profile distinct from other viruses but similar to hemophagocytic syndromes. 2014; 9(1): e85422.

20. De Paschale M and Clerici P. Serological diagnosis of Epstein-Barr virus infection: problems and solutions. World J Virol 2012; 1(1): 31-43.

21. Lee CC, Lin LL, Chan WE, Ko TP, Lai JS, Wang AH. Structural basis for the antibody neutralization of Herpes simplex virus. Acta Crystallogr D Biol Crystallogr 2013; 69(10): 1935-45.

22. Caignard G, Leiva-Torres GA, Leney-Greene M, Charbonneau B, Dumaine A, Fodil-Cornu N, Pyzik M, Cingolani P, Schwartzentruber J, Dupaul-Chicoine J, Guo H, Saleh M, Veillette A, Lathrop M, Blanchette M, Majewski J, Pearson A, Vidal SM. Genome-wide mouse mutagenesis reveals CD45-mediated T cell function as critical in protective immunity to HSV-1. PLOS Pathogens 2013; 9(9): e1003637.

23. Wang K, Kappel JD, Canders C, Davila WF, Sayre D, Chavez M, Pesnicak L, Cohen JI. A herpes simplex virus 2 glycoprotein D mutant generated by bacterial artificial chromosome mutagenesis is severely impaired for infecting neuronal cells and infects only Vero cells expressing exogenous HVEM. J Virol 2012; 86(23): 12891–12902.

24. Connolly SA, Jackson JO, Jardetzky T, Longnecker R. Fusing structure and function: a structural view of the herpesvirus entry machinery. Nat Rev Microbiol 2011; 9(5): 369–381.

25. Spear PG and Longnecker R. Herpesvirus entry: an update. J Virol 2003; 77(19): 10179–10185.

26. Manservigi R, Argnani R, Marconi P. HSV recombinant vectors for gene therapy. The Open Virol J 2010; 4: 123-156.

27. Flowerdew SE, Wick D, Himmelein S, Horn AK, Sinicina I, Strupp M, Brandt T, Theil D, Hüfner K. Characterization of neuronal populations in the human trigeminal ganglion and their association with latent herpes simplex virus-1 infection. PLOS ONE 2013; 8(12): e83603.

28. Zaichick SV, Bohannon KP, Smith GA. Alphaherpesviruses and the cytoskeleton in neuronal infections. Viruses 2011; 3: 941-981.

29. Gilden D, Nagel MA, Cohrs RJ, Mahalingam R. The variegate neurological manifestations of varicella zoster virus infection. Curr Neurol Neurosci Rep 2014; 13(9): 374(ePub).

30. Zerboni L, Sen N, Oliver SL, Arvin AM. Molecular mechanisms of varicella zoster virus pathogenesis. Nat Rev Microbiol 2014; 12(3): 197–210.

31. Baird NL, Yu X, Cohrs RJ, Gilden D. Varicella zoster virus (VZV)-human neuron interaction. Viruses 2013; 5: 2106-2115.

32. Fryer JF, Heath AB, Anderson R, Minor PD and the collaborative study group. Collaborative study to evaluate the proposed 1st WHO International Standard for human cytomegalovirus (HCMV) for nucleic acid amplification (NAT)-based assays. WHO ECBS Report 2010; WHO/BS/10.2138.

33. Fryer JF, Heath AB, Wilkinson DE, Minor PD and the collaborative study group. Collaborative study to evaluate the proposed 1st WHO International Standard for Epstein-Barr virus (EBV) for nucleic acid amplification (NAT)-based assays. WHO ECBS Report 2011; WHO/BS/11.2172.

34. Raggam RB, Wagner J, Bozic M, Michelin BDA, Hammerschmidt S, Homberg C, Kessler HH. Detection and quantitation of Epstein-Barr virus (EBV) DNA in EDTA whole blood samples using automated sample preparation and real time PCR. Clin Chem Lab Med 2010; 48(3): 413-418.

35. Raggam RB, Bozic M, Salzer HJF, Hammerschmidt S, Homberg C, Ruzicka K, Kessler HH. Rapid quantitation of cytomegalovirus DNA in whole blood by a new molecular assay based on automated sample preparation and real-time PCR. Med Microbiol Immunol 2010; 199: 311-316.

36. Raggam RB, Saldanha J, Kessler HH. Qualitiy assurance and quality contol. In: Kessler HH (Ed.). Molecular diagnostics of infectious diseases, 3rd edition. Berlin, Boston: Walter de Gruyter 2014; 3: 41-55.

37. Hayden RT, Hokanson KM, Pounds SB, Bankowski MJ, Belzer SW, Carr J, Diorio D, Forman MS, Joshi Y, Hillyard D, Hodinka RL, Nikiforova MN, Romain CA, Stevenson J, Valsamakis A, Balfour HH Jr; U.S. EBV Working Group. Multicenter comparison of different real-time PCR assays for quantitative detection of Epstein-Barr virus. J Clin Microbiol 2008; 46(1): 157-63.

38. Pang XL, Fox JD, Fenton JM, Miller GG, Caliendo AM, Preiksaitis JK. Interlaboratory comparison of cytomegalovirus viral load assays. Am J Transplant 2009; 9: 258-268.

39. Preiksaitis JK, Pang XL, Fox JD, Fenton JM, Caliendo AM, Miller GG. Interlaboratory comparison of Epstein-Barr virus viral load assays. Am J Transplant 2009; 9(2): 269-79.

40. Zweygberg-Wirgart B, Brytting M, Linde A, Wahren B, Grillner L. Sequence variation within three important cytomegalovirus gene regions in isolates from four different patient populations. J Clin Microbiol 1998; 36(12): 3662-3669.

41. Ishii H, Ogino T, Berger C, Köchli-Schmitz N, Nagato T, Takahara M, Nadal D, Harabuchi Y. Clinical usefulness of serum EBV DNA levels of BamHI W and LMP1 for nasal NK/T-Cell lymphoma. J Med Virol 2007; 79: 562-572.

42. Tsai DE, Douglas L, Andreadis C, Vogl DT, Arnoldi S, Kotloff R, Svoboda J, Bloom RD, Olthoff KM, Brozena SC, Schuster SJ, Stadtmauer EA, Robertson ES, Wasik MA, Ahya VN. EBV PCR in the diagnosis and monitoring of posttransplant lymphoproliferative disorder: results of a two-arm prospective trial. Am J Transplant 2008; 8: 1016-1024.

Figures and Tables

Fig.1: A cartoon depicting the structure of the HCMV virion. Image from (http://www.virology.net/big_virology/bvdnaherpes.html) Dr. Marko Reschke, Marburg, Germany.

Fig.2-Fig.4: Crough T and Khanna R. Immunobiology of human cytomegalovirus: from bench to bedside. Clin Microbiol Rev 2009; 22(1): 76–98.

Fig.5: Balfour Jr., Odumade OA, Hogquist KA, Henry H. Progress and problems in understanding and managing primary Epstein-Barr virus infections. Clin Microbiol Rev 2011; 24(1): 193–209.

Fig.6: Medical Books Online; http://www.cixip.com/index.php/page/content/id/942; Chapter 397 - Herpes Simplex Virus Infections.

Fig.7: Zerboni L, Sen N, Oliver SL, Arvin AM. Molecular mechanisms of varicella zoster virus pathogenesis. Nat Rev Microbiol 2014; 12(3): 197-210.

Table 1-4: Ciotti M and Kessler HH. Pathogens relevant in transplantation medicine. In: Kessler HH (Ed.). Molecular diagnostics of infectious diseases, 3rd edition. Berlin, Boston: Walter de Gruyter 2014: 125-143.