Quantitative PCR in the Diagnosis and Monitoring of Human Cytomegalovirus Infection in Organ Transplant Patients

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Academic Dissertation

To be presented, with the permission of the Faculty of Biosciences of the University of Helsinki, for public discussion in the Auditorium IV of Main Building, University of Helsinki, Unioninkatu 34, Helsinki, on June 4th at 12 o'clock noon.

Helsinki 2004

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ISBN 952-91-7278-8 (paperback)
ISBN 952-10-1883-6 (PDF)
http://ethesis.helsinki.fi
Helsinki University Printing House
Helsinki 2004

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List of original publications

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals.

I. Piiparinen H, Höckerstedt K, Grönhagen-Riska C, Lappalainen M, Suni J and Lautenschlager I (2001). Comparison of plasma polymerase chain reaction and pp65-antigenemia assay in the quantification of cytomegalovirus in liver and kidney transplant patients. Journal of Clinical Virology 22: 111-116.

II. Piiparinen H, Höckerstedt K, Lappalainen M, Suni J and Lautenschlager I (2002). Monitoring of viral load by quantitative plasma PCR during active cytomegalovirus infection of individual liver transplant patients. Journal of Clinical Microbiology 40: 2945-2952.

III. Piiparinen H, Helanterä I, Lappalainen M, Suni J, Koskinen P, Grönhagen-Riska C and Lautenschlager I. Quantitative PCR in the diagnosis of CMV infection and in the monitoring of viral load during the antiviral treatment in renal transplant patients. (submitted).

IV. Piiparinen H, Höckerstedt K, Grönhagen-Riska C and Lautenschlager I (2004). Comparison of two quantitative CMV PCR tests, Cobas Amplicor CMV Monitor and TaqMan assay, and pp65-antigenemia assay in the determination of viral loads from peripheral blood of organ transplant patients. Journal of Clinical Virology (in press).

Abbreviations

CMV	cytomegalovirus
CPE	cytopathic effect
cps/ml	copies/ml
dsDNA	double stranded
DNAE	early gene product
EIA	enzyme-linked immunosorbent assay
FRET	fluorescence resonance energy transfer
GCV	ganciclovir
HCMV	human cytomegalovirus
HCV	hepatitis C virus
HHV-6	human herpesvirus 6
HHV-7	human herpesvirus 7
IE	immediate early gene product
IR	inverted repeat
ISH	in situ hybridization
L	late gene product
NASBA	nucleic acid sequence based amplification
PMNL	polymorphonuclear leukocytes
RT	reverse transcriptase
TR	terminal repeat
U_L	unique long region of CMV genome
U_S	unique short region of CMV genome

Summary

Human cytomegalovirus (HCMV) is a member of the betaherpesviruses. It is a ubiquitous virus, the seroprevalence of which varies between 30 to 100 % in different countries. Acquisition of the virus in the general population mainly occurs early in life. Transmission of the virus can occur vertically or horizontally via direct contact with infectious bodily fluids or blood. The virus can also be transmitted by blood products or transplanted organs. After the primary infection, the virus will remain in a latent state in the host lifelong but may reactivate later. Infections in an immunocompetent individual are mainly asymptomatic or mild, mononucleosis-like syndromes. However, in immunocompromised patients, severe HCMV infections may occur.

HCMV is a significant pathogen in transplant patients causing symptomatic infections and end-organ diseases which may, without antiviral treatment, be life-threatening. Many risk factors for the development of symptomatic infection have been suggested. Viral load has been shown to be a major factor in the development of HCMV disease. The assessment of viral load has an important value in the diagnosis and monitoring of HCMV infection, and also in the prevention and treatment strategies of HCMV disease with antiviral drugs. Since the late 1980's, the semi-quantitative CMV pp65 antigenemia assay has been a commonly used method for these purposes. Within recent years, the applications of nucleic acid amplification techniques, especially quantitative modifications of PCR (polymerase chain reaction), have been developed for HCMV. PCR-based methods, both commercial and in-house applications, are widely used in clinical laboratories, and their utility in the HCMV diagnostics of transplant patients is now evaluated.

In the present study, the value of a commercial quantitative PCR test in the diagnosis of HCMV infection in organ transplant patients was investigated. The test was compared with the pp65 antigenemia assay, and a good correlation between the tests was shown. The clinical utility of quantitative PCR was further evaluated. The suitability of quantitative PCR for the diagnosis of symptomatic infection was proven. In addition, quantitative PCR was shown to have a clinical value in the monitoring of both asymptomatic and symptomatic HCMV infection in individual liver and kidney transplant patients. Also the response to the antiviral treatment was easily followed using the quantitative PCR assay. The next step was to develop a real-time PCR based assay, because the need for faster and more automated technique with a greater capacity for the quantitation of HCMV load was obvious. The clinical value of the developed real-time assay was also shown in the diagnosis and monitoring of HCMV infection in transplant patients.

Review of the literature

1. Human cytomegalovirus

1.1. Virus structure and genome organization

Human cytomegalovirus (HCMV) is a member of the *Betaherpesvirinae* subfamily, which belongs to the family *Herpesviridae* (Mocarski and Courcelle, 2001). Betaherpesviruses, which also includes human herpesvirus 6 (HHV-6) and human herpesvirus 7 (HHV-7), are more closely related to each other than to the other herpesviruses. HCMV is strictly species specific, as are the other cytomegaloviruses. HCMV has a linear double-stranded DNA genome of approximately 230 kbp encoding more than 200 proteins. The virion, which is 150-200 nm in diameter, consists of an icosahedral capsid surrounded by a tegument or matrix, which is further surrounded by a lipid-protein envelope. The viral genome is divided into two unique components, unique long (U_L) and unique short (U_S) regions (Figure 1.). The unique regions are flanked by terminal (TR) and inverted repeat (IR) sequences, U_L by TR_L and IR_L, and U_S by IR_S and TR_S, respectively. DNA sequence-based analyses demonstrate a great genomic heterogeneity within HCMV worldwide.

The capsid of the virion is composed of seven proteins. The major (MCP) and minor capsid proteins (mCP) are encoded by *UL86* and *UL85*, respectively. The minor capsid binding protein (mC-BP) is encoded by *UL46* and the smallest capsid protein (SCP) by *UL48.5*. Three assemblin protein –related proteins associate with capsids and are the products of *UL80*, *UL80a* and *UL80.5* genes. A tegument contains at least 25 proteins, of which many are phosphorylated. The basic phosphoprotein (pp150) encoded by *UL32* and lower matrix protein (pp65) encoded by *UL83*, are the most abundant proteins expressed during virus replication. The other prominent tegument proteins are ppUL99 (pp28), ppUL82 (pp71) and ppUL48 (huge tegument protein). Several transcriptional transactivator proteins have also been localized to the tegument. The envelope consists of two prominent glycoprotein complexes, a complex of dimers of glycoprotein B (gB), encoded by *UL55*, and a complex of the gH, gL, and gO, encoded by *UL75, UL115* and *UL74*, respectively. These glycoprotein complexes are assumed to play an important role in virus entry.

1.2. Virus replication

HCMV exhibits a restricted host range in cell culture. Although *in vitro* the most commonly used cells are primary differentiated human fibroblasts derived from skin or lung, HCMV can also replicate in other cell types including endothelial and epithelial cells, smooth muscle cells and leukocytes (Pass, 2001; Sinzger and Jahn, 1996). After the attachment of the virus to the cell surface, fusion of the virion envelope and the cell membrane occurs (Mocarski and Courcelle, 2001). The cellular receptor(s) for HCMV is still unclear, but it has been suggested to be widely distributed in cells (Mocarski and Courcelle,

2001). Epidermal growth factor receptor has recently been suggested to serve as a receptor for HCMV (Wang et al., 2003). The virus capsid is rapidly transported to the cell nucleus and, after the proteolysis of the capsid proteins, the viral DNA is released into the nucleus.

Viral gene expression in productive replication occurs in a temporally ordered cascade (Mocarski and Courcelle, 2001; Sinclair, 2000). Immediate-early (IE) gene expression is first activated followed by early and late gene expression which ultimately leads to virus assembly and release from the infected cells. The most abundantly expressed IE genes are transcribed from the major IE (MIE) locus, located in the U_L region (Figure 1.). The gene products of *UL122/123,* IE1 and IE2, are generated by differential splicing of a primary transcript. The IE proteins are transactivators of gene expression and play an important role in controlling both viral and cellular gene expression. One function of these proteins is to optimise the cellular environment for viral replication and viral gene expression. The expression of IE proteins is also crucial for the next step of the temporal cascade, correct expression of early genes. This is followed by the expression of late genes. The early (E) proteins are involved in the replication of the viral DNA, whereas late (L) gene expression provides the structural proteins/glycoproteins of the viral capsid, tegument and envelope.

DNA replication, formation of capsids and packaging of viral DNA occur in the nucleus. Subsequently, nucleocapsids acquire a primary envelopment by budding at the nuclear membrane. They further mature through a de-envelopment/re-envelopment process in the cytoplasm before leaving the cell via an exocytic-like pathway (Mettenleiter, 2002; Mocarski and Courcelle, 2001). The whole replication cycle of human CMV is slow, requiring approximately 48-72 hours. The cytopathic effect in response to HCMV is characteristically cell enlargement with intranuclear inclusions.

1.3. Epidemiology and transmission

HCMV is an ubiquitous virus and it infects humans of all ages. Acquisition of the virus in the general population usually occurs early in life, mainly during the first two decades, and often already during the first year. Based on sero-epidemiological studies, the seroprevalence varies between 30 % to 100 % in different countries (Ho, 1990). The great seroprevalence and also early acquisition of the virus have been associated with lower socioeconomic circumstances, developing countries and countries with crowded populations (Ho, 1990). However, in Scandinavian countries seroprevalence is as high as 60-80 %, and in Finland even 70-80 %, despite good hygiene and a high socioeconomic level.

After the primary infection, which is usually asymptomatic or very mild in immunocompetent individuals, the virus will remain in a lifelong latent state in the host. Later in life, the virus can reactivate or an individual can have a reinfection. Transmission of the virus can occur vertically from mother to fetus or newborn, or horizontally from one individual to another (Griffiths and Emery, 1997). During primary infection, the virus is secreted into the urine, saliva, semen and cervical secretions for weeks to months, as well as intermittently during reactivations and reinfections. Vertical transmission can occur

transplacentally or perinatally. An intra-uterine infection is the result of maternal viremia and perinatally acquired infection can occur at the time of delivery from infected maternal genital secretions or later via breast milk. Later in life, infection usually occurs by close contact via infected bodily fluids, e.g. from child to child or from child to susceptible adult via saliva or urine. In the adult population, a major route of infection is sexual transmission. HCMV can also be transmitted by blood transfusions or by organ or bone marrow transplantations.

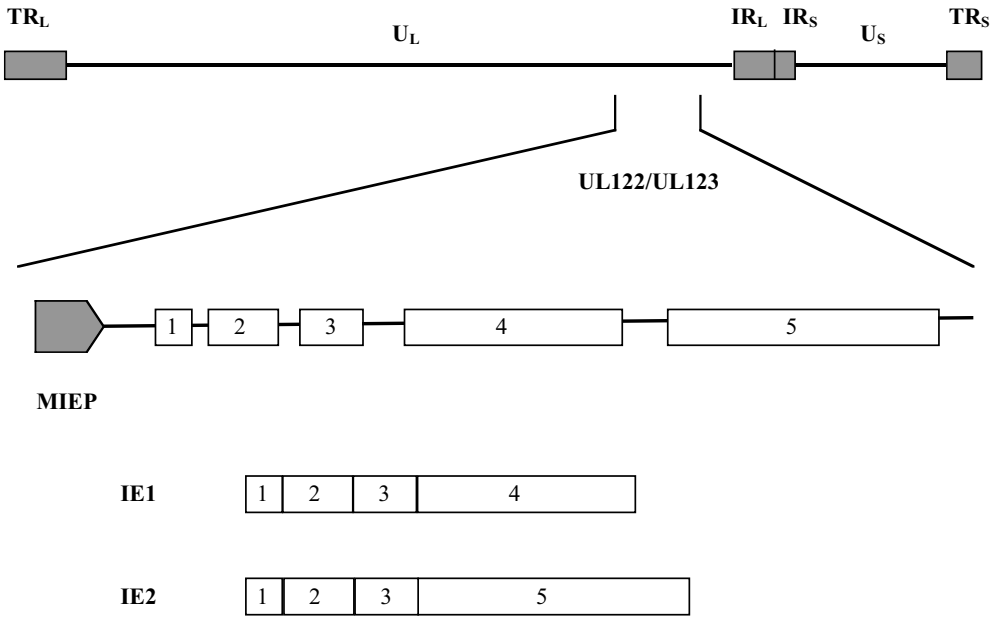

Figure 1. Simplified structure of the CMV genome
(U_L = unique long region, U_S = unique short region, TR_L and TR_S = terminal repeat sequence, IR_L and IR_S = inverted repeat sequence). The most abundantly expressed viral genes at immediate early time are transcribed from the major IE locus (MIE), located in U_L region (MIEP = major IE promotor). The primary transcript is differentially spliced to generate two major proteins, IE1 and IE2.

1.4. Pathogenesis

It has been suggested that epithelial cells of the upper alimentary, respiratory and genito-urinary tracts can be the site of primary replication after the direct contact of infectious virus and mucosal surface (Pass, 2001). In addition, it has been suggested that a fetus can be infected hematogenously (Halwachs-Baumann et al., 1998; Hemmings et al., 1998). In any case, once HCMV infection is established, leukocyte-associated and endothelial cell-associated viremia appears to play an important role in the passage of the virus between the various tissues of the host. Viremia may last for several weeks during the primary infection. Hematogenous spreading typically results in the infection of ductal epithelial cells at the initial site. HCMV can infect a wide range of tissues including

salivary glands, gastrointestinal tract, lung, liver, brain, kidney, spleen, pancreas, eye, heart, adrenals, thyroid and genital tract and then replicate in diverse cell types, including fibroblasts, epithelial cells, macrophages, vascular smooth muscle and endothelial cells (Pass, 2001). HCMV is also suggested to infect the vascular wall and play a role in the development of atherosclerosis (Bruggeman, 2000).

After the primary infection, long-term cellular and humoral immunity develops. The cell-mediated immunity, including both natural killer cells and cytotoxic-T lymphocytes, plays an important role in the control of HCMV infection (Mocarski and Courcelle, 2001). Humoral immune response also develops but it has been suggested to be less important in the control of HCMV infection (Mocarski and Courcelle, 2001). However, the immune defence is unable to clear the virus completely from the host, and the virus remains latent lifelong within the host cell. The ability of the virus to evade the host immune system may be due to various HCMV gene products, which show sequence similarity to, or functional relatedness with, host proteins. The proteins may have an ability to engage host proteins, which lead to alterations in host immune responses (Beck and Barrell, 1988; Mocarski, 2002; Reyburn et al., 1997). These proteins can interfere in classical or non-classical major histocompatibility complex protein function, modify leukocyte migration, activation and cytokine responses, or modulate host cell susceptibility to apoptosis.

HCMV can not usually be isolated from the blood of healthy individuals (Jordan, 1983). However, transmission can occur via blood transfusion. The risk can be reduced by removal of blood leukocytes before transfusion (de Graan-Hentzen et al., 1989; Lang et al., 1977). Thus, latently infected blood leukocytes probably are an important reservoir for transmission of the virus in organ transplantation and blood transfusion. Peripheral blood mononuclear cells in the myeloid lineage have been mainly found to support latent infection, instead of lymphocytes and polymorphonuclear leukocytes (Taylor-Wiedeman et al., 1993; Taylor-Wiedeman et al., 1991). The viral genome is maintained as a circular plasmid in the nucleus of these latently infected CD14+ monocytes (Bolovan-Fritts et al., 1999). Viral DNA has also been detected in the early bone marrow haematopoietic progenitors (CD34+ cells) (Mendelson et al., 1996; von Laer et al., 1995), and the common precursors of dendritic and myeloid cells (CD33+/CD14+ and CD33+/CD15+, along with the dendritic cell markers CD1a and CD10) (Hahn et al., 1998). It has been suggested that HCMV is maintained in the myeloid progenitor cells when they differentiate into peripheral blood mononuclear cells in the absence of lytic gene expression. This helps the virus to evade the host immune response. HCMV latency-associated transcripts have also been found in myeloid cells in both experimental and natural infection, but the role of these transcripts is still unclear (Hahn et al., 1998; Jenkins et al., 2004; Kondo et al., 1994; Kondo et al., 1996). Reactivation of a latent virus by allogeneic stimulation of peripheral blood mononuclear cells has been demonstrated (Soderberg-Naucler et al., 1997). Differentiation of latently infected monocytes into macrophages by activation of the immune response, seems to lead to virus reactivation and productive infection (Jarvis and Nelson, 2002; Soderberg-Naucler et al., 1997; Taylor-

Wiedeman et al., 1994). Activation of cytokines and signal transduction pathways have been suggested to play an important role in reactivation of the virus (Fietze et al., 1994; Ritter et al., 2000). The differentiation state of the cell seems to be critical for the silencing of IE gene expression and thus for the maintenance of latency.

In immunocompetent individuals, primary infection (and also reactivation/reinfection) usually remains asymptomatic or results in benign clinical symptoms such as fever, elevated levels of transaminases and/or mononucleosis syndrome. Severe clinical symptoms are observed in congenital infections, and among immunocompromised patients (such as transplant recipients and AIDS-patients) (Griffiths and Emery, 1997), in whom HCMV infections are an important factor for developing HCMV-associated disease.

2. Human cytomegalovirus infections in organ transplant patients

2.1. Clinical manifestations

The updated definitions of CMV infection and disease have recently been developed and published (Ljungman et al., 2002). "CMV infection" is defined as the isolation of cytomegalovirus or detection of viral proteins or nucleic acid in any body fluid or tissue specimen. The minimum requirements for definition of CMV disease are the documented presence of fever, the presence of neutropenia or thrombocytopenia, and the detection of CMV in the blood. An end-organ disease needs documented organ involvement by CMV.

Many risk factors have been reported for the development of symptomatic HCMV infection in organ transplant recipients. Viral load has been shown to be a major factor in the pathogenesis of HCMV (Cope et al., 1997; Emery, 1999; Hassan-Walker et al., 1999). Traditionally primary infections have been associated with the more severe forms of disease than reactivations or reinfections, i.e. the recipients who are HCMV-negative (R-) and obtain an organ from HCMV-positive donor (D+) (Gorensek et al., 1990; Hassan-Walker et al., 1999; Rubin, 1990). The type and intensity of immunosuppressive therapy are of primary importance. Administration of anti-thymocyte antibodies and rejection treatment with high doses of agents depressing cell-mediated immunity are a risk factor (Eckhoff et al., 1998; Emery et al., 2000a; Hibberd et al., 1992; Mathew, 1998; Portela et al., 1995; Rubin, 1990). The other factors known to have an influence include the donor-recipient HLA matching and the type and amount of blood products used (Sia and Patel, 2000). In general, the incidence of HCMV disease varies among type of transplantation. Lung and heart-lung recipients (50-80 %) as well as pancreas and kidney-pancreas recipients (50 %) have a greater risk of developing the disease than do heart, liver and kidney recipients (8-35 %) (Ljungman, 2002; van der Bij and Speich, 2001).

The symptoms of HCMV infection in immunocompromised patients usually include prolonged fever, the presence of leukopenia and/or thrombocytopenia, and increased levels of serum transaminases. In addition, HCMV may also cause severe end-organ diseases, such as hepatitis, gastrointestinal infections and pneumonitis (Patel and Paya, 1999), which may be life-threatening without antiviral treatment. HCMV infection of the transplanted organ may be an additional complication, which represents itself as impaired graft function.

In addition to direct consequences, various indirect effects caused by HCMV have been suggested (Fishman and Rubin, 1998; Rubin, 1990). Many clinical studies have shown an association between HCMV infection and acute or chronic rejection in heart, kidney, liver and lung transplantation (Evans et al., 2000; Grattan et al., 1989; Koskinen et al., 1993a; Kroshus et al., 1997; Lautenschlager et al., 1997; Luckraz et al., 2003; Pouteil-Noble et al., 1993; Reinke et al., 1994; Tong et al., 2002; Valantine, 1999). HCMV infection has been proven to be a risk factor for allograft atherosclerosis and rate of graft loss in heart transplantation (Grattan et al., 1989; Valantine, 1999). However, the impact of HCMV on chronic rejection in other organs is suggestive but not conclusive, and the opinions are still controversial (Cainelli and Vento, 2002; Lautenschlager, 2003). In addition to transplant rejection, HCMV is a risk factor for other opportunistic infections, such as bacterial and fungal infections (George et al., 1997; Husain et al., 2003) and has also been associated with other viruses, such as HHV-6, HHV-7 and HCV (Dockrell et al., 1997; Humar et al., 2002b; Lautenschlager et al., 2002; Paya, 1999; Razonable and Paya, 2002).

2.2. Prevention and treatment of HCMV disease

Since primary infections are more likely to cause disease than reactivation/reinfection, the patients whose serostatus is defined as D+/R- are at the highest risk of developing disease. Therefore, the determination of HCMV serostatus of an organ donor and recipient pre-transplantation is important. Also, leukocyte free blood products are used for transplant patients. There is no effective vaccine against HCMV.

Before the development of suitable antiviral drugs against HCMV, a reduction in the immunosuppressive regimen was used to prevent HCMV disease in transplant patients. However, this often led to an increased incidence of graft rejection (Sia and Patel, 2000). HCMV-immunoglobulins were also used then. Currently, good availability of antiviral agents allows maintenance of the level of immunosuppression required to prevent graft rejection.

Two different strategies, prophylaxis and pre-emptive therapy, are used for the prevention of symptomatic HCMV infection after transplantation. In prophylaxis, antiviral drugs are administered before any evidence of the virus, and in pre-emptive therapy, antiviral drugs are administered when there is laboratory evidence of active but asymptomatic infection.

The antiviral strategies for treatment and prevention of HCMV disease are outlined in more detail below.

2.2.1. Treatment of the disease

The three antiviral drugs that are currently licensed for the treatment of HCMV infection are ganciclovir (GCV), foscarnet and cidofovir. Ganciclovir is the drug of choice in the treatment of symptomatic infections. Ganciclovir is a specific nucleoside analog which first is phosphorylated before it gains antiviral activity (Littler et al., 1992; Sullivan et al., 1992). The initial phosphorylation is mediated by virus encoded phosphotransferase (product of *UL97*). The monophosphate form is further phosphorylated by the host's cellular kinases into the active triphosphate form, which inhibits viral DNA polymerase by competing with deoxyguanosine triphosphate (Noble and Faulds, 1998). For the treatment of HCMV disease, ganciclovir is administered intravenously (i.v.) for at least two weeks.

Intravenous foscarnet may be used under conditions of failure of ganciclovir treatment, ganciclovir resistance, or excessive side effects, such as leukopenia (Crumpacker, 1996; Noble and Faulds, 1998). Foscarnet is an inorganic pyrophosphate analog and directly inhibits viral DNA polymerase (Chrisp and Clissold, 1991; Crumpacker, 1992). However, foscarnet is nephrotoxic, which limits its use in kidney transplantation. Intravenous cidofovir may also be used, but experience of this agent in solid organ recipients are still very limited. Cidofovir is a cytosine analog which does not require the virus encoded phosphotransferase activity. Instead host cellular enzymes carry out the phosphorylations needed for activation (Lalezari et al., 1995; Snoeck et al., 1988).

Valganciclovir, a pro-drug of ganciclovir, is a new promising compound which is administered orally and has an increased bioavailability compared to that of oral ganciclovir (Pescovitz et al., 2000). Nowadays valganciclovir is used in therapy of HCMV disease, however, more clinical trials are needed. The HCMV hyperimmunoglobulin may also be used in some patient groups in combination with antiviral drugs, e.g. lung transplant patients with HCMV pneumonia (Patel and Paya, 1999; Zamora, 2001).

2.2.2. Prophylaxis

Prophylaxis consists of administration of antiviral drugs, immediately after transplantation, either to all transplant recipients or only to those who are at the highest risk for the HCMV disease, such as seronegative recipients receiving seropositive organ or patients who are treated with anti-T-lymphocyte antibodies. The practice and guidelines for prophylactic therapy vary widely, depending on the patient population and the transplant center (Batiuk et al., 2002; Patel et al., 1996). Also hyperimmunoglobulin in combination with ganciclovir has been used in certain patient groups, e.g. lung transplant recipients (Weill et al., 2003).

The first antiviral drug used for prophylaxis was high-dose oral acyclovir, an analog of deoxyguanosine. The efficacy of oral acyclovir has been demonstrated in studies showing reductions in the incidence of HCMV disease compared with no treatment or placebo (Balfour et al., 1989; Couchoud et al., 1998; Mollison et al., 1993). A pro-drug of acyclovir, valacyclovir, has been found to be more effective than acyclovir for prevention of HCMV disease (Fiddian et al., 2002; Lowance et al., 1999). Valacyclovir has increased oral bioavailability compared to that of acyclovir (Weller et al., 1993), and in vivo it is first converted to acyclovir. The action of acyclovir (valacyclovir) is dependent on phosphorylation, in which thymidine kinase is important (Talarico et al., 1999). However, CMV does not encode this kinase (unlike herpes simplex and varicella zoster viruses), and the phosphorylation, which may be allowed by *UL97* product (Talarico et al., 1999) is less specific. For this reason, these drugs are not potent enough for the treatment of HCMV infection.

Currently, the most widely used and specific drug for HCMV prophylaxis is ganciclovir, administered orally or intravenously. Intravenous ganciclovir has been proven to be a potent prophylactic agent in many studies (Merigan et al., 1992; Paya, 2001; Winston et al., 1995). The availability of the oral form has increased the use of this agent (Speich et al., 1999; Winston and Busuttil, 2004; Yango et al., 2003), although the bioavailability of oral ganciclovir is lower when compared to that of intravenous ganciclovir. A pro-drug of ganciclovir, valganciclovir, is a new, promising option also for prophylaxis. The drug is effectively absorbed and it is nowadays widely used although the published reports are still limited (Lake, 2003; Paya et al., 2004).

Although the efficacy of prophylaxis has been proven in many studies, there are still some controversial issues. It has been suggested that prophylaxis may only delay the development of HCMV disease in some patients (Brennan et al., 1997; Emery, 2001). Long term and/or sub-optimal antiviral therapy may also induce resistant HCMV strains (Chou, 2001; Drew, 2000). Also the fact that prophylaxis exposes all patients to potentially toxic drugs may diminish the utility of this approach (Emery, 2001). In addition, although the prophylaxis strategy has been found as a cost-effective intervention against HCMV (Das, 2003; Legendre et al., 2000), the surveillance strategies may be less costly (Emery, 2001; Singh, 2001).

2.2.3. Pre-emptive therapy

The advantage of pre-emptive therapy is the targeting of antiviral drugs only to those patients who have laboratory evidence of active HCMV infection. This minimises the number of patients who are exposed to antiviral drugs. Currently, the drug of choice for pre-emptive therapy is ganciclovir. Recent studies have shown the efficacy of pre-emptive therapy by the low rate of HCMV disease in different patient groups (Daly et al., 2002; Sagedal et al., 2003; Torre-Cisneros et al., 2002). The effectiveness of pre-emptive therapy

has also been proven in a randomized, placebo-controlled trial in liver transplant patients, in which pre-emptive therapy was reported to decrease HCMV disease (Paya et al., 2002). In the future, the effectiveness of new oral antiviral agents comparable or superior to intravenous ganciclovir (e.g. valganciclovir) remains to be seen.

In pre-emptive therapy only patients who have laboratory evidence of an active HCMV infection are treated with antiviral drugs. Therefore, the diagnostic test should predict impending symptomatic infection sufficiently early. In addition, because pre-emptive therapy requires frequent surveillance of all patients, the test should also be specific as well as rapid and easy to perform. Since viral load has been shown to be a major factor in the pathogenesis of HCMV (Cope et al., 1997; Emery, 1999; Hassan-Walker et al., 1999), quantitative measurements have been considered to have greater clinical value in a pre-emptive therapy strategy. Various diagnostic methods, virological and/or DNA/RNA-based, may be used for this purpose (Daly et al., 2002; Gerna et al., 2003; Grossi et al., 1996; Sagedal et al., 2003; Sia and Patel, 2000).

2.2.4. Drug resistance

The occurrence of antiviral drug resistant HCMV strains have been documented also among organ transplant patients (Isada et al., 2002; Limaye, 2002; Limaye et al., 2002; Lurain et al., 2002). The major risk factors for drug resistance include the long duration of drug exposure and high amounts of viral load (Chou, 2001; Drew, 2000). These factors are most prevalent in patients with primary infection, i.e. in D+/R- population, in which resistance has most often been observed (Rosen et al., 1997; Singh, 2001). The mutations in both the viral phosphotransferase gene (*UL97*) and the viral polymerase gene (*UL54*), may confer antiviral drug resistance in HCMV. The mutations of the *UL97* coding sequence, which may confer resistance only to ganciclovir, occur mainly in the region including codons 460-607 (Chou et al., 1995; Hanson et al., 1995; Lurain et al., 1994). The more rare mutations in the *UL54* coding sequence may confer resistance to any or all of the three most commonly used drugs (ganciclovir, foscarnet or cidofovir) (Cihlar et al., 1998a; Cihlar et al., 1998b; Mousavi-Jazi et al., 2003). Mutations in *UL54* appear to occur in regions between codons 300 and 1000, and they are often accompanied by mutations in *UL97*, showing higher levels of resistance to ganciclovir with possible cross-resistance to foscarnet and/or cidofovir (Jabs et al., 2001).

Management of ganciclovir resistant HCMV infections is difficult. Increased dosages of ganciclovir in combination with HCMV hyperimmunoglobulin and reduction of immunosuppression, or combination therapy with ganciclovir and foscarnet have been used (Limaye, 2002; Mylonakis et al., 2002). New drugs are also under development, many of which have different targets than currently available DNA polymerase inhibitors (De Clercq, 2003; Emery and Hassan-Walker, 2002), but their suitability for clinical use will be seen in the near future.

3. Laboratory diagnostics of HCMV in transplant patients

An accurate and rapid diagnosis of HCMV in transplant patients is of utmost importance. Different laboratory methods based on either serology or identification of virus/virus components in clinical specimens are now available. Historically HCMV infection has been detected by cytomegalic inclusion bodies in tissue specimens. The sensitivity of the histological examination has been enhanced by using immunostaining or *in situ* hybridization (ISH). However, these techniques have limited usefulness with transplant patients and they are mainly used for diagnosing HCMV in an organ involvement. Serological testing is mainly limited to determination of serostatus of recipient and donor before transplantation. It lacks sensitivity as an indicator of active HCMV infection in the post-transplant period. The infectious HCMV virus can be isolated from various clinical samples by using conventional tube cell culture or more rapid shell vial culture methods. These methods are time-consuming and cumbersome, and their sensitivity is relatively low, which diminishes the usefulness of these tests in rapid diagnosis and in the monitoring of HCMV infection in transplant recipients. Since the late 1980's, the pp65 antigenemia test has been the most common method for the diagnosis of HCMV infection. Because of its quantitative nature, it has proven the clinical utility, not only in the diagnosis of HCMV infection, but also in guiding pre-emptive therapy, as well as in monitoring the response to antiviral treatment. However, in recent years, the tendency has been towards replacement of the antigenemia assay with PCR methods, especially with quantitative modifications of PCR, and also with other nucleic acid amplification techniques.

3.1. Histological methods

Traditionally the recognition of cytomegalic inclusion bodies in histological specimens has been used for the diagnosis. In organ specific HCMV infection, such as HCMV pneumonitis or hepatitis, characteristic viral inclusions may be seen. The large inclusions are intranuclear and have a characteristic owl-eye appearance in haematoxylin and eosin stained tissue specimens. The positive results correlate well with active HCMV infection of the organ, e. g. hepatitis, but the sensitivity of the histopathological finding is relatively low (Colina et al., 1995; Mattes et al., 2000; Paya et al., 1989). Immunostaining with specific polyclonal or monoclonal antibodies against HCMV antigens has increased the sensitivity of the method compared to conventional staining (Barkholt et al., 1994; Colina et al., 1995; Paya et al., 1990). However, false-negative results may occur because of the focal and scarce distribution of HCMV positive cells in tissue samples (Colina et al., 1995). ISH methods, described for the identification of viral DNA in infected cells, have also been employed to improve the histological diagnosis of infection (Barkholt et al., 1994; Colina et al., 1995; Einsele et al., 1989; Espy et al., 1991; Musiani et al., 1996; Paya et al., 1990). HCMV specific probe, usually labelled by biotin, is used in these assays.

Although the histological methods may have an important role in detecting HCMV organ involvement, they are not suitable for the early diagnosis of HCMV infection. In addition, these assays are very laborious, and biopsy specimens are only taken when an end-organ disease is suspected.

3.2. Serology

Humoral response to primary HCMV infection is manifested by the production of IgG and IgM antibodies. Thus a diagnosis of HCMV infection can be obtained indirectly through serology. A variety of laboratory tests with different degrees of sensitivity have been described for the measurement of HCMV antibodies in human sera. The methods include complement fixation, indirect hemagglutination, latex agglutination, radioimmunoassay, immunofluorescence and enzyme immunoassay (Mendez et al., 1999a; Sia and Patel, 2000). In enzyme-linked immunosorbent (EIA) assays many different antigens have successfully been used as targets for detecting specific antibody production (Monte et al., 1996). EIA-based methods can be easily automated, they are fast and convenient to perform. They usually have good sensitivity and they are also commercially available.

However, the rise in serum antibody levels is an insensitive sign of actual HCMV infection in transplant patients (Halling et al., 2001; Marsano et al., 1990; Schmidt et al., 1995; Tanabe et al., 1997; Tong et al., 1998). The seroprevalence is high and the presence of IgG antibodies is only informative of the patient's past history regarding HCMV infection. Furthermore, there is a time lag between primary infection and IgM antibody production (IgM level can remain undetectable because of delayed seroconversion owing to immunosupressive agents), and IgM antibodies can also persist for a long time after infection in some healthy individuals. The results of the serological tests can also be confused by blood transfusions and/or antibody based therapy. Furthermore, increase or decrease in antibody levels, in general, does not provide an actual diagnosis of HCMV infection in the immunosuppressed patient population, due to frequent reactivations of the virus. Therefore serology has a limited diagnostic value in the transplant patient group.

Nevertheless, HCMV IgG antibody testing has a significant role in the evaluation of organ and recipient prior to transplantation. Since the solid organ recipient who belongs to the D+/R- group is considered to have the highest risk for the development of symptomatic HCMV infection (Ljungman, 2002; Razonable et al., 2001b), it is important to identify the recipients and donors that have been previously infected.

3.3. Viral culture

Infectious virus can be isolated from various clinical materials, such as bodily secretions, tissue or peripheral blood, in cell cultures which are permissive for HCMV replication. Human fibroblast cell cultures, obtained from the foreskin or from the embryonic lung tissues, have been used for conventional and shell vial methods for the isolation of HCMV

(Mendez et al., 1999a). In conventional viral isolation methods, commonly used in earlier times, determination of viral replication is based on typical cytopathic effects (CPE) produced by HCMV. The time required for the development of CPE usually varies from 2 to 4 weeks, even up to six weeks.

Several less time-consuming methods have been developed for the isolation of infectious virus (Mendez et al., 1999a; Sia and Patel, 2000). One of the most commonly used rapid method is the shell vial culture (Gleaves et al., 1984). In this method, for the improvement of absorption of the virus, the specimen is centrifuged onto the cell culture. Fibroblasts monolayers cultured in vials containing coverslips are used. These shell vial culture methods utilize indirect immunofluorescence to detect the immediate-early (IE) viral antigen after incubation of culture for one to three days (Lautenschlager et al., 1989).

The isolation of HCMV from the blood or target organ specimen by cell culture methods has a high correlation with disease. However, the relatively low sensitivity of detection of the virus in blood samples compared to nucleic acid-based and antigenemia methods, limits the use of these assays in the diagnosis and monitoring of HCMV infection in transplant patients (Badley et al., 1996; Evans et al., 1999; van der Bij et al., 1988; Wirgart et al., 1996).

Isolation of the virus from peripheral sites (such as urine or saliva) may be used to demonstrate asymptomatic shedding of HCMV in the throat or urinary tract. On the other hand, viral culture may be helpful in detecting HCMV from bronchoalveolar lavage (BAL) or biopsy specimens in the organ specific infection. Nevertheless, detection of the virus from the blood of immunocompromised patient is a sign of viremia and is always considered as a serious finding. Furthermore, positive virus isolation from any site of a seronegative recipient is a clear indication of primary infection, which may rapidly progress to a severe disease. However, viral culture is a qualitative method with a limited role in the diagnosis of HCMV infection.

3.4. Antigen detection

The antigenemia assay, first described by professor The and his associates (van den Berg et al., 1989; van der Bij et al., 1988), has been a major advance in the diagnosis of HCMV infection in transplant patients. In this test, monoclonal antibodies to pp65 (*UL83*) (Depto and Stenberg, 1989; Gerna et al., 1992; Grefte et al., 1992), the lower matrix protein, are used for the direct immunostaining of blood polymorphonuclear leukocytes (PMNL). However, it has been shown that the pp65 antigen in PMNLs is not a direct indication of virus replication *in vivo*, since the virus and viral material detected in PMNLs are transferred from other infected cells, e.g. endothelial cells, mainly by microfusion events (Gerna et al., 2000). Nevertheless, the pp65 antigenemia assay has been successfully used in the diagnosis of HCMV in transplant patients since the late 1980's.

The pp65 antigenemia assay consists of numerous steps, including isolation of PMNL, fixation, immunostaining, and microscopic evaluation and quantitation (The et al., 1995; The et al., 1990; van den Berg et al., 1989). Dextran sedimentation or direct erythrocyte lysis can be used for isolation of leukocyte populations of blood. The isolated leukocytes are then cytocentrifuged onto glass slides and indirect immunoperoxidase or immunofluorescense staining is used for immunodetection of the HCMV antigen. After microscopic evaluation, quantitative results are expressed as the number of HCMV-positive leukocytes per number of cells evaluated, usually per 50 000 or 200 000 leukocytes. The whole process can be performed within one day.

The antigenemia assay is more sensitive than cell culture methods in detecting HCMV in the blood (Landry and Ferguson, 1993; van den Berg et al., 1991; van der Bij et al., 1988). It is also highly specific for HCMV but a positive result does not always predict the onset of symptomatic infection (Landry and Ferguson, 1993; Sia and Patel, 2000; Tanabe et al., 1997). Nevertheless, when using the test in a quantitative manner, the assay can be used to predict and diagnose HCMV infection: at least, a higher degree of antigenemia is correlated with symptomatic disease, as has been shown by many groups (Hadaya et al., 2003; Humar et al., 1999; Kim et al., 2003; van den Berg et al., 1991; van den Berg et al., 1989).

One disadvantage of the antigenemia assay is that the blood samples should be processed within a certain time, preferably within six hours, for optimal results (Boeckh et al., 1994; Schafer et al., 1997). In addition, though there have been attempts to simplify the method (Gratacap-Cavallier et al., 2003; Ho et al., 1998), it is still quite time-consuming and laborious, at least with large specimen numbers. The automation of the test is also difficult because of the number of steps and the subjective evaluation of the infected leukocytes. Although there have been attempts to standardize the assay (Gerna et al., 1998; The et al., 1995; Verschuuren et al., 1999), there are a number of various in-house and commercial modifications available. This makes comparison of the results between different centers difficult. The clinically significant threshold of the number of positive leukocytes seems to vary also among different types of transplant populations (Baldanti et al., 1998; Razonable et al., 2002b; Sia and Patel, 2000). In any case, the pp65 antigenemia test has shown its clinical utility in the diagnosis of HCMV infection, in guiding of pre-emptive therapy, and also in the monitoring of response to antiviral treatment (Baldanti et al., 1998; Grossi et al., 1996; Kim et al., 2003; Kusne et al., 1999; van den Berg et al., 1993).

3.5. Nucleic acid amplification methods

The widespread introduction of the application of nucleic acid amplification technologies has been the most important improvement in HCMV diagnostics in the past decade. The most frequently used methods are based on PCR detecting HCMV DNA (or RNA) in whole blood or in different blood compartments. Both qualitative and quantitative assays have been used. However, understanding of the correlation between viral loads and clinical symptoms has diminished the significance of qualitative methods. Different

kind of quantitative PCR assays, both in-house and commercial ones, have been employed. The in-house assays, based on competitive PCR, are usually technically complicated. Commercial and real-time based applications, which are more convenient and faster, are more widely used at present. Also other nucleic acid-based techniques, such as nucleic acid sequence based amplification (NASBA) and signal amplification (Hybrid Capture System), have successfully been used in the detection of viral mRNA or DNA, respectively.

3.5.1. Principles of PCR

The invention of PCR has been one of the major advances in the area of molecular-based methods (Mullis and Faloona, 1987; Saiki et al., 1988; Saiki et al., 1985). PCR is a technique which allows a specific target DNA fragment to be exponentially amplified *in vitro* in a very easy way. In theory, a millionfold increase in yield of the desired target sequence can be achieved in a few hours, or even faster nowadays. PCR utilises a pair of oligonucleotides (primers), each hybridising to one strand of a double stranded DNA (dsDNA) target. The primers flank the region that will be amplified. The hybridised primer acts as a substrate for a thermostable DNA polymerase (most commonly derived from *Thermus aquaticus* and called *Taq* polymerase) that synthesises a complementary strand via the sequential addition of deoxyribonucleotides. The process includes repetitive cycles of three steps, denaturation of dsDNA, annealing of the primers and extension of the DNA fragments, which are accomplished by cyclic temperature changes in the reaction (Figure 2). The number of repetitive cycles varies usually from 25 to 50 in PCR tests used for diagnostic purposes. In addition to suitable primers, template DNA, polymerase enzyme, and deoxyribonucleotide mixture, optimal conditions for the enzyme are needed for successful PCR. If the starting material is RNA, a further step with a reverse transcriptase (RT) enzyme is needed before amplification. This technique is then referred to as RT-PCR. At present, various programmable thermal cyclers, which vary in many technical aspects such as capacity and rapidity, are available for the performance of a PCR run.

Conventional detection of amplified DNA products relies on electrophoresis combined with ethidium bromide staining. The sensitivity and specificity of the detection can be improved using hybridisation methods either after, or instead of, electrophoresis. Traditionally, the detection has been performed after the completion of the PCR run, but the recent development of real-time PCR instruments allows monitoring of the accumulation of PCR products during the run.

3.5.2. Basics of quantitative PCR

PCR can also be used for the quantitation of nucleic acids. Until the introduction of real-time PCR, competitive PCR (Gilliland et al., 1990; Siebert and Larrick, 1992) had been the most common method to measure the amount of DNA or RNA in a clinical sample. In this technique, a reference (competitor), which is usually a synthetic nucleic acid

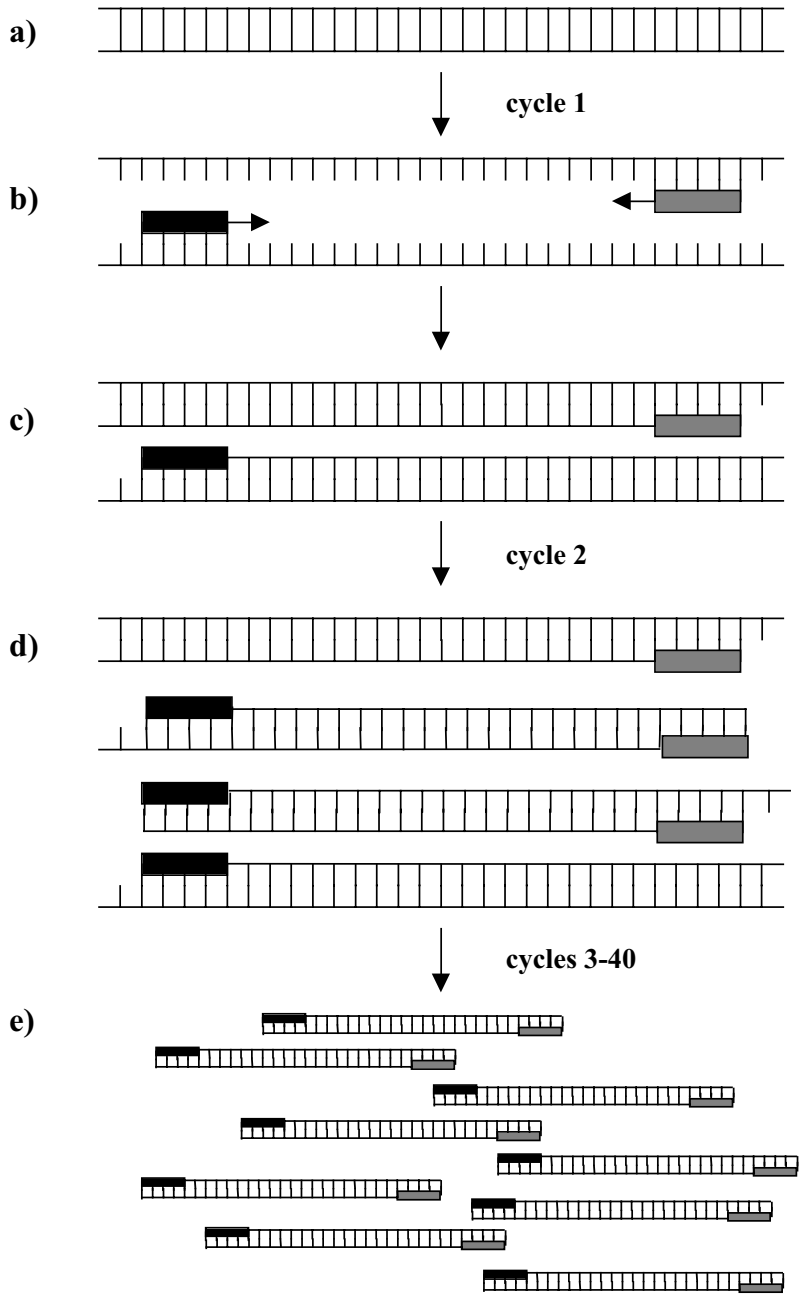

Figure 2. The principle of PCR

a) The double stranded template DNA is first denaturated at high temperature (~ 94-96°C). b) The lower temperature of reaction allows the primers to hybridise to the complementary sequences of the target DNA (~ 37-72°C). c) Extension of the primers by *Taq* polymerase enzyme occurs at 72°C. d)-e) One cycle consists of these three reactions in different temperatures. Cycles are repeated and the amount of amplified sequence is increased exponentially.

fragment with the same primer annealing sites as the specific target, is co-amplified with the target of interest. After completion of PCR, amplification products of the specific target and of the competitor are separated on the basis of minor modifications in their sequences, and the ratio of their quantities is calculated. Successful quantitation is accomplished only if the specific conditions are met in the reactions, i.e. the target and competitor sequences have to be amplified with equal efficiencies, which ensures that the initial ratio of target to competitor remains constant through the amplification. The initial amount of target DNA in a sample can be determined when the initial amount of the competitor is known.

One drawback of competitive PCR is the narrow linear range of quantitation, 2-3 logs at best, that can be achieved. In addition to this, in-house competitive quantitative methods are usually complicated and time-consuming, which has diminished their attractiveness in clinical laboratories. Availability of commercial competitive tests has improved the situation for quantitative PCR.

In real-time PCR (also called kinetic PCR), the accumulation of the PCR products is monitored continuously during the PCR run, compared with the end-point measurements that quantitate the final PCR product. At present, several instruments are available for real-time PCR, in which the accumulation of the product is monitored by measuring the fluorescence in each cycle. The measured fluorescence is plotted against the cycle number (Heid et al., 1996). The cycle number, in which the exponential amplification (threshold cycle C_T) is first detected over background, has an inverse linear relationship to the amount of target in the initial reaction. Absolute quantitation of the amount of target in the initial sample can be accomplished by measuring its C_T value and using the external standard curve to determine the starting copy number of the target genome. This system allows a very wide dynamic range of quantitation, at least 5-6 logs.

Currently, various chemistries for the detection of PCR products during real-time PCR are available (Mackay et al., 2002). These can be classified into amplicon sequence specific or non-specific detection methods. The most commonly used detection methods in the virus diagnostic assays are based on the use of specific fluorogenic oligoprobes. These methods rely upon fluorescence resonance energy transfer (FRET), which is the interaction of two fluorescent dyes. TaqMan probes (Holland et al., 1991), also called 5′ nuclease or hydrolysis oligoprobes, were the first ones used in special real-time instruments (Heid et al., 1996). These probes are linear oligonucleotides that have a reporter fluorescent dye at the 5′ end and a quencher dye at the 3′ end. When the probe is intact, two labels are close to each other and the quencher dye greatly reduces the light emission of the reporter dye. During the extension phase of PCR, if the target is present, the probe anneals downstream from one of the primer sites (Figure 3a) and is cleaved by the 5′ nuclease activity of *Taq* DNA polymerase as this primer is extended (Figure 3b). Cleavage removes the probe from the target, and at the same time also separates the reporter dye from the quencher dye, allowing the light emission of the reporter dye to occur (Figure 3c). This light emission can be detected by a real-time instrument, for which the ABI Prism 7000, 7700 or 7900HT sequence detection systems (Applied Biosystems) are the most used ones at present. The other commonly used probes are hybridization probes,

which have become the method of choice for the LightCycler real-time instrument (Roche Molecular Biochemicals). In this technique, two adjacent probes are used, one labelled with a 3′ fluorescein and the other with 5′ dye (Wittwer et al., 1997). When both oligoprobes are hybridized during the PCR process near to each other, FRET can occur between the labels and the acceptor dye emits light.

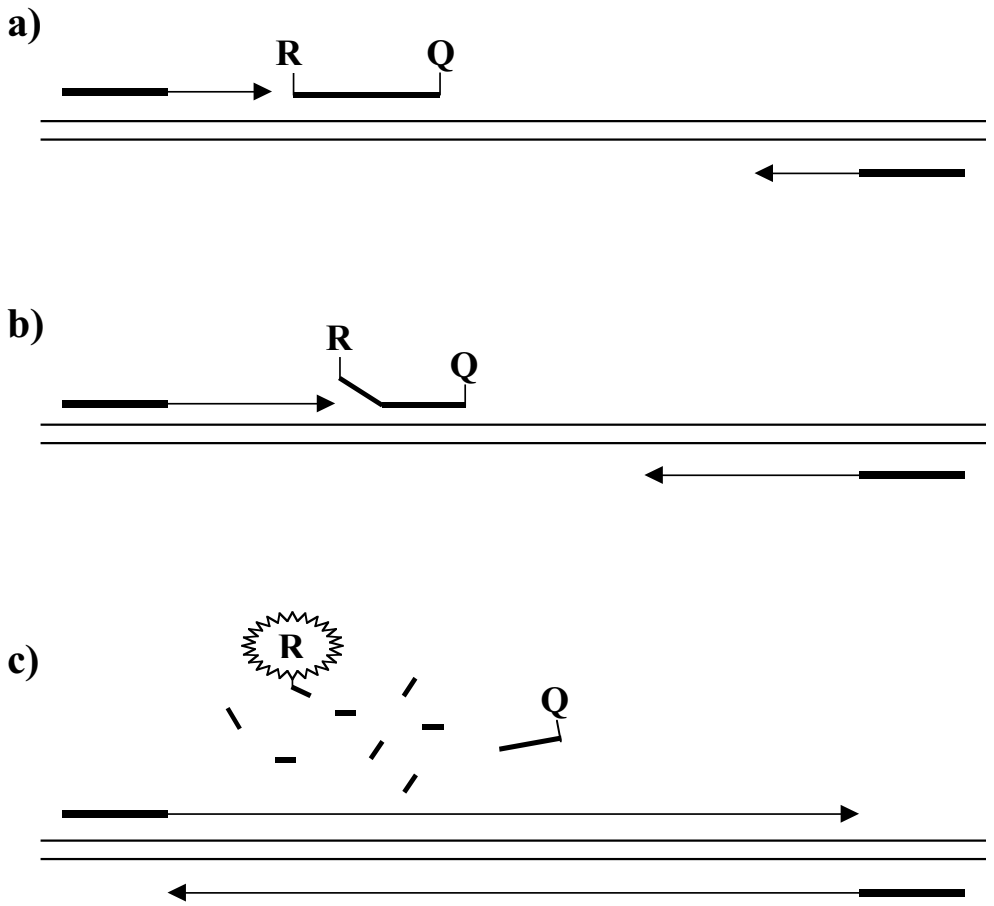

Figure 3. Stepwise representation of nuclease activity of *Taq* polymerase with the 5′ nuclease oligoprobe during extension phase of PCR (R = reporter, Q = quencher)

a) During of the extension phase the probe anneals downstream from one of the primer sites. b) The probe is cleaved by the 5' nuclease activity of *Taq* polymerase as this primer is extended. c) Cleavage removes the probe from the target, and at the same time also separates the reporter dye from quencher dye allowing the light emission of reporter dye to occur.

3.5.4. Qualitative PCR in the laboratory diagnostics of HCMV

Qualitative PCR has been proven to be more sensitive than antigenemia test or cell culture assay on the detection of HCMV infection (Cunningham et al., 1995; Evans et al., 1998; Hiyoshi et al., 1997; Rowley et al., 1991; Weinberg et al., 2000). Despite extreme sensitivity and specificity in detecting HCMV DNA, qualitative assays usually have a low positive predictive value for symptomatic infection, at least when peripheral blood leukocytes have been used as a specimen (Delgado et al., 1992; Gerna et al., 1991; Tanabe et al., 1997). This is due to the capacity of these tests to detect HCMV DNA even in the case of latent infection. It has also been reported that the demonstration of HCMV DNA in plasma may correlate more closely with disease, and also with the antigenemia test, than that in leukocytes or whole blood (Patel et al., 1994; Wolf and Spector, 1993; Wolff et al., 1996). In any case, a negative PCR result strongly indicates against HCMV infection, and a positive result in a seronegative person speaks for primary infection.

Several in-house protocols for the qualitative PCR detection of HCMV have been described with wide variability in the performance (e.g. differences in nucleic acid extraction methods, target sequences, primers, instruments, amplification protocols, reagent concentrations, detection methods) (Cunningham et al., 1995; Gerna et al., 1991; Rowley et al., 1991; Wolf and Spector, 1993). This has made the results non-comparable between different laboratories (Grundy et al., 1996). In addition, at least when using conventional instruments for amplification and gel electrophoresis and hybridisation for detecting the amplification products, these techniques are laborious and time-consuming. The use of commercial assays (Hiyoshi et al., 1997) has simplified qualitative PCR technically, but it has not removed the problem in its clinical use, i.e. the possible detection of latent infection in seropositive recipients.

3.5.5. Quantitative PCR in the laboratory diagnostics of HCMV

The first developed in-house quantitative PCR tests were laborious methods, based on the co-amplification of a DNA standard that competed with a target sequence using the same primer sites (Barber et al., 1999; Boom et al., 1999; Cope et al., 1997; Mendez et al., 1998b; Toyoda et al., 1997; Zipeto et al., 1993). Whilst these assays are still in clinical use (Jordan et al., 2002; Preiser et al., 2003; Weinberg et al., 2000), the availability of more automated quantitative PCR systems has changed HCMV diagnostics.

The most widely used semi-automated commercial method, Cobas Amplicor CMV Monitor test (Roche Diagnostics), has simplified the laboratory practise significantly compared with in-house quantitative tests, and also compared with pp65 antigenemia assay (Boivin et al., 2000; Caliendo et al., 2000; Humar et al., 1999; Rollag et al., 2002; Sia et al., 2000a; Tong et al., 2000). The development of real-time methodology and the availability of automated real-time instruments, have further simplified quantitative PCR assays and reduced the turnaround time needed for the test performance. In recent years,

many applications usually based on TaqMan or LightCycler technologies, have been described for the measurement of HCMV DNA in the blood samples of transplant patients (Gault et al., 2001; Kearns et al., 2001; Pang et al., 2003; Sanchez and Storch, 2002; Schaade et al., 2000).

Many technical factors may induce variability between different quantitave PCR assays, including nucleic acid extraction method, amplification instrument, quantitation standard, or detection method. Furthermore, it has been suggested that the sensitivity of the quantitative assay may be dependent on the target sequence and the selected oligonucleotides (Mendez et al., 1998b). The polymerase gene (*UL54*) is the target region in commercially available Cobas Amplicor CMV Monitor assay (Roche Diagnostics). In addition to this, many different target sequences and oligonucleotides have successfully been used in in-house quantitative assays, including gB (*UL55*), IE (*UL122/123*), pp65 (*UL83*), *US17* gene and HindIII-X fragment (Gault et al., 2001; Guiver et al., 2001; Ikewaki et al., 2003; Li et al., 2003; Mendez et al., 1998b; Mengelle et al., 2003a; Pang et al., 2003; Stocher and Berg, 2002). So far, the most appropriate region for amplification of HCMV DNA has not been established. In addition, one of most important issues which may cause variability in the results of different tests is the choice of specimen type.

The measurement of viral load has successfully been used also in the diagnostics of HCMV in the transplant patients. Both commercially available and in-house quantitative HCMV DNA assays are currently used in clinical laboratories. Quantitative PCR tests can be used for the distinction of symptomatic and asymptomatic HCMV infection (Caliendo et al., 2002; Caliendo et al., 2000; Razonable et al., 2002b). In recent years, quantitative PCR tests have also been used in the guiding of pre-emptive therapy (Daly et al., 2002; Paya et al., 2002), though the optimal threshold of viral load which predicts the likelihood of HCMV disease, has not yet been defined. In any case, it is generally accepted that higher HCMV DNA load predicts clinical progression to HCMV disease (Razonable et al., 2002b). Recently, quantitative PCR has also proven its utility in the monitoring of response to antiviral treatment (Hadaya et al., 2003; Jordan et al., 2002; Pang et al., 2003). The persistence of HCMV DNA at the end of therapy has been demonstrated to be a risk factor for a recurrence (Jordan et al., 2002; Razonable et al., 2002a; Sia et al., 2000b; Weinberg et al., 2000). In addition to testing viral load, applications of viral load kinetics (i.e., rate of change of viral load) have been found to be a useful early predictor of patients at risk for developing HCMV disease, and also to predict recurrence (Emery et al., 2000b; Humar et al., 2002a). Viral load kinetics have also been reported to serve as an early indicator of viral resistance (Emery and Griffiths, 2000).

3.5.6. Other nucleic acid amplification methods

It has been suggested that the detection of HCMV mRNA would correlate better with symptomatic HCMV infection than DNA, because of the ability of the virus to remain latent in the host cell. Previously, in-house RT-PCR methods were used for the detection of mainly immediate early or late transcripts in blood samples of transplant patients

(Bitsch et al., 1993; Gaeta et al., 1997; Nelson et al., 1996; Randhawa et al., 1994). The sensitivities, specificities and correlations with HCMV disease of these tests varied considerably. Later, many applications based on NASBA technique have been employed. NASBA is an isothermal amplification process which involves the coordinated activities of three different enzymes: ribonuclease H, reverse transcriptase and DNA-dependent RNA polymerase (Compton, 1991). The final product of the process is a specific single-stranded RNA, instead of the dsDNA of PCR. The high sensitivity in detecting HCMV infection has been shown by the NASBA assay with the IE-1 target (Blok et al., 1999; Blok et al., 2000; Goossens et al., 1999; Oldenburg et al., 2000). In recent years, many applications of the NASBA technique based on the detection of pp67 mRNA have been developed and the test application is also commercially available (Blok et al., 2000; Gerna et al., 2003; Gerna et al., 1999; Merlino et al., 2002; Witt et al., 2000). These assays detect pp67 mRNA, which is transcribed late in the viral replicative cycle, in a qualitative manner. pp67 NASBA assay may have a clinical utility in both early diagnosis of symptomatic HCMV infection and monitoring transplant patients with active disease, as well as in guiding pre-emptive therapy (Caliendo et al., 2002; Gerna et al., 2003; Witt et al., 2000). Recently, the pp67 NASBA has also been combined with the quantitative detection of IE-1 mRNA, which may improve the clinical utility of this technique (Greijer et al., 2002).

HCMV DNA has also been detected by a signal amplification method, Hybrid Capture System, from blood samples of transplant patients (Aitken et al., 1999; Bhorade et al., 2001; Ho et al., 2000; Mazzulli et al., 1999; Norris et al., 2002). The Hybrid Capture System is a solution hybridization assay that involves amplified detection instead of amplification of the desired nucleic acid fragment. The assay uses a specific RNA probe which targets 17 % of the HCMV genome. The RNA-DNA hybrid is captured by a monoclonal antibody and is detected by a luminometer. Both qualitative and quantitative applications are commercially available. The quantitative form has clinical utility in the diagnosis and monitoring of HCMV infection (Aitken et al., 1999; Bhorade et al., 2001; Ho et al., 2000; Norris et al., 2002).

3.6. Antiviral susceptibility testing

After the more widespread use of antiviral drugs, resistant HCMV strains havebeen reported also in the transplant patient population (Isada et al., 2002; Limaye, 2002; Limaye et al., 2002; Lurain et al., 2002). The methods used for antiviral susceptibility testing can be divided into phenotypic and genotypic assays (Erice, 1999). Phenotypic assays rely on the suppression of viral growth in the presence of antiviral agents. Various modifications have been employed (Erice, 1999; Landry et al., 2000; McSharry et al., 1998), of which the plaque reduction assay is considered as a standard method. A major limiting factor of the phenotypic methods, is that they require viral culture from the clinical sample, followed by several passages to reach the necessary viral titres for the performance of the assay, which can take up 4-6 weeks. Because the assay is burdensome and it lacks standardization, it is not routinely undertaken in the majority of centers.

The fact, that specific mutations in the *UL97* and *UL54* genes are associated with antiviral drug resistance, have led to the development of genetic methods. These are based on PCR amplification of the specific region of the genome followed by restriction enzyme analysis or direct sequencing of the amplification product/products (Alain et al., 1997; Lurain et al., 2002; Mendez et al., 1999b; Wolf et al., 1995). An obvious advantage of these assays is the possibility to use clinical specimens directly, rather than virus isolates, which significantly shortens the time required for performance of the test. In addition, mixtures of the different strains are not always detected in viral culture methods (Lurain et al., 2002). However, standardization and validation of these methods are needed for clinical use.

Aims of the study

The aims of the present study were:

To evaluate the value of quantitative PCR in the diagnosis of HCMV infection in organ transplant patients (I)

To evaluate the clinical utility of quantitative PCR in the monitoring of active HCMV infection and in the monitoring of the response to antiviral treatment in individual liver and kidney transplant patients (II, III)

To develop a faster and more high-throughput quantitative PCR method for the determination of HCMV DNA viral loads in the peripheral blood of organ transplant patients and to evaluate its value in the diagnosis and monitoring of HCMV infection in transplant patients (IV).

Materials and methods

1. Clinical specimens

The clinical material consisted of consecutive EDTA-blood samples from adult liver and kidney transplant patients sent to the Department of Virology, Helsinki University Central Hospital. The number of specimens analysed were 253, 243, 342 and 270 in the studies I, II, III and IV, respectively. The more detailed descriptions of each patient population are found in the Results and Discussion.

2. Shell vial culture assay (III)

A modification of the rapid shell vial culture (Gleaves et al., 1984) was used for the isolation of HCMV in blood specimens, as described previously (Lautenschlager et al., 1989). Indirect immunofluorescence against the HCMV immediate early antigen (BioSoft, Varilhes, France) was used for the detection of HCMV positive cells in the fibroblast cultures.

3. pp65 antigenemia assay (I, II, III, IV)

The standard HCMV pp65 antigenemia test was used for the detection of HCMV viral proteins in the leukocytes, as described previously (Koskinen et al., 1993b; The et al., 1990; van den Berg et al., 1989). In short, the leukocytes were isolated from blood samples and cytocentrifuged onto microscope slides, which were dried and fixed in cold acetone. Immunoperoxidase staining and a monoclonal antibody against HCMV pp65 antigen (Biotest, Frankfurt, Germany) were used for the demonstration of viral proteins in cells. The positive results were quantified by counting the number of pp65-positive cells per 50 000 leukocytes on the slide.

4. Quantitative HCMV PCR test (I, II, III, IV)

As a commercial quantitative PCR assay, the Cobas Amplicor CMV Monitor test (Roche Diagnostics) was used for the quantitation of HCMV DNA in plasma samples. Plasma was isolated by centrifugation from the EDTA-blood specimens within 24 h after collection. The test was run according to the manufacturer's instructions. Briefly, a known number of the plasmid quantitation standard, with the same primer binding sites as the HCMV target, was added to each sample before DNA extraction. DNA isolation from 200 µl of plasma was performed by lysis of virus particles followed by alcohol precipitation. PCR amplification was performed using a biotinylated primer pair, selected from the amino terminus of the DNA polymerase gene, spanning the 365 bp fragment. The PCR products of HCMV and quantitation standard were denatured and serially diluted to achieve a larger dynamic range for quantitation. Specific probes, on the basis of the

unique probe binding sites for both HCMV and quantitation standard, were used in hybridization of HCMV and quantitation standard PCR products. Target-probe complexes were detected spectrophotometrically using avidin-horseradish peroxidase conjugate and its substrates, and the levels of HCMV DNA in the test samples were calculated by comparing the HCMV signal to the quantitation standard signal. The linear range of the assay was 400-100 000 copies/ml (cps/ml) according to the manufacturer's recommendations.

5. Real-time HCMV PCR test (IV)

In the present study, the quantitative real-time HCMV PCR test based on TaqMan technology was developed. The assay was used for the quantitation of HCMV DNA in plasma samples.

5.1. DNA extraction

DNA was extracted from the plasma samples by the automated MagNA Pure LC instrument (Roche Diagnostics), which uses magnetic bead technology, and the MagNA Pure LC Total Nucleic Acid Kit (Roche Diagnostics). DNA extraction was performed from 200 µl of plasma according to the manufacturer's instructions. Briefly, the samples were dissolved and stabilized by proteinase K incubation and by lysis buffer containing denaturising agents. The released nucleic acids were bound to the silica-coated magnetic particles. After several washing steps, the purified nucleic acids were eluted in 50 µl of low salt elution buffer. Ten µl of the eluted nucleic acid solution was further used for each real-time reaction.

5.2. Standards

Plasmid pCMV1 was prepared to achieve a quantitative standard curve for a real-time PCR. The 421 bp fragment, which consisted of the region amplified in real-time assay, from the pp65 gene (GenBank accession number M15120), was amplified using primers p1 and p2 (Table 1). The product was subsequently cloned into the pGEM-T vector (Promega), and after the purification the plasmid was linearized. The concentration of the plasmid was measured by spectrophotometry and the amount of DNA was calculated corresponding to copies of DNA. The plasmid was diluted to obtain the 10-fold series from 10 to 10^6 copies per reaction to create a standard curve.

5.3. Amplification

The primers 1 and 2, and the probe (Table 1), were selected from the pp65 gene (GenBank accession number M15120) using the PrimerExpress software program (Applied Biosystems). The length of the amplified product was 57 bp. The amplifications were carried out in the ABI PRISM 7900HT sequence detector instrument (Applied Biosystems) in a final volume of 50 µl. The PCR mixture contained 300 nM of primer 1, 900 nM of primer 2, 250 nM of probe and 1 x TaqMan universal mix (Applied Biosystems). In the reaction, dUTP was used instead of dTTP. An aliquot of 10 µl template was added to each reaction and all samples were run in duplicates. The first step of the amplification program was 2 min at 50°C, which allowed uracil-N-glycosylase (included in the TaqMan Universal mix) to destroy possible contaminating pre-amplified PCR products containing dUTP. In incubation of 10 min at 95°C, AmpliTaq Gold enzyme was allowed to activate and nucleic acids were allowed to denature. Forty-five cycles of denaturation at 95°C for 15 s and of annealing-extension at 60°C for 1 min were carried out for the amplification of HCMV DNA.

6. Statistical analysis

Quantitative results of pp65 antigenemia and quantitative PCR assays were expressed as means ± SD (I) or as medians (II, III, IV). Student's *t*-test (I) or Mann-Whitney *U*-test (II, III, IV), and Pearson correlation and linear regression (IV) were used for the statistical analysis of the quantitative results, and *P*-values <0.05 were considered statistically significant.

Table 1. Primers and probe used in real-time HCMV PCR.

Primer or probe	**Sequence (5′-3′)**[a]
p1	CGA CGA CGA CGT CTG GAC CAG
p2	CTG CCA TAC GCC TTC CAA TTC G
1	TCG CGC CCG AAG AGG
2	CGC CCG GAT TGT GGA TT
probe	*FAM*-CAC CGA CGA CGA TTC CGA CAA CG-*TAMRA*

[a]Modifications: *FAM*, 6-carboxy-fluorescein; *TAMRA*, 6-carboxy-tetramethyl-rhodamine

Results and discussion

1. Comparison of quantitative PCR assay, Cobas Amplicor CMV Monitor test, and pp65 antigenemia assay (I)

In our preliminary work (I), the suitability of the commercially available quantitative PCR test, Cobas Amplicor CMV Monitor test (Roche Diagnostics), was evaluated for the detection of HCMV DNA in plasma samples of transplant patients by comparing the results of PCR with those determined by pp65 antigenemia assay. Totally 253 consecutive blood samples both from adult liver and kidney patients were investigated by both methods in parallel from the concurrently obtained samples.

1.1. Sensitivities of the assays

The results of individual samples by the quantitative tests were treated in a qualitative manner for the assessment of sensitivity and specificity of the Cobas Monitor assay. A sample was considered positive if HCMV DNA was detected at any measured level by Cobas test, and if ≥1 positive cell(s) were demonstrated by pp65 antigenemia test.

HCMV positivity by either one or both of the quantitative methods used were detected in 35 % (89/253) of the samples investigated (Table 2.). 164 samples were negative by both tests. The PCR assay found 88 % (78/89) (range 274-165000 cps/ml) and the pp65 test 89 % (79/89) (range 1-1500 positive cells) of the positive findings, respectively. Eleven out of 89 PCR negative/pp65 positive samples were found. All of these samples had low-level antigenemia, 1-5 positive cells in pp65 test. On the other hand, 10 PCR positive/pp65 negative samples were detected. Six of these samples were obtained during GCV treatment and four of these before antigenemia appeared. The greatest amount of PCR positive/pp65 negative samples had a low-level DNAemia. Very similar findings have been reported also by other groups evaluating the Cobas CMV Monitor assay (Caliendo et al., 2000; Flexman et al., 2001; Humar et al., 1999; Razonable et al., 2001a). However, the commercial quantitative plasma Cobas Monitor PCR test detected all the HCMV infections which required specific antiviral treatment corresponding to the previously reported cut-off level of 10 positive cells/50 000 leukocytes in pp65 antigenemia test (The et al., 1990; van den Berg et al., 1989).

Table 2. Comparison of the qualitative results of the Cobas Monitor and pp65-antigenemia assays.

	pp65 positive	pp65 negative	Total
Cobas positive	68	10	78
Cobas negative	11	164	175
Total	79	174	253

1.2. Correlation of quantitative PCR assay and pp65 antigenemia test

The good correlation between the quantitative results of individual blood samples measured by pp65 antigenemia assay and by Cobas Monitor assay was shown (I: Fig. 1). The samples were divided into four different groups on the basis of the results of the pp65 antigenemia test (<10, 10-<100, 100 -<1000 and 1000-<10000 positive cells /50 000 leukocytes). The HCMV DNA levels obtained by the Cobas Monitor assay in the different groups were 3137±5158 cps/ml, 20 372±43 496 cps/ml, 37 920±32 300 cps/ml and 100450±28 179 cps/ml, respectively. The differences were statistically significant between the groups <10 and 10-<100, as well as 100-<1000 and 1000-<10 000.

We also studied the viral load values prior to and at the time of GCV administration by the Cobas Monitor test. The DNA levels before the treatment varied between 392 and 3440 cps/ml, except in one patient with 16 500 cps/ml. On the other hand, those patients who received antiviral treatment due to symptomatic infection, demonstrated higher plasma DNA levels (1420-103 000 cps/ml) when treatment was started. Previously it had been reported that also higher levels of antigenemia correlate with symptomatic HCMV infection (Boeckh and Boivin, 1998; van den Berg et al., 1989). Our PCR findings were quite similar to those of a previously published study, in which the Cobas Monitor assay and pp65 antigenemia test were used (Humar et al., 1999). The mean peak viral load at the time of HCMV diagnosis was significantly higher than that of the patients with asymptomatic infection as measured by the Cobas assay (73 715 cps/ml vs 3615 cps/ml) (Humar et al., 1999). Elevated antigenemia levels in patients with symptomatic infection were also observed in that study. Higher HCMV DNA levels in the leukocyte fraction from patients with symptomatic infection than of those with asymptomatic infection, assessed by different quantitative nucleic acid-based methods, have also been shown recently (Aitken et al., 1999; Mendez et al., 1998a; Roberts et al., 1998).

The follow-up curves of two liver transplant patients were shown (I: Fig. 2). The strong temporal relationship between antigenemia and DNAemia was observed in both patients. The high antigenemia level (100 positive cells) correlated with the clinical symptoms as well as with the results of quantitative PCR (25 100 cps/ml) in one patient, who needed antiviral treatment (GCV) due to symptomatic HCMV infection (I: Fig. 2a). The patient (I: Fig. 2b) with no clinical symptoms and without antiviral treatment, showed only moderate level DNAemia (4330 cps/ml) at the time of low antigenemia peak (5 positive cells). In both cases, the DNAemia, and also antigenemia, disappeared during the follow-up period.

Our results are in agreement with those reported by other studies supporting clinical use of the Cobas CMV Monitor assay (Caliendo et al., 2000; Humar et al., 1999). In these studies, a good correlation between the Cobas Monitor test and the pp65 antigenemia assay has been shown. On the basis of that, and due to our own encouraging results, it

was supposed that the quantitative Cobas Monitor assay may be used in the detection of HCMV infection, and also in the monitoring of HCMV infection and of the response to the antiviral treatment in individual patients, in a similar way as with the pp65 antigenemia assay.

1.3. Cobas Amplicor CMV Monitor test

Despite the fact that the Cobas Monitor PCR test measures plasma DNA while the antigenemia assay measures pp65 antigen positivity in leukocytes, both quantitative methods were shown to be equally useful in the diagnosis of clinically significant HCMV. However, the quantitative Cobas Monitor assay had several advantages over the pp65 antigenemia test. It was more automated, including the manual nucleic acid extraction step followed by the automated amplification and detection steps. The hands-on time needed for performance of the test decreased significantly when using the semi-automated Cobas Monitor system. In addition, the Cobas Monitor assay used only 200 µl plasma instead of the 3-5 ml of whole blood required for the antigenemia test. The processing and handling of plasma samples were easier compared with the leukocyte preparation required for the pp65 test. The use of quantitative semi-automated PCR assay allowed larger amounts of samples to be investigated within a shorter time, and also by a more convenient manner.

Although there have been attempts to standardize the pp65 antigenemia method (Gerna et al., 1998; The et al., 1995), various modifications of the test are in clinical use. Major advantages of the introduction of the commercial Cobas CMV Monitor test was that it offered a standardized method and introduced comparability between centers. Although in-house quantitative PCR assays for the quantitation of HCMV DNA in blood samples have also been developed and used in clinical studies (Barber et al., 1999; Boom et al., 1999; Cope et al., 1997; Mendez et al., 1998a; Toyoda et al., 1997), these tests have not been well standardized either. These tests may differ in many factors, such as target sequences, oligonucleotides, nucleic acid extraction methods and instruments used for amplification, which may cause differences in the performance of the test, detection limits, sensitivity and linear range. As a result, high variability between viral load values of different in-house quantitative assays and Cobas Monitor test have been reported (Caliendo et al., 2001; Razonable et al., 2001a). This has made comparison of quantitative results between centers difficult.

2. Diagnostic validation and use of the Cobas Amplicor CMV Monitor assay (II, III)

Although quantitative PCR assays were already widely used in HCMV diagnostics, the clinical correlation of the findings was not as clear as that with the pp65 antigenemia test. The antigenemia test has been the most commonly used method for 15 years in the detection of HCMV infection, and also for the assessment of viral load and the monitoring of response to antiviral treatment (Boeckh and Boivin, 1998; Gerna et al., 1998; Sia

and Patel, 2000; The et al., 1990; van den Berg et al., 1989). In the present study, the clinical utility of the Cobas Amplicor CMV Monitor assay was further evaluated in parallel with pp65 antigenemia test in liver (II) and kidney (III) transplant patient groups, not only in the diagnosis of HCMV infection but also in the monitoring of individual patients during HCMV infection and antiviral treatment.

2.1. Comparison of the quantitative assays and shell vial culture assay (III)

The comparison of the qualitative results of 342 consecutive blood samples of kidney transplant patients (III) between the Cobas Monitor and the pp65 antigenemia assays, were performed in a similar way as in our previous work (I).

The sensitivities of 78 % by Cobas Monitor test and 88 % by pp65 antigenemia assay, respectively, were observed in the detection of HCMV positive findings. Instead, shell vial culture assay was far less sensitive (38 %) for the detection of the virus in blood than the other two methods, as reported previously (Evans et al., 1999; Wirgart et al., 1996). Only small differences similar to those of our previous work (I) between Cobas Monitor test and pp65 antigenemia assay were observed. In general, the Cobas Monitor test detected all of the infections which needed antiviral treatment (>16 positive cells in pp65 test). On the other hand the shell vial culture assay remained negative through the follow-up period even in a case with a symptomatic infection.

2.2. Diagnosis of symptomatic HCMV infection by the Cobas Amplicor CMV Monitor assay (II, III)

In our preliminary work (I), we already showed the correlations between the viral loads in DNA and pp65 levels after transplantation and at the time when specific antiviral treatment was started. In studies II and III, the peak viral load values were assessed in the patients with symptomatic HCMV infection and in the patients with asymptomatic infection, using the Cobas Amplicor CMV Monitor test and the pp65 antigenemia test. In the liver patient group (II), altogether 243 consecutive blood samples from 27 patients were investigated. DNAemia and antigenemia were detected in 20 patients. In addition, a very low antigenemia level (3 positive cells) was observed in one more patient 76 days after transplantation. The subsequent samples, however, were all negative. On the basis of this, the patient was classified into the HCMV negative group. Thirteen out of 20 PCR positive/pp65 positive patients developed symptomatic HCMV infection 27 to 52 days after transplantation and 7 patients remained asymptomatic. All symptomatic patients were treated with i.v. GCV. In the kidney patient group (III), altogether 342 blood samples were investigated. Thirty-one out of 116 patients had at least one positive finding by PCR or pp65 test or by both tests. Fourteen out of these patients developed symptomatic infection and were treated with i.v. GCV.

The peak viral loads observed in the patient groups II and III measured by Cobas Monitor test and by pp65 antigenemia are shown in Figure 4. In both patients groups, the median peak viral load values of the patients with symptomatic infection were statistically higher than those of the patients without symptomatic infection by both quantitative tests. These results are in agreement with other groups evaluating Cobas Monitor assay (Caliendo et al., 2002; Caliendo et al., 2000; Flexman et al., 2001; Humar et al., 1999). Very similar results had also been shown by in-house quantitative PCR methods, and other commercial quantitative methods or modifications, using either plasma samples or leukocytes (Aitken et al., 1999; Ferreira-Gonzalez et al., 1999; Mendez et al., 1998a; Roberts et al., 1998; Weinberg et al., 2000).

2.3. Monitoring of HCMV infection in individual patients (II, III)

The liver transplant patients (II) were frequently monitored during the first three months after transplantation. The response to the antiviral treatment was monitored in the symptomatic patient group and, in the case of asymptomatic infection, the patient was monitored until pp65 antigenemia disappeared. Five of the 13 symptomatic infections were found from patients with primary infections (D+/R-), usually with high peak viral loads measured by both methods (in four cases >10 000 cps/ml and ≥50 positive cells and in one case 526 cps/ml and 80 positive cells) (II: Fig. 2). Six patients with symptomatic infection had reactivations/reinfections (II: Fig. 3). The peak viral loads in this D+/R+ group, as assessed by the Cobas Monitor test, were high (>10 000 cps/ml) in three cases, and lower (range 2290-7730 cps/ml) in the other three cases. The peak viral loads as determined by pp65 assay were high (≥50 positive cells) in all of these cases. One patient in the D-/R+ group who had symptomatic reactivation/reinfection, had moderate peak viral load by the Cobas Monitor test (6510 cps/ml and 100 positive cells), and another one had lower peak values by both methods (1120 cps/ml and 25 positive cells) (II: Fig. 4). All liver recipients with asymptomatic HCMV infection belonged to the serostatus group of D+/R+ (reactivations/reinfections) (II: Fig. 5), and were not treated with antivirals. The overall peak viral loads of the asymptomatic patient group were significantly lower than those of symptomatic infections. In this study, there were PCR negative patients in each serostatus groups: D+/R- (n = 1), D+/R+ (n = 2), D-/R+ (n = 3), and D-/R- (n =1). Table 3 shows the peak viral load values demonstrated by the Cobas Monitor and pp65 assays in each serostatus group.

Table 3. Peak viral loads demonstrated by the Cobas Monitor and pp65 antigenemia assays in the different serostatus groups.

Serostatus	**No. of Patients**	**Cobas peak range, cps/ml (median)**	**pp65 peak range, pos cells/50 000 leukocytes (median)**
D+/R- (symptomatic)	5	526-21 600 (16 100)	50-800 (100)
D+/R+ (symptomatic)	6	2520-126 000 (8965)	50-300 (185)
D-/R+ (symptomatic)	2	1120-6510 (3815)	25-100 (62)
D+/R+ (asymptomatic)	7	915-5490 (2240)	15-60 (29)

In the liver patient group (II), the high peak viral loads (>10 000 cps/ml) were seen especially in the D+/R- group with primary HCMV infections. In addition, the median peak viral load of the symptomatic infections were significantly higher than those of the symptomatic infections (10 200 cps/ml versus 2240 cps/ml). Instead, the peak viral loads of the asymptomatic D+/R+ patients did not exceed 5500 cps/ml, and only two of these patients had viral loads over 5000 cps/ml. Thus, it was supposed that the cut-off level of 2000-5000 cps/ml might be optimal for predicting HCMV disease in seropositive recipients, at least in this transplant patient group, as was proposed also by other groups using the Cobas Monitor test (Caliendo et al., 2002; Humar et al., 1999).

The kidney transplant patients (III) were monitored weekly to follow the response to antiviral treatment and, in the case of asymptomatic infection, until the pp65 antigenemia subsided. Five of the symptomatic cases belonged to the D+/R- group, one case to the R-/D- group, seven cases to the D+/R+ group and one case to the D-/R+ group, respectively. All asymptomatic patients had reactivations or reinfections being seropositive before transplantation (D+/R+ or D-/R+). In this patient group, we did not observe significantly higher peak viral loads in the patients with primary infections than in the reactivation/reinfection groups. However, the overall peak viral loads were significantly higher in the symptomatic patients than in the asymptomatic patients as was the case with the liver transplant patient group (II) (Figure 4.). Therefore, a similar cut-off level may be considered appropriate for predicting HCMV disease also in this patient group.

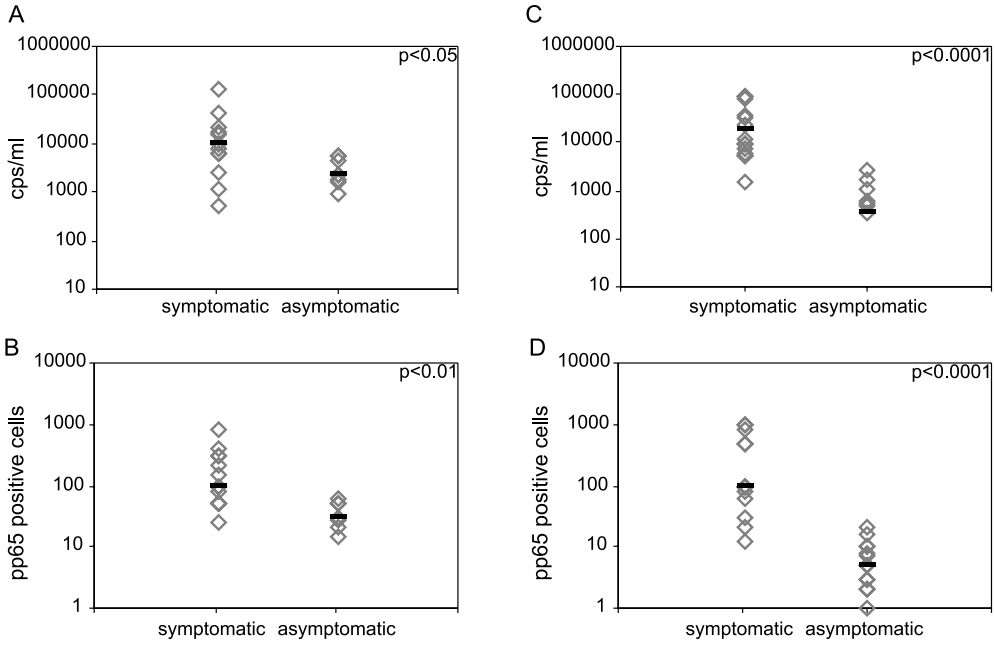

Figure 4. Median values of peak viral loads of symptomatic and asymtomatic HCMV infections assessed by PCR test and by pp65 antigenemia assay.
A and B in liver patient group (II), C and D in kidney patient group (III).

In both patient groups, time-related viral load values assessed by the Cobas Monitor test and pp65 assay correlated well in symptomatic and asymptomatic infections (as shown in more detail in the original publications, II: Fig. 2, 3, 4 and 5; III: Fig. 2). The response to the antiviral treatment was easy to follow by both methods. This indicated the suitability of the Cobas Monitor assay, not only for the diagnosis of symptomatic HCMV infection, but also for the monitoring of individual liver and kidney transplant patients.

3. HCMV real-time PCR assay (IV)

The real-time PCR assay was developed in the present study because the need for a faster and more automated assay with greater capacity for the quantitation of HCMV viral load in plasma samples was obvious. The real-time technology based on TaqMan chemistry and the MagNA Pure LC nucleic acid extraction system (Roche Diagnostics) were selected for allowing the improvements. The nucleotide sequence region for amplification was chosen from the pp65 gene (*UL83*) of HCMV, instead of the DNA polymerase region used in the Cobas Amplicor CMV Monitor assay. The primers and probe used are described in the Materials and Methods.

3.1. Technical characteristics of HCMV real-time assay

3.1.1. Detection limit, linearity and reproducibility of HCMV real-time assay

In the developed assay 10 plasmid copies per reaction were reproducibly detected, and the 250 HCMV cps/ml plasma was assessed as the detection limit of the test. A broad linear range, from 250 up to 25 000 000 cps/ml plasma, was observed. This was much broader than that of the Cobas Monitor assay (from 400 to 100 000 cps/ml plasma). Good amplification efficiency of the standard plasmid was indicated by the correlation efficient values of at least 0.9930 and the slope values between –3.3 and –3.4 within different PCR runs, when the 10-fold dilution series of the standard plasmid was used.

The reproducibility of the TaqMan test was assessed using the clinical plasma specimens, in which the HCMV copy number varied, as specified by the Cobas Monitor assay, representing the whole linear range of the real-time test. Four different samples were used for both intra-assay and inter-assay analyses. The samples were investigated as triplicates for intra-assay analysis, and in three different test runs for inter-assay analysis. The coefficient of variations (%) ranged from 5.12 % to 13.1 % in intra-assay analysis and from 4.50 % to 19.6 % in inter-assay analysis, being the highest in the low-copy samples. The results were comparable with those of other groups employing real-time HCMV tests (Gault et al., 2001; Machida et al., 2000; Sanchez and Storch, 2002) indicating a good accuracy of the real-time PCR.

3.1.2. Automated extraction of nucleic acids

Turnaround time of a clinical PCR test depends widely on the method used for nucleic acid extraction. Conventional extraction with an organic solution, such as phenol, is efficient but it is also very laborious and time-consuming, and therefore it is not suitable for the processing of a large number of samples. Commercial nucleic acid isolation kits have been developed to reduce time and they have also been widely used in the quantitative HCMV PCR assays described (Gault et al., 2001; Mendez et al., 1998a; Michaelides et al., 2003; Pang et al., 2003; Razonable et al., 2002a). Also the commercially available Cobas Amplicor CMV Monitor test has been assessed to use manual nucleic acid extraction (Roche Diagnostics). However, also these kits usually need long hands-on time and are usually not suitable for automation. In this study, we described the use of an automated nucleic acid extraction device, MagNA Pure LC (Roche Diagnostics), and the Total Nucleic Acid Isolation Kit (Roche Diagnostics) for the processing of plasma specimens. In this purification system released nucleic acids are bound to the silica-coated magnetic particles, washed and then eluted. Technically the most important advantage of the MagNA Pure instrument was the short hands-on time needed for preparing samples, about 15 minutes for the set-up of the instrument. Thirty-two samples could be processed within 1.5 hours, which greatly increased the capacity of the test. In recent years, automated nucleic acid extraction instruments have been combined with HCMV real-time assays also by other groups (Geddes et al., 2003; Mengelle et al., 2003b).

Good performance of a diagnostic PCR test with purified nucleic acid is also dependent on the method of nucleic acid extraction. Many potential inhibitory compounds derived from the clinical samples have been reported (Wilson, 1997), including also components of blood, such as hemoglobin, heme and IgG antibody (Akane et al., 1994; Al-Soud et al., 2000; Al-Soud and Radstrom, 2001), or added anticoagulants such as heparin (Satsangi et al., 1994). In recent years, the MagNA Pure LC instrument has been proven to be an efficient nucleic acid extraction system in various sample types (Espy et al., 2001; Kessler et al., 2001; Rabenau et al., 2002). In our hands, the MagNA Pure instrument was proven to present an efficient method for the processing of clinical plasma specimens. In our evaluation study of 160 plasma samples, the results after the conventional phenol extraction and after the MagNA Pure system were comparable with each other, indicating a good efficiency of the automated extraction system. Recently, MagNA Pure LC was shown to provide an efficient and reproducible DNA purification method in the other real-time HCMV test that analysed clinical plasma specimens (Stocher and Berg, 2002).

3.1.3. Turnaround time of HCMV real-time assay

In addition to the development of automated nucleic extraction devices, the introduction of real-time technology has been an important element for improvements of diagnostic quantitative PCR assays. The use of real-time technology has reduced the entire time needed for PCR significantly when compared with PCR using conventional instruments, and also when compared with commercially available kits using competitive quantitative PCR, in which amplification and detection follow each other. Modern real-time

instruments allow amplification, detection of PCR products and quantitation of the viral DNA to occur simultaneously and quickly. In our work, the use of the ABI PRISM 7900HT Sequence Detector instrument (Applied Biosystems) and TaqMan technology reduced the entire testing time significantly when compared with Cobas Monitor test, being 3-4 hours instead of 7-8 hours, depending on the number of samples analysed.

3.2. Diagnostic validation and use of HCMV real-time PCR assay

The clinical utility of the developed HCMV real-time assay in the detection and monitoring of HCMV infection was evaluated by investigating 270 consecutive blood samples from 23 adult liver and four kidney recipients. The results were compared with those of Cobas Amplicor CMV Monitor assay, and also with those of the pp65 antigenemia assay. In the qualitative comparisons, ≥250 cps/ml and ≥400 cps/ml, the detection limits of the quantitative PCRs, were considered HCMV DNA positive for real-time and Cobas Monitor assays, respectively. In the pp65 antigenemia assay ≥1 positive cell(s) in an individual sample was considered positive.

3.2.1. Detection of HCMV DNA by real-time assay

The sensitivities of each method in the different patient populations (in studies I, III and IV) in the detection of HCMV in blood samples are summarised in Table 4.

The developed HCMV real-time PCR assay was found to be the most sensitive assay in this patient population (IV), detecting 92 % of the positive findings. Instead, the Cobas Monitor test was less sensitive than the pp65 antigenemia assay in this study, as well as in our study III, and as sensitive as pp65 in our study I. Recently, the better sensitivities of the quantitative plasma PCR tests compared with the pp65 antigenemia assay have also been reported by other groups using real time PCR (Pang et al., 2003; Tanaka et al., 2002) or modified Cobas Monitor assay (Hadaya et al., 2003). In all of these reports the detection limit of PCR assay has been lower than that of Cobas test (400 cps/ml). The equivalent of the sample volume added to each amplification reaction is one of the most important factors which can affect the sensitivity of the test. In our work the sample equivalent in the real-time assay was larger than in the Cobas Monitor test (40 µl in real-time versus 25 µl in Cobas). The detection limits of the described plasma real-time HCMV assays are usually lower than that of the Cobas Monitor assay (Leruez-Ville et al., 2003; Pang et al., 2003; Tanaka et al., 2002), which has increased the sensitivity of these tests in the detection of HCMV DNA in blood samples.

Comparisons of the qualitative results of HCMV real-time, Cobas Monitor and pp65 assays in patient group IV are outlined in Table 5. The very similar findings as in our own studies (I, III) and as previously reported studies by other groups (Caliendo et al., 2001; Caliendo et al., 2000; Humar et al., 1999) concerning the differences between Cobas Monitor test and pp65 antigenemia assay, were also shown in this patient group (IV).

All 20 PCR negative/pp65 positive (PCR negative at least by one of the PCRs used) samples had low-level antigenemia, 1-5 positive cells. Many of these samples were detected before the HCMV episode of the patient and the rise of DNAemia, or the samples were obtained during GCV treatment. On the other hand, all 14 PCR positive/pp65 negative (PCR positive at least by one of the PCRs used) samples were obtained in the patients with GCV treatment. Most of these samples had a low-level DNAemia.

Also some inconsistency between the results of pp65 and PCR methods was observed. Six out of 14 PCR positive/pp65 negative samples had a higher level of DNAemia by both PCR assays (646-21 400 cps/ml by Cobas and 8015-73 530 cps/ml by real-time). All of these samples were obtained during or soon after the antiviral treatment. Similar differences, the high HCMV DNA copies with non-detectable or very low antigenemia in some samples, have also been observed by other groups (Gault et al., 2001; Griscelli et al., 2001; Nazzari et al., 2000). The clinical significance of such findings is unclear.

In this study, the Cobas Monitor and real-time assays were compared also with each other. Only small differences between these tests were observed when qualitative results were evaluated, mainly because of different sensitivities of the assays. DNAemia was observed in 15 samples only by real-time test, in which the levels of DNA were under or near the detection limit of the Cobas test (303-861 cps/ml), except in one sample with 1637 cps/ml. On the other hand, only one sample, obtained during antiviral treatment, was real-time negative/Cobas positive (1930 cps/ml).

Table 4. The sensitivities of each detection method in different studies.

	Detection method		
Study No.	**Real-time PCR**	**Cobas Monitor test**	**pp65 assay**
I	-	88 % (78/79)	89 % (79/89)
III	-	78 % (121/156)	88 % (138/156)
IV	92 % (106/115)	80 % (92/115)	88 % (101/115)

Table 5. Comparison of the qualitative results of the real-time, Cobas Monitor and pp65- antigenemia assays.

	Method		
No of samples	**Real-time**	**Cobas Monitor**	**pp65-ag**
81	+	+	+
10	+	+	-
11	+	-	+
4	+	-	-
1	-	+	+
0	-	+	-
8	-	-	+
155	-	-	-
Total 270	Total 106 pos.	Total 92 pos.	Total 101 pos.

3.2.2. Correlation between quantitative tests

Good correlation between pp65 antigenemia and the Cobas Monitor assays ($R = 0.80$, $P < 0.0001$), between pp65 and real-time assays ($R = 0.84$, $P < 0.0001$), and between the Cobas Monitor and real-time assays ($R = 0.64$, $P < 0.0001$) was found (IV: Fig. 1). The correlations were similar if the samples from liver and kidney transplant recipients were evaluated separately. The lower correlation between the Cobas Monitor and real-time assays may be due to one patient with an unusually prolonged antiviral treatment. The value increased if this patient was not included ($R = 0.71$). Also the narrower linear range of the Cobas Monitor assay could have had an effect on the lower correlation of these two tests. The upper limit of the linear range of Cobas assay was guaranteed up to 100 000 cps/ml by the manufacturer, however, it has been reported to be lower (Caliendo et al., 2001).

Different types of specimens, including peripheral whole blood and different blood compartments (plasma/serum, PBLs, PMNLs, PBMCs) have been used for the quantitation of HCMV DNA in blood samples with varying results (Guiver et al., 2001; Hadaya et al., 2003; Li et al., 2003; Mengelle et al., 2003a; Mengelle et al., 2003b; Razonable et al., 2002a). Which is the most appropriate sample type for the measurement of viral load, is still debated. In general, viral loads have been shown to be higher in whole blood or leukocytes than in plasma (Gerna et al., 1994; Mengelle et al., 2003b; Razonable et al., 2002a; Sia et al., 2000a). Thus, cell-containing specimens have been found to have a higher sensitivity in the qualitative detection of HCMV compared with cell-free specimens (plasma/serum) (Mengelle et al., 2003b; Razonable et al., 2002a). However, the simultaneous high levels of DNA in the plasma and other blood compartments has been shown during HCMV disease (Michaelides et al., 2003; Razonable et al., 2002a). A good correlation between plasma and cell-containing samples, has also been reported in quantitative PCR (Michaelides et al., 2003; Razonable et al., 2001a; Sanchez and Storch, 2002). In addition, quantitative PCR assays with low detection limits have been developed for the detection of HCMV DNA in plasma samples (Hadaya et al., 2003; Pang et al., 2003), and also a good correlation between these tests and pp65 antigenemia test has been shown. These results are in agreement with ours indicating the suitability of plasma samples for the measurement of viral loads in blood. Easy handling of plasma samples is an advantage in the laboratory. Delayed separation of plasma has been reported to increase the frequency of DNA positivity for HCMV probably due to latently infected leukocytes (Schafer et al., 2000). However, a recent report indicated that a 24-hour delay in plasma isolation did not affect viral quantitation by real-time PCR (Nesbitt et al., 2004). In addition, the test is not dependent on the number of leukocytes, and thus it may also be used in patients with leukopenia because of immunosuppressive therapy or during GCV treatment.

3.2.3. Diagnosis and monitoring of HCMV infection in individual patients by HCMV real-time assay

All liver transplant patients were frequently monitored. The liver patients with symptomatic infections were treated with i.v. GCV and were monitored until pp65 antigenemia and DNAemia subsided. All kidney recipients had symptoms already before the first sample was obtained, and were also frequently monitored for response to the antiviral treatment in a similar way to the liver recipients. Nineteen out of 27 patients (23 liver and 4 kidney transplant patients) demonstrated some degree of pp65 antigenemia and DNAemia by both PCR tests. Thirteen patients developed symptomatic infection. Six patients remained asymptomatic within the follow-up period. All four kidney recipients were included in the symptomatic patient group.

In our previous studies with liver (II) and kidney (III) transplant patients, the peak viral loads in the symptomatic HCMV patients were shown to be statistically higher than in the patients with asymptomatic infection as studied by Cobas Monitor and pp65 assays. This was also observed in this patient group (IV), and in addition, a statistically significant difference was also demonstrated with the results obtained by the developed real-time assay (53 291 cps/ml versus 1915 cps/ml).

The longitudinal monitoring curves observed by real-time, Cobas Monitor and pp65 antigenemia assays of the two treated liver transplant and one treated kidney transplant patients were demonstrated (IV: Fig. 2). The follow-up curves of two different PCR tests and pp65 assay correlated well and significant differences between these methods in the response to antiviral treatment were not observed. However, the clinical management of kidney transplant patients was different to that of the liver transplant patients. The liver transplant patients were frequently monitored from the day of transplantation, having several negative samples before the HCMV episode. Instead, the kidney transplant patients already had symptomatic HCMV infection before the first sample was obtained.

In our previous studies (I, II and III) we showed the clinical utility of the Cobas Monitor CMV test in the diagnosis and monitoring of HCMV infections in transplant patients. In this study (IV) a good correlation between developed real-time HCMV PCR test and the Cobas Monitor test was shown. However, the real-time assay had advantages over Cobas Monitor test. It was a rapid and labor-saving method in the quantitation of HCMV DNA. Larger capacity enabled larger numbers of samples to be analysed in a shorter time. Also, the wider linearity of real-time assay compared with the Cobas test, made it clinically easier to assess the patients with very low or very high DNAemia. This proved that real-time PCR assay can be a good alternative method for the diagnosis of HCMV in clinical laboratories where a large amount of specimens should be analysed every day.

Acknowledgements

This study was carried out in the Department of Virology, Haartman Institute, University of Helsinki. I thank professors Klaus Hedman, Antti Vaheri and Carl-Henrik von Bonsdorff for the opportunity to complete my study. I also thank professor Martti Vaara and docent Maija Lappalainen for providing good working facilities in HUSLAB. I warmly thank Maija also for her support and friendship during all these years. The head of General Microbiology, University of Helsinki, professor Timo Korhonen, is thanked for providing a nice atmosphere for graduate and post graduate studies.

I warmly thank my supervisor, docent Irmeli Lautenschlager, for guiding me through these years.

I thank docents Raija Vainionpää and Hannu Jalanko for reviewing this work, and for their constructive and valuable comments.

I wish to thank all my co-authors: docent Maija Lappalainen, MD Jukka Suni, MD Petri Koskinen and MD Ilkka Helanterä. Professors Krister Höckerstedt and Carola Grönhagen-Riska are warmly thanked for providing clinical material, without which this work would not have been possible.

I sincerely thank Kaija Sopenlehto and Teija Tekkala, for the great years working together, as well as for expert technical assistance. Marjatta Palovaara and Miia Mäkijouppila are also thanked for excellent technical assistance. I thank Stephen Venn for correcting the language. Lauri Ylimaa is thanked for the layout of this book.

I warmly thank my friend Hannimari Kallio-Kokko for all kind of help and company during these years. Hanni, thank you!

I wish to thank Laura Mannonen for her friendship and help in PCR world. I also would like to thank Raisa Loginov for her support and friendship during these years.

I warmly wish to thank the whole PCR group. Without you I would not be here! Satu Cankar is thanked for all kind of help I have needed, and also for her friendship. I also want to thank Maria, Leena, Eeva, Heli, Olli, Suvi, Mirja, Minna and all the other friends inside the department for many nice moments and good company, and for their kind help.

I warmly thank my parents for excellent facilities for spending my leizure time. I wish to thank Leena, Pike and Kati for many wonderful moments outside the lab. Timo, Matti, Juha, Eija and Kids, as well as all my friends, are thanked for good company and support. Finally, I thank Kimmo for all moments we have spent together.

Helsinki, May 2004

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