POLYMERASE CHAIN REACTION FOR DETECTION OF HUMAN BETAHERPESVIRUSES DNA IN SERA

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Abstract

The clinical importance of HCMV, especially in immunocompromised patients such as transplant recipients, is very well recognised whereas that of newly discovered herpesviruses, HHV-6 and HHV-7, remains to be fully elucidated. The biological and pathogenic characteristics of these viruses are similar and their clinical significance in this group of patients is equally expected. In this study, PCR was used to detect HCMV, HHV-6 and HHV-7 DNA in serum samples from renal and bone marrow transplant recipients and also in tissue sections and skin biopsy specimens from cases of *hydrops fetalis* and chronic psoriasis. These PCRs were performed retrospectively on 463 serum specimens from 124 renal transplant recipients and 97 serum specimens from 66 bone marrow transplant recipients, tissue sections (liver, kidney, heart, lungs and placenta) from 4 cases of *hydrops fetalis* and 11 control non-hydrops cases and skin biopsies from 10 cases of chronic psoriasis. In addition, HHV-6 PCR products were digested with restriction enzymes for the purpose of typing into variant A or B. In the renal transplant group (n=124), HCMV was detected in 31, HHV-6 in 29 and both HCMV and HHV-6 (dual infection) in 16 patients but none of these patients was positive for HHV-7. In the bone marrow transplant patients (n=66), HCMV was detected in 2 patients, HHV-6 in 11, HHV-7 in 1 patient and dual infection in 2 patients (HCMV and HHV-6 in 1 patient and HCMV and HHV-7 in the other). In addition, HHV-6 DNA was detected in 1 of 4 cases of *hydrops fetalis* in all tissue sections tested (liver, heart, lungs, kidney and placenta) but in none of the 11 controls (non-*hydrops fetalis* cases). With respect to chronic psoriasis, only 1 of 10 cases was positive for HHV-6 DNA in 2 biopsies representing the involved and uninvolved skin. All the HHV-6 positive specimens in this study were typed as HHV-6 variant A. In conclusion, HHV-6 and HCMV are important pathogens in bone marrow and renal transplant recipients and coinfection with both viruses should be expected in these groups of patients but the importance of HHV-7 could not be established in either group. HHV-6 may be an important aetiological agent in cases of *hydrops fetalis* and future work should directed towards understanding this association. Interestingly, the clinical importance of variant A of HHV-6 may be underestimated as both variants may be equally pathogenic.

Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Osama Hussain jiffri

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Dedication

This thesis is dedicated to my parents, along with loving members of my family who have been there for me at every turn; my wonderful wife Fadwa and children Shereen, Hussain, Marwan and Sultan for their enduring love and support, my brothers and sisters.

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1. INTRODUCTION

1.1. Herpesviridae

The family *Herpesviridae* comprises a group of over 100 viruses that infect a wide range of vertebrates. The family of these viruses is classified on the basis of their biological differences, cell pathology in cell culture, tissue tropism *in vivo* and latency site into three sub-families, the *Alpha-*, *Beta-* and *Gamma-herpesvirinae* (Roizman and Sears, 1993).

The alphaherpesviruses are characterised by the widest host range and rapid cytopathic effect in cell culture. Viruses within the alphaherpesvirus subfamily are neurotropic and establish persistence in the sensory ganglia that innervate the skin. In contrast, the betaherpesviruses have a narrower host range and slower growing in cell culture and have a tendency to give a cytomegalic type of pathology and are primarily lymphotropic in nature. Gammaherpesviruses are somewhat intermediate between the alpha- and beta-herpesviruses, being significantly slower growing in cell culture than alphaherpesviruses but quicker than betaherpesviruses and do not tend towards cytomegalic pathology (Table 1).

The virus particle has a size varying from 120 to nearly 300 nm and is arranged in four concentric layers; a nucleic acid genome (core), a protein capsid, a tegument and a lipid envelope. The virus has a linear, double stranded DNA genome between 80-150 million daltons molecular weight approximately 120 to 230 (Kbp). The genome is enclosed within an icosadeltahedral capsid. The tegument is an ill-defined, amorphous area between the envelope and the nucleocapsid and is probably important in virus replication. The envelope surrounding the virus particle has an almost identical appearance to the host nuclear membrane, which suggests the envelope may be acquired as the virus exits the nucleus. Variable length glycoprotein spikes protrude from the lipid envelope either discretely or clustered. These provide important antigenic determinants to distinguish the different members of the family (Stannard *et al.*, 1987).

Table 1. Pathogenic profiles of the Human herpesviruses

Virus	Subfamily	Disease Association for:			Latency or persistence
		Primary infection	Reactivation	Chronic infection	
HSV-1	α	5/6 subclinical, 1/6 oral lesions, neonatal infections	Oral lesions, encephalitis	Minimal	Neurons of trigeminal ganglia
HSV-2	α	5/6 subclinical, 1/6 genital lesions	Genital lesions, encephalitis	Minimal	Neurons of sacral ganglion
VZV	α	Varicella	Herpes zoster	Minimal	Cells of trigeminal and dorsal root ganglia
HCMV	β	5% of infectious mononucleosis, congenital CMV	?	Atherosclerosis (?), restinosis (?)	Monocytes or granulocytes-macrophage progenitors (?)
EBV	γ	>85% of infectious mononucleosis	?	Nasopharyngeal carcinoma, Burkitt's lymphoma	B cells
HHV-6A	β	?	?	MS (?)	Skin, lymphocytes
HHV-6B	β	ES in 30% of children	?	MS (?)	Macrophages, lymphocytes
HHV-7	β	Subset of ES	?	?	Infectious virus in saliva
HHV-8	γ	?	?	KS, multicentric Castleman's disease, primary effusion lymphomas	Sperm?, lymphocytes?

HSV-1=herpes simplex virus type 1; HSV-2=herpes simplex virus type 2; VZV=Varicella Zoster virus; HCMV= human cytomegalovirus; EBV=Ebstein Barr virus; HHV-6A= human herpesvirus 6 variant A; HHV-6B= human herpesvirus 6 variant B; HHV-7= human herpesvirus 7; HHV-8= human herpesvirus 8; ES=Exanthum subitum; MS=multiple sclerosis; KS=Kaposi's sarcoma.
Modified from Braun et al., (1997)

There are eight members of the *Herpesviridae* commonly causing infection in humans (human herpesvirus 1 to 8). According to their site of latency, the human herpesviruses can be designated neurotropic or lymphotropic where latency is established in the nervous system or in the lymphocytes, respectively (Roizman and Sears, 1993).

Human herpesvirus 1 or herpes simplex virus type 1 (HSV 1) is usually transmitted non-venereally and affects non-genital sites including the mouth, lips, skin, eyes and brain. Human herpesvirus 2 or herpes simplex virus type 2 (HSV 2) is most often transmitted venereally causing genital herpes with occasional severe infection in neonates (Whitley, 1996). Varicella-zoster virus (VZV) or human herpesvirus 3 results in chicken pox, a common childhood illness that may reoccur as shingles in later life (Arvin, 1996). Epstein-Barr virus (EBV) or human herpesvirus 4 infection is asymptomatic if acquired in infancy, but can cause infectious mononucleosis or glandular fever in later life and is also associated with Burkitt's lymphoma and nasopharyngeal carcinoma (Rickinson and Kieff, 1996). Human cytomegalovirus (HCMV) or human herpesvirus 5 give rise to little or no ill-effects in healthy individuals and is only of concern as a congenital infection or in immunocompromised patients causing pneumonitis, severe infectious mononucleosis, deafness and mental retardation (Britt and Alford, 1996). Human herpesvirus 6 (HHV-6) has two subtypes, type A isolated from immunocompromised patients (Secchiero and Cleghorn, 1998) and type B which causes *exanthem subitum* (ES) in children (Dewhurst *et al.,* 1993a). Human herpesvirus 7 (HHV-7) has been isolated from peripheral blood mononuclear cells but has been associated with some cases of ES (Tanaka *et al.,* 1994). Human herpesvirus 8 has been suggested as the causative agent of Kaposi's sarcoma (KS) in patients with acquired immune deficiency syndrome (AIDS) (Chang *et al.*, 1994).

1.2. Human Cytomegalovirus

The designation cytomegalovirus was proposed by Weller *et al.,* (1960). The word cytomegalovirus is derived from the cytopathic effects induced by the virus and its role in congenitally acquired cytomegalic inclusion disease. HCMV is the prototype of the betaherpesvirus group and the importance of this virus as a human pathogen has dramatically increased over the past two decades in the immunocompromised including AIDS and transplant patients. In this group of patients, the virus is responsible for life-threatening acute disease due to primary or reactivation of latent infection (Van der Meer *et al.,* 1996).

1.2.1. Epidemiology

Humans are the only reservoir for HCMV. The transmission of HCMV infection occurs through direct and indirect person-to-person contact and several factors have been recognised to affect the transmission of this virus. These include prolonged shedding of the virus in oropharyngeal, ocular, cervical and vaginal secretions, urine, semen, breast milk, faeces and blood (Alford, *et al.,* 1980; Kumar *et al.,* 1973; Klemola *et al.,* 1969; Reynolds *et al.,* 1973; Stagno *et al.,* 1975).

The sources of transmission include community-acquired infection, sexual transmission and hospital-acquired infection. Community-acquired infection is usually caused by close contact with young children as evident from the higher rates of infection observed in children (Hutto *et al.,* 1985; Pass *et al.,* 1986; Pass and Kinney, 1985) where oral and respiratory spread appears to be the predominant routes of transmission. In addition, due to the shedding of the virus in the genital tract (Chandler *et al.,* 1985; Hutter *et al.,* 1989), genital transmission of HCMV is considered to be another major source of viral transmission in the adult population.

The excretion of the virus is increased in immunocompromised patients including those undergoing allograft transplantation and individuals with AIDS (Drew *et al.,* 1984; Pollard, 1988; Gallant *et al.,* 1992; Pertel *et al.,*

1992). The source of the infection in these groups includes transplanted organs, blood products, and reactivation of endogenous virus in transplant recipients or reactivation and/or reinfection in the case of AIDS patients.

Recurrent infection with HCMV is defined as intermittent excretion of virus from single or multiple sites for a number of years and results from one or more of three mechanisms. First, virus excretion during a mild chronic infection manifested following a primary infection. Second, reinfection in the immune host with a new HCMV strain as evident from antigenic variation of HCMV strains (Spector *et al.*, 1984a). The third mechanism is reactivation of HCMV that had become latent during the primary infection (Huang *et al.*, 1980).

After a primary infection, HCMV remains dormant in a variety of cells throughout the body and is capable of being reactivated under conditions of immunosuppression where the virus emerges from its latent state and causes clinical disease (Rubin, 1994). HCMV has been found in neutrophils, monocytes, epithelial cells of salivary glands, kidneys, breasts, testes, and prostate with the transplanted kidney as an established source of latent infection. Studies on HCMV at the cellular level indicate that, following penetration of a susceptible cell, cellular function is not significantly impaired initially and the cell retains its viability, despite continual shedding of virus, for a prolonged period (Mocarski *et al.*, 1979).

HCMV infection is endemic in nature without any seasonal variation but higher rates of infection have been observed owing to ill-defined socioeconomic factors which may include hygienic status and degree of close contact. Up to 100% of HCMV infection in developing countries is acquired in early childhood (Gold and Nankervis, 1982) whereas approximately 40 to 80% of children in the developed world do not become infected until well into puberty making sexual transmission an important factor for infection spread (Gold and Nankervis, 1982).

1.2.2. Pathogenesis

The virus enters the host cell by fusion of the viral envelope with the cell membrane. After the envelope is shed and capsid removed, the viral nucleic acid enters the nucleus, where it serves as a template for production of mRNA and more DNA. The viral proteins and nucleic acid self-assemble and, finally, the complete virus particle is wrapped in an envelope as it buds through the inner nuclear membrane. The enveloped particles burst the host cell as they exit (Naraqui, 1984).

The replication cycle of HCMV is divided into three phases as determined by the production of specific proteins: (1) an immediate early phase, which begins after penetration of cells by the virus resulting in immediate early proteins which are responsible for transcription of early genes; (2) an early phase during which viral DNA polymerase is synthesised; and (3) a late phase characterised by synthesis of structural viral proteins, assembly of the whole virion and release of infectious virus. The whole replication cycle takes an average of 18-24 hours (Chou, 1990).

Active HCMV infection has been detected in several organs including lungs and gastrointestinal tract (Sinzger *et al.*, 1995), liver (Theise *et al.*, 1993), retina (Rummelt *et al.*, 1994), central nervous system (CNS) (Schmidbauer *et al.*, 1989) endomyocardium (Arbustini *et al.*, 1992), kidneys (Gnann *et al.*, 1988), and the peripheral blood leukocytes (Gerna *et al.*, 1992).

The mechanism of tissue destruction is either direct or indirect. Direct cytopathogenicity occurs due to extensive viral replication (Heinemann, 1992) whereas indirect destruction can occur as seen in bone marrow transplant recipients owing to immunopathological mechanisms (Grundy *et al.*, 1987). A number of pathogenetic mechanisms for HCMV-associated tissue damage (indirect cytopathogenicity) have been suggested. These include cytopathic effects of cytotoxic T cell reactions against HCMV-infected target cells (Grundy *et al.*, 1987), blood vessel occlusions due to vasulitic alterations resulting in necrosis (Kyriazis *et al.*,

1992), and enhancement of either the frequency of activated lymphocytes or the extent of major histocompatibility complex (MHC) expression which might result in rejection episodes or graft versus host reactions in cases of allogeneic transplantation (Sedmak and Orosz, 1991).

Asymptomatic infection is a major characteristic of HCMV in the normal host. Whether this reflects true latency or low-level productive infection is still unresolved. HCMV has been shown to downregulate the expression of MHC class I molecules on infected cells which has been suggested as a mechanism of immune evasion (Warren *et al.,* 1994; Steinmassl and Hamprecht, 1994) whereas limited viral gene expression in non-permissive cell types has been suggested as a mechanism of viral latency by which these cells might escape from the host's cytotoxic immune reaction (Poland *et al.,* 1994; Gilbert *et al.,* 1993). Although the sites of HCMV latency or persistence are still cryptic, the monocyte/macrophage system and vascular endothelial cells have been considered to be potential sites of HCMV persistence (Fish *et al.,* 1995; Sinclair and Sissons, 1996).

The immune response to HCMV infection is an important factor in preventing HCMV disease. This is clear from the subclinical nature of the infection in normal individuals compared to severe and fatal disseminated disease in immunocompromised patients. Both humoral and cellular immunity has been recognised to target a number of HCMV-encoded proteins (Britt and Alford, 1996).

Primary HCMV infection is followed by a series of humoral and cell-mediated immune responses involving production of IgM and IgG antibodies, cytotoxic cell responses and activation of natural killer cells and antibody-dependent killer cells (Rook, 1988). The fact that reactivation of disease can occur in the presence of intact humoral immunity that cell-mediated immunity is the major factor in limiting HCMV infection (Simmons *et al.,* 1977). Thus, it seems that the cytotoxic cellular response to CMV infection determines the outcome of CMV disease.

The cells that target virus-infected target cells may be either cytotoxic T cells or non-T lymphocytes. HCMV-specific cytotoxic T cells are antigen specific and are human lymphocyte antigen (HLA)-restricted. The non-T lymphocytes target HCMV-infected cells in an MHC-unrestricted fashion and have the properties of natural killer (NK) cells and antibody-dependent killer cells (ADCC) which possess receptors for the Fc portion of an immunoglobulin.

In bone marrow transplant recipients, low levels of HCMV-specific cytotoxic T-lymphocytes, NK and ADCC responses were associated with severe and fatal HCMV disease and a positive correlation between effective HCMV-specific cytotoxic response and recovery from infection was demonstrated indicating that these cells mediate recovery from the infection (Quinnan *et al.*,1982).

HCMV reactivation in seropositive transplant recipients is usually mild and subclinical. However, during aggressive immunosuppressive therapy, reactivation of HCMV may have a serious outcome. The mechanism of reactivation of latent virus in transplant recipients is not clear but may represent a combination of both allogenic activation and iatrogenic immunosuppression (Rubin *et al.*, 1994).

The frequency of superinfection in allograft recipients with HCMV has led to the hypothesis that HCMV infection itself could cause immunosuppression by immunomodulation. HCMV can decrease both humoral and cell-mediated immune response in human transplant recipients (Hirsch *et al.*, 1984) thereby increasing the susceptibility to superinfection with a variety of pathogens and, possibly, playing a role in the pathogenesis of allograft injury. HCMV causes depression of cell-mediated immunity by downregulation of class 1 MHC antigens on the infected cells and thereby impairing the presentation of viral antigen by MHC class 1 antigens which is vital in mediating cytotoxic T-cell response (Barnes *et al.*, 1992). Lymphocyte blastogenesis in response to mitogens and herpesvirus antigens has been shown to be depressed during acute HCMV infections (Rinaldo *et al.*, 1980). This is probably caused by

suppression of antigen presentation and release of interleukin-1 as a result of infection of macrophages (Dudding *et al.,* 1987). Later studies indicated that clinical isolates could suppress the functions of large lymphocytes, including γ-interferon production, which are the cells primarily concerned with NK cell activity (Rook *et al.,* 1985).

1.2.3. Clinical manifestations

1.2.3.1. Infections in immunocompetent individuals

In normal and immunocompetent individuals the infection by HCMV is usually asymptomatic but in some patients infectious mononucleosis syndrome may be encountered. Infectious mononucleosis manifestations include fever, myalgia and cervical adenopathy and complications such as pneumonia, hepatitis and meningitis occur in a few cases (Klemola *et al.*, 1970). Another manifestation of HCMV disease in immunocompetent individuals is post-perfusion syndrome, which arises after HCMV infection acquired by blood transfusion. The disease is characterised by sudden fever, atypical lymphocytosis, splenomegaly and hepatitis (Reyman, 1966).

1.2.3.2. Congenital infections

HCMV is a common cause of congenital viral infection leading to cytomegalic inclusion disease in up to 7% of cases (Stagno *et al.,* 1990). The infection could be primary or recurrent. The clinical manifestations of the symptomatic congenital infection are growth retardation, thrombocytopenia, hepatosplenomegaly, hepatitis, microcephaly, encephalitis, and focal neurological symptoms (Boppana *et al.,* 1992) whereas asymptomatic infection may lead to hearing defects and impaired intellectual capabilities (Griffiths, 1995).

1.2.3.3. Infection of transplant recipients

HCMV is a common cause of illness in solid-organ transplant recipients occurring in up to 85% of kidney, heart, and liver transplant recipients

(Dummer *et al.,* 1983; Lumbreras *et al.,* 1993; Reyes *et al.,* 1992). In these patients, the outcome of the infection is controlled by the degree of immunosuppression and the serological status of the recipient and to a lesser extent by other factors such as histocompatibility and the source of allograft (Kirklin *et al.,* 1994). In most cases, however, the infection is acquired as primary infection, reactivation or one that is acquired through the transplanted organ by a seropositive recipient (Chou, 1986; Grundy *et al.,* 1987, Singh *et al.,* 1988).

1.2.3.3.1 HCMV in renal transplantation

HCMV is an important and common opportunistic infectious agent after renal transplantation causing both subclinical infection and potentially life-threatening illness (Peterson *et al.,* 1980; Fryd *et al.,* 1980). These include pneumonitis, colitis, hepatitis, mononucleosis, retinitis, encephalitis, superinfections and allograft dysfunction (Rubin *et al.,* 1994). HCMV disease usually presents 1-4 months post-transplant in 90% of cases, but HCMV disease as late as two years post-transplant has been recorded (Linneman *et al.,* 1978).

Previous studies have shown that 37-72% of renal transplant recipients develop HCMV infection, but many of these remain asymptomatic and only 2-23% develop clinical illness (overt HCMV disease) with a mortality of 1-3% (Metselaar *et al.,* 1989). HCMV disease begins insidiously with constitutional symptoms of malaise, generalised weakness, nasal stuffiness and fever, often accompanied by myalgia and arthralgia. In about a third of patients, prolonged fever is the presenting feature and approximately 60% of fever during the first 6 months posttransplant may be due to HCMV infection (Suwansirikul *et al.,* 1977; Peterson *et al.,* 1980). Pyrexia associated with chills and night sweats has been reported to be present in 95% of patients with CMV disease, but this may be absent in patients with tissue-invasive HCMV disease of the gastrointestinal tract, liver and pancreatic allografts (Sutherland *et al.,* 1979; Mayoral *et al.,* 1991; Escudero-Fabre *et al.,* 1992).

During the second week of illness, orthostatic hypotension may develop which occurs even in patients who were hypertensive previously requiring treatment (Simmons *et al.,* 1977). In about one third of patients, a dry non-productive cough develops within a few days which can be associated with tachypnoea and dyspnoea suggesting HCMV pneumonitis. The respiratory rate is considered to be the best indicator of pulmonary involvement (Rubin, 1990; Peterson *et al.,* 1980).

The gastrointestinal tract is the second major organ system to be involved and may be life-threatening. Diffuse inflammation, ulceration and motility disorders lead to subjective symptoms of abdominal fullness, nausea, pain and vomiting (Mayoral *et al.*, 1991; Sutherland *et al.*, 1979). Oesophagitis leads to odynophagia. Colonic ulceration leading to haemorrhage and perforation is a well-recognised complication. Appendicitis (Perkal *et al.,* 1992) and haemorrhoiditis (Shutze *et al.,* 1991) due to HCMV has been reported more recently. HCMV involvement of the liver and pancreas has also been reported (Peterson *et al.,* 1980).

Uncommon presentations of HCMV disease include encephalitis, transverse myelitis (Spitzer *et al.,* 1987), acute polyradiculoneuritis (Pouteil-Noble *et al.,* 1993), epididymitis (McCarthy *et al.,* 1993), endometritis (Sayage *et al.,* 1990), and skin ulceration from vasculitis (Minars *et al.,* 1977). Chorioretinitis, which is the major ophthalmic manifestation of HCMV infection in AIDS patients, is uncommon in renal transplant recipients (Peterson *et al.,* 1980).

HCMV-seronegative recipients of transplants from seropositive donors are at greatest risk of developing primary infection and are prone to severe HCMV disease (Rubin *et al.,* 1979; Suwansirikul *et al.,* 1977). However, reactivation of infection in seropositive recipients may be equally high as they form a large population of transplant recipients. There are a number of modes for transmission of active infection. These are HCMV-infected allografts from seropositive donors (Betts *et al.,* 1975; Chou, 1986; Grundy *et al.,* 1988); viable leucocyte-containing blood products from seropositive donors which harbour latent HCMV (Schrier *et al.* 1985);

reactivation of endogenous virus in seropositive transplant recipients and by acquisition of virus from the community as a result of intimate contact with an actively infected individual (Rubin, 1993; Grundy *et al.,* 1987).

Primary HCMV infection occurs when an individual who has not been previously infected with the virus becomes infected due to viral transmission from a donor who has latent HCMV infection. The source of infection is the allograft in 90% of cases and in the remainder leukocyte-containing blood products are the source (Betts *et al.,* 1975; Grundy *et al.,* 1987; Rubin, 1993). Approximately 90% of seronegative recipients who receive kidneys from seropositive cadaveric donor, as opposed to 70% of seronegative recipients of kidneys from seropositive living-related donors, demonstrated posttransplant seroconversion. This difference in the incidence is probably related to the increased level of rejection and added amount of immunosuppressive therapy in cadaveric recipients (Rubin, 1985).

Secondary infection occurs due to reactivation of latent virus or reinfection during the immunosuppressed state (Suwarnasirikul *et al.,* 1977). In a seropositive recipient, the source of HCMV is thought to be reinfection or superinfection with donor strain virus rather than reactivation and is supported by typing of virus isolates (Grundy *et al.,* 1988). Since reinfection occurs despite the presence of specific antibody, cell-mediated immunity is probably important for the control of HCMV infection. In one study (Grundy *et al.,* 1988), reinfection was associated with symptomatic disease in 40% of individuals whereas none of those with endogenous reactivation became symptomatic.

The serological status of the donor and recipient is the most important risk factor influencing the incidence of CMV infection and disease (Rubin, 1993; Peterson *et al.,* 1980; Johnson *et al.,* 1988). In patients receiving kidneys from seropositive donors, the incidence of HCMV disease was 61% in HCMV seronegative recipients compared to 24% in seropositive recipients. In recipients of seronegative donors, those who were seropositive had a disease incidence of 20% compared with 2% who were

seronegative (Smiley *et al.,* 1985). In addition, simultaneous transplantation of organs (kidney-pancreas transplantation) is considered as a risk factor for HCMV infection and disease (Dunn *et al.,* 1991; Pouteil-Noble *et al.,* 1993) which is highly likely to be due to the increased magnitude of immunosuppression used in this group of patients. Likewise, a significantly high incidence of HCMV infection was observed in recipients of cadaveric organs compared to living-related sources and is probably due to the increased intensity of immunosuppression required to prevent and treat rejection in the former group (Johnson *et al.,* 1988; Peterson *et al.*, 1980; Lewis *et al.,* 1988; Dunn *et al.,* 1991). The age, sex and diabetic status of the recipient had no influence on HCMV infection or disease (Fryd, 1980).

The risk of HCMV infection after renal transplantation is determined by the interaction of two factors: the state of immunosuppression and exposure to the virus. These two factors have a semiquantitative relationship: if the state of immunosuppression is sufficient, even a trivial exposure to HCMV can cause severe clinical illness; conversely, if the viral load is sufficient, even an under immunosuppressed patient can develop life-threatening illness (Tolkoff-Rubin, 1994).

Acute rejection episodes occur concomitantly with HCMV infection and this relationship is well established. Moreover, HCMV infection may be associated with both acute and chronic rejection (Pouteil-Noble *et al.,* 1990). The incidence of acute rejection after HCMV infection is reported to be 45% among infected versus 10.6% among non-infected renal transplant recipients (Pouteil-Noble *et al.,* 1993).

The exact sequence of events regarding HCMV infection and acute rejection still remains controversial and cause or effect is debated. However, most data suggest that allograft rejection precedes active HCMV infection and that the increased immunosuppression given for the treatment of allograft rejection may be responsible for the infection, but that continuous allogenic stimulus by the graft may also be important. The replication of the latent virus in renal parenchymal cells could result in

new antigens in or on renal parenchymal cells being presented, which could add to the mismatch of the kidney and possibly contribute to rejection (Betts *et al.,* 1975).

Simmons *et al.,* (1970) first suggested that HCMV infection could be associated with an increased risk of allograft dysfunction from acute rejection and graft loss and that the rejection was related to tapering of immunosuppression during an active HCMV infection. Deterioration in renal function following HCMV infection could be due to systemic viral infection and associated allograft infection causing moderate non-specific dysfunction unrelated to rejection. However, this possibility seems unlikely since HCMV could not be cultured from renal biopsy specimens during the acute episodes of the disease (Lopez *et al.,* 1974). It was also postulated that allograft rejection was initiated by HCMV-specific, MHC-restricted T lymphocytes which are primarily directed against HCMV. These T lymphocytes could mediate rejection because of their cross-reactive recognition of the allogenic MHC antigens (Gaston *et al.,* 1985).

During rejection, the expression of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), and MHC molecules, are upregulated by several cytokines, such as interleukin-1, tumour necrosis factor-alpha, and γ-interferon, elaborated by activated immunocompetent cells. These adhesion molecules provide signal during antigen recognition for lymphocyte activation and have important functions in the pathogenesis of allograft recognition (Goral *et al.,* 1994).

HCMV upregulates both MHC class 1 and ICAM-1 expression as a direct effect of the virus on HCMV-infected proximal tubular epithelial cells (Grundy *et al.,* 1988; van Drop *et al.,* 1991). It has also been shown that MHC class II antigens, which are no longer detectable in renal allograft after successful transplantation, presumably due to administration of corticosteroids, are upregulated by HCMV via γ-interferon release by the activated T cells (Von Willebrand *et al.,* 1986). HCMV, by itself, acts as an independent source of γ-IF (Glasgow, 1974). γ-IF activates resting

monocytes into tissue macrophages which, in turn, release interleukin-1 (IL-1). These cascades of events may thus result in an increased antigen presentation and amplification of the immune response and clinical manifestations of acute rejection (Von Willebrand *et al.*, 1986).

Chronic rejection is a leading cause of graft loss and graft failure representing up to 35% of graft losses at 5-10 years following renal transplantation resulting in a progressive decline in renal function (Matas, 1994). The aetiology of chronic rejection is multifactorial involving both immune and non-immune endothelial injuries. HCMV disseminates through the blood stream and infection of both vascular smooth muscle and endothelium is a regular occurrence forming the foundation of chronic allograft injury in transplant recipients (Tumilowicz *et al.*, 1985; Smiley *et al.*, 1988).

1.2.3.3.2 Infection in bone marrow transplant recipients

HCMV disease is an important issue in bone marrow transplantation as demonstrated by the high mortality rate and occurrence of graft versus host disease (GVHD). A mortality rate of up to 90% has been reported due to untreated HCMV pneumonia (Ljungman *et al.*, 1990; Enright *et al.*, 1993; Foot *et al.*, 1993). The incidence of HCMV infection in bone marrow transplantation has reached up to 50% (Ljungman *et al.*, 1990; Nomura *et al.*, 1990; Rubie *et al.*, 1993) although higher incidence has been found in seropositive recipients of seropositive donors (up to 80%) comparing to 40% incidence observed in seronegative recipients of seropositive donors (Paulin *et al.*, 1986; Bowden, 1991). In general, however, the serological status of the donor and the recipient and the use of blood products represent important risk factors for HCMV infection in bone marrow transplantation (Miller *et al.*, 1991).

Clinical manifestations of HCMV disease in this population include haematological and hepatic abnormalities, gastrointestinal tract involvement but more significantly pneumonia, which progresses eventually leading to death. HCMV pneumonia demonstrated a mortality

rate of 80% although proper patient management and installation of appropriate therapy has led to reduction in the mortality rate down to 10 to 20% (Enright *et al.,* 1993; Goodrich *et al.,* 1993; Goodrich *et al.,* 1991).

1.2.3.4. Infections in HIV-infected hosts

HCMV plays a major role as one of the most important opportunistic infections in AIDS patients as 90% of AIDS patients develop active HCMV infection. This is probably due to the high seropositivity to HCMV in this population and the frequent risk of reinfection (Gallant *et al.*, 1992).

The clinical syndromes of HCMV infection in AIDS patients included almost every organ system but the lungs, CNS and the gastrointestinal tract are the most frequently affected systems (Heurlin *et al.,* 1991; Klatt and Shibata, 1988; Morgello *et al.,* 1987). The most severe and frequent clinical manifestations are pneumonitis, retinitis (Gallant *et al.,* 1992; Jabs *et al.,* 1989), encephalitis (Vinters *et al.,* 1989), gastritis (Francis *et al.,* 1989) and enterocolitis (Dieterich and Rahmin, 1991).

1.2.4. Laboratory diagnosis

The cornerstone of laboratory diagnosis remains the direct demonstration of virus in blood, respiratory secretions, urine, serum or tissues. Serological techniques are most useful for assessing past exposure to the virus, and thus for prediction of subsequent clinical disease in transplant patients.

HCMV can be cultured from specimens of blood, urine, bronchoalveolar lavage, throat or genital swabs on human embryonic lung (HEL) or foreskin fibroblasts. However, the conventional cell culture technique demonstrating cytopathic effect (CPE) takes 1-8 weeks for isolation of HCMV and is far too slow to allow therapy to be instituted. Moreover, the sensitivity of the method was found to be 76% (Stirk, 1987) which is not ideal.

Detection of early antigen fluorescent foci (DEAFF) as described by Griffiths *et al.,* (1984) allows a reduction in processing time by using short-term viral culture, followed by immunocytochemical detection with monoclonal antibodies directed against early or immediate early antigens of HCMV. Although the cells appear morphologically normal by light microscopy, under ultraviolet illumination the infected nuclei stain brightly. This technique enables a virological diagnosis within 24-48 hours (Griffiths *et al.,* 1984) accompanied by a sensitivity of 80% and a specificity of 100% when compared with the result of virus isolation. Griffiths *et al.,* (1984) reported that this method gave positive results 27 hours after inoculation of specimen instead of the mean of 17.5 days with the method based on the detection of cytopathic effect.

Other studies have used the DEAFF test incorporating centrifugation of the fibroblast monolayer inoculated with the clinical specimen, assisting in adsorption of the virus and thereby resulting in a four-fold increase in the sensitivity of the test (Hudson, 1988). Unfortunately, the sensitivity is reduced on buffy coat cultures because of leukocyte toxicity on monolayers and from the effect of the centrifugation procedure (Marsano *et al.,* 1990).

Van der Bij *et al.,* (1988) described an immunoperoxidase technique which is based on the detection of nuclear HCMV late antigen (HCMV-LA) in peripheral blood leukocytes (polymorphonuclear leukocytes and monocytes) with monoclonal antibodies to the specific HCMV antigen. The technique has a sensitivity of 93% and specificity of 92% for the diagnosis of active HCMV infection in addition to the ease of performance (Van der Bij *et al.,* 1988). This antigenaemia assay involves isolation of leukocytes by dextran sedimentation and cytocentrifugation onto microscope slides; indirect immunoperoxidase staining using a combination of monoclonal antibodies, followed by a counterstaining with haematoxylin and microscopic examination of the slides and counting of the number of positive cells. The test can be carried out within 3 hours as compared to ≥16 hours for DEAFF test (Griffiths *et al.,* 1984) and 24-48 hours for DNA-DNA hybridisation of leukocytes (Spector *et al.,* 1984b).

Advances in molecular hybridisation techniques have made it possible to detect HCMV genetic material using viral DNA probes in clinical specimens of urine, infected tissues and human leukocytes (Spector *et al.*, 1984b; Saltzman *et al.*, 1988; Yoshimura *et al.*, 1989). In one study, HCMV DNA was detected by dot-blot hybridisation in approximately 58% of blood specimens when no infectious HCMV could be recovered in culture (Saltzman *et al.*, 1988). Similarly, Spector *et al.*, (1984b) detected CMV DNA in 21/53 (40 %) of buffy coat samples obtained from patients without documented viraemia.

In situ hybridisation (ISH) is based on the ability of labelled probes to bind target DNA in thin formalin-fixed paraffin-embedded tissue sections or cytology smears on fine-needle aspiration biopsy or peripheral blood specimens. This technique reveals not only the presence of a specific sequence but also its spatial distribution within tissues or individual cells. ISH detects HCMV-infected cells even in the absence of typical nuclear inclusions in tissue sections (Ulrich *et al.*, 1986). ISH is found to be highly sensitive and specific compared to classical virus serology and can be an important diagnostic tool in differentiating between rejection and HCMV infection of the allograft (Arndt *et al.*, 1990).

More recently, the PCR for selective amplification of HCMV DNA has been shown to be a sensitive and specific method. The PCR has been applied to detect HCMV DNA in different clinical specimens. These include tissue sections, blood cells, plasma, serum, CSF, bronchoalveolar lavage and urine (Smith and Dunstan, 1993). The main controversy encountered in this application is the relevance of the positive result to the clinical manifestation investigated. The test may not be efficient in discriminating active infection from a latent infection. The latter has been partially overcome by the use of PCR tests with reduced sensitivity, detection of HCMV-specific mRNA by reverse transcriptase PCR (Patel *et al.*, 1995), the use of serum as opposed to whole blood and the application of quantitative PCR (Fox *et al.*, 1995).

Storch *et al.*, (1994) examined serial blood specimens for HCMV from 46 solid organ transplant recipients using PCR, pp65 antigenaemia assay and a quantitative shell vial culture (QSVC) technique. In a total of 535 specimens tested, HCMV was detected in 41 by PCR and in 37 patients by QSVC of 43 recipients at risk of HCMV infection. In a comparison of the 3 tests in a total of 395 specimens, HCMV was detected in 30, 32, and 35 of 38 patients by antigenaemia assay, QSVC, and PCR, respectively. The study concluded that PCR was indicative of active HCMV infection in their study as no HCMV DNA was detected in healthy HCMV seropositive volunteers although the need for quantitative PCR to distinguish clinically significant and insignificant HCMV infection was highlighted.

Several studies have demonstrated the significance of PCR on serum samples from patients at risk of HCMV disease as a sensitive approach for detecting symptomatic infection (Brytting *et al.*, 1992, Patel *et al.*, 1995, Tokimuatsu *et al.*, 1995; Eckart *et al.*, 1997; Cunningham *et al.*, 1995; Nelson *et al.*, 1995). In the study by Cunningham *et al*, (1995), a PCR for the detection of HCMV DNA in serum and leukocytes of 12 renal transplant recipients was developed. The PCR was compared to an enzyme immunosorbent assay for detection of IgM and virus isolation. In a total of 4 IgG positive patients and 8 seronegative patients, 2 of the 4 patients had reactivation of HCMV disease confirmed by culture, and 3 of the 8 seronegative patients had a primary infection confirmed by serology in 1 patient and by serology and culture in the other 2 patients. The PCR was reported as positive in 3 cases earlier than serology or cell culture and concurrently positive in 2 cases. All the 7 culture and serology-negative patients were negative by PCR. The study suggested that detection of HCMV DNA in serum may be a stronger indication of active infection and may provide a sensitive and an early indicator for primary and reactivation infections in renal transplant recipients.

In a prospective study of 40 renal transplant recipients, Eckart *et al.*, (1997) compared PCR for detection of HCMV DNA in sera to that in leukocytes, pp65 leukocytic antigenaemia assay and virus isolation.

HCMV infection could be established in 26 patients of whom 11 patients developed symptomatic infection. PCR in serum was positive in 21 patients but that in leukocytes was positive in all infected patients. The antigenaemia assay was positive in 21 patients, and viraemia was detected by cell culture in 13 patients. All the symptomatic patients were positive by PCR in sera and leukocytes whereas the antigenaemia was negative in 1 symptomatic patient and virus isolation was reported negative in 3 symptomatic patients. The study concluded that PCR in serum has the optimal specificity for predicting HCMV disease and that both PCRs are the most sensitive tests.

In urine samples, Demmler *et al.*, (1988), studied HCMV-culture positive samples of 44 congenitally infected newborns utilising PCR. The PCR was positive in all tested samples. The study suggested that the virus could be detected in urine as early as the first week of life, which may help in prompt diagnosis of CMV-associated disease such as hearing loss and mental retardation. In another report, Hsia *et al.*, (1989) utilised PCR for detection of HCMV DNA in 37 clinical urine samples. The test produced identical results to virus isolation in 21 as negative and in 14 as positive. In addition, the PCR was reported positive in the remaining 2 samples which were cell culture negative.

The PCR has also been used as a method to monitor urinary excretion of HCMV in patients undergoing bone marrow transplantation (Ohshima *et al.*, 1996). In a total of 61 patients, 15 were positive by PCR and only 4 were positive by culture. The study concluded that monitoring of virus excretion by PCR is a useful means by which prompt detection of HCMV infection may be achieved. This would allow for proper institution of antivirals in patients undergoing bone marrow transplantation.

1.2.5. Treatment

A number of antiviral drugs have been used for treatment of HCMV-associated diseases. These include interferon, interferon stimulators, transfer factors, and nucleoside drugs (iododeoxyuridine,

fluorodeoxyuridine, cytosine arabinoside, adenosine arabinoside and acyclovir) and more recently ganciclovir, foscarnet, cidofovir, labucavir and adefovir. Clinical disease targeted include congenital infections, mononucleosis syndrome in normal hosts, transplant recipients and AIDS patients (Griffiths and Whitley, 1997; de Clercq, 1998; de Jong *et al.*, 1998).

Antivirals such as acyclovir, ganciclovir, and foscarnet have been utilised in prophylactic regimens to prevent HCMV disease in transplant recipients. The preliminary data indicated encouraging results with ganciclovir and foscarnet (Noble and Faulds, 1998; Birkeland *et al.*, 1998).

Another prophylactic approach is the use of passive and active immunisation. Passive immunisation is based on the use of intravenous immunoglobulins but revealed discordant results and its role requires further investigation (Snydman *et al.*, 1987). Active immunisation utilises live attenuated viral particles and subunit vaccines. The latter demonstrated immunogenic nature *in vitro* and induced both cellular responses and virus-neutralising antibodies *in vivo* (Gonczol and Plotkin, 1990; Plotkin *et al.*, 1990; Spaete, 1991).

Another approach of managing HCMV disease is the use of antivirals to prevent HCMV disease in high-risk patients prior to the appearance of disease. This strategy is known as pre-emptive therapy (Patel *et al.*, 1997; Griffiths and Whitley, 1997).

1.3. Human herpesvirus 6

The first description of HHV-6 was by Salahuddin *et al.,* (1986) and Josephs *et al.,* (1986) from people with AIDS and lymphoproliferative diseases. The virus has distinct growth properties, antigenicity and genetic composition that differed from the previously characterized herpesviruses. The virus is lymphotropic in nature and has been isolated from both B and T lymphocytes (Ablashi *et al.,* 1987 Agut *et al.,* 1988; Becker *et al.,* 1988; Downing *et al.,* 1987; Lopez *et al.,* 1988; Lusso *et al.,* 1988; Takahashi *et al.,* 1989; Tedder *et al.,* 1987).

1.3.1. Epidemiological features

HHV-6 is acquired at an early stage of life and up to 83% are infected by the age of 13 years (Briggs *et al.,* 1988; Brown *et al.,* 1988; Huang *et al.,* 1992; Knowles and Gardner, 1988; Okuno *et al.,* 1989; Yoshikawa *et al.,* 1989). Using PCR, up to 10% of infants less than 1 month of age were positive for HHV-6 DNA which is significantly less than observed at 1 year of age (66%) (Hall *et al.,* 1994). Several studies have shown that the seropositivity to HHV-6 declined with age among adults (Brown *et al.,* 1988b; Enders *et al.,* 1990; Levy *et al.,* 1990a; Niederman *et al.,* 1988; Yanagi *et al.,* 1990). However, Briggs *et al.,* (1988) and Okuno *et al.,* (1989) found no significant difference with age and Yamanishi, (1992) demonstrated an increase in this value after age of 62 years.

The seroprevalence also differed depending on the location in which the study was performed. It has been reported as low as 20% in pregnant Moroccan women (Ranger *et al.,* 1991) and as high as 100% among asymptomatic Chinese adults (Levy *et al.,* 1990a). Among pregnant women in sub-Saharan Africa the prevalence is between 60 and 90% (Ranger *et al.,* 1991) and varies in Tanzania, Malaysia, Thailand, and Brazil from approximately 39 to 80% (Balachandra *et al.,* 1989; Buchbinder *et al.,* 1989; Essers *et al.,* 1991; Levine *et al.,* 1992b; Linhares *et al.,* 1991; Yadav *et al.,* 1991). The seroprevalence has been reported to be higher in females than males in some studies (Linhares *et al.,* 1991;

Clark *et al.,* 1990, Buchbinder *et al.,* 1989; Briggs *et al.,* 1988) but at least one study by Saxinger *et al.,* (1988) showed no difference in seroprevalence between males and females.

Two groups of this virus have been identified as HHV-6 variants A and B (Ablashi *et al.,* 1993). Although these variants show extensive cross hybridisation with each other and have identical sizes and base composition (Lindquester *et al.,* 1991; Martin *et al.,* 1991b), they have shown different epidemiological features, growth properties, sero-reactivity and nucleotide sequences (Di-Luca *et al.,* 1998; Aubin *et al.,* 1991; Ablashi *et al.,* 1991; Aubin *et al.,* 1993; Dewhurst *et al.,* 1992; Dewhurst *et al.,* 1993b; Schirmer *et al.,* 1991).

HHV-6 variant A was isolated from a 17-year-old with acute lymphoblastic leukaemia (Salahuddin *et al.,* 1986) and has been obtained mostly from chronically ill adults (Aubin *et al.,* 1991; Ablashi *et al.,* 1991; Downing *et al.,* 1987) and also in cases of Kaposi's sarcoma (Bovenzi *et al.,* 1993), chronic fatigue syndrome (Di Luca *et al.,* 1995b; Yalcin *et al.,* 1994) and AIDS patients (Knox and Carrigan, 1996b; Salahuddin *et al.,* 1986). The variant B of HHV-6 has been frequently isolated from children especially those with exanthem subitum (ES) (Dewhurst *et al.,* 1993a; Schirmer *et al.,* 1991; Pellett *et al.,* 1990).

HHV-6 transmission has been linked with saliva. HHV-6 variant B isolates have been obtained from saliva (Black *et al.,* 1993; Levy *et al.,* 1990a; Mukai *et al.,* 1994) but the occurrence of HHV-6 variant A isolates in saliva has yet to be described. Thus, the saliva represents a possible mode of HHV-6 transmission from mothers to their infants (Mukai *et al.,* 1994; Suga *et al.,* 1995).

Intra-uterine or prenatal transmission as a mode of HHV-6 transmission has also been speculated. In the study by Hall *et al.,* (1994), approximately 13% of infants with primary HHV-6 infection were younger that 2 months of age. Aubin *et al.,* (1992) found that one of 52 aborted foetus was positive for HHV-6 DNA. In this case the virus was detected in peripheral

blood lymphocytes, thymus, liver, spleen, brain, and CSF and also in abortive chorionic villous tissue of women who experienced spontaneous abortions. In another study by Okuno *et al.,* (1995), HHV-6 DNA was detected in the cervices of about 20% of women during late pregnancy, compared to 6% in non pregnant controls which might suggest HHV-6 reactivation during pregnancy and possible prenatal transmission of the virus. Mendel *et al.,* (1995) described two cases of HHV-6-associated fulminant hepatitis in neonates (3 and 5 days old) which is clearly suggestive of pre-or peri natal transmission.

Supporting the possibility of both prenatal and sexual spread, using PCR, Leach *et al.,* (1994) detected HHV-6 DNA in the vaginal secretions of 10% of women attending a sexually transmitted diseases clinic.

Suga *et al.,* (1995) demonstrated that HHV-6 DNA can be detected by PCR in stool specimens for up to 6 months after an episode of ES. This indicates that the faecal-oral route could be another potential mode of transmission among children.

1.3.2. Pathogenesis

HHV-6 replicates most efficiently *in vitro* in activated primary T cells and continuous T-cell lines. Although the primary target of the virus is the CD4+ T cells, HHV-6 has been detected in lymph nodes (Levine *et al.,* 1992a) lymphocytes (Jarrett *et al.,* 1990; Cuende *et al.,* 1994), macrophages and monocytes (Kondo *et al.,* 1991), kidney tubule endothelial cells (Okuno *et al.,* 1990a), salivary glands (Fox *et al.,* 1990; Krueger *et al.,* 1990); and in the central nervous system (Luppi *et al.,* 1994; Luppi *et al.,* 1995). In these cells the virus does not shut off host cell protein synthesis (Balachandran *et al.,* 1989). In contrast, increased expression of α-interferon (Kikuta *et al.,* 1990a), $CD4^+$ (Lusso *et al.,* 1991a), interleukin-1β, and tumor necrosis factor-α has been demonstrated (Flamand *et al.,* 1991). In addition, HHV-6 infection resulted in elevation of the expression of $CD4^+$ molecules which was found to increase the susceptibility of lymphocytes to infection with HIV-

1 (Lusso *et al.,* 1991a). Indeed, HHV-6 and HIV-1 can productively infect the same types of cells and may productively coinfect human $CD4^+$ T lymphocytes (Carrigan *et al.,* 1990; Levy *et al.,* 1990b; Lusso *et al.,* 1989). HHV-6 can trans-activate the transcription of the long terminal repeats of HIV-1 (Ensoli *et al.,* 1989; Di Luca *et al.,* 1991; Horvat *et al.,* 1989) and superinfection with HHV-6 of cells that are latently infected with EBV can activate EBV lytic replication (Flamand *et al.,* 1993).

HHV-6 infection results in both humoral and cellular immune response. The humoral immune response is protective in nature as suggested by the protective role of residual maternal antibodies (Hall *et al.,* 1994; Huang *et al.,* 1992; Okuno *et al.,* 1990b; Knowles and Gardner, 1988) and neutralising antibodies that are virus-specific (Asano *et al.,* 1989).

The importance of the cellular immune responses may be highlighted by the activity of T cell clones against HHV-6 infection *in vitro* (Yasukawa *et al.,* 1993) and the occurrence of either primary or reactivated HHV-6 infection in immunocompromised patients. The latter could be illustrated by HHV-6 viraemia or increase in antibody titre after renal transplantation (Yoshikawa *et al.,* 1992a; Merlino *et al.,* 1992; Okuno *et al.,* 1990a) and isolation of HHV-6 or detection of its DNA from the blood, bone marrow, and bronchial lavage (BAL) or oral lavage specimens (Carrigan *et al.,* 1991; Carrigan and Knox, 1994; Drobyski *et al.,* 1993; Kadakia *et al.,* 1996; Wilborn *et al.,* 1994; Cone *et al.,* 1993) from bone marrow transplant (BMT) recipients. In addition, widespread dissemination of HHV-6 in various tissues of AIDS patients has been demonstrated (Corbellino *et al.,* 1993; Knox and Carrigan, 1994a).

Other anti-HHV-6 non-specific immunological responses including the production of interferon α (IFN-α) (Kikuta *et al.,* 1990a; Takahashi *et al.,* 1992), and up regulation of natural killer cell cytotoxicity (Flamand *et al.,* 1996) may be important factors in controlling HHV-6 infection.

A number of studies have suggested that HHV-6 is an immunosuppressive virus. HHV-6 variant A infection of T cell clones *in vitro* resulted in

decreased expression of surface CD3 (Furukawa *et al.,* 1994; Lusso *et al.,* 1991b) and both HHV-6 variants have been demonstrated to possess an inhibitory effect on the lymphoproliferative responses of peripheral blood mononuclear cells to mitogen stimulation (Flamand *et al.,* 1995; Horvat *et al.,* 1993). Thus, HHV-6 infection-caused immunosuppression could be the ultimate cause of the concurrent infections of HHV-6 with other human pathogens such as measles, EBV, HCMV, HSV-1, adenovirus, parvovirus B19, and Legionella (Balachandran *et al.,* 1991; Chou and Scott, 1990; Irving *et al.,* 1990a; Knox *et al.,* 1995; Portolani *et al.,* 1993; Russler *et al.,* 1991; Suga *et al.,* 1990a).

HHV-6 persistence and latency in the host is the matter of current investigation. Consistent with persistence is the finding that 5% of the adult population without recent or concomitant illness have HHV-6 IgM antibodies (Suga *et al.,* 1992). The increase in IgG titre in immunocompromised patients is suggestive of HHV-6 reactivation (Okuno *et al.,* 1990a; Merlino *et al.,* 1992; Yoshikawa *et al.,* 1992a; Hall *et al.,* 1994). The latter has been suggested to be triggered by infection with other herpesviruses (Ward *et al.,* 1991; Torigoe *et al.,* 1995; Ueda *et al.,* 1994; Hidaka *et al.,* 1994). In the study by Hall *et al.,* (1994), about 7% of children with acute HHV-6 infection had sequential HHV-6 PCR results that changed from positive to negative to positive in peripheral blood mononuclear cells over a 1 to 2 year follow-up period which might indicate either reactivation or reinfection. Yoshikawa *et al.,* (1992b) demonstrated an identical HHV-6 DNA restriction fragment pattern in lymphocytes from a bone marrow transplant patient both before and after transplantation which strengthen the argument for persistent infection. Other suggested latency sites include macrophages (Kondo *et al.,* 1991), salivary glands (Fox *et al.,* 1990; Krueger *et al.,* 1990), CNS (Challoner *et al.,* 1995), kidneys (Hoshino *et al.,* 1995), lungs (Cone *et al.,* 1993a; Cone *et al.,* 1996), cervix and vaginal secretions (Leach *et al.,* 1994; Okuno *et al.,* 1995). However, cell lines in which HHV-6 remains in a latent state without viral replication have yet to be developed. Thus, whether HHV-6 establishes a true latency or persistent infection is still to be determined.

1.3.3. Clinical Syndromes

The childhood disease ES (roseola infantum or sixth disease) is the classical manifestation of the HHV-6 primary infection (Yamanishi *et al.*, 1988). The disease is characterised by high fever (up to 5 days), erythematous and macular or maculopapular rash (up to 3 days), lymphocytosis and neutropenia (Krugman *et al.*, 1993; Balachandra *et al.*, 1991). Clinical findings such as diarrhoea, cough, oedematous eyelids, bulging fontanelle, non-specific prodrome and febrile convulsions are not uncommon (Asano *et al.*, 1994). However, these symptoms are not HHV-6 specific (Suga *et al.*, 1989) and the clinical manifestations of ES described above may resemble that of measles or rubella (Black *et al.*, 1996; Tait *et al.*, 1996; Halawani and Ayed, 1998).

A number of studies have shown that not all HHV-6 primary infection manifest as classical ES. Yoshida *et al.*, (1995) described a child with vesicular lesions which were similar to those of varicella and were found to be HHV-6 positive by PCR. Pruksananonda *et al.*, (1992) studied the clinical presentation of 34 febrile children with HHV-6 viraemia. Of these children, 3 (9%) developed rash following resolution of fever, 22 (65%) had high temperature, 28 (82%) had malaise and irritability and 21 (62%) manifested with tympanic membrane inflammation.

Other studies presented as case reports have shown various clinical manifestation that could be associated with primary HHV-6 infection. These include hepatosplenomegaly (Huang *et al.*, 1991), fatal fulminant hepatitis (Mendel *et al.*, 1995), liver dysfunction (Tajiri *et al.*, 1990; Takikawa *et al.*, 1992), infectious mononucleosis-like illnesses (Hanukoglu and Somekh, 1994; Kanegane *et al.*, 1995; Vanzeijl *et al.*, 1995) intussussception (Asano *et al.*, 1991a), thrombocytopenic purpura (Kitamura *et al.*, 1994; Saijo *et al.*, 1995) fatal haemophagocytic syndrome (Huang *et al.*, 1990), meningo-encephalitis (Ishiguro *et al.*, 1990; Yoshikawa *et al.*, 1992c) encephalopathy (Jones *et al.*, 1994), and fatal disseminated infection (Prezioso *et al.*, 1992).

Although relatively rare, HHV-6 primary infection in adults has also been reported and appears to be more severe. This includes mononucleosis-like syndrome (Akashi *et al.*, 1993; Goedhard *et al.*, 1995; Steeper *et al.*, 1990), prolonged lymphadenopathy (Niederman *et al.*, 1988) and fulminant hepatitis (Sobue *et al.*, 1991).

1.3.3.1. Renal transplant recipients

Different studies have aimed to investigate the role of HHV-6 in renal transplant recipients. Okuno *et al.*, (1990a) showed that 8 of 21 renal allograft recipients had significant post-transplantation rises in HHV-6 titre with concurrent severe rejection in contrast to only 5 patients of the 13 recipients, who did not have significant increase of antibody titre, experienced rejection. Merlino *et al.*, (1992) demonstrated no correlation between HHV-6 infection and rejection in a study of 53 renal allograft recipients. In another study, Yoshikawa *et al.*, (1992a) reported that HHV-6 viraemia or significant antibody titre increases to HHV-6 in 55% of 65 renal transplant recipients had no correlation with transplant rejection.

Yalcin *et al.*, (1994) investigated 16 renal transplant patients and detected HHV-6 DNA by PCR in the peripheral blood mononuclear cells of 10 (63%) patients compared with 7 (44%) of 16 healthy controls. HHV-6 variant A and B were detected in 3 and 7 patients of the 10 transplant recipients. HHV-6 was detected in 5 transplant patients who experienced rejection.

Utilising immunohistochemical techniques, Hoshino *et al.*, (1995) performed retrospective investigation on 105 biopsy samples of 76 renal transplant recipients and detected HHV-6 in 63 (61%) samples with significant correlation with the transplant rejection. HHV-6 antigens were detected in 28 (73.7%) of 38 specimens from patients with acute rejection, 3 (75%) of 4 specimens from patients with accelerated rejection, 8 (72.7%) of 11 specimens from patients experiencing cyclosporine nephropathy and in 11 (46%) of 24 samples from transplant recipients with chronic rejection.

1.3.3.2. Bone marrow transplant recipients

In bone marrow transplant (BMT) recipients, HHV-6 infection has been associated with various clinical manifestations including rash, graft versus host disease, pneumonitis, sinusitis, febrile episodes and suppression of graft outgrowth.

Drobyski *et al.,* (1994) associated HHV-6 variant B detection with fatal encephalitis in bone marrow transplant recipients. Yoshikawa *et al.,* (1991) studied, prospectively, 25 pediatric patients who received bone marrow transplantation to determine the relationship between bone marrow transplantation and HHV-6 infection by virus isolation from peripheral blood mononuclear cells (PBMC) or bone marrow mononuclear cells and by determining neutralisation antibodies to HHV-6 during the 2 months following the transplant. All the patients were immune to HHV-6 at the time of the transplant showing negative result by virus isolation. HHV-6 was detected by virus isolation and/or neutralisation test for detection of antibodies from 12 (48%) of the recipients between day 14 and day 22 of the transplant. Of the 12 positive patients, 4 developed a skin rash resembling GVHD. In the 13 patients who were HHV-6 negative, none developed a skin rash. The study suggested a frequent infection with HHV-6 only a few weeks after the transplant that was closely associated with development of skin rashes.

Asano *et al.,* (1991) reported detection of HHV-6 in 3 children with leukemia after bone marrow transplantation. The virus was isolated from the blood of the recipients on day 15 after transplantation. Of the 3 patients, 2 had fourfold rises in neutralising antibody titers to HHV-6. The authors suggested reactivation of HHV-6 in these patients since both recipients and donors had neutralising antibodies but no virus was detected in their blood at the time of transplantation. Reactivation of the virus may occur during neutropenia a few weeks after bone marrow transplantation and the infection may cause episodes of fever and skin rash that resemble those of GVHD in some patients.

Knox and Carrigan, (1992) demonstrated in an *in vitro* study a possible suppression of bone marrow progenitor cell differentiation by HHV-6. Normal bone marrow mononuclear cells were infected with HHV-6 and the impact of the infection on cell differentiation and proliferation was evaluated using methylcellulose-based colony formation assay. The assay showed that the outgrowth of colony-forming units of granulocyte and macrophage lineage was decreased by approximately 43% and that of granulocyte, erythrocyte, macrophage and megakaryocyte lineage was inhibited by an average of 71% whereas the erythroid burst-forming unit was decreased by 73%. In addition, outgrowth of the marrow stromal layer was reduced by 74%. The virus was detected in bone marrow monocytes although cell-free virus could not be detected in infected culture supernatants. Furthermore, addition of a neutralising monoclonal antibody specific for interferon-α to the infected cultures resulted in significant reversal of the viral suppressive effects.

Drobyski *et al.,* (1993) investigated HHV-6 infection in 16 allogeneic bone marrow transplant recipients for the first 100 days after transplantation. Analysis of the clinical course of these patients revealed the occurrence of posttransplant marrow suppression in 5 patients. The suppression occurred more frequently in patients with HHV-6 viremia (4/6) than in those from whom the virus was not isolated (1/10). This effect was confirmed by the isolation of the virus from the bone marrow of all 4 patients at the time of marrow suppression and by *in vitro* colony-forming unit assay. All the clinical isolates were typed as variant B. The study concluded that HHV-6 might be implicated as a novel cause of bone marrow suppression in marrow transplant recipients.

Wilborn *et al.,* (1994) evaluated the potential role of HHV-6 infection in patients after bone marrow transplantation. PCR was utilised to investigate HHV-6 DNA in urine specimens, buffy coat leukocytes and oral lavage fluid from 60 bone marrow transplant recipients. HHV-6 was detected in 36 patients (60%). The majority of the positive patients had positive results only sporadically. A total of 23 patients were positive in 1 week after transplant, 5 patients after 2 weeks, 6 patients were positive after 3 to

5 weeks and in 2 patients the frequency of positive tests was high in 7 of 7 weeks and 10 of 10 weeks analysed. A total of 24 patients (40%) remained PCR negative throughout the post-transplant period. There was a correlation between the results of HHV-6 PCR and the occurrence of acute GVHD. Typing of the PCR products from 18 patients showed HHV-6 variant B in 16 (88.9%) and variant A in 2 cases (11.1%). The study concluded that detection of HHV-6 DNA is associated with moderate and severe acute GVHD.

Michel *et al.,* (1994) have provided a detailed description of the exanthems in an infant who had three successive bone marrow transplants due to severe combined immunodeficiency (SCID). GVHD with skin and liver involvement occurred after the third transplant, which resolved after long-term steroid therapy. Chronic dermatitis but with no GVHD was observed 12 months later which was consistent with the detection of HHV-6 DNA in tissue samples (the samples were negative for parvovirus B19 and other herpesviruses). In another study by Appleton *et al.,* (1994), the dermatological manifestation exanthematous papular rashes were observed in 6 BMT recipients treated for SCID or osteopetrosis who were positive for HHV-6 DNA by PCR. Of the 6 patients, 2 had dermatitis and 4 had osteopetrosis and GVHD.

Carrigan *et al.,* (1991) suggested an association of HHV-6 with pneumonitis in BMT recipients. In this study, HHV-6 was isolated from the blood and bone marrow of an adult who received an autologous transplant for testicular carcinoma and died of progressive pneumonitis. Immunohistochemical staining showed widespread HHV-6 infection.

Pitalia *et al.,* (1993) aimed to investigate paraffin-embedded lung tissue specimens from 7 bone marrow transplant recipients who died of interstitial pneumonitis. Immunohistochemical staining demonstrated HHV-6 antigens in pneumocytes from 6 of the patients. In addition, the 6 patients were positive for adenovirus antigen and 4 were positive for HCMV.

Using quantitative PCR, Cone *et al.,* (1993) found that 6 (40%) of 15 bone marrow transplant recipients had significantly higher levels of HHV-6 DNA in lung biopsy specimens compared to immunocompetent controls. The severity of acute GVHD in these bone marrow transplant recipients correlated with the levels of HHV-6 DNA. However, only 1 of the 6 patients died of pneumonitis whereas 6 of the remaining 9 recipients died of acute lung disease. The latter finding might suggest that pneumonitis due to HHV-6 may not be as severe as that caused by other infectious agents.

Kadakia *et al.,* (1996) studied 15 allogeneic and 11 autologous marrow transplantation patients. Post-transplantation, HHV-6 variant B was isolated from the peripheral blood mononuclear cells of 12 patients (6 allogeneic and 6 autologous) and the infection was detected in another 11 patients by salivary shedding and increased antibody titre. Thus, 23 patients showed evidence of active HHV-6 infection either by virus isolation, salivary shedding or increase in antibody titre. The isolation of HHV-6 was associated with active HCMV infection but no association was found between detection of HHV-6 and GVHD, pneumonia, delay in engraftment or marrow suppression.

Rieux *et al.,* (1998) provided a case report of a recipient of an unrelated allogeneic bone marrow transplantation with neurological symptoms. The authors described a case of a woman with acute myeloid leukemia in second remision who developed febrile meningoencephalitis 8 months after a second unrelated bone marrow transplantation. Computed tomography and magnetic resonance images of the brain were non-specific but HHV-6 DNA was detected by PCR in CSF, peripheral blood mononuclear cells and bone marrow. The patient was treated with ganciclovir and foscarnet for 3 months resulting in resolution of the symptoms and PCR negative result 40 days after the beginning of antiviral therapy.

Wang *et al.,* (1999) utilised PCR to detect HHV-6 DNA in CSFs from allogeneic bone marrow transplant patients. The test was applied on CSF

from 22 bone marrow transplant patients and 107 patients who were immunocompromised but did not have CNS symptoms as controls. HHV-6 DNA was detected in 5 (23%) of 22 cases and in CSF specimens from 1 (0.9%) of 107 controls. None of the 5 cases with HHV-6 DNA detected in CSF samples had any other identified cause of the CNS symptoms. In only 3 cases was the variant of HHV-6 identified (HHV-6 variant B). The use of acyclovir as a prophylactic regime did not prevent the occurrence of HHV-6-associated CNS disease but 4 of the 5 cases improved or they were cured after treatment with either ganciclovir or foscarnet.

Cone *et al.,* (1999) prospectively evaluated the DNA levels of HHV-6 in peripheral blood mononuclear cells of 20 CMV-seronegative allogeneic bone marrow transplant patients and 10 healthy control subjects. A quantitative PCR was used to evaluate blood and saliva specimens collected weekly for 3 months after transplantation. Of the 20 patients, 1 experienced a primary HHV-6 infection after transplantation as determined by seroconversion, HHV-6 viraemia and the appearance of skin rash; 18 patients had increased peripheral blood mononuclear cells HHV-6 DNA levels that were consistent with asymptomatic HHV-6 reactivation and 1 patient developed a reactivation-associated skin rash. HHV-6 variant B was detected in all cases. Installation of acyclovir or intravenous immunoglobulin was not correlated with lower HHV-6 DNA levels. The authors concluded that HHV-6 reactivation may be common following allogeneic marrow transplantation and that the infection could be associated with self-limited clinical symptoms such as diffuse maculopapular rash.

Maeda *et al.,* (1999), using nested PCRs, compared the prognosis of HHV-6 infection in 16 allogeneic peripheral blood stem cell transplantation and 22 bone marrow transplantation cases. HHV-6 DNA was detected most frequently at 3 weeks post-transplantation. The detection rates of HHV-6 after bone marrow transplantation were significantly higher that those after peripheral blood stem cell transplantation. The authors suggested an advantage for the latter

transplantation over the former in terms of suppression of HHV-6 reactivation and prevention of subsequent complications.

1.3.3.3. Other transplants recipients

In liver transplant recipients, HHV-6 infection has been demonstrated on the basis of seroconversion and viraemia during a febrile episode in liver transplant recipient from a seropositive donor (Ward *et al.,* 1989). In another study by Singh *et al.,* (1995), clinical manifestations of fever, rash, thrombocytopenia and encephalopathy have been associated with HHV-6 viraemia. Sutherland *et al.,* (1991) demonstrated seroconversion or a significant rise in antibody titre to HHV-6 in 7 patients of 50 liver transplant recipients.

1.3.3.4. Clinical manifestation in HIV-infected patients

Several studies have aimed to investigate for HHV-6 in HIV-infected individuals and produced rather contrasting results. In some studies no difference in seroprevalence and antibody titre to HHV-6 between HIV-infected and uninfected individuals was observed (Brown *et al.,* 1988; Essers *et al.,* 1991; Fox *et al.,* 1988). In the study by Spira *et al.,* (1990) lower prevalence was found among those infected with HIV whereas Ablashi *et al.,* (1988) found higher prevalence when compared with uninfected controls.

Another criterion of HHV-6 investigation in AIDS patients is the correlation of seroprevalence with AIDS progression. Spira *et al.,* (1990) found no correlation between HHV-6 seroprevalence and progression of AIDS. In contrast, Chen *et al.,* (1992) showed that slow progressors have decreased HHV-6 seroprevalence when compared with rapid progressors.

The frequency of HHV-6 detection in HIV-seropositive individuals is also varied in different publications. It ranged from 3 to 63% in saliva from HIV-negative individuals and from 0 to 96% from HIV-infected

individuals (Di Luca *et al.*1995a; Fairfax *et al.,* 1994; Gautheret *et al.,* 1995; Gopal *et al.,* 1990) without any significant difference in the quantity of the viral DNA between the two groups (Cone *et al.,* 1993b; Fairfax *et al.,* 1994). The corresponding figure in peripheral blood mononuclear cells in both groups ranged from 10 to 49% and 10 to 75%, respectively (Fairfax *et al.,* 1994, Gautheret *et al.,* 1995; Gopal *et al.,* 1990). In contrast to this variation observed, Gopal *et al.,* (1990) detected a higher frequency of HHV-6 DNA in HIV-seronegative than in HIV-seropositive individuals while Gautheret *et al.,* (1995) demonstrated no difference between the two groups.

A number of studies have suggested that HHV-6 may have a pathogenic role in HIV infection progression. Madea *et al.,* (1990) detected HHV-6 DNA with low frequency in cells from the lymph nodes using *in situ* hybridisation. Dolcetti *et al.,* (1996) detected HHV-6 DNA in lymph nodes of 13 (65%) of 20 HIV-seropositive patients with nonneoplastic reactive lymphadenopathy and in only 2 (20%) of 10 HIV-seronegative patients. All the 13 HHV-6-positive specimens revealed follicular hyperplasia. Knox and Carrigan, (1996b) demonstrated active HHV-6 infection in lymph nodes of HIV-seropositive patients who presented with lymphadenopathy. HHV-6 antigens were detected using immunohistochemistry in lymph node specimens demonstrating follicular hyperplasia with histiocytosis and reactive lymphadenitis.

HHV-6 infection in AIDS patients could manifest as disseminated infection with the virus being detectable in many organs of the body. Corbellino *et al.,* (1993) detected HHV-6 DNA by PCR in 85% of diverse specimens obtained from 5 patients who died of AIDS while only 54% of similar tissues were positive in two HIV-negative controls. Knox and Carrigan, (1994a) detected HHV-6 antigens using immunohistochemical staining in 34 samples from the lungs, lymph nodes, spleen, liver and kidneys of 9 AIDS patients.

HHV-6 DNA and antigens have been detected in AIDS-associated retinitis although in the presence of those from HCMV and HIV (Qavi *et al.,*

half of 17 normal-appearing retinas of AIDS patients and in 5 retinas showing microlesions of uncertain aetiology. HCMV was not detected in any of these cases.

1.3.3.5. Other clinical syndromes

The association of HHV-6 with other clinical manifestations has also been reported. These include pneumonitis (Cone *et al.,* 1994; Portolani *et al.,* 1996; Lusso and Gallo, 1994), CNS disease (Saito *et al.,* 1995; Knox and Carrigan, 1995; Patnaik and Pater, 1995) and neoplasia (Fillet *et al.,* 1995; Dolcetti *et al.,* 1996; Kempf *et al.,* 1995). HHV-6 has also been associated with other illness with still uncertain aetiology. These include multiple sclerosis (Spira *et al.,* 1990; Wilborn *et al.,* 1994; Challoner *et al.,* 1995) non-Hodgkin's lymphomas (Borisch *et al.,* 1991) Hodgkin's disease (Torelli *et al.,* 1991) and chronic fatigue syndrome (Yalcin *et al.,* 1994; Di Luca *et al.,* 1995b).

1.3.4. Laboratory Diagnosis

HHV-6 can be detected in different clinical specimens using cell culture isolation (Drobyski *et al.,* 1993a; Carrigan and Knox, 1994), serological techniques (Chou and Scott, 1990) and more efficiently using the PCR (Drobyski *et al.,* 1993b; Wilborn *et al.,* 1994; Secchiero *et al.,* 1995a).

As the case with most viruses, cell culture is usually expensive and needs up to 20 days for the cytopathic effect to be observed. Confirmation of HHV-6 in cell culture also requires the use of monoclonal antibodies, oligonucleotide probes, or sometimes amplification of specific DNA by PCR (Campadelli *et al.,* 1993). In order to reduce the time of cell culture positivity, a rapid shell vial assay that detects HHV-6 early antigen within 72 hours has been developed but the test has poor sensitivity (Carrigan *et al.,* 1996).

In serological methods, HHV-6-specific IgG and IgM antibodies can be detected using different test formats which include anti-complement immunofluorescence (Couillard *et al.,* 1992; Robert *et al.,* 1990),

immunofluorescence (Couillard *et al.,* 1992; Robert *et al.,* 1990), neutralization (Suga *et al.,* 1990b; Suga *et al.,* 1992), competitive radioimmunoassay (Coyle *et al.,* 1992), and EIA (Asano *et al.,* 1990; Parker and Weber, 1993). However, the test might not be useful in a clinical setting due to the high prevalence of the antibodies in the adult population (Briggs *et al.,* 1988) or the presence of maternal antibodies for the first six months of life and cross reactivity of HHV-6 with HCMV and HHV-7 (Adler *et al.,* 1993; Berneman *et al.,* 1992). In addition, a test format that discriminates between the two variants of HHV-6 has yet to be established.

In formalin-fixed paraffin-embedded tissues, HHV-6 has been detected using immunohistochemical stains (Pitalia *et al.,* 1993). The test has the potential of detecting productive infection as a marker of active infection (Drobyski *et al.,* 1994; Knox and Carrigan, 1996b).

The diagnostic methods that are based on detection of viral antigens or genome are more rapid and have potential to support of supporting clinical investigation (Lusso and Gallo, 1995). The PCR as a method of detecting HHV-6 DNA has proved useful in this concept. However, the advantages of HHV-6-PCR can be offset by the presence of HHV-6 in the saliva of laboratory personnel which can lead to false-positive PCR results if anti-contamination measures are not strictly implemented (Cone *et al.,* 1990) and more important is the discrimination power of the test to discriminate between active and latent infection.

Detection of HHV-6 DNA by PCR in blood cells or tissue has the limitation of the difficulty in interpreting positive result with respect to disease. This is because of the possibility of latent HHV-6 infection rather than active infection. In this setting even quantitative PCR (Cone *et al.,* 1993a; Secchiero *et al.,* 1995) had difficulty diagnosing active infection. In contrast, detection of HHV-6 DNA by PCR in serum or plasma specimens that are cell-free may be the only means to achieve reliable diagnosis of active infection. In support of this is the finding that HHV-6 DNA was detected in serum specimens from 16 (94%) of 17 ES patients, 3

(23%) of 13 BMT recipients and 4 (22%) of 18 HIV-infected patients but in none of serum specimens taken from 57 healthy adults (Huang *et al.*, 1992; Secchiero *et al.*, 1995).

Bland *et al.*, (1998) compared PCR-based method in serum with serology and antigen detection method on specimens from 67 febrile children. Of these children 19 (28.4%) had no evidence of HHV-6 infection and remained PCR negative, with no IgM or IgG. Thirty-three of the children (49.2%) were seropositive and negative PCR and IgM. Four of the children (6%) were only PCR positive. Eleven of the children (16.4%) demonstrated IgG seroconversion or four-fold rise in antibody titre but only 5 were PCR positive of whom only one had IgM. The remaining 6 of the 11 patients showed IgM but were PCR negative. The study concluded that rapid diagnosis of HHV-6 could be achieved by application of PCR in serum specimens along with the use of IgM serology.

1.3.5. Treatment

Based on results obtained in cell culture and on patients with disease, treatment of HHV-6 infections has a variant specific-pattern. HHV-6 variant B produced the same antiviral susceptibility as HCMV and has been shown to be more sensitive to ganciclovir and foscarnet than to acyclovir. In contrast, HHV-6 variant A is susceptible to acyclovir and foscarnet but seems to be resistant to ganciclovir (Drobyski *et al.*, 1993; Jacobs *et al.*, 1994; Braun *et al.*, 1997).

1.4. Human herpesvirus 7

The description of HHV-7 was established by Frenkel *et al.,* (1990a) during a study involving HIV-1 growth in cell culture. Spontaneous cytopathic effect was observed in cell cultures of uninfected $CD4^+$ cells that were purified from the peripheral blood mononuclear cells of a healthy donor. The cells remained negative for HIV-1 and a new herpesvirus with distinct properties was isolated. The virus isolate differed from other known herpesviruses including HHV-6 in restriction enzyme and blot hybridisation analysis of the virus DNA. The virus was designated HHV-7 (RK strain).

In another study, a new herpesvirus distinct from HHV-6 was also isolated from the peripheral blood mononuclear cells of a chronic fatigue syndrome patient and was designated HHV-7 strain JI (Berneman *et al.,* 1992). The virus DNA produced a distinct restriction enzyme digestion pattern and showed only partial homology with other herpesviruses and also differed from HHV-6 in its antigenic properties (Frenkel *et al.,* 1990a; Berneman *et al.,* 1992b; Wyatt *et al.,* 1991; Tomasi *et al.,* 1994).

The target cell of HHV-7 was found to be $CD4^+$ T cells and its replication cycle is similar to that of HHV-6. The virus can be isolated (9 to 14 days) in stimulated human cord blood mononuclear cells and immature T-cell line SupT1 producing a cytopathic effect that has the appearance of typical rounded enlarged cells (Lusso *et al.*, 1994; Berneman *et al.,* 1992b; Frenkel and Roffman, 1996).

1.4.1. Epidemiology

Preliminary studies have investigated the seroprevalence of HHV-7 infection and demonstrated high seropositivity in the population. Wyatt *et al.,* (1991) showed up to 96% of healthy adults had HHV-7 IgG antibody titres ranging between 1:80 to more than 1:640, by indirect immunofluorescence assay. The virus infects during childhood but possibly later than the age documented for HHV-6. This is supported by

the finding that the children investigated acquired antibodies to HHV-6 by the age of 25 months, while none of them acquired antibodies to HHV-7 and that the percentage of children who acquired antibodies to HHV-7 showed a gradual increase throughout the childhood.

Yoshikawa *et al.,* (1993) demonstrated a prevalence rate of up to 60% in children between the ages of 9 and 17 years or adults between 20 to 29 years and 35% in individuals of the age between 30 to 39 years. In addition, 40% of infants (newborns to 1 month of age), had antibody to HHV-7 and only 10% had detectable antibody to HHV-7 by the time they were 10 to 11 months old. By the age of 1 to 2 years, 45% had antibody to HHV-7. In conclusion, the prevalence of HHV-7 infection increased slowly, after 6 months of age, reaching its maximum level of 60% at 13 years of age. In another study by Tanaka-Taya *et al.,* (1996), the detection rate of HHV-6 decreased with age after being the highest in children aged 1-year-old while that of HHV-7 increased with age and reached its maximum in adults.

Wyatt and Frenkel, (1992) reported that 75% of the saliva specimens from healthy adults tested positive, by isolation, for HHV-7. The identification was confirmed by restriction enzyme analysis of viral DNA and reactivity with monoclonal antibodies. Di Luca *et al.,* (1995a) reported that HHV-6 was present in 63% of salivary gland biopsies and in 3% of saliva from healthy persons whereas the figures for HHV-7 were, respectively, 75% and 55%. Wilborn *et al.,* (1995) reported that HHV-7 is present in 97.3% of adult blood donors.

Gautheret-Dejean *et al.,* (1997) studied HCMV, HHV-6, and HHV-7 by PCR in saliva and urine samples from 125 HIV-seropositive patients and 29 HIV-seronegative individuals. In saliva, the detection rates were 63%, 61%, and 43% for HHV-7, HCMV and HHV-6, respectively. In urine samples the overall detection rates were 6.5%, 37%, and 2% for HHV-7, HCMV, and HHV-6, respectively. The rate of detection did not differ according to $CD4^+$ cell count among HIV-positive patients for any of the three viruses. These results suggest that saliva may play a major role in

transmission of HHV-6 and HHV-7 whereas urine might not be an important route of transmission.

Consistent with prenatal transmission of HHV-7 and HHV-6, Okuno *et al.,* (1995) evaluated the presence of HHV-7 and HHV-6 in 72 cervical swabs from the cervices of women in the late stages of their pregnancies using PCR. The study demonstrated that 2 (2.7%) of the swabs contained HHV-7 DNA and 14 (19.4%) were positive for HHV-6. These finding suggest that both viruses may be an important cause of prenatal infection.

1.4.2. Pathogenesis

Little is known about the pathogenic characteristics of HHV-7. Early studies have concentrated on the interaction of the virus with its host cell. Berneman *et al.,* (1992) studied the cellular receptors of HHV-7 through infection of cord blood mononuclear cells and demonstrated that these cells expressed CD3 (93%), CD2 (97%), CD7 (95%), CD4 (42%) and CD8 (4%) but not CD1 or CD19. Both Lusso *et al.,* (1994) and Yasukawa *et al.,* (1995) reported the CD4 glycoprotein as a critical component of the receptor for HHV-7 infection. In the report by Lusso *et al.,* (1994), the CD4 expression was selectively down regulated during the course of HHV-7 infection. This was confirmed by murine monoclonal antibodies to human CD4 and recombinant soluble CD4 which resulted in a dose-dependent inhibition of HHV-7 infection. This in part might explain the finding of Crowley *et al.,* (1996) who suggested that HHV-7 is a powerful inhibitor of HIV-1 infection in cells of the mononuclear phagocytic lineage. The inhibition occurred at a very early stage, which is consistent with blocking at the level of virus attachment to the cells. In confirmation of this, Furukawa *et al.,* (1994) also demonstrated that infection with HHV-7 induced down-regulation of surface CD4, an essential molecule for T cell triggering. On the basis of these findings, it was speculated that HHV-7 infection might induce dysfunction of CD4+ T cells including the cytotoxic activity of CD4+ cytotoxic T cell.

The possibility that HHV-7 might result in reactivation of HHV-6 latent infection has been suggested. Katsafanas *et al.,* (1996) designed an *in vitro* system in which reactivation of HHV-6 and HHV-7 from latency was studied. HHV-7 was reactivated from latently infected peripheral blood mononuclear cells by T cell activation whereas HHV-6 could not be reactivated under similar conditions. Interestingly, latent HHV-6 could be reactivated and recovered after the same cells were infected with HHV-7. This resulted in an increase in the load of HHV-6 genomes and disappearance of that of HHV-7.

1.4.3. Association of HHV-7 with various clinical manifestations

1.4.3.1. Exanthum subitum

As another cause of ES, HHV-7 was isolated from the peripheral blood mononuclear cells of two infants with typical ES (Tanaka *et al.,* 1994). In the first infant, the virus was cultured from cells collected on the third febrile day and isolated within 10 days of incubation. The virus was identified using immunofluorescence antibody and restriction endonuclease analysis which showed that the isolate was very similar to HHV-7 but not HHV-6. In addition, only the antibody titres to HHV-7 increased after day 17 of illness while those of HHV-6 remained low. The second infant who had two episodes of the disease did not show any evidence of HHV-7 infection in the first episode but HHV-6 infection was identified. In the second episode, however, HHV-7 was isolated and correctly identified.

Asano *et al.,* (1995) reported a case of 13-month-old boy who had a prior history of ES at 6 months of age and who developed fever for 3 days and a skin rash accompanied with nonspecific signs and symptoms such as anorexia, irritability, mild diarrhoea, oedema, mild inflammation of the pharynx and mild occipital and cervical lymphadenopathy. HHV-7 was isolated and identified using monoclonal antibody to HHV-7. In addition, seroconversion to HHV-7 but not HHV-6 was demonstrated. However, both HHV-7 and HHV-6 DNAs were detected in peripheral blood

mononuclear cells with HHV-7 DNA being detected in saliva and stool and HHV-6 DNA in saliva only.

1.4.3.2. Other clinical manifestations

As a recently discovered virus, several reports have suggested an association of HHV-7 with different clinical manifestations. Kawa-Ha *et al.,* (1993) isolated HHV-7 from a 5-year-old boy suffering from fever, hepatosplenomegaly and pancytopenia with no other viruses being detected. Berneman *et al,* 1992a and Secchiero *et al.,* (1993) suggested an association of HHV-7 with chronic fatigue syndrome and reported high titre neutralising antibody to HHV-7 in sera from patients with the disease. In the study by Di Luca *et al.,* (1995b) no association was concluded as HHV-7 was detected in 83% of blood samples from healthy donors and in 82% of those from chronic fatigue syndrome patients.

A report by Hashida *et al.,* (1995) described a case of infantile hepatitis associated with HHV-7 infection. Clinical symptoms of fever, rash, liver dysfunction accompanied with leukocytopenia, thrombocytopenia, and hypogammaglobulinaemia and seroconversion to HHV-7 along with the detection of HHV-7 DNA by PCR in blood and liver were observed. In support of this, Torigoe *et al.,* (1997) isolated HHV-7 from peripheral blood mononuclear cells and saliva from an infant with ES complicated by liver dysfunction. The virus isolate was characterised by immunofluorescence assay and an increase of HHV-7 antibody titre. In addition, no other virus infection was identified.

Yalcin *et al.,* (1994) studied the occurrence of HHV-7 and HHV-6 in 16 renal transplant recipients and 16 healthy controls by virus isolation, serology, and PCR. HHV-7 was detected in 3 individuals (19%) for both groups whereas HHV-6 DNA was detected in 10 (63%) of 16 renal transplant recipients and in 7 (44%) of 16 healthy controls. Dual infection with HHV-7 and HHV-6 was detected by PCR in 2 patients and one control sample. The study highlighted the potential of dual infection with

the two viruses and may also reflect on the phenomenon of latency and reactivation of both viruses.

Torigoe *et al.,* (1995) demonstrated 22 cases of HHV-7 infection on the basis of seroconversion to HHV-7. HHV-7 infection occurred later than HHV-6 infection and caused ES in 47.1% of the children. HHV-7 was also postulated to be responsible for the other symptoms in these children, which included neurological complications such as febrile convulsion and acute hemiplegia. In support of the latter, Torigoe *et al.,* (1996) detected HHV-7 infection using cell culture, serology and PCR in 2 children with ES complicated with acute hemiplegia. Thus, HHV-7 may be an important aetiological agent of the latter clinical manifestation and possibly other neurological symptoms such as febrile convulsions.

HHV-7 has also been associated with the inflammatory disease pityriasis rosea. Drago *et al.,* (1997) and Kosuge *et al.,* (1998) using PCR, cell culture and serology have detected HHV-7 DNA sequences in plasma, peripheral blood mononuclear cells and the skin from patients with pityriasis rosea. In contrast, Kempf *et al.,* (1998) demonstrated that the detection rate of HHV-7 in skin biopsies from patients with pityriasis rosea (7.6%) is lower than that from normal individuals (14%) and suggested that HHV-7 might not play any role in the pathogenesis of this disease.

1.4.4. Laboratory diagnosis

As with HHV-6, the laboratory diagnosis of HHV-7 relies on the use of cell culture isolation, serology and more recently the use of PCR. The former utilises the human T-cell line, Sup T1 (Berneman *et al.,* 1992a). Antibody detection (IgG or IgM) using indirect immunofluorescence assay (Salahuddin *et al.,* 1986) or antigen detection utilising specific monoclonal antibodies have been described. In addition, a number of studies have utilised PCR for the detection of HHV-7 DNA in different clinical specimens (Berneman *et al.,* 1992b).

1.5. *Hydrops fetalis*

During pregnancy the fetus is susceptible to a number of pathogens owing to the decreased maternal resistance to infection (Anteby and Yagel, 1994). The infection occurs through transplacental transmission during maternal viraemia and the outcome of the infection is determined depending on the gestational age and the pathogenic characteristics of the causative pathogens. Viral infections are very well known causes of various foetal syndromes. These infections could result in asymptomatic or severe disease. The latter may include fetal or neonatal death or long-term sequelae.

Major viral infection-induced congenital symptoms include intrauterine growth restriction, *hydrops fetalis*, ascites, microcephaly, hydrocephaly and intracranial or intrahepatic calcification (Grose *et al.,* 1989). *Hydrops fetalis* is perhaps the most readily detectable ultrasound finding and is characterised by the presence of excessive fluid accumulation in at least two foetal cavities. The condition, in general, is caused by a variety of fetal, placental and maternal diseases (Santolaya *et al.,* 1992; Weiner, 1993).

Hydrops fetalis is usually divided into immune and non-immune *hydrops fetalis*. The former is caused by isoimmunization. Immune *hydrops fetalis* results from anaemia caused by the presence of circulating antibodies against the foetal red blood cell antigens. These antibodies are transferred from maternal blood to the fetal circulation. However, the incidence of immune *hydrops fetalis* is declining after the advent of Rh immunoglobin prophylaxis. This has resulted in a relative increase in the *hydrops fetalis* of non-immune aetiology.

Several mechanisms of *hydrops fetalis* have been suggested. These include haematological, genetic and chromosomal, cardiovascular, pulmonary and other organ abnormalities, although a number of cases remain to be classified as idiopathic (Forouzan, 1997).

As causes of non-immune *hydrops fetalis*, several infectious agents have been suggested although the association of other infections with this disease may be hampered by the lack of sensitive and appropriate diagnostic techniques (Weiner, 1997). The infectious agents associated with this disease so far include *Treponema pallidum* (Barton *et al.,* 1992), Toxoplasma gondii (Zones *et al.,* 1988), HCMV (Fadel and Ruedrich, 1988), HSV (Greene *et al.,* 1993), adenovirus (Ranucci-Weiss *et al.,* 1998), and more frequently parvovirus B19 (Berry *et al.,* 1992; Yaegashi *et al.,* 1998).

In general, the importance of infectious agents in such abnormality is likely to increase due to the advent of molecular diagnostics. The latter include the use of immunohistochemistry, PCR and *in situ* hybridisation or *in situ* PCR. An infectious aetiology is documented by virus isolation or serology in 5 to 15% of cases of *hydrops fetalis* but about 22% of cases remain as idiopathic (Machin, 1989; Boyd and Keeling, 1992). However, serology may lack sensitivity and can be difficult to interpret whereas cell culture is slow and its general consideration as the gold standard test may be hampered by relatively low sensitivity when compared to methods such as PCR. The latter technique has recently been demonstrated as an effective tool to detect viral nucleic acid in fetal infections. Van den Veyver *et al.,* (1998) utilised PCRs to detect HCMV, HSV, parvovirus B19, adenovirus, enterovirus, EBV and Respiratory syncytial virus (RSV) in amniotic fluid, fetal blood, pleural fluid and tissue from 303 abnormal pregnancies. Viral genomes were detected in 144 of 371 samples (39%) or 124 of the 303 patients (41%). The positive patients included 74 patients with adenovirus (24%), 30 patients with HCMV (10%) and 22 patients with enterovirus (7%) including concomitant infection with these viruses. Non-immune *hydrops fetalis* was clinically diagnosed in 91 patients. Of these, 50 patients (55%) were positive for viral nucleic acid by PCR in decreasing order of detection as adenovirus, enterovirus, parvovirus 19, HCMV, HSV, EBV and RSV (Table 2).

Table 2. Viral Etiologies of Fetal Disease

Fetal abnormality (Number of Patients)	Total of PCR +	Adenovirus	Enterovirus	Parvovirus	HCMV	HSV	EBV	RSV	Total
Nonimmune hydrops (91)	50	30*	7*	8*	8*	5*	2	1*	61*
Oligohydramnios (11)	4	2	0	0	2	0	0	0	4
Polyhydramnios (15)	7	5*	3*	0	0	0	0	0	8*
Hydrothorax/pleural effusion (18)	6	4	1	0	1	0	0	0	6
"Stuck twin" syndrome (25)	10	4*	5*	0	2*	2*	2*	0	15*
Ventriculomegaly (26)	7	6	0	0	1	0	0	0	7
Microcephaly (5)	1	1	0	0	0	0	0	0	1
Cytic hygroma (7)	0	0	0	0	0	0	0	0	0
Maternal infection (22)	12	7*	0	0	6*	1*	0	1*	15*
Choroid plexus cyst (5)	0	0	0	0	0	0	0	0	0
Echogenic bowel (22)	6	1	0	0	5*	1*	0	0	7*
Liver calcification (3)	1	0	0	0	1	0	0	0	1
Intrauterine growth restriction (22)	10	8*	1*	0	3*	0	0	0	12*
Thick placenta (7)	0	0	0	0	0	0	0	0	0
Hydronephrosis (3)	0	0	0	0	0	0	0	0	0
Multiple congenital anomalies (7)	0	0	0	0	0	0	0	0	0
Bradiacardia (1)	0	0	0	0	0	0	0	0	0
Tachycardia (1)	0	0	0	0	0	0	0	0	0
Myocarditis (autopsy-proven) (12)	10	6*	5*	0	1*	0	0	0	12*
Total (303)	124	74	22	8	30	9	4	2	149
"Controls" (154)	4	3	0	0	1	0	0	0	4

* Denotes multiple concomitant PCR-positive viral amplimers.

Modified from Van Den Veyver *et.al.*, (1998).

1.6. Psoriasis

Psoriasis is a common chronic disease, which manifests as red, scaly and indurated skin lesions. The lesions usually occur in characteristic areas of the body such as scalp, elbows, umbilicus, gluteal cleft, genital areas and knees. The patients may also present with itching, burning and soreness of the lesions and may be accompanied with joint pain or even true arthritis (Feldman and Clark, 1998). The condition affects 2-3% of the population in North West Europe and the United States but it is uncommon in Africa, Asia and the Far East (Fry, 1988).

Psoriasis should be clinically differentiated from other skin conditions that may present with this kind of lesions. These disorders include Tinea infections, pityriasis rosea, chronic forms of dermatitis (for example, nummular dermatitis or lichen simplex chronicus), secondary syphilis and cutaneous lupus erythematosus. The identification of classical psoriasis is straightforward while atypical cases require the search for specific criteria (Feldman and Clark, 1998)

The main cause of psoriasis is still to be found. The lesions are characterised by rapid growth resulting in skin turnover in as few as 4 days in comparison to approximately 1 month in the case of the normal skin. This phenomenon has led to the belief that the condition is primarily caused by an inflammatory process. Several factors have been suggested which include infection, heredity and other predisposing factors (Fry, 1988).

Recently, several studies have highlighted the involvement of an immunopathological process in the pathogenesis of psoriasis. The early lesion (plaques) of the disease are characterised by an influx of CD8+ T cells from the cutaneous blood vessels into the dermis and that certain subtypes (Vβ13) of the CD8+ T cells proliferate within the psoriatic plaque (Chang *et al.,* 1997). This activation of this subtype has also been observed in multiple sclerosis (Wucherpfennig *et al.,* 1990). The significance of this activation may be highlighted by the ability of these

cells to secret cytokines of the helper type such as IFN-γ, TNF-α, IL-2, IL-8 and IL-12 resulting in a relative decrease in other cytokines primarily IL-10 (Griffiths and Voorhees, 1996).

The proliferation of CD8+ in cases of psoriasis may be achieved as a response to super-antigens that still remained to be proved and defined (Griffiths and Voorhees, 1996). However, the secretion of Th1 cytokines may reflect infection with chronic viral infection or other chronic infection due to intracellular pathogens. These infections may act as stimuli to the activation of CD8+ T cells (Griffiths and Voorhees, 1996). It has been reported that several viral infection such as HCMV and HHV-6 may lead to *in vitro* production and release of cytokines such as IL-2, TNF-α and IL-12 (Geist *et al.*, 1991; Li *et al.*, 1997). Whether this would be implicated in any importance *in vivo* is still to be determined.

1.7. Aim of the study

Herpesviruses-associated disease is a very well known clinical problem in immunocompromised patients including transplant recipients. The clinical importance of HCMV is well established but that of HHV-6 and HHV-7 is still in its infancy. In addition, preliminary data in the laboratory where this study is performed suggested a possible association of HHV-6 with cases of *hydrops fetalis* whereas virological evidence for virus-associated chronic psoriasis has been suggested. The aim of this study is to:

1. Design and optimise PCR assays for the detection of HCMV, HHV-6 and HHV-7 DNAs. This involves the search for optimal PCR primers and alteration of PCR components.

2. Testing different methods to select the most efficient for the extraction of DNA from the clinical specimens.

3. Retrospective application and evaluation of these PCRs on serum samples from renal and bone marrow transplant recipients.

4. Screening of these viruses especially HHV-6 in cases with *hydrops fetalis* and cases of chronic psoriasis.

5. Optimisation and application of typing protocol for HHV-6 variants A and B using restriction endonuclease analysis on the HHV-6 PCR product.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals and reagents

Ethanol, glycogen, human DNA, isopropanol, ammonium peroxodisulphate, deoxyribonucleoside triphosphates (dNTPs), guanidinium thiocyanate, restriction endonucleases, proteinase-K, bovine serum albumin and acrylamide/bisacrylamide solution were purchased from Boehringer Mannheim Biochemica, Germany.

The DNA molecular weight markers 1 kilobase (1kb) plus DNA ladder and 1kb DNA ladder and loading buffer (10X BlueJuice) were purchased from Gibco BRL, Bethesda Research Lab, Middlesex.

Bromophenol blue, N,N,N,N,-tetra-methylethylene diamine (TEMED) and all standard laboratory chemicals were purchased from BDH. Ltd. Poole, England.

Hybaid recovery amplification kit was purchased from Hybaid Limited, Middlesex, London.

All oligonucleotide primers were purchased from Pharmacia Biotech.

AmpliTaq® DNA polymerase, AmpliTaq® Gold DNA polymerase, $MgCl_2$ and 10xPCR Buffer were obtained from Perkin Elmer Cetus, Norwalk, CT, USA.

Ethylenediaminetetraacetic acid (EDTA), sarcosine, sodium acetate, sodium chloride, sodium citrate, sodium phosphate, trizma base, ethidium bromide, dithiothreitiol (DTT), n-octane, phenol/chloroform, magnesium chloride, and mineral oil were from Sigma Chemical Company (St. Louis-USA).

DYNAL® DNA DIRECT Kit™ System II was purchased from Dynal A.S, Oslo, Norway.

QIAamp® HCV Kit, QIA plasmid maxi kit was purchased from QIAGEN, Germany.

2.1.2. Viral control DNAs

The plasmid vector pAT 153 containing the HCMV J fragment and the HHV-6 plasmid DNA were provided by Dr R. J. Cooper, Department of Pathological Sciences, University of Manchester.

Human T cell line (HSB-2) and HHV-6 infected HSB-2 cells were provided by Dr G. Toms, Department of Virology, Medical School, University of Newcastle.

HHV-7 strain H7-4 infected cell DNA was purchased from API Advance Biotechnology.

2.2. Methods

2.2.1. Clinical specimens

2.2.1.1. Serum samples

Serum samples from patients with renal, bone-marrow transplants, dialysis patients, neonates and children were provided by the Diagnostic Virology Laboratory. They had been stored at - 40 °C for up to 4 years after being subjected to routine laboratory tests. They were selected for this study either because they were from children less than 3 years old or from transplant recipients.

2.2.1.2. Skin biopsies

Biopsies from the involved and the uninvolved skin of patients with psoriasis attending Dermatology Clinic in North Manchester General Hospital were kindly provided by Dr. Brian Kirby.

2.2.1.3. Tissue sections

Paraffin-embedded tissue sections (heart, lung, liver, kidneys, and placenta) from foetal hydrops and non-hydrops cases were obtained from the histocytopathology laboratory from Manchester Royal Infirmary (MRI) and were provided by Dr Lynette Moore.

2.2.2. Preparation and extraction of DNA from serum specimens

2.2.2.1. Guanidinium thiocyanate (GuSCN) extraction method

DNA was extracted from serum samples using the method of Casas *et al.*, 1995. The sample (50µl) was added to 200µl of extraction buffer (4M GuSCN, 0.5% N-lauryl sarcosine, 1mM dithiothreitol (DTT), 25mM sodium citrate and 100µg/ml glycogen) and incubated at room temperature for 10 minutes. The extract was adjusted to 0.3M sodium acetate. Cold (-20°C) isopropanol (250µl) was then added to precipitate the nucleic acid. After 5 minutes, the samples were centrifuged for 10 minutes at 14,000g. The supernatant was removed and the pellet washed by addition of 70% ethanol (500µl). After further centrifugation for 10 minutes at 14,000g, the ethanol was removed and the pellet air-dried before being dissolved in sterile distilled water (20µl). Samples were stored at -20°C. An extraction control of sterile distilled water or bovine serum albumin (50µl) was extracted with each batch of samples using identical conditions and reagents to ensure no cross contamination between samples has occurred.

2.2.2.2. HYBAID protocol

Serum samples (5µl) was added to15µl of the Hybaid reagent, the tubes were vortexed briefly and then microwaved for 7 minutes at maximum power (650 w).

2.2.2.3. DYNAL extraction method

In this method, 1ml of serum was centrifuged for 2 minutes at 14,000g. Supernatant was removed and 100µl of magnetic beads was added to the pellet. A volume of 50µl of 6M NaCl and 500µl ethanol was added and mixed. Magnetic separation was performed by applying the magnet for 3 minutes. Supernatant was aspirated and the target material specifically bound to the magnetic beads in the pellet was washed 3 times with sterile distilled water (1 ml) and centrifuged for 2 minutes at 14,000g. The pellet was resuspended in 20µl of sterile distilled water.

2.2.2.4. QIA amp HCV Kit

Serum samples (140µl) was mixed with 560µl of prepared buffer AVL/carrier RNA and incubated at room temperature for 10 minutes when 560µl of absolute ethanol was added. The mixture, in two equal volumes of 630µl was then added to QIAamp spin column and centrifuged at 6000g for 1 minute. Buffer AW (2x500µl) was added to the column and centrifuged at 6000g for 1 minute. The DNA was eluted with 50µl of RNase-free water that has been preheated to 80°C. The DNA preparation was then centrifuged at 6000g for 1 minute. The composition of AVL and AW buffers have not been released by the manufacturers.

2.2.2.5. Preparation of paraffin-embedded tissues

DNA was extracted from tissue sections by a modification of the method of Wright and Manos (1990). The tissue sections were deparaffinised with 1ml octane at room temperature for 30 minutes followed by centrifugation for 5 minutes and the supernatant discarded. After repeating this procedure, the pellet was washed with 2x 500µl of 100% ethanol, each wash followed by centrifugation for 5 minutes. The supernatant was removed and the pellet was then air-dried. Digestion buffer (100µl) containing 200µg / ml of proteinase K was added and incubated overnight at 37°C. The reaction was then heated at 95°C for 10 minutes to inactivate proteinase K and centrifuged for 30 seconds. The supernatant was

removed and extracted with Hybaid reagent as described in section 2.2.2.2.

2.2.2.6. Extraction of Skin biopsies

All skin biopsies were first snap frozen in liquid nitrogen and stored at –80°C. These were disrupted during thawing by a glass homogeniser. DNA was then extracted by the guanidinium thiocyanate method as described in section 2.2.1.1.

2.2.3. PCR protocols

All PCR reactions were prepared under strict measures for contamination prevention as described by Kwok & Higuchi (1989). These included the use of PCR-dedicated rooms (DNA-free and DNA-preparation rooms), handling of PCR products in an area separated from the PCR rooms, the use of plugged tips, gloves, face masks and surgical hoods. In addition, all surfaces were cleansed with 1N HCl before and after preparation of PCR mixtures.

2.2.3.1. Oligonucleotide primers

All oligonucleotide primers utilised in this study, their sizes, nucleotide sequences, target DNAs and the size of the PCR products generated are shown in Table 3.

2.2.3.2. PCR mixture

Except for the type of primer pair used, all the PCR reaction mixtures were identical. In a final volume of 50µl, each reaction contained 0.2µM of each of the respective primer pair, dNTPs (200µM each), 1x PCR buffer (10mM tris-HCl pH 8.3, 50mM KCl, 1.5mM $MgCl_2$) and 1.25 units of AmpliTaq® DNA polymerase or AmpliTaq® Gold DNA polymerase in the case of the hot start method. A drop of mineral oil was added to prevent evaporation and 5µl of the test sample or sterile distilled water (negative control or extraction negative control) was added.

Table 3. Properties of oligonucleotide primers

Target	Primer	Primer Sequence (5'→3')	Length (mer)	Position	Product size (bp)	Reference
HCMV	233C	TGCAGTTTGGTCCCTTAAAG	20	2641-2622[a]	171	McElhinney et al., (1995)
	724C	AAGAATCCTCACCTGGCTTA	20	2471-2490[a]		
HHV-6	H6-6	AAGCTTGCACAATGCCAAAAAACAG	25	17627-17603[b]	223	Gopal et al. (1990)
	H6-7	CTCGAGTATGCCGAGACCCCTAATC	25	17405-17429[b]		
	DROB1	GTTCCAGGCGGCATGAATTC	20	86136-86155[b]	385	Drobyski et al., (1993)
	DROB2	GACACGGCCTCTCTACATCAC	21	86519-86499[b]		
HHV-7	HV7	TATCCCAGCTGTTTTCATATAGTAAC	26	1-26[c]	186	Berneman et al., (1992)
	HV8	GCCTTGCGGTAGCACTAGATTTTTTG	26	186-161[c]		
β-globin	GH20	GAAGAGCCAAGGACAGGTAC	20	β-globin	268	Bauer et al., (1991)
	PCO4	CAACTTCATCCACGTTCACC	20			

a = according to the numbering system of Jahn *et al.*, (1987); b = according to the numbering system of Lawrence *et al.*, (1990); C = according to the numbering system of Berneman *et al.*, (1992).

2.2.3.3. Thermocycling parameters

All the PCR reactions were carried out using the thermocycling machine Techne-Ori-Block PHC-1 (Techne-Cambridge-England). The thermocycling program comprised of 40 cycles. In the first cycle, the reaction was held at 94°C for 7 minutes when AmpliTaq® polymerase was used or for 10 minutes for the hot start method to activate the enzyme AmpliTaq® Gold DNA polymerase, at 55°C for 1 minute and at 72°C for 1 minute. In the remaining 39 cycles, every cycle consisted of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and polymerisation at 72°C for 1 minute.

2.2.3.4. Analysis of PCR products

PCR products (10µl) were mixed with 2 µl of blueJuice™ gel loading buffer (65% (w/v) sucrose; 1 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.3% (w/v) bromophenol blue) and 10 µl of this mixture along with the molecular weight marker 1kb plus DNA ladder were loaded into 8% polyacrylamide gels and electrophoresed in TPE buffer (0.09 M tris-phosphate, pH 8.0 and 0.002 M EDTA) at 140 V for 45 minutes using the electrophoresis power supply EPS500/400 (Pharmacia Biotech, St. Albans, Hertfordshire). The gels were then stained in ethidium bromide solution (1µg/ml) for 1 minute, washed in SDW for 2 minutes and visualised with a UV transilluminator using the computerised system E.A.S.Y. Enhanced System Analysis; (Herolab, Germany).

2.2.3.5. Restriction endonuclease analysis

The restriction endonuclease reactions were carried out as described by the manufacturer. In a total volume of 20 µl, the PCR product (5µl) was digested with 1 µl of enzyme (5units/µl) in presence of the appropriate buffer supplied by the manufacturers depending on the enzyme. Uncut control reaction, which excluded the enzyme, was also carried out. Resulting fragments were separated on 8% polyacrylamide gels as described for the PCR products (section 2.2.3.4).

2.2.4. Diagnostic Virology laboratory techniques

The Diagnostic Virology Laboratory does not perform any investigation for the presence of HHV-6 or HHV-7 in clinical specimens. In contrast, HCMV infection is diagnosed using different techniques in various clinical specimens including serum, urine, CSF and throat swabs. The techniques include cell culture isolation, DEAFF test and IgM and IgG serology. In addition, PCR is performed only if requested by the clinician.

3. RESULTS

3.1. Optimisation of PCRs

The hot start method amplitaq-gold-based PCR was evaluated against amplitaq DNA polymerase-based PCR for the detection of HCMV, HHV-6 and HHV-7 DNAs. Control DNAs were titrated in sterile distilled water and the sensitivities of the three PCRs were determined. All the PCR reactions contained 1.5 mM $MgCl_2$, 1.25 units of enzyme and 200 μM of each dNTP.

3.1.1. HCMV-PCR

The primers 233C and 724C derived from the DNA sequence of the HCMV phosphoprotein gene generating a PCR product of 171 bp were used. The target HCMV plasmid DNA, which represents the plasmid DNA pAT153 containing the fragment J of HCMV (AD169) DNA (Oram *et al.,* 1982), was titrated and tested by the HCMV-PCR.

In the titration experiment using amplitaq DNA polymerase, the sensitivity of the PCR was 100 copies per 5 μl (Figure 1). In the figure, lane 1 is a positive PCR with 10^6 copies of the plasmid DNA and lane 5 represents the sensitivity level of the PCR (100 copies) whereas lane 7 is the PCR negative control.

Using identical reaction conditions, the hot start (amplitaq-gold)-based PCR resulted in sensitivity of 10 copies per 5 μl. As shown in Figure 2, HCMV DNA was titrated in lanes 1 to 8. Lane 7 represents the sensitivity level (10 copies) and lane 9 is the PCR negative control. Lane L is the molecular weight marker (1kb DNA ladder).

3.1.2. HHV-6-PCR

The HHV-6 plasmid DNA (fragment HD5x of HHV-6 DNA cloned into plasmid vector pUC13) (Martin *et al.,* 1991a) was titrated (10-fold serial dilution) in sterile distilled water and amplified utilising the primers H6-6

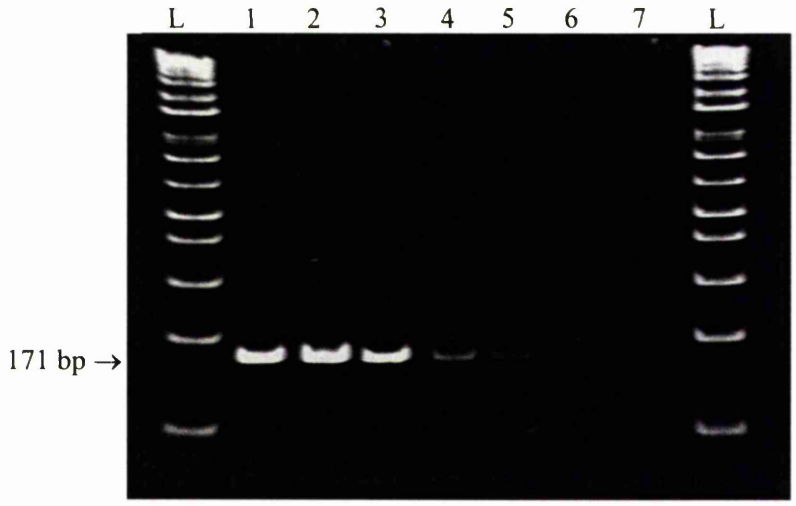


Figure 1. Sensitivity of HCMV–PCR.

Lanes L= molecular weight marker (1kb plus DNA ladder).

Lane 1= 10^6 copies/5 µl of the HCMV plasmid control DNA.

Lanes 2-6= 10-fold serial dilutions of Lane 1.

Lane 7= negative control.

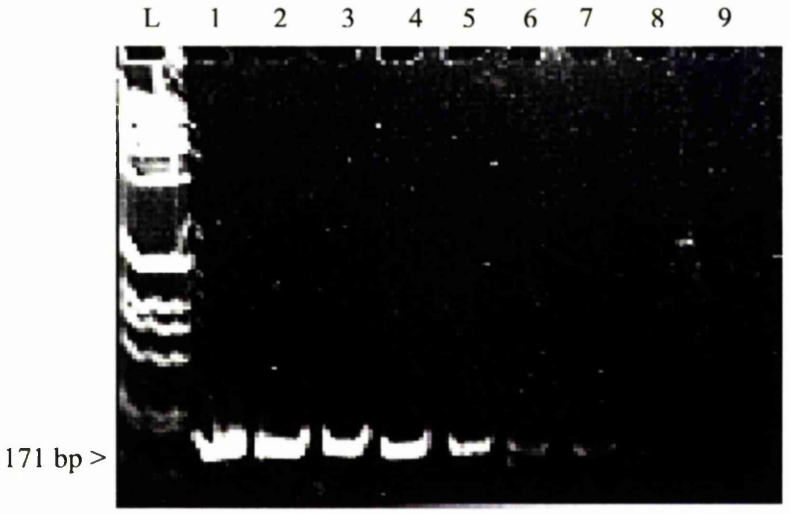


Figure 2. Sensitivity of hot start HCMV –PCR.

Lane L= molecular weight marker (1kb DNA ladder).

Lane 1= 10^7 copies/5 µl of the HCMV plasmid control DNA.

Lanes 2-8= 10-fold serial dilutions of Lane 1.

Lane 9= negative control.

and H6-7 in both HHV-6 PCR formats (amplitaq-based- and amplitaq-gold-based HHV-6-PCRs).

Figure 3 shows the sensitivity of the HHV-6-PCR using amplitaq DNA polymerase as 100 copies per 5 µl. Lane 5 indicates this level of sensitivity and lane 9 represents the PCR negative control.

The hot start HHV-6 PCR resulted in sensitivity of 10 copies per 5 µl of control plasmid DNA (lane 6). Lanes 1 to 8 in Figure 4 are 10-fold serial dilutions of the control plasmid DNA. The PCR product size (223 bp) is indicated. Lane 9 is the PCR negative control and lane L represents the molecular weight marker (1kb plus DNA ladder).

3.1.3. HHV-7-PCR

The primers HV-7 and HV-8 were used to amplify the 186 bp PCR product from 10-fold serial dilutions of HHV-7-infected cell DNA. The sensitivity of the PCR was found to be 10^{-5} dilution per 5 µl when the amplitaq-based PCR was used (Figure 5). In contrast, a sensitivity of 10^{-6} dilution of HHV-7-infected cell DNA was obtained with the hot start (amplitaq gold)-based HHV-7-PCR (Figure 6).

Table 4 summarises all the sensitivities obtained with all PCRs (HCMV, HHV-6 and HHV-7) using both formats (amplitaq- or amplitaq gold-based PCR). As, in all cases, the "hot start" format with amplitaq gold was ten-fold more sensitive, it was used for all the subsequent experiments.

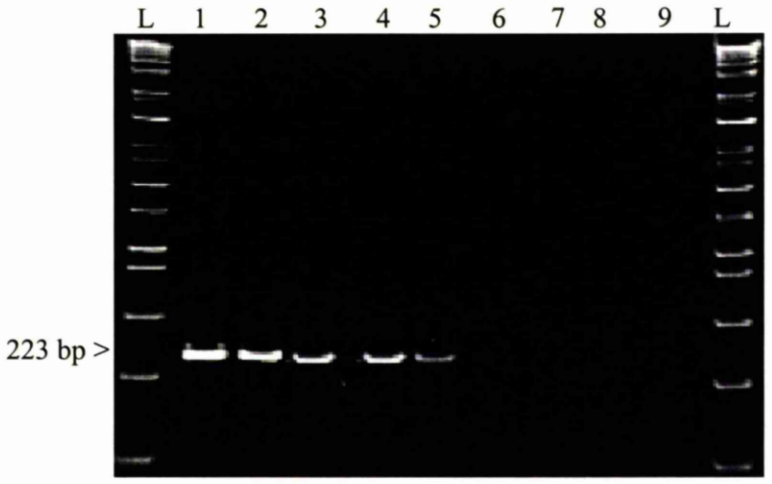


Figure 3. Sensitivity of HHV-6-PCR.

Lanes L= molecular weight marker (1kb plus DNA ladder).

Lane 1= 10^6 copies/5 µl of the HHV-6 plasmid control DNA.

Lanes 2-8= 10-fold serial dilutions of Lane 1.

Lane 9= negative control.

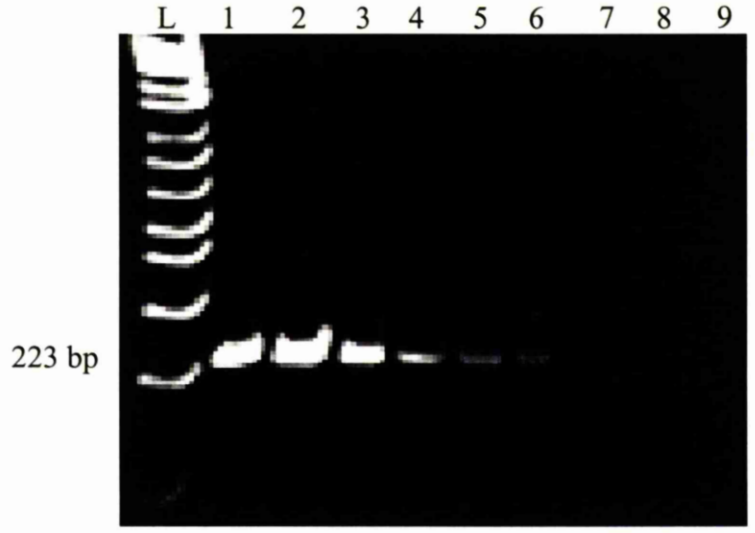


Figure 4. Sensitivity of hot start HHV-6-PCR.

Lanes L= molecular weight marker (1kb plus DNA ladder).

Lane 1= 10^6 copies/5 µl of the HHV-6 plasmid control DNA.

Lanes 2-8= 10-fold serial dilutions of Lane 1.

Lane 9= negative control.

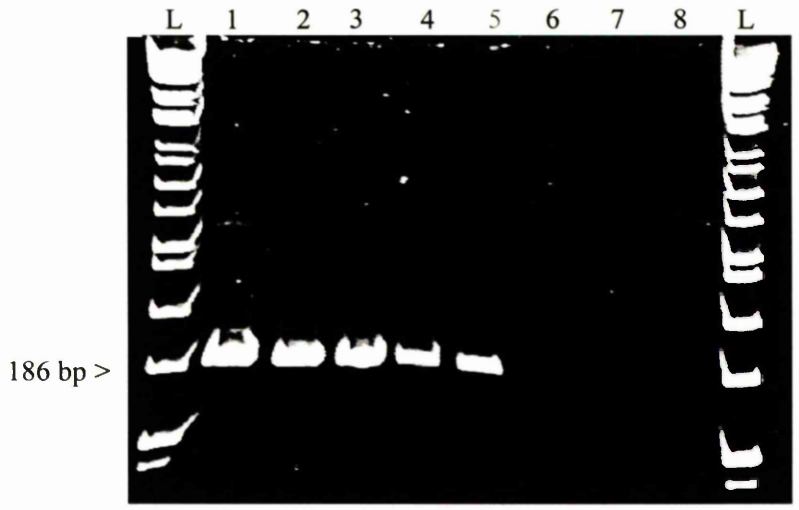


Figure 5. Sensitivity of HHV-7-PCR.

Lanes L= molecular weight marker (1kb DNA ladder).

Lane 1= 10^{-1} dilution of HHV-7-infected cell DNA.

Lanes 2-8= 10-fold serial dilutions of Lane 1.

Lane 9= PCR negative control.

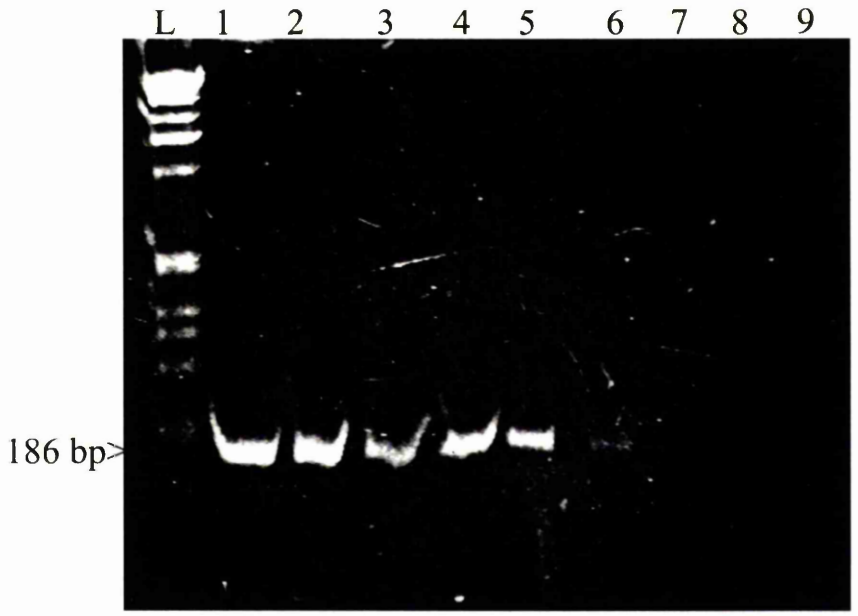


Figure 6. Sensitivity of hot start HHV-7-PCR.

Lanes L= molecular weight marker (1kb DNA ladder).

Lane 1= 10^{-1} dilution of HHV-7-infected cell DNA.

Lanes 2-8= 10-fold serial dilutions of Lane 1.

Lane 9= PCR negative control.

Table 4. Sensitivities of HCMV-, HHV-6- and HHV-7-PCR

PCR	Product size (bp)	PCR based on:	Sensitivity (per 5 μl)*	Figure
HCMV	171	Amplitaq	100	1
		Amplitaq gold	10	2
HHV-6	223	Amplitaq	100	3
		Amplitaq gold	10	4
HHV-7	186	Amplitaq	10^{-5}	5
		Amplitaq gold	10^{-6}	6

* Sensitivity readings for HHV-7 PCRs are in dilution of HHV-7-infected cell DNA

3.2. Evaluation of DNA preparation methods on serum samples

In order to study the effect of different DNA extraction techniques on serum specimens on the sensitivity and specificity of the PCR, serum specimens were first extracted by the guanidinium thiocyanate method and tested by the HCMV PCR.

A total of 10 serum samples extracted with guanidinium thiocyanate and negative by the HCMV PCR were pooled. This was then spiked with 1000 plaque-forming units (pfu) of HCMV-infected HEL cells and serial 10-fold dilutions made in serum. These dilutions were then subjected to the different extraction methods which included guanidinium thiocyanate, Hybaid Recovery Kit, DYNAL method and QIA amp HCV Kit. All the extracts were amplified with the HCMV PCR see (Table 5).

In another experiment, the pooled serum was also spiked with the HCMV plasmid DNA, serially diluted in serum, extracted with the guanidinium thiocyanate method only and tested with the HCMV-PCR.

3.2.1. Guanidinium thiocyanate method

The sensitivity of the HCMV-PCR on serial dilutions of HCMV-infected cells in serum was 0.01 pfu per 5 µl (Figure 7). The total time required for this extraction method was approximately 45 minutes.

When HCMV plasmid DNA was titrated in serum, the HCMV PCR sensitivity was identical to that described for HCMV plasmid DNA in sterile distilled water, i.e. 10 copies of the plasmid DNA per 5µl (data not shown).

This method of extraction was then evaluated on the same 10 serum specimens, individually spiked with 1000 pfu of HCMV. All of the serum specimens were positive by the HCMV PCR (10/10) as shown in Figure 8.

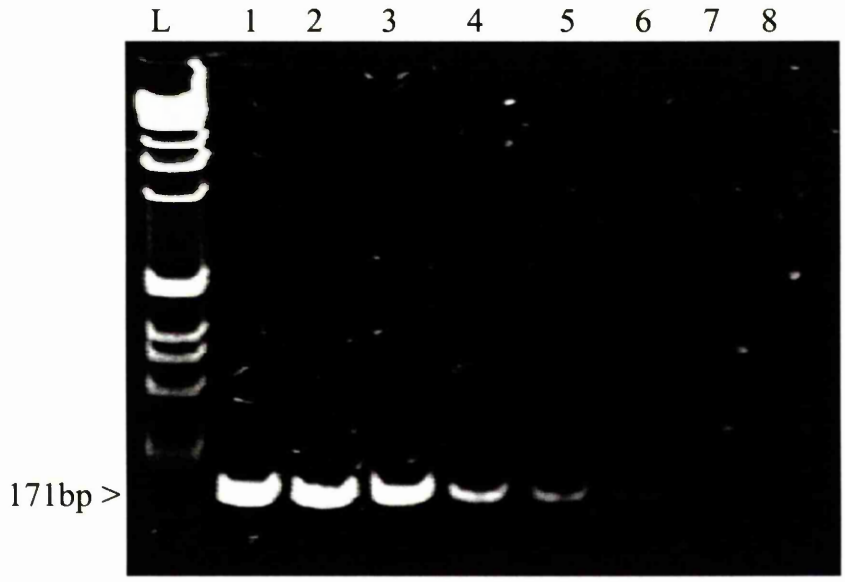


Figure 7. Sensitivity of HCMV-PCR on HCMV-diluted in serum and extracted by the guanidinium thiocyanate method.

Lane 1= 1000 plaque forming units (pfu) of HCMV infected cells in serum

Lanes 2-7= 10-fold serial dilutions of lane 1 in serum.

Lane 8= PCR negative control.

Figure 8: Detection of HCMV DNA in serum samples spiked with HCMV and extracted by the guanidinium thiocyanate method.

Lanes L= molecular weight marker (1kb plus DNA ladder).

Lanes 1-10= serum samples spiked with HCMV infected cell.

Lanes 11= negative control.

Lane 12= PCR positive control.

Lane 13= extraction negative control.

3.2.2. Hybaid Recovery Kit

Figure 9 shows the dilution of HCMV in the pooled sera extracted by the Hybaid Recovery Kit and tested by the HCMV PCR. The sensitivity of the test was 0.1-1 pfu per 5 µl. The Hybaid Recovery Kit was also evaluated on the individual serum samples tested above and only 6 of the 10 samples were positive for HCMV DNA by the PCR as shown in Figure 10.

3.2.3. DYNAL extraction method

As shown in Figure 11, amplification of HCMV DNA from dilutions of HCMV extracted by this method resulted in sensitivity level of 1-10 pfu per 5 µl. This method of extraction resulted in the least number of positive HCMV-spiked serum samples, i.e only 3 of the 10 samples were positive by the HCMV-PCR (Figure 12).

3.2.4. QIA amp HCV Kit

A sensitivity of 1-10 pfu per 5 µl was achieved when the QIA amp HCV Kit was used to extract HCMV DNA from 10-fold dilutions of HCMV in serum (Figure 13). The method is relatively complex due to the multiple centrifugation steps required and the time required to prepare DNA extracts by this method is approximately 1 hour.

Figure 14 shows the results of this method on the 10 HCMV-spiked serum specimens. Only 4 samples were positive by this method.

The results obtained with the evaluation of the different extraction methods are summarised in Table 5. The guanidinium thiocyanate method is the most sensitive method for extracting HCMV from serum samples (0.01 pfu/5µl) and it takes about 45 minutes to complete. The second method, the Hybaid Recovery Kit is faster and simpler (excludes any centrifugation steps) but it is at least 10-fold less sensitive than the guanidinium thiocyanate method. Both the DYNAL and the QIA amp HCV are equally sensitive but are 100-fold less sensitive than the

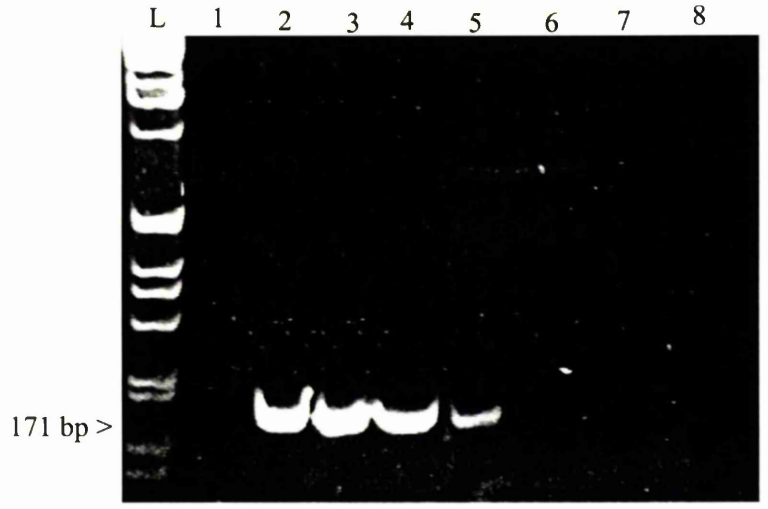


Figure 9. Sensitivity of HCMV-PCR on HCMV-infected cell titrated in serum extracted by the Hybaid recovery kit.

Lane 2= 1000 plaque forming units (pfu) of HCMV in serum

Lanes 3-6= 10-fold serial dilutions of lane 2 in serum.

Lane 7= PCR negative control.

Lane 8= extraction negative control.

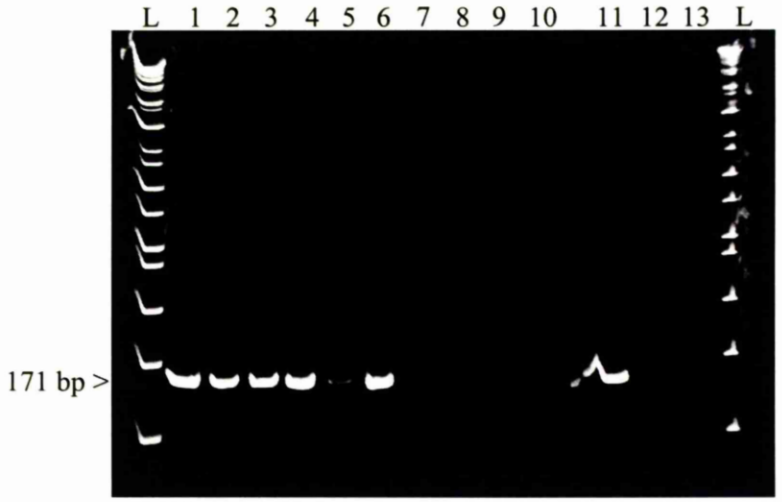


Figure 10: Detection of HCMV DNA in serum samples spiked with HCMV and extracted by Hybaid Recovery Kit.

Lanes L= molecular weight marker (1kb plus DNA ladder).

Lanes 1-10= serum samples spiked with HCMV.

Lane 11= PCR positive control.

Lane 12= negative control.

Lane 13= extraction negative control.

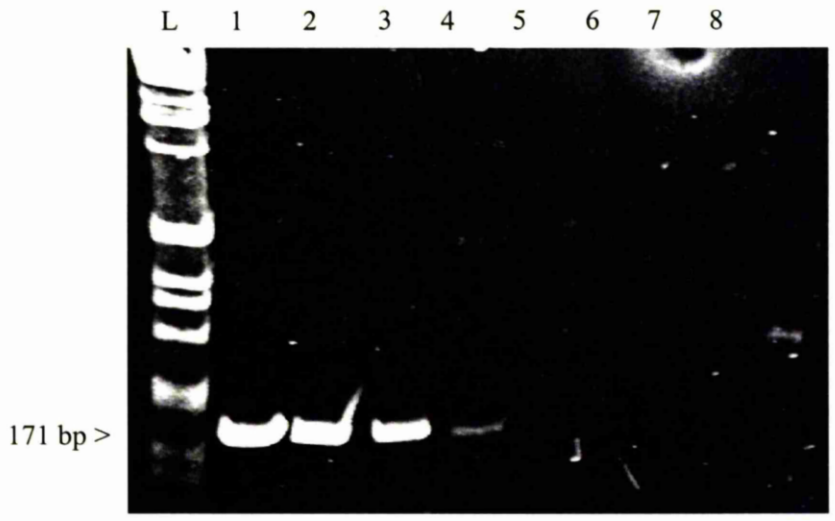


Figure 11. Sensitivity of HCMV-PCR on HCMV-diluted in serum and extracted by the DYNAL method.

Lane 1= 1000 plaque forming units (pfu) of HCMV in serum

Lanes 2-6= 10-fold serial dilutions of lane 1 in serum.

Lane 7= PCR negative control.

Lane 8= extraction negative control.

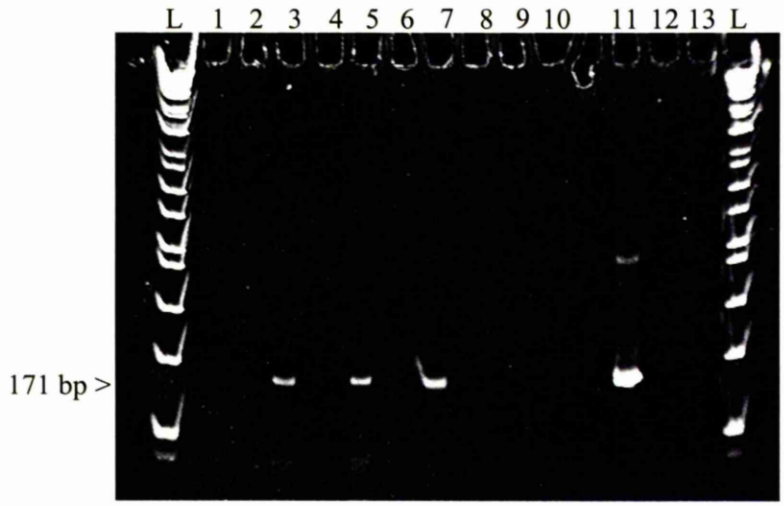


Figure 12: Detection of HCMV DNA in serum samples spiked with HCMV and extracted by DYNAL method.

Lanes L= molecular weight marker (1kb plus DNA ladder).

Lane 1-10= serum samples spiked with HCMV.

Lane 11= PCR positive control.

Lane 12= negative control.

Lane 13= extraction negative control.

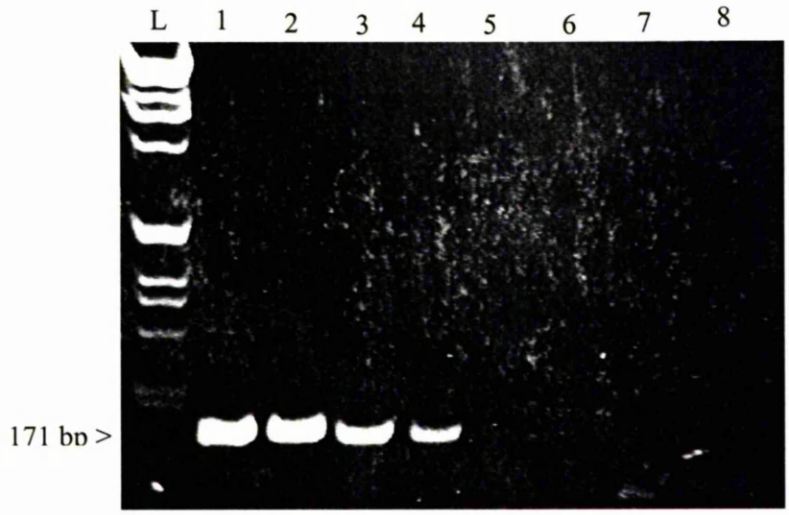


Figure 13. Sensitivity of HCMV-PCR on HCMV infected cell titrated in serum extracted by the QIA Amp kit method.

Lane 1= 1000 plaque forming units (pfu) of HCMV infected cells in serum

Lane 2-6= 10-fold serial dilution of lane 1 in serum.

Lane 7= PCR negative control.

Lane 8= extraction negative control.

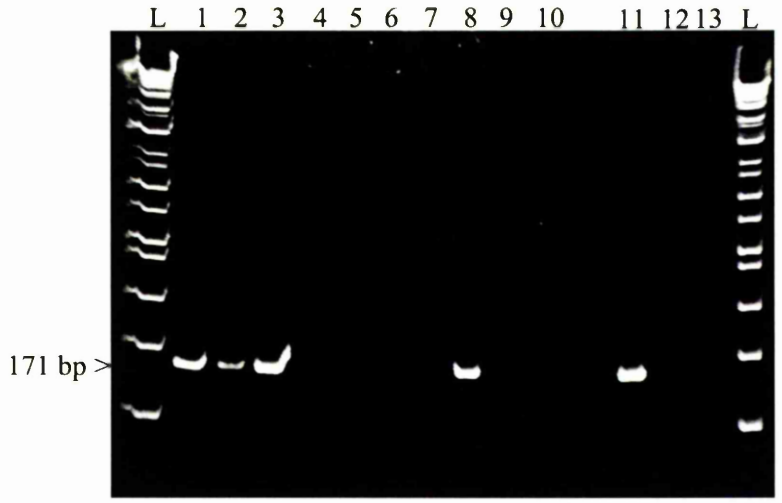


Figure 14: Detection of HCMV DNA in serum samples spiked with HCMV and extracted by the QIA amp HCV Kit.

Lanes L= molecular weight marker (1kb plus DNA ladder).

Lanes 1-10= serum samples spiked with HCMV infected cell.

Lane 11= PCR positive control.

Lane 12= negative control.

Lane 13= extraction negative control.

Table 5. Comparison of 4 methods of extracting DNA from serum.

Method	Sensitivity*		Volume required (μl)	Centrifugation steps	Time required (minutes)
	PFU	Samples			
Guinidinium thiocyanate	0.01	10/10	50	2	45
Hybaid Recovery Kit	0.1-1	6/10	5	0	15
DYNAL	1-10	3/10	1000	2	45
QIA amp HCV	1-10	4/10	140	3	45

* The sensitivity of each method was evaluated as the number of plaque forming units (pfu) that could be detected by any method or the number of positive samples from a total of 10 serum specimens spiked with HCMV. Each assessment was repeated on 3 occasions.

guanidinium thiocyanate method. The DYNAL and the QIA amp HCV methods both take 45 minutes to complete. However, the DYNAL method requires 1 ml of serum sample to be processed.

3.3. Detection of HHV-6, HCMV and HHV-7 by PCR in renal transplant recipients

3.3.1. Application of PCR on serum specimens

Serum samples that were stored (-20°C) for a period ranging from 1 month to 5 years were extracted using the guanidinium thiocyanate method and tested using the HHV-6, HHV-7 and HCMV PCRs.

Figure 15 shows the application of HCMV PCR on some of these serum samples. The test is positive for the 171bp PCR product in lanes 1, 2 and 5. The remaining samples are negative (lanes 3, 4 and 6-9) and also the negative controls (lane 10 and 11) which indicates the validity of the results.

Examples of positive serum samples for HHV-6 DNA are shown in Figure 16. Lanes 2, 3, 6, 8 and 9 are serum samples extracted by the guanidinium thiocyanate method demonstrating the presence of the 223 bp HHV-6 PCR product.

A total of 463 serum samples from renal transplant recipients at stages before and after transplantation was tested. HHV-6 DNA was detected in 111 serum samples, HCMV DNA in 65 specimens and dual HHV-6 and HCMV infection were detected in 38 serum samples. None of the samples were positive for HHV-7 DNA and the remaining 249 serum samples were negative for all the three targets (Table 6).

The serum samples were collected from 124 renal transplant recipients. Of these, 31 patients were positive for HCMV, 29 for HHV-6, and 16 were positive for both HHV-6 and HCMV (dual infection). None of the patients had HHV-7 infection detectable by PCR. In the remaining 48 patients none of the three agents were detected (Table 7).

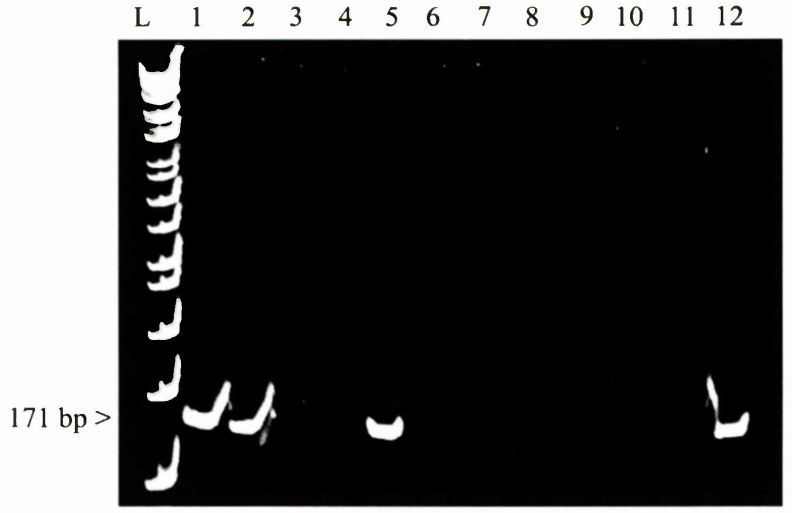


Figure 15. Detection of HCMV DNA in serum samples from renal transplant recipients.

Lane L= 1 kb plus DNA ladder

Lanes 1, 2, and 5= serum samples positive for HCMV DNA.

Lanes 3, 4, 6-9= serum samples negative for HCMV DNA.

Lane 10= PCR negative control.

Lane 11= extraction negative control.

Lane 12= PCR positive control.

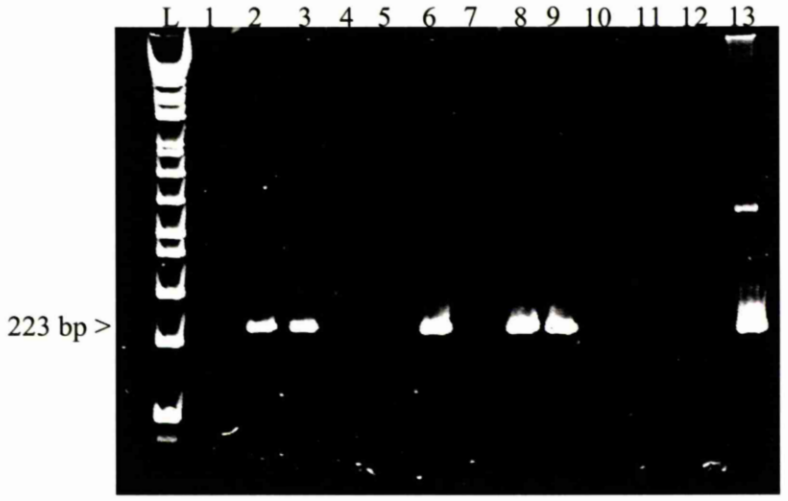


Figure 16. Detection of HHV-6 DNA in serum samples from renal transplant recipients.

Lane L= 1 kb plus DNA ladder

Lanes 2, 3, 6, 8 and 9= serum samples positive for HHV-6 DNA.

Lanes 1, 4, 5, 7 and 10= serum samples negative for HHV-6 DNA.

Lane 11= PCR negative control.

Lane 12= extraction negative control.

Lane 13= PCR positive control.

Table 6. HCMV, HHV-6 and HHV-7 DNA in serum specimens from renal transplant patients

HCMV	HHV-6	HHV-7	No of specimen
+	-	-	65
-	+	-	111
+	+	-	38
-	-	+	0
-	+	+	0
+	-	+	0
+	+	+	0
-	-	-	249
Total			436

Table 7. HCMV, HHV-6 and HHV-7 in renal transplant patients

HCMV	HHV-6	HHV-7	No of Patients
+	-	-	31
-	+	-	29
+	+	-	16
-	+	+	0
-	-	+	0
+	-	+	0
+	+	+	0
-	-	-	48
Total			124

3.3.2. Analysis of PCR results with clinical manifestations in selected cases

In 5 patients, viral DNA was detected in their serum for prolonged periods. Some clinical information was obtained for these patients. In all cases loss of function after transplantation ranged from months to years. In some cases detection of HCMV and HHV-6 DNAs coincided with loss of function and episodes of kidney rejections. Dual infection with HCMV and HHV-6 were detected in some cases but none of the cases was positive for HHV-7 DNA. In other cases, the PCR was positive for HCMV and/or HHV-6 DNA in serum samples collected at different stages of transplantation where cell culture and DEAFF test remained negative for HCMV in urine. The description of the clinical details and the results of the laboratory investigation of these cases are described, respectively in the corresponding figure and table of each case.

None of the other patients showed this prolonged shedding of virus in their sera. Virus appeared sporadically or else only a few were available from each patient.

3.3.2.1. Patient No. R7

In this case, a chronic loss of function occurred 6 years after the first transplant, which led to a second transplant. This also failed due to rejection episodes and complete loss of function leading to a third transplant. The third transplant failed after about 5 years (Figure 17). Within this period a total of 22 serum samples were available and tested for the DNAs of HHV-6, HCMV and HHV-7 by PCR and the results are shown in Table 8. In the table, the first 11 serum samples showed positivity to HHV-6 by PCR which was negative in the following 3 specimens which showed positivity for HCMV DNA. After this point, 3 serum samples were positive for HHV-6 and HCMV (dual infection). However, the PCR was negative for HCMV in the remaining samples with only HHV-6 being detected in the following 3 serum samples. In none of the samples was HHV-7 DNA detected. Overall, in a 2 year period 17 of

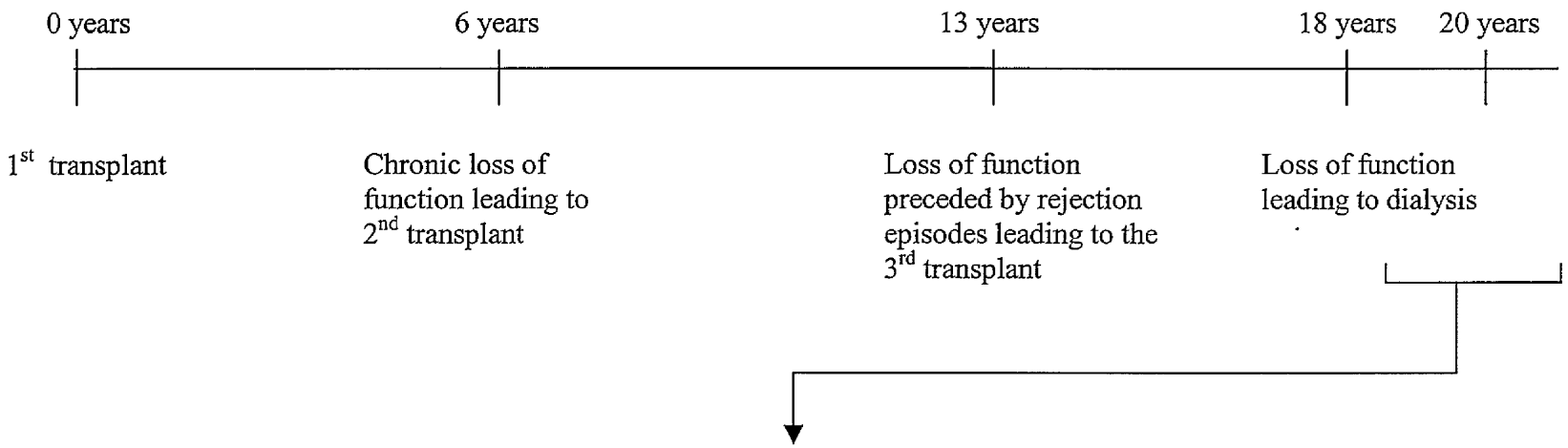


Figure 17. Data representation from Table 8 displayed on a time scale.

Table 8. Detection of HCMV and HHV-6 in serum by PCR in Patient No. R7

No of sample	Date of collection	PCR status in serum for		
		HCMV	HHV-6	HHV-7
1	10/6/96	-	+	-
2	6/8/96	-	+	-
3	3/9/96	-	+	-
4	20/9/96	-	+	-
5	5/11/96	-	+	-
6	3/12/96	-	+	-
7	7/1/97	-	+	-
8	30/4/97	-	+	-
9	12/5/97	-	+	-
10	3/6/97	-	+	-
11	2/9/97	-	+	-
12	5/11/97	+	-	-
13	5/11/97	+	-	-
14	1/12/97	+	-	-
15	28/1/98	+	+	-
16	2/3/98	+	+	-
17	18/3/98	+	+	-
18	6/4/98	-	+	-
19	4/5/98	-	+	-
20	1/6/98	-	+	-
21	13/6/98	-	-	-
22	17/6/98	-	-	

22 sera were positive for HHV-6, 6 were positive for HCMV, all in a 4 month period and on a 8 week period where there were dual infections.

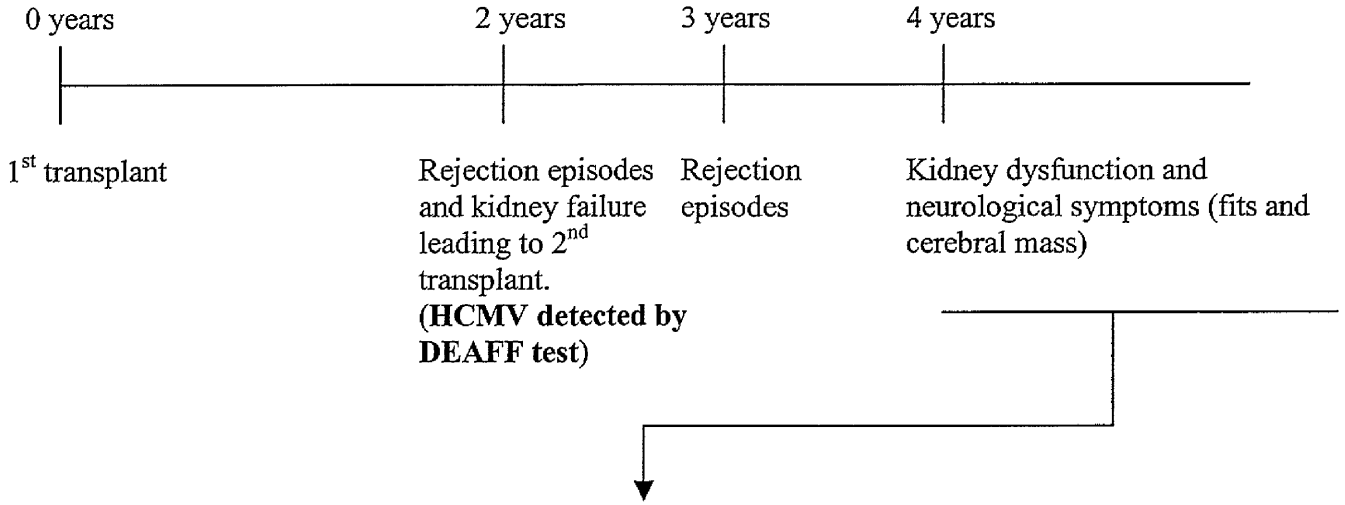
3.3.2.2. Patient No. R36

The patient required transplantation at age of 47 years. Rejection episodes and complete kidney failure occurred within 2 years of the first transplant (Figure 18) This led to a second transplant. At this point DEAFF test was positive for HCMV. Approximately 1 year after the second transplant, rejection episodes manifested with complete kidney dysfunction. The complete loss of function coincided with appearance of neurological symptoms including fits and appearance of cerebral mass. A total of 9 serum samples collected over a period of 24 months on the second to fourth year of the second transplant revealed HHV-6 and HCMV DNA (dual infection) in the first 5 serum samples (Table 9). However, HCMV DNA was not detected on the last 4 samples with HHV-6 DNA being detected in the last 2 serum samples. HHV-7 was not detected in these samples. In addition, virus isolation and DEAFF test remained negative for HCMV on a urine sample collected at a point when both HHV-6 and HCMV DNAs were detected in serum (Figure 18).

Overall, in a 2 year period, 7 of 9 sera were positive for HHV-6 DNA and 5 were positive for HCMV DNA. There was a dual infection for a bout 1 year.

3.3.2.3. Patient No. R41

In this case rejection episodes occurred within the first year of transplant leading to kidney failure (Figure 19). No serum samples were available during this period but a total of 50/53 serum samples collected over 5 years starting on the third year post-transplant showed positivity for HHV-6 and/or HCMV DNA as described in Table 10. None of the samples was positive for HHV-7 DNA. In addition, 3 urine samples collected on 3 points were positive for HCMV by DEAFF test but not by virus isolation (Figure 19).



A total of 9 serum samples collected over this period were tested by PCRs for HCMV, HHV-6 and HHV-7 DNAs. The results are described in Table 9

Figure 18. Data representation from Table 9 displayed on a time scale.

Table 9. Detection of HCMV and HHV-6 in serum by PCR in Patient No. R36

No of sample	Date of collection	PCR status in serum for		
		HCMV	HHV-6	HHV-7
1	1/4/96	+	+	-
2	10/5/96	+	+	-
3	6/9/96	+	+	-
4	3/1/97	+	+	-
5	4/4/97	+	+	-
6	18/7/97	-	-	-
7	10/10/97	-	-	-
8	16/1/98	-	+	-
9	24/4/98	-	+	-

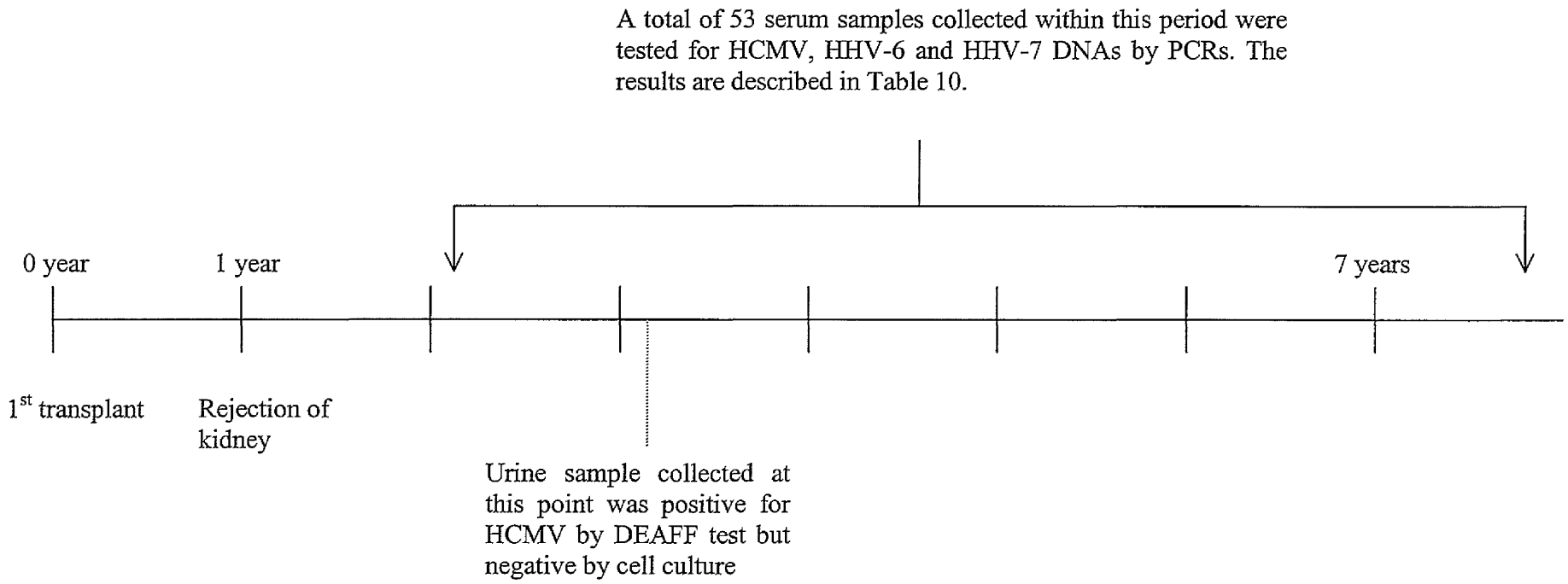


Figure 19. Data representation from Table 10 displayed on a time scale.

Table 10. Detection of HCMV and HHV-6 in serum by PCR in Patient No. R41

No of sample	Date of collection	PCR status in serum for		
		HCMV	HHV-6	HHV-7
1	4/8/93	+	-	-
2	3/11/93	-	+	-
3	8/12/93	-	+	-
4	12/1/94	-	-	-
5	9/2/94	-	+	-
6	9/3/94	+	+	-
7	6/4/94	-	+	-
8	11/5/94	-	+	-
9	6/7/94	+	-	-
10	3/8/94	+	+	-
11	5/10/94	-	+	-
12	9/11/94	-	+	-
13	6/12/94	-	+	-
14	10/1/95	-	+	-
15	15/2/95	-	+	-
16	8/3/95	+	+	-
17	5/4/95	+	+	-
18	3/5/95	-	+	-
19	8/8/95	-	+	-
20	5/9/95	+	+	-
21	3/10/95	-	+	-
22	7/11/95	-	+	-
23	5/11/95	-	+	-
24	10/1/96	+	+	-
25	14/2/96	+	+	-
26	5/3/96	-	+	-
27	2/4/96	-	+	-
28	7/5/96	-	+	-
29	4/6/96	-	+	-
30	3/7/96	-	+	-
31	5/8/96	-	+	-
32	3/9/96	-	+	-
33	8/10/96	-	+	-
34	5/11/96	-	+	-
35	3/12/96	+	+	-
36	7/1/97	-	+	-
37	4/2/97	-	+	-
38	5/3/97	-	+	-
39	26/3/97	+	+	-
40	13/5/97	+	+	-
41	4/6/97	+	+	-
42	8/7/97	-	+	-

Table 10. Continued

No of sample	Date of collection	PCR status in serum for		
		HCMV	HHV-6	HHV-7
43	5/8/97	-	+	-
44	2/9/97	-	+	-
45	7/10/97	-	+	-
46	4/11/97	-	+	-
47	3/12/97	-	+	-
48	6/1/98	-	+	-
49	3/2/98	-	-	-
50	3/3/98	-	-	-
51	7/4/98	-	+	-
52	5/5/98	-	+	-
53	4/6/98	+	+	-

Overall, during the five year period, 48/53 sera were positive for HHV-6 DNA and 14/53 were positive for HCMV DNA. Dual infections occurred in 12 specimens. This patient appeared to have chronic HHV-6 infection with a sporadic appearance of HCMV.

3.3.2.4. Patient No. R22

In this patient, 3 serum samples collected within the year prior to the first transplant were found to be positive for HHV-6 DNA but were negative for HCMV and HHV-7 DNA (Table 11). Another 2 serum samples taken a few months after the first transplant were positive for HHV-6 in one sample and for both HHV-6 and HCMV in the other sample (dual infection). Within this period a urine sample was found positive for HCMV by DEAFF test but negative by virus isolation. Rejection episodes occurred by the end of the first 2 years of the transplantation finally leading to transplant failure that accompanied with severe ophthalmic zoster and herpetic neurologia by the third year. No serum samples were available at this point of transplantation (Figure 20). All six sera, collected over a 14 month period, were positive for HHV-6 DNA.

3.3.2.5. Patient No. R21

Figure 21 shows the clinical and virological findings in this case. Rejection episodes started on the second month of the transplantation and continued throughout the first year. A total of 10 serum samples collected at this stage were positive for HHV-6 only in 5 samples, for HCMV only in 1 sample and for both (dual infection) in 4 serum specimens (Table 12). All the samples were negative for HHV-7 DNA. Urine samples (2 samples) collected approximately 1 year post-transplant were found positive by DEAFF test but not by virus isolation. No serum samples were available at this point but the kidney was reported as "still functioning". Again, this patient appears to exhibit a chronic HHV-6 infection with 9/10 sera positive.

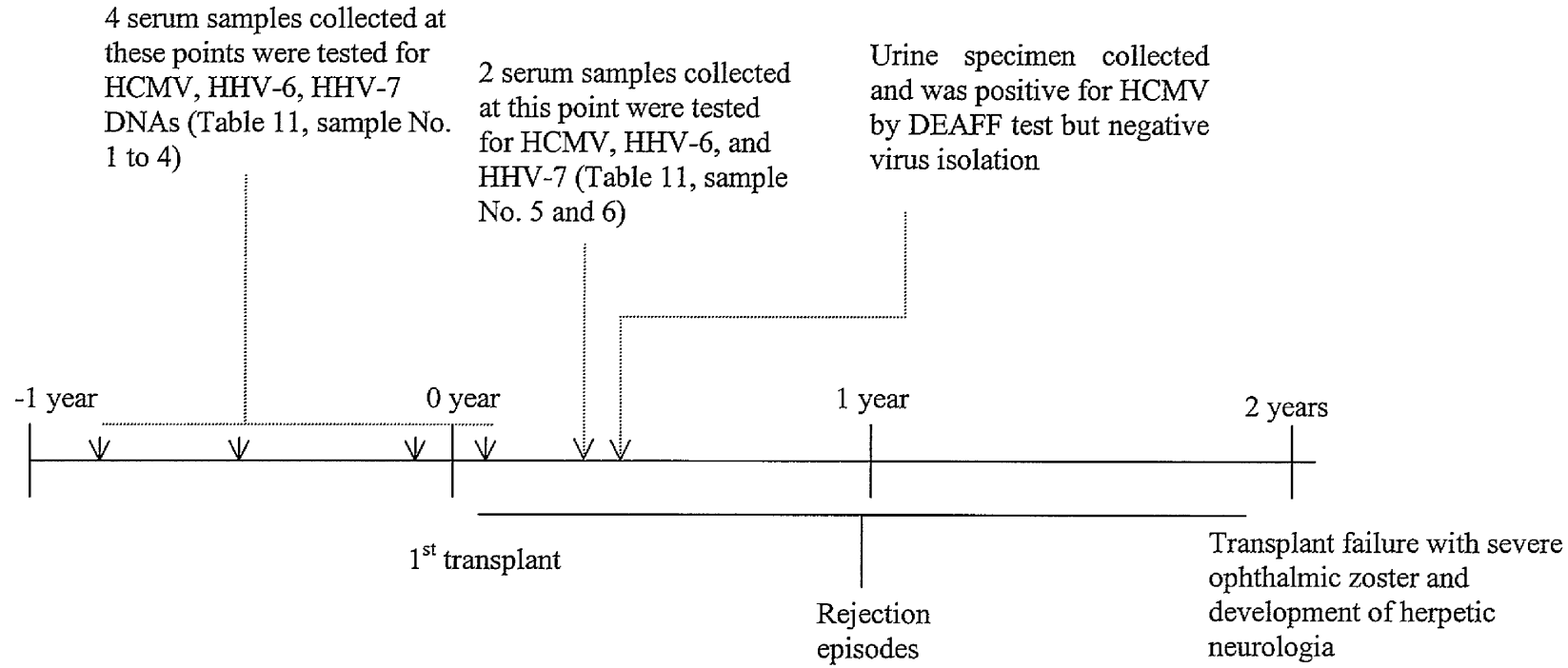


Figure 20. Data representation from Table 11 displayed on a time scale.

Table 11. Detection of HCMV and HHV-6 in serum by PCR in Patient No. R22

No of sample	Date of collection	PCR status in serum for		
		HCMV	HHV-6	HHV-7
1	22/2/94	-	+	-
2	14/6/94	-	+	-
3	14/11/94	-	+	-
4	16/1/95	-	+	-
5	20/4/95	+	+	-
6	20/4/95	+	+	-

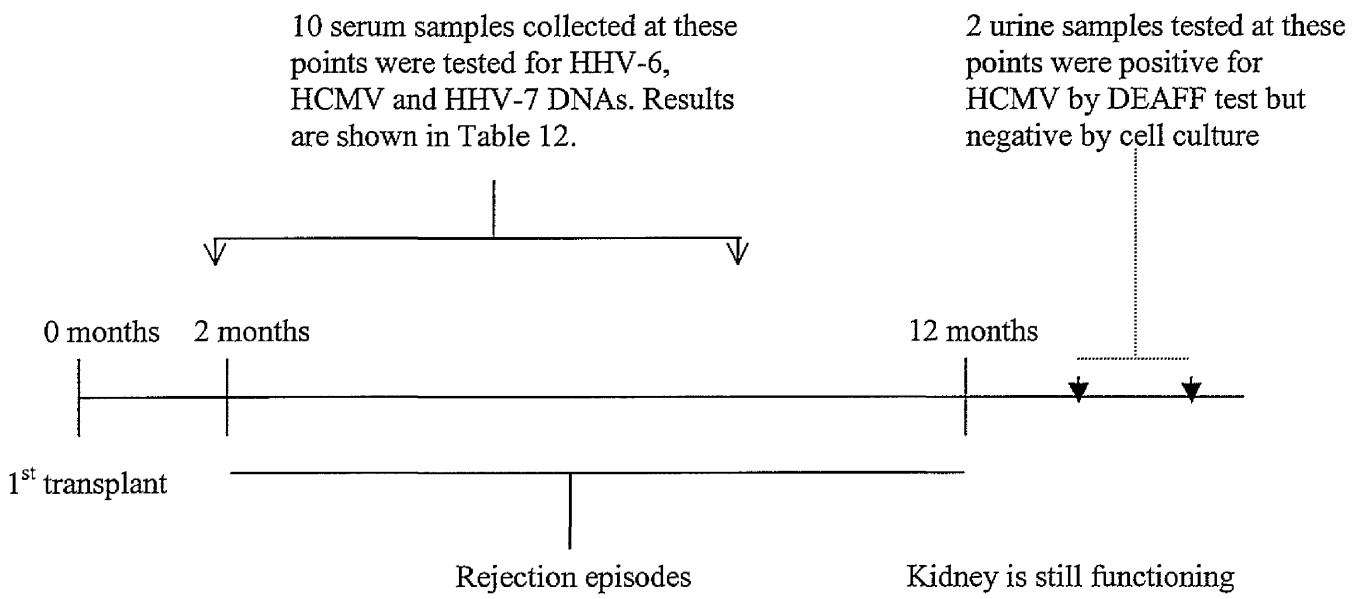


Figure 21. Data representation from Table 12 displayed on a time scale.

Table 12. Detection of HCMV and HHV-6 in serum by PCR in Patient No. R21

No of sample	Date of collection	PCR status in serum for		
		HCMV	HHV-6	HHV-7
1	29/6/93	+	+	-
2	23/1/93	+	+	-
3	29/7/93	+	-	-
4	4/1/94	+	+	-
5	25/1/94	-	+	-
6	29/3/94	+	+	-
7	28/6/94	-	+	-
8	4/10/94	-	+	-
9	12/12/94	-	+	-
10	17/12/94	-	+	-

3.3.3. Comparison of PCR in serum with virus detection in urine specimens

In a total of 8 patients positive for HHV-6 and/or HCMV DNA in serum samples, diagnostic laboratory results of HCMV isolation in urine samples taken at different points of transplantation were available. As shown in Figure 22, the PCR was positive for HCMV in serum significantly earlier than in urine by virus isolation and DEAFF test. In case 1, the PCR was positive for HCMV DNA at least 1 year prior to detection by virus isolation or DEAFF test in urine. In case 2, the detection of HCMV DNA preceded virus isolation positivity by 2 months and HHV-6-PCR was also positive in the same serum samples positive for HCMV indicating dual infection. In case 3, 6 and 8, DNAs of HCMV and HHV-6 were detected concomitantly in serum samples at least 1-2 years before the detection of HCMV by isolation and DEAFF test in urine. In case 7, the patient was positive for HCMV in a total of 5 serum samples by PCR and urine samples by cell culture and DEAFF test. Serum samples were also positive for HHV-6 DNA by PCR. In another 2 cases (case 4 and 5), the PCR was positive for HHV-6 DNA at least 4 months prior to the positivity to HCMV by PCR. In these 2 cases, HCMV PCR positivity coincided with results of virus isolation and DEAFF test.

The PCR results for HHV-6 and HHV-7 could not be compared with conventional techniques due to the fact that the Diagnostic Virology Laboratory does not test for either virus in any clinical specimens.

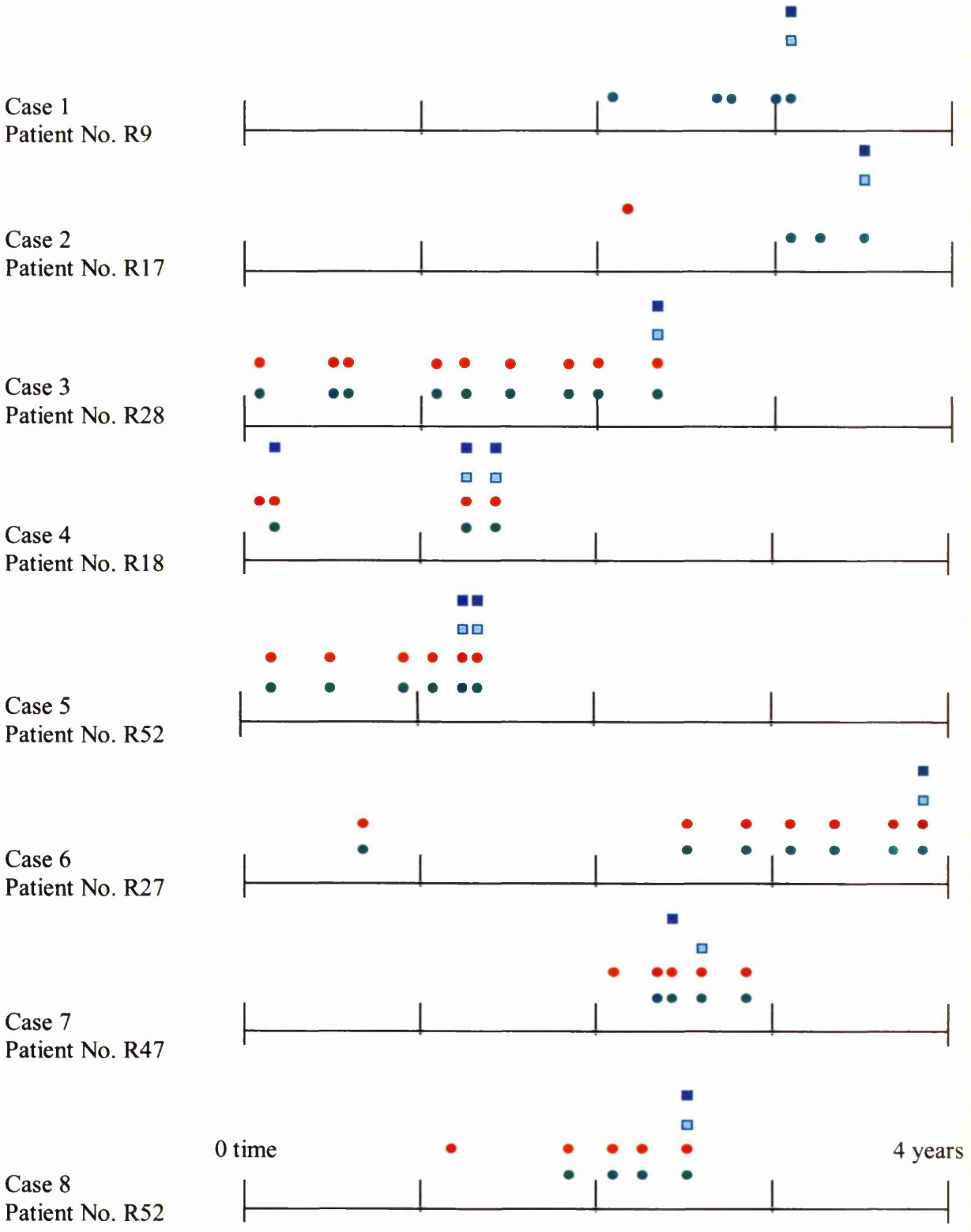


Figure 22. Correlation of PCR in serum with virus detection in urine specimens. ● = HHV-6 by PCR; ● = HCMV by PCR; ■ = HCMV by DEAFF; ■ = HCMV by culture.

3.4. HHV-6, HCMV and HHV-7 in bone marrow transplant recipients

3.4.1. Detection rates

Serum samples from bone marrow transplant recipients were extracted using the guanidinium thiocyanate method and tested retrospectively by the PCRs for the three viruses (HHV-6, HHV-7 and HCMV). The total samples tested were 97 collected from 66 patients and stored at –20°C for a period of 2 to 5 years. These samples were collected at various points during the pre- and post-transplant periods.

The application of HHV-6 PCR in this group of patients is shown in Figure 23, HCMV-PCR in Figure 24 and HHV-7 PCR in Figure 25. Each figure shows examples of positive specimens.

HHV-6 DNA alone was detected in 16 serum samples, HCMV DNA alone in 3 samples and HHV-7 DNA in 1 sample. Both HHV-6 and HCMV DNA were detected in 2 samples and HCMV and HHV-7 DNA in 1 sample. None of the samples were positive for both HHV-6 and HHV-7 DNAs. Of the total of 97 serum samples, 23 were positive for at least one virus and the remaining 74 samples were negative for all three viruses (Table 13).

Of the 66 patients tested, 18 were positive for virus DNA. HCMV DNA was detected in 2 patients, HHV-6 in 11 patients and HHV-7 in 1 patient. Dual infection was detected as HHV-6 and HCMV in 3 patients and as HCMV and HHV-7 in 1 patient (Table 14).

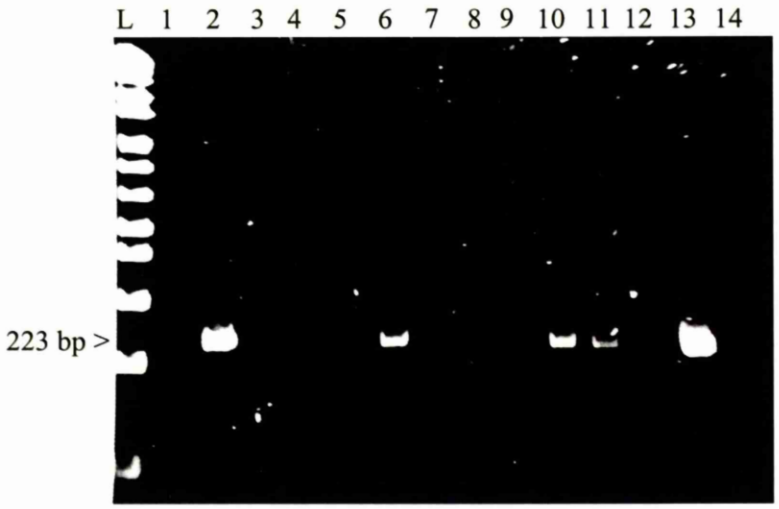


Figure 23. Detection of HHV-6 DNA in serum samples from bone marrow transplant recipients.

Lane L= 1 kb plus DNA ladder

Lanes 2, 6, 10 and 11= serum samples positive for HHV-6 DNA.

Lanes 1, 3, 4, 5, 7-9= serum samples negative for HHV-6 DNA.

Lane 12= PCR negative control.

Lane 13= PCR positive control.

Lane 14= extraction negative control.

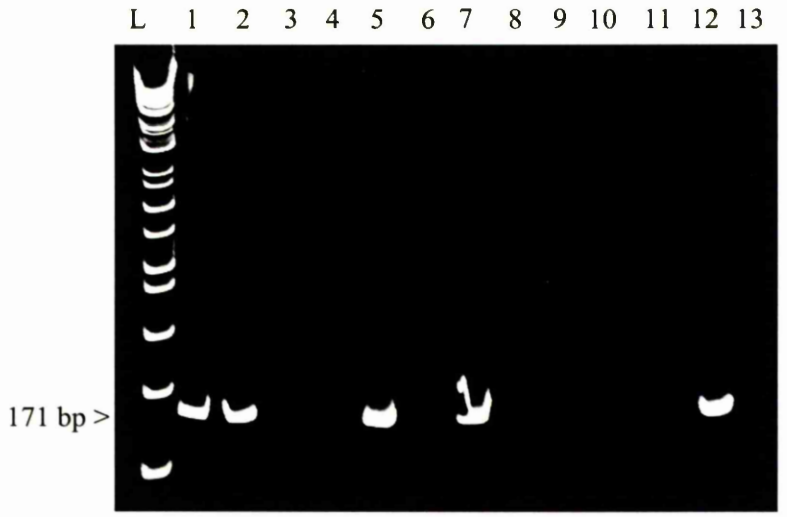


Figure 24. Detection of HCMV DNA in serum samples from bone marrow transplant recipients.

Lane L= 1 kb plus DNA ladder

Lanes 1, 2, 5 and 7= serum samples positive for HCMV DNA.

Lanes 3, 4, 6, 8-10= serum samples negative for HCMV DNA.

Lane 11= PCR negative control.

Lane 12= PCR positive control.

Lane 13= extraction negative control.

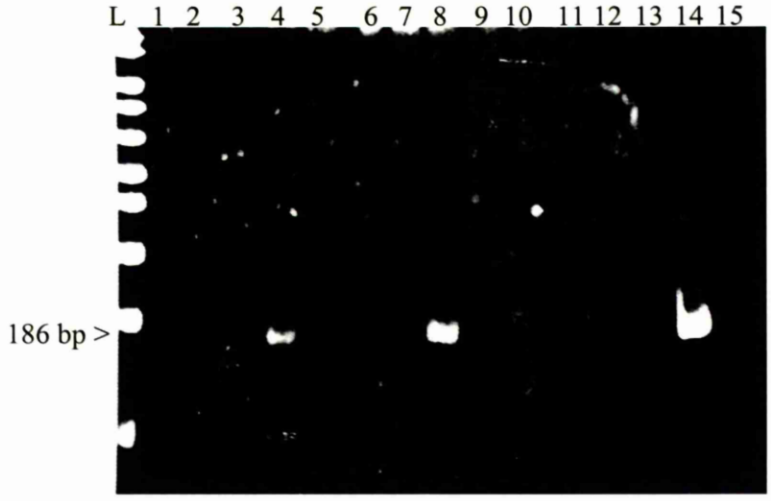


Figure 25. Detection of HHV-7 DNA in serum samples from bone marrow transplant recipients.

Lane L= 1 kb plus DNA ladder

Lanes 4 and 8= serum samples positive for HHV-7 DNA.

Lanes 1-3, 5-7 and 9-12= serum samples negative for HHV-7 DNA.

Lane 13= PCR negative control.

Lane 14= PCR positive control.

Lane 15= extraction negative control.

Table 13. HCMV, HHV-6 and HHV-7 DNA in serum specimens from bone marrow transplant patients

HCMV	HHV-6	HHV-7	No of specimen
+	-	-	3
-	+	-	16
+	+	-	2
-	-	+	1
+	-	+	1
-	+	+	0
+	+	+	0
-	-	-	74
Total			97

Table 14. HCMV, HHV-6 and HHV-7 in bone marrow transplant patients

HCMV	HHV-6	HHV-7	No of Patients
+	-	-	2
-	+	-	11
+	+	-	2
-	+	+	0
+	-	+	1
-	-	+	1
+	+	+	0
-	-	-	49
Total			66

3.4.2. Correlation between virus detection and transplantation period

Clinical details were available for 6 cases which were correlated with the detection of the 3 viruses by PCR as described below.

3.4.2.1. Three cases with Hurler's syndrome (Patients No. B54, B28 and B45)

One of the patients with Hurler's syndrome (Patient No. B54) required 2 transplants. HCMV was detected in a serum sample collected one month after the first transplant and in another serum sample collected two months after the transplant both HHV-6 and HCMV DNAs (dual infection) were detected. During this time, HCMV was also positive by cell culture isolation in urine. In the second transplant, only HHV-6 was detected in 3 serum samples collected after 10 days, 1 month and 2 months post-transplant. HCMV PCR was negative in the latter serum samples.

In another 2 serum samples collected from the other 2 patients with Hurler's syndrome (Patient No. B28 and B45) during the pre-transplant period, HHV-6 DNA was detected in both patients who remained negative for HCMV and HHV-7. No serum samples were tested during the post-transplant period.

3.4.2.2. Patient No. B59

In this patient, HHV-6 DNA was detected in 2 serum samples collected 1 and 2 months post-transplant. The patient had symptoms of respiratory failure, rash, conjunctivitis and stomatitis. Other agents were screened for including chlamydia, mycoplasma, RSV, adenovirus, HSV but all remained negative. The samples were also negative for HCMV and HHV-7 DNA.

3.4.2.3. Patients No. B40 and B48

In 2 serum samples collected during the post transplant period of 2 patients, HHV-7 infection was detected in one patient and dual infection with HCMV and HHV-7 was detected in the other. Both patients had symptoms consistent with respiratory manifestations.

3.4.3. Prospective evaluation of one bone marrow transplant patient (Patient No. B19)

An additional patient, a 2 years old male child required bone marrow transplantation due to X-linked lymphoproliferative syndrome. Pre-transplant screening was negative for viruses by cell culture, electron microscopy, antigen detection techniques and serology. A total of 2 serum samples collected during this period were also negative for HHV-6, HCMV and HHV-7 DNAs by PCR.

The patient received the transplant but developed acute neurological symptoms and manifestations including diffuse high signal compatible with an infection on magnetic resonance imaging (MRI) scan approximately 2 months post-transplant. At this point, serum and CSF samples were collected and tested for viruses including HCMV and HSV by the Diagnostic Virology Laboratory but the samples were negative. Using the PCRs described in this thesis, HHV-6 DNA was detected in 2 serum samples and 2 CSF samples. HCMV and HHV-7 PCR were negative but no follow-up was conducted.

3.5. Association of HHV-6 DNA detection with *hydrops fetalis*

Non-immune *hydrops fetalis* is a clinical entity that can be classified as idiopathic in some cases although several viral agents have been implicated as the aetiological agents in these cases. These viruses include parvovirus B19, HCMV and adenovirus. In this section, cases with *hydrops fetalis* were screened for the presence of HHV-6, HHV-7 and HCMV.

A total of 4 cases and 11 controls were studied. Tissue sections including heart, lung, liver and kidneys from the fetus and the placenta from the mother were prepared and screened for the presence of HHV-6, HHV-7 and HCMV DNAs.

3.5.1. *Hydrops fetalis* Case 1

3.5.1.1. Clinical details and post-mortem examination

The mother of the fetus in this case was 30-years old with no previous clinical history and a gestation of 17 weeks (17/40) by ultrasound which revealed extensive fetal hydrops. The placenta showed small amount of marginal haemorrhage but no focal lesions were observed on slicing. Histological examination of the fetus showed normal organs and tissues except for the lungs which had an immature appearance. Placental microbiology showed haemolytic streptococcus group B while the liver culture was negative and virus isolation of the pleural fluid from the fetus was also negative. Blood samples were also taken from the fetus and revealed *Toxoplasma gondii* and HCMV antibodies which were not indicative of current infection. Autopsy examination demonstrated growth parameters consistent with the gestational age, nuchal oedema and skeletal abnormalities. The final diagnosis in this case was Trisomy 21.

3.5.1.2. Detection of HHV-6 DNA in paraffin-embedded tissue sections

HHV-6 DNA had been previously detected by the PCR in tissue sections of the kidney, liver, heart and lungs of this case (Ashshi 1999). This result has been confirmed in this study (Figure 26). In addition, sections of the placenta were prepared as described in section 2.2.2.5. This was also found to contain HHV-6 DNA as shown in Figure 27 where duplicates are clearly PCR positive in lanes 1 and 2.

All the tissue sections of this case (kidney, liver, heart, lung and placenta) were negative for HHV-7 and HCMV DNAs.

3.5.2. Screening for HHV-6, HCMV and HHV-7 in other cases of *hydrops fetalis* and non-hydrops controls

A further 3 cases of *hydrops fetalis* were also tested. These are summarised together with case 1 in Table 15. Paraffin embedded tissue sections of placentas were available from two mothers; liver, kidney, and lung sections from 2 cases and liver, kidney and heart sections from one case. All were extracted and amplified with the HHV-6, HHV-7 and HCMV PCRs. All PCRs remained negative in all cases. Figure 28 shows the negative HHV-6 PCR on all these tissue sections tested from case 3 and 4.

A total of 11 controls were also studied (Table 16). These included non-hydrops cases with clinical final diagnosis of chorioamnionitis in 4 cases and streptococcal B sepsis in 1 case. Gestation periods, where available, ranged from 17 to 41 weeks. In each case, tissue sections from heart, lung, liver, kidney and placenta were available and all were tested for the three viruses by PCR. All the 11 control cases were negative in the three PCRs (HHV-6, HHV-7 and HCMV) in all tissue sections tested.

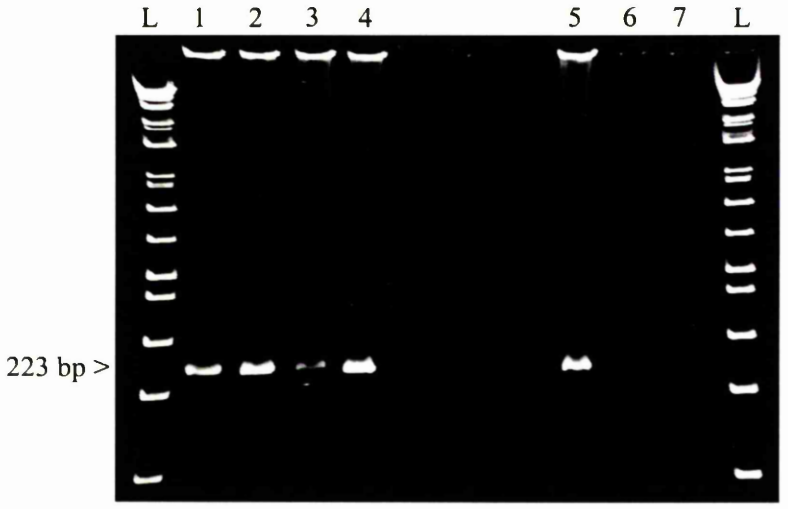


Figure 26. Detection of HHV-6 DNA in tissue sections from the *hydrops fetalis* case 1.

Lane L= 1kb plus DNA ladder.

Lane 1= Kidney tissue sections positive for HHV-6 DNA

Lane 2= Liver tissue sections positive for HHV-6 DNA

Lane 3= heart tissue sections positive for HHV-6 DNA

Lane 4= Lungs tissue sections positive for HHV-6 DNA

Lane 5= PCR positive control.

Lane 6= PCR negative control.

Lane 7= extraction negative control.

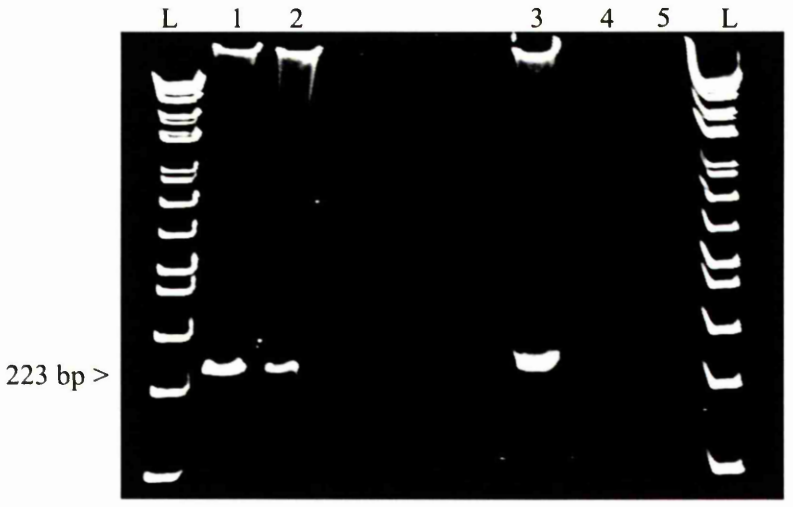


Figure 27. Detection of HHV-6 DNA in tissue sections from the placenta of case 1.

Lane L= 1kb plus DNA ladder.

Lane 1 and 2= Duplicates of placenta sections positive for HHV-6 DNA

Lane 3= PCR positive control.

Lane 4= PCR negative control.

Lane 5= extraction negative control.

Table 15. Tissues tested for virus in fetal hydrops cases

Case No	Tissue					Diagnosis
	Heart	Lung	Liver	Kidney	Placenta	
1	✓	✓	✓	✓	✓	Turner's syndrome
2		✓	✓	✓		Down's syndrome
3	✓		✓	✓	✓	Unexplained
4		✓	✓	✓	✓	Unexplained

Table 16. Gestation and diagnosis of non-hydrops cases used as controls

Case No	Sex	Gestation	Diagnosis
1	F	41/40	Group B streptococcus infection
2	F	40/40	Unexplained
3	F	19/40	Chorioamnionitis
4	M	24/40	Chorioamnionitis (Coliform)
5	M	20/40	Hydrocephalus
6	F	19/40	Trisomy 20
7	F	19/40	Chorioamnionitis (Coliform)
8	NA	18/40	Villitis (unknown cases)
9	F	20/40	Chorioamnionitis (Bacteriodes)
10	M	17/40	Pseudomonas
11	F	21/40	Hanhart syndrome

NA= not available

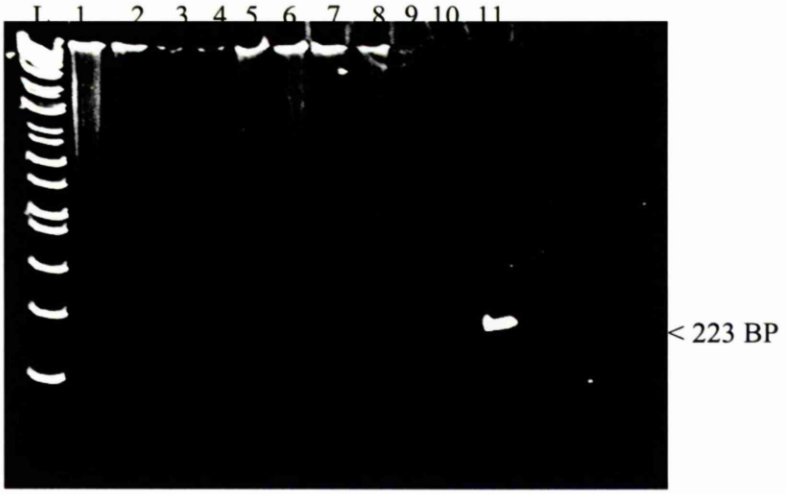


Figure 28. HHV-6 PCR on tissue sections from 2 cases of *hydrops fetalis*.

Lane L= 1 kb plus DNA ladder.

Lane 1-4= negative tissue sections of heart, liver, kidney, and placenta of case 3

Lanes 5-8= negative tissue sections of lung, liver, kidneys and placenta of case 4.

Lanes 9= PCR negative control.

Lanes 10= extraction negative control.

Lanes 11= PCR positive control

3.5.3. Screening for PCR inhibition

In order to test for the presence of non-specific inhibitors of PCR, the primers GH20 and PCO4 were used to amplify a fragment of the human β-globin. The test resulted in a PCR product of 268 bp in all tissue sections from the *hydrops fetalis* and control cases. Examples are shown with two of the fetal hydrops cases in Figure 29. This result excludes the possible occurrence of non-specific PCR inhibition giving false negative results.

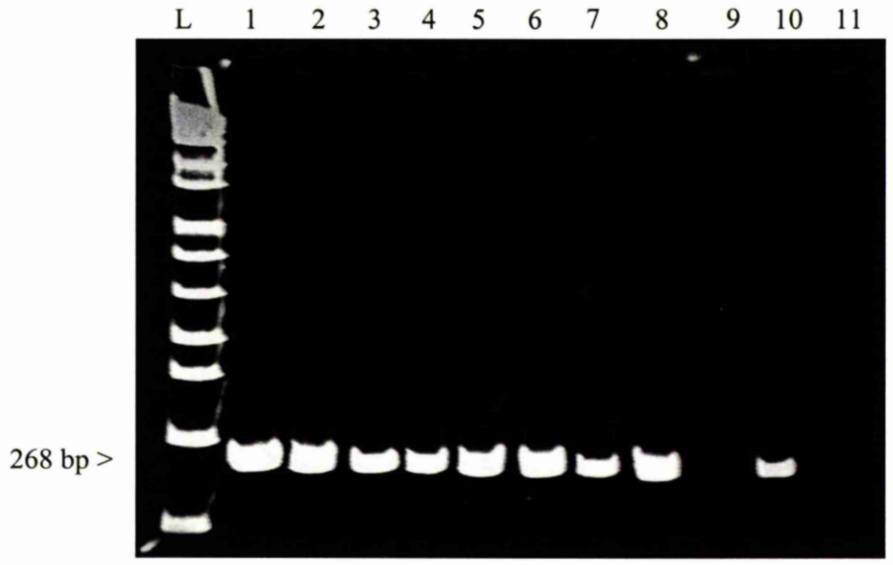


Figure 29. Amplification of human β-globin gene from cases of *hydrops fetalis* that were negative for HHV-6 DNA by PCR.

Lane L= molecular weight marker (1kb plus DNA ladder)

Lane 1-4= tissue sections of the liver, kidney, heart and placenta of case 3 of *hydrops fetalis*, respectively.

Lanes 5-8= tissue sections of the liver, kidney, lung and placenta of case 4 of *hydrops fetalis*, respectively.

Lane 9= extraction negative control

Lane 10= PCR positive control.

Lane 11= PCR negative control.

3.6. Detection of HHV-6 DNA in chronic psoriasis

A total of 10 patients (mean age 45±2SE range 23-69) with untreated chronic plaque psoriasis had 4 mm diameter skin biopsies taken from involved and uninvolved skin under lignocaine local anaesthetic. The skin biopsies were initially snap frozen in liquid nitrogen and stored at –80°C. These were then extracted as described in section 2.2.2.6.

HHV-6 DNA was detected in 2 skin biopsies representing the involved and uninvolved skin of one patient. The test was repeated 3 times on both biopsies and remained positive in every case.

Figure 30 illustrates the results obtained with the HHV-6 PCR on these biopsies. Lane 1 and 2 shows, respectively, the involved and the uninvolved skin biopsy positive for HHV-6 DNA. HHV-7 and HCMV was not detected in either the involved or uninvolved skin biopsies in any of the patients.

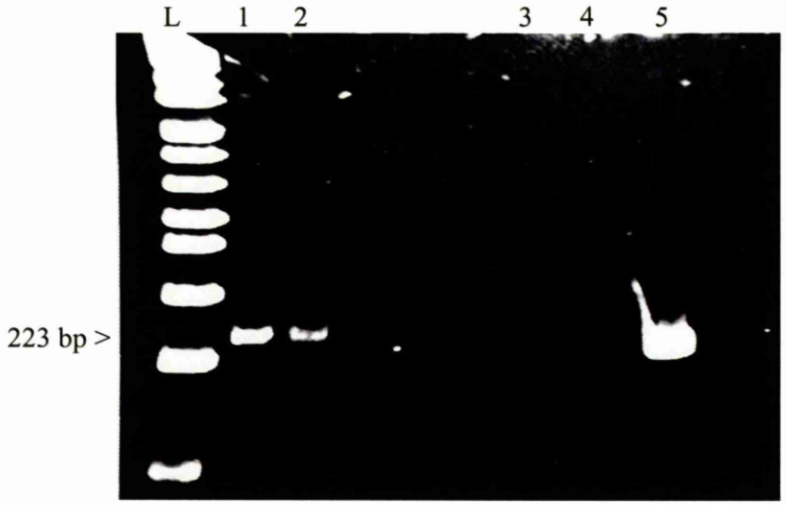


Figure 30. HHV-6 PCR on skin biopsy from the positive case of chronic psoriasis.

Lane L = 1 kb plus DNA ladder.

Lane 1 = involved skin biopsy.

Lane 2 = uninvolved skin biopsy.

Lane 3 = PCR negative control.

Lane 4 = extraction negative control.

Lane 5 = PCR positive control.

3.7. Identification of HHV-6 variant A and B

3.7.1. Typing of the 223 bp HHV-6 PCR products by *Ava*II

3.7.1.1. Renal transplant recipients

All the HHV-6 serum positive samples were typed as HHV-6 variant A using the restriction endonuclease *Ava*II on the 223bp PCR product. Figure 31 demonstrates the typical restriction pattern produced on these samples. The DNA fragments produced are consistent with type A (138 bp and 85 bp in lanes 5 and 7). In the figure, lane 1 is the PCR product from HHV-6 type A-infected HSB-2 cells and lane 3 is that of HHV-6 type A plasmid DNA cut with *Ava*II as controls for the enzyme activity.

3.7.1.2. Bone marrow transplant recipients

All the serum samples positive for HHV-6 were typed using the restriction endonuclease *Ava*II which cuts the PCR products of HHV-6 variant A but not those of B. All the HHV-6 detected in the serum samples from the bone marrow transplant group were typed as variant A. This is clear from the restriction pattern of 138 bp and 85 bp DNA fragments in lanes 5, 7 and 9 in Figure 32. Variant B is not cut by the enzyme as shown in lane 2 of the figure.

3.7.1.3. *Hydrops fetalis*

The HHV-6 PCR products (223 bp) from the tissue sections of kidney, liver, heart, lung and placenta of *hydrops fetalis* case 1 were treated with the enzyme *Ava*II. Figure 33 shows that all the tissue sections contained HHV-6 DNA of variant A. Lanes 1 and 2 are treated and untreated PCR products from HHV-6 type A-infected HSB-2 cells, respectively. Lanes 3 and 4, 5 and 6, 7 and 8, 9 and 10 are, respectively treated and untreated PCR products from the tissue sections of kidney, liver, heart and lungs. Lanes 11 and 12, 13 and 14 are treated and untreated replicates of the PCR products from the placenta tissue sections. All the treated PCR products

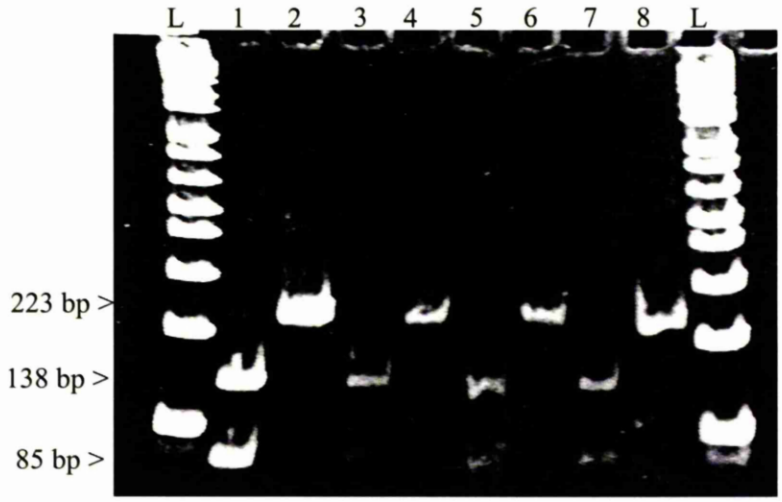


Figure 31. Typing of HHV-6 PCR products from serum specimens of renal transplant recipients using the restriction endonuclease *Ava*II.

Lanes L= molecular weight marker (1kb plus DNA ladder).

Lane 1= PCR products from HHV-6 type A-infected HSB-2 cells cut with *Ava*II.

Lane 2= PCR products from HHV-6 type A-infected HSB-2 cells uncut.

Lane 3= PCR products from HHV-6 type A plasmid DNA cut with *Ava*II.

Lane 4= PCR products from HHV-6 type A plasmid DNA uncut.

Lane 5 and 6= PCR products from positive serum sample cut and uncut with the enzyme *Ava*II, respectively.

Lane 7 and 8= PCR products from positive serum sample cut and uncut with the enzyme *Ava*II, respectively.

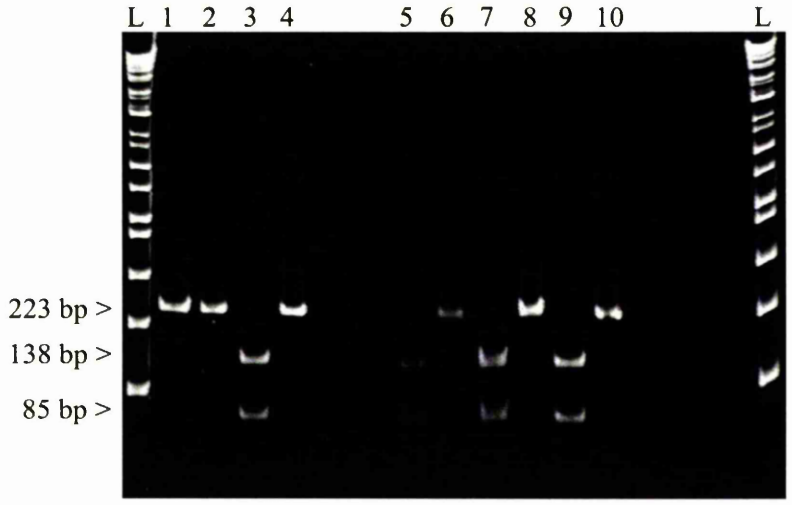


Figure 32. Typing of HHV-6 PCR products from serum specimens of bone marrow transplant recipients using the restriction endonuclease *Ava*II.

Lanes L= molecular weight marker (1kb plus DNA ladder).

Lane 1= PCR products from HHV-6 type B.

Lane 2= PCR products from HHV-6 type B treated with *Ava*II.

Lane 3= PCR products from HHV-6 type A plasmid DNA cut with *Ava*II.

Lane 4= PCR products from HHV-6 type A plasmid DNA uncut with *Ava*II.

Lane 5, 7 and 9= PCR products from positive serum samples cut with the enzyme *Ava*II.

Lane 6, 8 and 10= PCR products from positive serum samples not treated with *Ava*II.

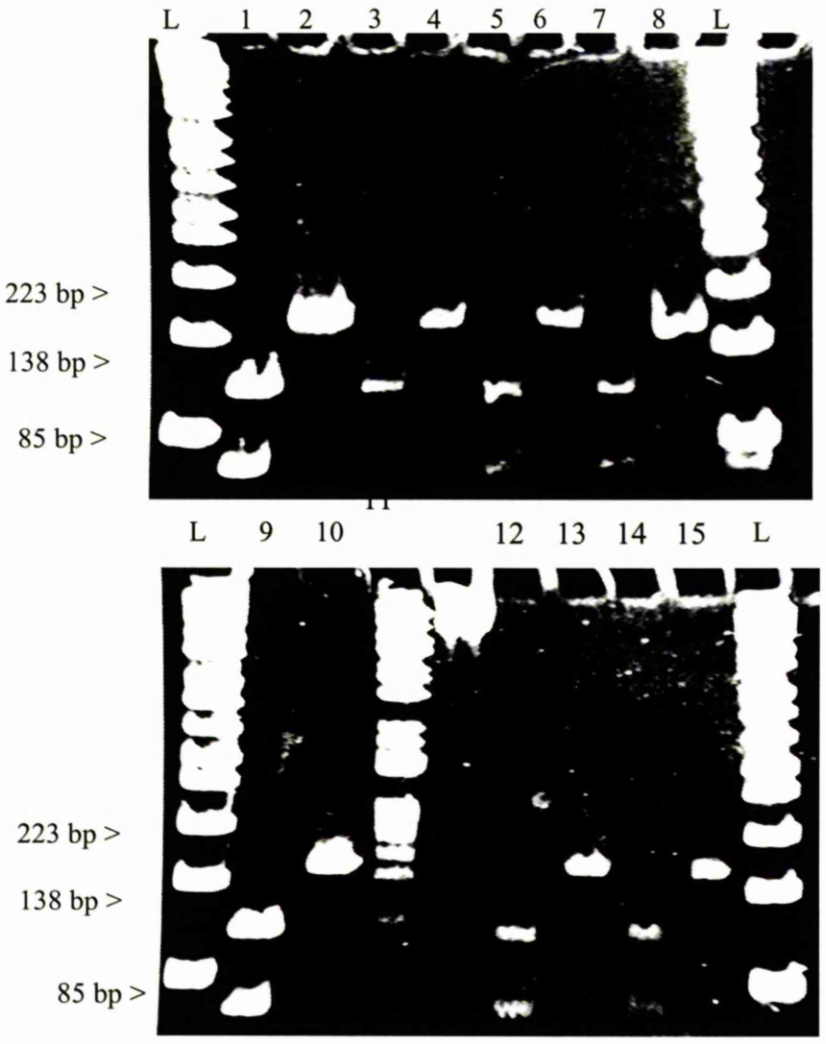


Figure 33. *Ava*II cleavage of HHV-6 PCR products from *hydrops fetalis* case 1.

Lane L= 1 kb plus DNA ladder

Lane 11= 1Kb DNA ladder.

Lane 1 and 2= cut and uncut HHV-6 type A-infected HSB-2 cells

Lane 3-4, 5-6, 7-8, 9-10= cut and uncut PCR products from the tissue sections of kidney, liver, heart and lungs, respectively.

Lanes 12-13 and 14-15= cut and uncut PCR products from the placenta tissue sections.

revealed the restriction pattern of 138 and 85 bp which indicates that all the PCR products obtained were from variant A of HHV-6.

3.7.1.4. Chronic psoriasis

The DNA fragments produced are also consistent with type A (138 bp and 85 bp) as shown in lanes 1 and 3 of Figure 34 representing, respectively, the involved and the uninvolved skin. In the figure, lanes 2 and 4 are the uncut 223 bp HHV-6 PCR products as controls for the enzyme activity.

3.7.2. Confirmation of the typing protocol using *Hae*III

The available nucleotide sequences of the 223 bp PCR products of HHV-6 variant A and B were aligned with each other utilising the Basic Local Alignment Search Tool (BLAST) family of programs at the National Centre for Biotechnology Information (NCBI). All the sequences analysed were 223 bp in length and Figure 35 provides a detailed description for the results obtained.

The nucleotide sequences of both HHV-6 variants were analysed for restriction endonuclease recognition sequences utilising the software WebCutter Version 2.0. Only a total of 3 commercially available restriction endonucleases (*Hae*III, *Ava*II and *Hin*fI) were found to be discriminative. The enzyme *Ava*II, as described earlier, cuts variant A but not B whereas *Hin*fI cuts variant B but not A. The restriction endonuclease *Hae*III cuts both the variants but producing distinguishable restriction patterns.

In Figure 35, the recognition sequences and the cut sites of these enzymes on both variants of HHV-6 are shown. The enzyme *Hae*III recognises the sequence GG/CC. In the figure, the green-highlighted bases represent this recognition sequence. *Ava*II recognises the sequence G/GTCC (yellow-highlighted bases) whereas *Hin*fI identifies the nucleotide sequence G/AATC (violet-highlighted bases). The enzyme *Hae*III has one cut site at position 185 on variant A but two cut sites at positions 83 and 185 on

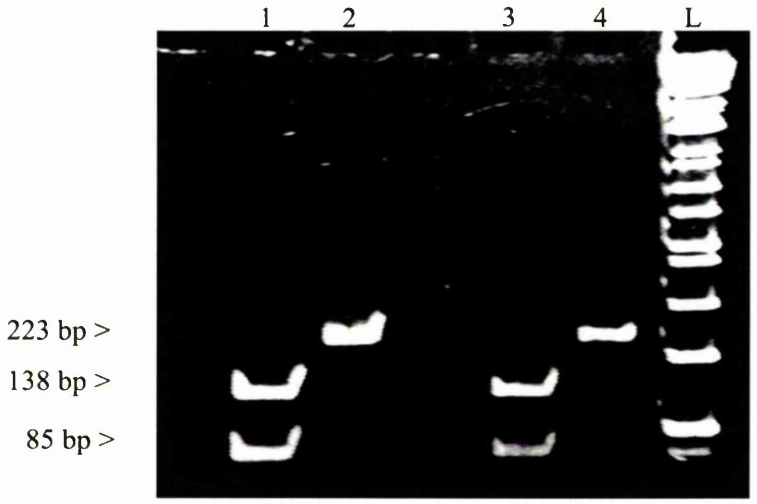


Figure 34. Typing of HHV-6 PCR products from the case of chronic psoriasis.

Lane L = 1 kb plus DNA ladder.

Lane 1= *Ava*II cut involved skin.

Lane 2 = uncut involved skin.

Lane 3 = *Ava*II cut uninvolved skin.

Lane 4 = uncut uninvolved skin.

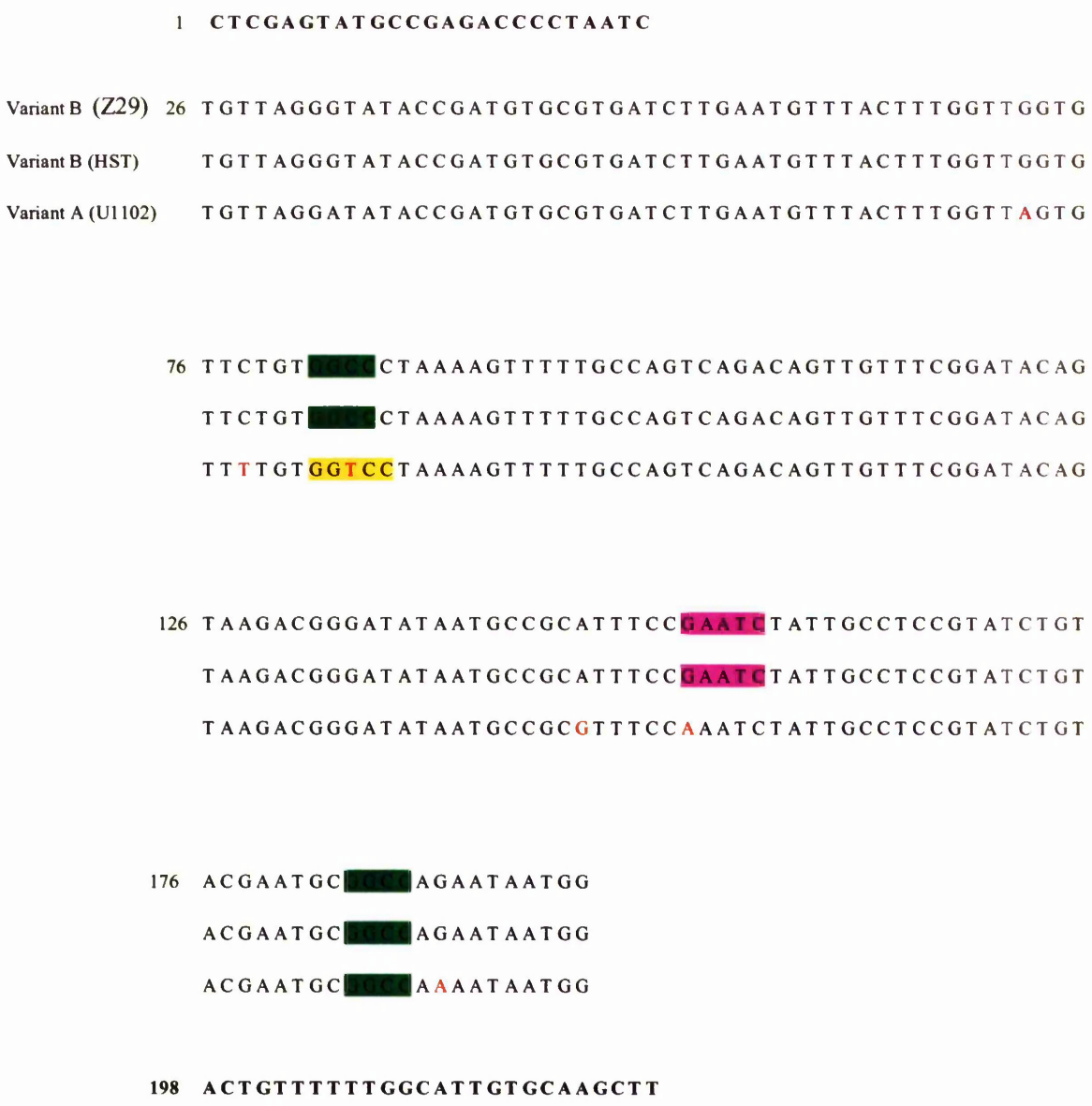


Figure 35. Alignment of the 223 bp HHV-6 PCR products nucleotide sequence of variant A and B. Nucleotide sequences of HHV-6 variant A (U1102) or B (Z29 and HST). The nucleotide sequences of the primers H6-6 and H6-7 are also shown (Bold bases). All the sequences analysed are 223 base pair (bp) in length. Comparison of the nucleotide sequences of both variants shows a total of 7 base mismatches as G↔A (at position 33, 72, 147, 153 and 189) and C↔T (at position 78 and 84). The bases GGCC; GAATC and GGTCC represent, respectively, the recognition sequences of the restriction endonucleases *Hae*III, *Ava*II and *Hinf*I.

variant B. The enzyme *Ava*II has a cut site at position 82 of variant A but it does not cut the sequence of variant B whereas *Hinf*I has a cut site at position 153 of variant B but it does not cut the sequence of variant A.

The specificity of the enzyme *Ava*II to discriminate between variant A and B shown above (section 3.7.1) was confirmed using *Hae*III. A total of 10 HHV-6 positive serum samples from the bone marrow transplant group were reamplified and subjected to the restriction endonuclease *Hae*III. All the tested samples were typed as variant A confirming the results obtained by the enzyme *Ava*II. Figure 36 demonstrates the variant-specific restriction patterns for HHV-6 variants A and B produced by the enzyme *Hae*III (185 and 38 bp DNA fragments for variant A and 102, 83 and 38 bp DNA fragments for variant B). The specificity of *Hinf*I was not confirmed in the laboratory.

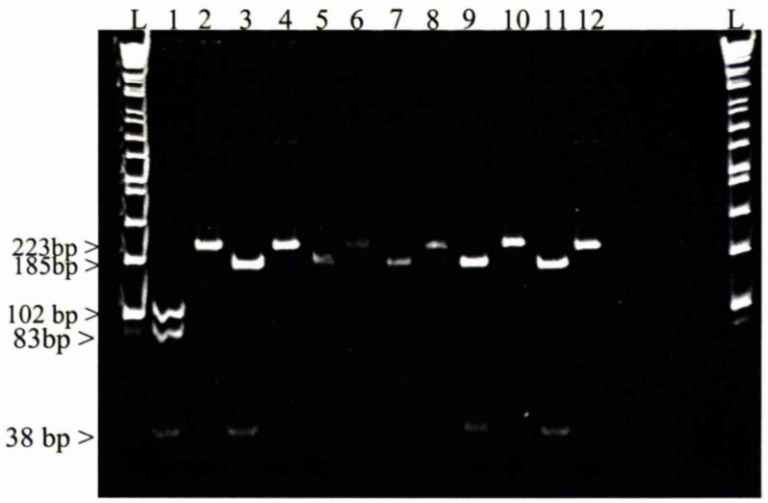


Figure 36. Typing of HHV-6 PCR products from serum samples by the enzyme *Hae*II.

Lanes L= molecular weight marker (1kb plus DNA ladder).

Lane 1= PCR products from HHV-6 type B cut with *Hae*III.

Lane 2= PCR products from HHV-6 type B not treated with *Hae*III.

Lane 3= PCR products from HHV-6 type A cut with *Hae*III.

Lane 4= PCR products from HHV-6 type A not treated with *Hae*III.

Lane 5, 7, 9 and 11= PCR products from positive serum samples of bone marrow transplant patients cut with the enzyme *Hae*III.

Lane 6, 8, 10 and 12= PCR products from positive serum samples of bone marrow transplant patients not treated with *Hae*III.

3.8. Reamplification of HHV-6 from serum samples using the primers DROB1 and DROB2

Due to the unexpected presence of HHV-6 variant A in the serum samples tested, it was necessary to confirm the presence of HHV-6 in these samples using different set of primers. The primers DROB1 and DROB2 produced a PCR product of 385 bp in size from different region of the genome. A total of 40 serum samples that were positive (20 samples) and negative (20 samples) for HHV-6 DNA using the primers H6-6 and H6-7 were reamplified using the HHV-6 PCR based on the primers DROB1 and DROB2. In all 40 samples, 20 were found positive and the remaining 20 samples were found negative by the primers DROB1 and DROB2. These results were in complete agreement of those obtained by the primers H6-6 and H6-7.

Figure 37 demonstrates examples of the positivity of the DROB1/DROB2-based HHV-6 PCR in 4 serum samples from bone marrow transplant recipients as shown by the occurrence of the band 385 bp in lanes 1, 2, 3 and 4. Another 3 samples remained negative for the expected product.

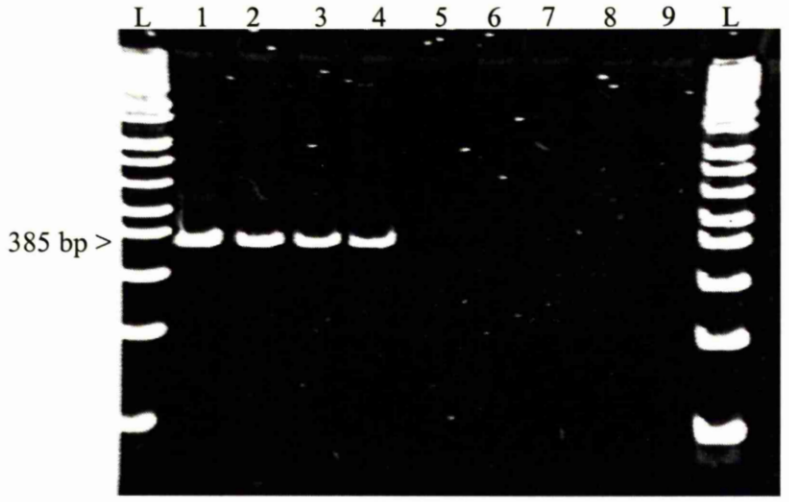


Figure 37. Reamplification of HHV-6 positive serum samples using the primers DROB1 and DROB2.

Lane L= molecular weight marker (1kb plus DNA ladder)

Lane 1-4= serum samples that were positive for HHV-6 using the primers H6-6 and H6-7.

Lanes 5-7= serum samples that were negative for HHV-6 by the primers H6-6 and H6-7.

Lane 8= PCR negative control.

Lane 9= extraction negative control.

4. DISCUSSION

4.1. Development and optimisation of PCRs

HCMV disease is a very well known clinical problem in immunocompromised patients including transplant recipients and the virus is a well-established cause of congenital infection. The clinical importance of HHV-6 is increasing while HHV-7 is acquired early in childhood but its association with clinical disease is still under ongoing investigation. Both HHV-6 and HHV-7 have been suggested to act as cofactors for HCMV disease progression (Chan *et al.,* 1997; Osman *et al.,* 1996; Herbein *et al.,* 1996; Desjardin *et al* 1998) and coinfection or reactivation of HHV-6 and HCMV in immunocompromised patients may be the trigger for severe viral disease (Ratnamohan *et al.,* 1998).

Conventional techniques, such as virus isolation, antigen detection techniques and serology can be insensitive when compared to DNA amplification techniques such as the PCR. This study aimed to develop and optimise PCRs for the detection of HHV-6, HCMV and HHV-7 DNAs in serum samples from transplant recipients. The study is mainly based on retrospective analysis of stored frozen serum samples collected from renal and bone marrow transplant recipients.

Oligonucleotide primers that have been already shown to produce satisfactory results for the detection of the 3 targets were utilised in this study. HCMV PCR uses the primers 233C and 724C described by McElhinney *et al.,* (1995), HHV-6 PCR contains the primer pair H6-6/H6-7 designed by Gopal *et al.,* (1990) and HHV-7 DNA is amplified using the primers HV7 and HV8 described by Berneman *et al.,* (1992). In the presence of the usual concentrations of PCR components (1.5 mM $MgCl_2$, 1.25 units of enzyme and 200 μM of each dNTP), the PCRs produced sensitivities of 100 copies of HCMV or HHV-6 plasmid DNAs and 10^{-5} dilution of HHV-7 infected cells.

PCR components such $MgCl_2$, enzyme and dNTPs concentrations are of vital importance to the performance of any PCR. Increasing these reactants may lead to production of non-specific signals while the use

of low concentrations may lead to reduction in the yield or even failure of the test (Cha and Thilly, 1993; Robertson and Walsh-Weller, 1998).

As one of the developments in PCR technology, the hot start method aimed to reduce or even eliminate non-specific interactions between the primers (primer dimers). Primer dimers arise due to annealing of primers together and extension at low temperature owing to the activity of taq polymerase at low temperatures (Chou *et al.,* 1992).

Although previous versions of the hot start method utilised mechanical means in which one of the key components of the test is not added, a recent protocol utilises an enzyme (amplitaq gold) that is activated only when the mixture is heated at 94°C for approximately 10 minutes (Brownie *et al.,* 1997; Birch *et al.,* 1996). This prevents the chance of contamination noticed in the mechanical versions of the method and significantly facilitates automation. The hot start PCR method (amplitaq gold-based) utilised in this study resulted in at least 10-fold improvement in the sensitivities of the PCRs to detect their respective targets i.e., 10 copies for HCMV DNA, 10 copies of HHV-6 DNA and 10^{-6} dilution of HHV-7-infected cell DNA were detected. These limits were consistent over a series of experiments.

4.2. The choice of serum as clinical sample

Detection of HCMV in clinical samples has been faced in several studies with the concept of insignificance. This is mainly due to the possibility of detection of latent HCMV DNA in seropositive patients in the absence of clinical disease (Ratnamohan *et al.,* 1992; Stanier *et al.,* 1989). Antiviral drugs such as ganciclovir and foscarnet, used alone or in combination with intravenous immunoglobulins have clinical benefits, either preventing or curing HCMV disease (Emanuel *et al.,* 1988; Goodrich *et al.,* 1991; Schmidt *et al.,* 1991). However, these therapeutic agents are associated with toxicity and excess cost and must therefore be used to treat only patients at high risk of HCMV disease. Thus, the optimal diagnostic approach would be that which is the most predictive of the occurrence of HCMV disease.

After infection, the virus may be excreted in urine for months or years in subjects with or without clinical symptoms (Peterson *et al.,* 1980; Meyers *et al.,* 1986) and the viral load may be an important criterion to consider when detecting HCMV DNA in urine specimens (Cope *et al.,* 1997). Likewise, the significance of qualitative detection of HCMV by PCR in other clinical samples such as blood differed in several reports. A number of reports have demonstrated that HCMV viraemia has a predictive value for severe HCMV disease (Peterson *et al.,* 1980; Meyers *et al.,* 1986; The *et al.,* 1990) but HCMV DNA has been detected in the peripheral blood mononuclear cells of healthy blood donors (Stanier *et al.,* 1989; Bevan *et al.,* 1991).

To overcome this difficulty several studies have shown the importance of quantitative PCR (Boeckh and Boivin, 1998; Boom *et al.,* 1999) but the threshold level of discriminating active infection from clinically insignificant values remains to be identified. Indeed, quantitative monitoring may not be more predictive to HCMV disease than qualitative PCR detection (Aitken *et al.,* 1999).

Reverse transcription PCR has also been utilised to detect HCMV late mRNA as a method to indicate symptomatic infection but was less sensitive than detection of HCMV DNA by PCR. However, both methods were equally useful for identification of bone marrow transplant recipients at highest risk of HCMV disease (Gozlan *et al.,* 1996). Moreover, recent studies have focused on the monitoring of the expression of HCMV immediate early and late mRNA using nucleic acid sequence-based amplification (NASBA) with preliminary encouraging results but more evaluation of this method is required (Blok *et al.,* 1998; Blok *et al.,* 1999).

Another interesting approach to define and detect symptomatic HCMV infection is the detection of HCMV DNA in serum by PCR (Cunningham *et al.,* 1995). The principle of this approach is that active viral replication may lead to release of virus from cells into serum. Detection of HCMV DNA by PCR in plasma or serum has been shown to be a sensitive and specific early marker for HCMV infection and HCMV disease in bone marrow transplant recipients (Aspin *et al.,* 1994; Hebart *et al.,* 1996; Ishigaki *et al.,* 1991; Wolf and Spector, 1995), renal transplant recipients (Brytting *et al.,* 1992; Cunningham *et al.,* 1995; Freymuth *et al.,* 1994; Wolf and Spector, 1993), liver transplant recipients (Freymuth *et al.,* 1994; Patel *et al.,* 1994; Patel *et al.,* 1995; Schmidt *et al.,* 1995), congenitally infected newborns (Nelson *et al.,* 1995) and HIV-seropositive patients (Dodt *et al.,* 1997). Likewise, detection of HHV-6 DNA by PCR in the serum has been shown to be a valuable tool for the diagnosis of acute and/or active viral infection (Osman *et al.,* 1997). Huang *et al.,* (1992) studied 30 subjects; 10 were experiencing acute HHV-6 infections and 20 were healthy and served as controls. HHV-6 DNA was detected in the serum of the 10 cases with acute HHV-6 infections but all 20 controls had no HHV-6 DNA in their sera. In addition, the time for serum to become PCR-positive coincided with the appearance of IgG HHV-6 antibody.

Based on these data the study described in this thesis aimed to use serum as the diagnostic specimen for the detection of HCMV, HHV-6 and HHV-7 DNAs in bone marrow and renal transplant recipients. In addition, other

advantages of utilising serum include the fact that it is one of the commonest specimens sent to virology laboratories, it requires little preparation before DNA extraction and retrospective banks of serum samples are readily available for validation. Moreover, the use of anticoagulants such as heparin in the case of plasma or whole blood has been shown to result in PCR inhibition (Wilson, 1997).

4.3. The GuSCN protocol as DNA extraction method from serum

One of the possible shortcomings of PCR is the occurrence of false negative results. False negative result could arise due to inefficient cell lysis required for extraction of nucleic acids, degradation of DNA, and loss of the enzyme activity due to inhibitors in the tested sample. The choice of the extraction method has been shown to significantly affect the outcome of PCR protocols on different clinical samples. This is mainly due to the different efficiency of these methods to remove PCR inhibitors, lysis of cellular components to extract DNA and/or to retrieve extracted DNA from other cellular components (Behzadbehbahani *et al.,* 1997; Evans *et al.,* 1998).

In serum samples, the DNA extraction method is of great importance. Although simple pre-treatment (e.g., proteinase digestion and heat treatment or treatment with alkali) may prove sufficient for detection of high viral loads, low loads may remain undetected (Aspin *et al.,* 1994; Ishigaki *et al.,* 1991). In this study, 4 extraction methods were evaluated for their ability to extract HCMV DNA from serum samples. The methods are GuSCN, QIA amp HCV kit, Hybaid recovery kit and DYNAL extraction method. These techniques vary in terms of simplicity and time required for complete sample preparation. The criterion with most consideration in this thesis is the sensitivity of the method to extract DNA to be detected by PCR. In other words, although the GuSCN protocol is relatively time consuming when compared with, for example, the Hybaid recovery kit, the method was the most sensitive for extraction of HCMV or HCMV DNA titrated in serum samples. Indeed, the sensitivity obtained with HCMV DNA titrated in serum was identical to that obtained with the same DNA titrated in sterile distilled water.

The GuSCN method should be performed with care and special precautions should be implemented for its disposal to avoid contact with acid and the consequent release of cyanide gas. In addition, the DNA pellet expected may not be visible and thus the supernatant should

aspirated with care. However, the other protocols may also have their own disadvantages. For example, the Hybaid method is prone to formation of a very viscous suspension with serum making it difficult to handle. This is presumably due to high protein content of serum.

4.4. HCMV, HHV-6 and HHV-7 in renal transplant recipients

A total of 463 serum samples from 124 renal transplant recipients at stages before and after transplantation was tested by PCRs for the detection of HCMV, HHV-6 and HHV-7 DNAs. HHV-6 DNA was detected in 111 serum samples, HCMV DNA in 65 specimens and dual HHV-6 and HCMV infection were detected in 38 serum samples. None of the samples were positive for HHV-7 DNA and the remaining 249 serum samples were negative for all the three targets. Of the 124 patients, 31 were positive for HCMV, 29 for HHV-6, and 16 were positive for both HHV-6 and HCMV (dual infection) but none of the patients had HHV-7 infection detectable by PCR.

In organ transplantation, HCMV primary disease with organ involvement, usually occurs within the first 6 weeks while the less severe secondary disease, which occurs 2-3 months after transplantation, may be caused by reactivation or reinfection with the virus (Grundy *et al.,* 1988).

Reactivation of HHV-6 during periods of intense immunosuppression has been reported (Knox and Carrigan, 1994b; Fairfax *et al.,* 1994). Infection with HHV-6 is common following solid organ transplantation, affecting up to 82% of transplant recipients (Herbein *et al.,* 1996; Okuno *et al.,* 1990a; Yoshikawa *et al.,* 1992; Morris *et al.,* 1989; Drobyski *et al.,* 1993; Hoshino *et al.,* 1995; Ward *et al.,* 1989). This reactivation has been associated, in several studies, with hepatitis (Singh *et al.,* 1997), interstitial pneumonitis (Cone *et al.,* 1993), encephalitis (Drobyski *et al.,* 1994), symptomatic HCMV infection and graft rejection (Lautenschlager *et al.,* 1998).

During this study, long term detection of HHV-6 DNA in serum samples was observed in some patients. This is highly likely to indicate HHV-6 chronic infection rather than PCR contamination. The latter is remote as all PCR negative controls remained negative and all sera were tested chronologically where the results obtained were identified with patients after all serum samples have been tested by the PCRs. Thus, more than

after all serum samples have been tested by the PCRs. Thus, more than one serum sample from an individual patient were only rarely tested in the same PCR batch. These patients had long term problems associated with their transplant, including rejection episodes. It is possible, therefore, that the chronic HHV6 infection demonstrated here had a role to play in these problems.

Clinical details, where available, were correlated with virus detection in serum. Loss of function or occurrence of rejection episodes after transplantation ranged from months to years which in some cases coincided with detection of HCMV and HHV-6 DNAs. Primary infection and reactivation of HCMV are commonly encountered after organ transplantation surgery and immunosuppressive therapy (Armstrong *et al.*, 1976; Grundy, 1990) and HHV-6 has been shown to infect the renal tissues. In this group of patients, the infection may be correlated with immunosuppressive therapy, severe allograft dysfunction (Jacobs *et al.*, 1994) and/or rejection (Okuno *et al.*, 1990a).

With respect to virus-associated rejection, most studies have reported on the role of HCMV in the rejection process and most concluded that this virus is not associated with organ rejection (Pouteil Noble, 1993). In contrast, the association of HHV-6 with the rejection phenomenon is yet unclear although cytokines such as interferons induced by HHV-6 infection may lead to interaction of graft cells with lymphocytes of the transplant recipient (Takahashi *et al.*, 1992). In addition, Wade *et al.*, (1998) found that children with no prior immunity to HHV-6 appear to have the highest risk for HHV-6-associated rejection with 100% of such patients rejecting their kidneys after the appearance of IgM antibody to HHV-6. In addition, renal transplant recipients of cadaveric donors are more likely to reject their kidneys after reactivation of HHV-6 than recipients of living donors.

Dual infection with HCMV and HHV-6 were detected in some cases but none of the cases was positive for HHV-7 DNA. Dual infection could have contributed to the progression of disease associated with either virus.

In support of this speculation, Irving *et al.,* (1990a) reported serological evidence of simultaneous reactivation of HCMV and HHV-6 after renal transplantation and other reports demonstrated dual infection with HCMV and either HHV-6 or HHV-7 in transplant patients (Knox *et al.,* 1994; Chan *et al.,* 1997). Osman *et al.,* (1996) reported that DNAemia due to HCMV, HHV-6 and HHV-7 was found in 50%, 36%, and 39% of renal allograft recipients, respectively, at some time during the post-transplant period. Of the patients positive for HCMV, the risk of progression to HCMV disease was increased in patients with concurrent DNAemia to all 3 viruses. The study concluded that in patients with HCMV DNAemia, concurrent infection or reactivation with either HHV-6 or HHV-7 is associated with an increased risk of progression to HCMV disease.

DesJardin *et al.,* (1998) demonstrated a potential association of HHV-6 and HCMV following renal transplantation by retrospectively testing serial serum specimens for HHV-6 IgG and IgM antibody. Reactivation of HHV-6 occurred in 35 (66%) of 53 transplant recipients. Although no association was found between HHV-6 reactivation and graft rejection or loss and mortality, the reactivation of HHV-6 was associated with primary HCMV infection and HCMV syndrome (HCMV-related hepatitis, HCMV-related neutropenia and serious HCMV disease).

In a recent study, Ratnamohan *et al.,* (1998) examined prospectively reactivation or infection with HCMV and HHV-6 by PCRs in serum and urine specimens. The study also evaluated the relative contribution of the two viruses towards disease during renal transplantation and whether active infection of both viruses together may predict either the frequency or severity of disease. The authors examined the incidence of HCMV and HHV-6 infection in a prospective blinded consecutive series of 30 renal and renal/pancreas transplant patients. Of the 30 patients, 15 had a clinical diagnosis of viral syndrome. Of the 15 patients, 3 were positive for HHV-6 DNA only in urine or serum and had fever and abnormal liver function but not neutropenia, 5 patients had moderate to severe disease and had HCMV and HHV-6 DNA in serum and urine and 7 patients developed disease and were positive for both HCMV and HHV-6 DNA in 6 patients

and for HCMV DNA only in 1 patient. The study demonstrated that disease associated with HHV-6 alone consisted of fever and hepatitis, but not neutropenia or other end-organ involvement while disease associated with HCMV alone was only observed in 1 patient and consisted of fever and neutropenia. In contrast, simultaneous detection of both HHV-6 and HCMV in serum or urine was the strongest predictor of disease occurrence and severity.

The increased risk of progression to, for example, HCMV disease in patients who are positive concurrently for both HCMV and HHV-6 could have many explanations. HCMV by itself is immunomodulatory, which may predispose to the reactivation of HHV-6. On the other hand, HHV-6 infects CD4+ positive T lymphocytes and the infection is associated with an alteration of cell surface markers with dysregulation of cell function (Furukawa *et al.,* 1994). HHV-6 can induce production of interleukin-1β and tumor necrosis factor-α (Flamand *et al.,* 1991), suppress T lymphocyte function due to reduced interleukin-2 synthesis (Flamand *et al.,* 1995) and suppress bone marrow by inducing interferon-α (Knox *et al.,* 1992). In addition, a bone marrow-suppressive effect of HHV-6 infection has been reported *in vivo* (Drobyski *et al.,* 1993; Carrigan and Knox, 1994; Knox and Carrigan, 1996a). It is conceivable, therefore, that HHV-6 infection could be responsible for a state of immunosuppression, predisposing to clinical disease in transplant recipients patients who have otherwise asymptomatic HCMV infection. Alternatively, it is possible that patients who were most severely immunosuppressed are those most likely to progress to symptomatic disease by one of the viruses, for example HCMV, and were also the ones most likely to reactivate the other virus (HHV-6 in this case). The latter would indicate that the detection of both viruses might merely reflect the more severely immunocompromised patient.

4.5. HHV-6, HCMV and HHV-7 in bone marrow transplant recipients

A total of 97 serum samples from 66 bone marrow transplant recipients were tested retrospectively for the presence of HHV-6, HHV-7 and HCMV DNAs using PCR. HHV-6 DNA was detected in 16 serum samples, HCMV DNA alone in 3 samples and HHV-7 DNA in 1 sample. Both HHV-6 and HCMV DNA were detected in 2 samples and HCMV and HHV-7 DNA in 1 sample but none of the samples were dually positive for HHV-6 and HHV-7 DNAs and the remaining 74 samples were all negative for the three viruses. Of the 66 patients tested, 18 were positive for virus DNA. HCMV DNA was detected in 2 patients, HHV-6 in 11 patients and HHV-7 in 1 patient. Dual infection was detected in 3 patients as HHV-6 and HCMV in 2 patients and HCMV and HHV-7 in 1 patient.

The clinical significance of HCMV detection in allogeneic bone marrow transplantation is very well established with HCMV pneumonia being the most significant manifestation, with a mortality rate of up to 90% (Winston *et al.,* 1990). Thus, more research is concentrated on evaluation of several approaches to treat or prevent the development of active HCMV disease. These include the early detection of patients at risk of developing HCMV-related disease (Meyers *et al.,* 1986), prophylactic and pre-emptive treatment strategies (Reed *et al.,* 1988; Forman and Zaia, 1994; Singhal *et al.,* 1994; Reddy *et al.,* 1999) and the concept of donor vaccination (Li *et al.,* 1994; Walter *et al.,* 1995).

Although the pathogenic potential of HHV-6 after bone marrow transplantation is undergoing investigation, recent studies demonstrated the possible clinical significance of this virus in this group of patients. Increased isolation rates of HHV-6 from blood and marrow after bone marrow transplantation suggested reactivation of the virus owing to the acute immunosuppression (Yoshikawa *et al.,* 1991; Kadakia *et al.,* 1996). This reactivation may have a pathogenic role in the respiratory system disease such as pneumonia (Cone, 1995; Cone, 1993), marrow dysfunction

(Carrigan and Knox, 1995; Knox and Carrigan, 1992; Drobyski *et al.*, 1993), encephalitis (Drobyski *et al.*, 1994), graft-versus-host disease (Appleton *et al.*, 1995; Wilborn *et al.*, 1994) and, in some cases, asymptomatic hematogenous reactivation of the virus with self-limited skin rashes has been reported (Asano *et al.*, 1991; Cone *et al.*, 1999)

In this study, a prospective evaluation of 2 years old male child who required bone marrow transplantation due to X-linked lymphoproliferative syndrome was performed. Pre-transplant screening was negative for viruses by cell culture, electron microscopy, antigen detection techniques, serology and by the 3 PCRs in a total of 2 serum samples. The patient developed acute neurological symptoms approximately 2 months post-transplant. At this point, serum and CSF samples were collected and tested for viruses including HCMV and HSV by the Diagnostic Virology Laboratory but negative results were reported. Using the PCRs described in this thesis, HHV-6 DNA was detected in 2 serum samples and 2 CSF samples but both HCMV and HHV-7 PCRs remained negative.

The incidence of neurological complications after bone marrow transplantation varies between 11 to 37% and are usually associated with fatal outcome (Furlong and Gallucci, 1994; Haire *et al.*, 1995; Gallardo *et al.*, 1996; Graus *et al.*, 1996) with aetiology of a large proportion of these cases remaining unexplained. HHV-6 can infect glial cell lines and both variants can productively infect primary astrocytes (He *et al.*, 1996) and cases of central nervous system infection with the virus have been described in both immunocompromised patients and immunocompotent individuals (Kondo *et al.*, 1993; Caserta *et al.*, 1994; McCullers *et al.*, 1995; Knox *et al.*, 1995; Singh *et al.*, 1995; Novoa *et al.*, 1997). However, HHV-6 DNA can be detected in normal brain tissues as well as in cellular components of CSF from patients with noninflammatory neurological symptoms (Caserta *et al.*, 1994; Luppi *et al.*, 1995; Liedtke *et al.*, 1995).

The detection of HHV-6 DNA in the CSF of the case described above clearly suggest a possible association of the virus with the acute

neurological manifestations observed. In support of this, a total of 4 cases of encephalitis with HHV-6 as the identified pathogen after allogenic bone marrow transplantation have been reported; 1 case was due to HHV-6 variant A and 3 were due to HHV-6 variant B (Drobyski *et al.,* 1994; Bosi *et al.,* 1998; Rieux *et al.,* 1998; Tsujimura *et al.,* 1998). More recently, Wang *et al.,* (1999) detected HHV-6 DNA in CSF samples from 5 (45%) of 11 patients with central nervous system symptoms of undefined origin after allogeneic bone marrow transplantation but in a CSF sample from only 1 (0.9%) of 107 immunocompromised patients without CNS symptoms (controls) and in none of 11 patients who underwent bone marrow transplantation for whom other causes of the CNS diseases were documented. Furthermore, HHV-6 DNA was not detected either in the CSF samples collected before bone marrow transplantation or in the CSF samples collected after the CNS symptoms had abated following antiviral therapy. These data clearly support HHV-6 reactivation which may lead to neurological symptoms.

Wang *et al.,* (1996) have shown that patients who undergo allogneic bone marrow transplantation frequently have several of the lymphotropic herpesviruses detectable in blood samples during the first months after bone marrow transplantation. HHV-7 as a closely related virus to HHV-6 has been reported as a cause of exanthem subitum and CNS symptoms (Ablashi *et al.,* 1995; Torigoe *et al.,* 1996). In the study described in this thesis, HHV-7 DNA along with HCMV DNA were detected in a serum sample from 1 bone marrow transplant patient. However, this could not be associated with any clinical manifestation but reactivation of both viruses has been documented and HHV-7 reactivation may increase the severity of HCMV disease as described above.

4.6. Possible association of HHV-6 with *hydrops fetalis*.

The prevalence of *hydrops fetalis* is between 1 in 2500 and 1 in 3500 deliveries (Im *et al.,* 1984). The aetiology of non-immune *hydrops fetalis* may involve a variety of factors, including some viruses. Parvovirus B19 is responsible for at least 10% of cases (Yaegashi *et al.,* 1998) and other viruses such as HCMV and adenovirus have also been associated with the disease (Ranucci-Weiss *et al.,* 1998; Barron and Pass., 1995).

A part of this thesis was aimed to investigate the role of the 3 viruses studied in the aetiology of this disease. Liver, kidney, heart, lungs and placenta tissue sections of 4 cases of *hydrops fetalis* and 11 controls remained negative for HCMV and HHV-7 DNAs but one case was positive for HHV-6 DNA. Paraffin embedded tissue sections of the liver, kidney, heart, lungs and the placenta of this case were all positive for HHV-6 DNA on at least 4 repeats. The positivity in this case represents the first description of HHV-6 DNA in tissues from cases of *hydrops fetalis*. In all cases, PCR for detection of β-globin was positive which clearly eliminates the possibility of PCR inhibition in those samples that were negative for viral DNAs.

The detection of HHV-6 DNA in one case of *hydrops fetalis* leads to the possibility of associating HHV-6 with this disease and that HHV-6 was probably the cause of foetal death in this case. In this study, however, it was very difficult to correlate the presence of HHV-6 DNA with histopathological findings due to the lack of the latter. Histological findings included normal organs and tissues but the lungs had an immature appearance and the final diagnosis for this case was Downs syndrome (trisomy 21).

The high sensitivity of the PCRs utilised in this study may raise the argument of contamination leading to false positivity in this case. This is highly unlikely as multiple repeated controlled experiments confirmed the positive result and all tissue sections from this case only were positive. Thus, this fetus may have had concomitant risk factors for *hydrops fetalis*

i.e., HHV-6 infection and Down's syndrome may have contributed to foetal death.

Clear evidence confirming HHV-6 as a cause of *hydrops fetalis* could not be demonstrated but one cannot rule out that HHV-6 may result in *hydrops fetalis* in a small number of cases. In part, the finding of HHV-6 in this case supports other studies which demonstrated intrauterine transmission of HHV-6 and occurrence of spontaneous abortion (Ando *et al.,* 1992; Okuno *et al.,* 1995; Maeda *et al.,* 1997).

Hall *et al.,* (1994) identified HHV-6 genome in 12 of 41 (29%) peripheral blood mononuclear cell samples of healthy neonates suggesting infection with the virus before birth. Dunne and Demmler, (1992) surveyed 799 cord blood sera and detected HHV-6 specific IgM in 2 samples indicating infection in utero. In another study, HHV-6 DNA could be detected in the specimens of 5 (1.6%) of 305 babies born to ostensibly healthy mothers, indicating that intrauterine infection and that congenital infection may be responsible for the HHV-6 seropositivity in children (Adams *et al.,* 1998).

Intrauterine transmission of HHV-6 was further confirmed through analysis of 52 foetuses from induced abortion from HIV seropositive women (Aubin *et al.,* 1992). In one foetus, the virus DNA was detected in peripheral blood lymphocytes, thymus, liver, spleen, brain, and CSF. In addition, HHV-6 specific antigen was demonstrated in abortive chorionic villous tissue in 2 of 3 pregnant women who experienced spontaneous abortions and who had positive IgM antibody to HHV-6 in serum (Aubin *et al.,* 1992).

4.7. Typing of HHV-6 PCR products

The two variants of HHV-6 (A and B) differ under DNA restriction analysis, tropism in cell culture, antigenicity and pathogenicity (Bovenzi *et al.,* 1993; Schirmer *et al.,* 1991). In addition, both variants display differential susceptibility to antivirals. HHV-6 variant B has ganciclovir susceptibility similar to that of HCMV whereas variant A is relatively less susceptible (Russler *et al* ., 1989). The HHV-6 PCR described in this thesis combines both detection and typing of HHV-6.

The typing protocol described in this study is based on restriction endonuclease analysis of the PCR products generated by the primers H6-6 and H6-7. Based on the finding of Kidd *et al.,* (1998), the restriction endonuclease *Ava*II differentiates between the variants because it cuts variant A but not B. All the HHV-6 positive samples in this study were typed as HHV-6 variant A.

HHV-6 B is the cause of *exanthem subitum* (Yamanishi *et al.,* 1988), febrile illness of children (Pruksananonda *et al.,* 1992) and it is the major type found in healthy children and adults (Dewhurst *et al.,* 1992, 1993; Yamamato *et al.,* 1994), transplant recipients (Yalcin *et al.,* 1994), immunosuppressed patients (Drobyski *et al.,* 1994), Sjogren's syndrome (Ranger-Rogez *et al.,* 1995), and oncology patients (Torelli *et al.,* 1991; Arivananthan *et al,* 1997). In contrast, HHV-6 type A has yet to be clearly associated with any disease process, although initially it was isolated from patients with lymphoproliferative disorders and AIDS (Salahuddin *et al.,* 1986; Arivananthan *et al,* 1997). The prevalence of infection with type A remains unclear and it has been suggested that the virus may have a greater propensity for latency, reactivation or sequelae (Dewhurst *et al.,* 1992; Arivananthan *et al,* 1997; Hall *et al.,* 1998).

HHV-6 variant A has been obtained primarily from adults, most of whom were chronically ill (Aubin *et al.,* 1991; Ablashi *et al.,* 1991; Downing *et al.,* 1987) and has been detected more frequently in two illnesses, Kaposi's sarcoma (Bovenzi *et al.,* 1993) and chronic fatigue syndrome (Di Luca *et*

al., 1995b; Yalcin *et al.,* 1994b) but has also been found in association with other conditions, including AIDS (Knox and Carrigan, 1996b; Salahuddin *et al.,* 1986) and cases of encephalitis in immunocompromised patients (Knox *et al.,* 1995). In addition, *in vivo* and *in vitro* coinfection of both variants has been described (Dewhurst *et al.,* 1992; Aubin *et al,* 1994; Arivananthan *et al.,* 1997).

Drobyski *et al.,* (1993) utilised variant-specific probe hybridisation protocol to examine 16 bone marrow transplant recipients and found that all patients had HHV-6 variant B which was in agreement with other reports which demonstrated variant B in bone marrow transplant recipients (Wilborn *et al.,* 1994; Cone *et al.,* 1999). Tanaka-Taya *et al.,* (1996) showed that all the samples that were positive for HHV-6 in throat swabs from 62 children and 28 adults were typed as variant B but coinfection with both variants was detected in 2 subjects. In another report, HHV-6 was found in 7 of 20 Kaposi's sarcoma biopsies, 6 of these isolates were typed as variant A and 1 was typed as B using restriction site analysis of the amplified product (Bovenzi *et al,* 1993).

Cone *et al,* (1996) studied 34 lung tissue specimens that were positive for HHV-6 DNA and demonstrated that 22 of the specimens showed coinfection with both variants, 2 had type A and 10 had type B. Using variant-specific primers, Aberle *et al,* (1996) studied saliva and peripheral blood mononuclear cells from 44 healthy young adults. Variant B was detected in 98% of the blood cells and in 95% of the saliva. HHV-6 A was detected in 16% of the peripheral blood mononuclear cells but in none of the saliva samples. Utilising these variant-specific primers, Knox *et al.,* (1995) demonstrated the presence of HHV-6 variant A in the brain tissue specimen of HIV-infected infant.

Most of the studies where HHV-6 was typed utilised peripheral blood samples as source sample. This has been shown to result in identification of type B HHV-6 in most cases whereas Secchiero *et al.,* (1995) showed that serum samples from bone marrow transplant recipients may be needed to identify type A of the virus. In this thesis, although the unexpected

detection of variant A only could be translated to a technical error in the PCR, for example due to contamination, or in the typing method, due to a pitfall in the identification protocol, both suggestions have been excluded.

For the former possibility (contamination), the positive samples remained positive for HHV-6 when repeated at least twice and all negative and extraction negative controls remained negative. In addition, the samples remained positive when reamplified with another set of primers which excludes the possibility of contamination due to carry over of PCR products.

With respect to the typing method, the enzyme *Ava*II does not cut the variant B of the virus but it produces a clear cut restriction pattern consistent with variant A. Although failure to cut the PCR products, due to technical error, would mean the PCR products could have been misidentified as variant B, this argument is not relevant in this study because all positive samples (PCR products) were cut and produced restriction patterns consistent with variant A.

As the nucleotide sequences of variant B was available (Dominguez *et al.,* 1999; Isegawa *et al.,* 1999) it was of diagnostic advantage to align the sequences of the PCR products from both variants. The nucleotide sequence of the 223 bp PCR products of both variants showed a total of 7 mismatches when compared to each other. The specificity of the enzyme *Ava*II on both sequences was confirmed but another restriction endonuclease (*Hae*III), which cuts both variants but differently, was identified (*Hae*III produces restriction pattern of 38 and 185 bp for variant A and 38, 83 and 102 bp for variant B). Reamplification of HHV-6 DNA from positive samples and typing of PCR products using this enzyme (*Hae*III) produced restriction profiles consistent with HHV-6 variant A (38 and 185 bp).

Another finding was the presence of base mismatches with the primer pair H6-6/H6-7 with respect to its recognition sequences of variant B. These primers were originally designed on the basis of the nucleotide sequence

of variant A where it produced a sensitivity of 1-10 copies of HHV-6 plasmid DNA (Gopal *et al.,* 1990). However, alignment of both primers to the newly available HHV-6 sequences of variant B resulted in recognition of at least 3 mismatches when compared with the primer H6-6 binding region of variant B (strain HST) or 5 mismatches when compared with that of strain Z29 of variant B. Most important is the finding that one of the mismatches (only in strain Z29 and not in strain HST) involved the 3' end of the primer. The latter has been demonstrated to affect PCR performance and result in PCR failure (Robertson *et al.,* 1998). However, both variants were successfully amplified in this study.

A number of techniques of typing HHV-6 variants have appeared in the literature. HHV-6 isolation by means of peripheral blood mononuclear cells coculture is time consuming and could favour the selection of one variant in mixed infections. Other methods utilised PCR-based protocols. These include variant-specific hybridisation of HHV-6 amplimers using variant-specific oligonucleotide probes (Aubin *et al.,* 1994; Gautheret *et al.,* 1996), variant- specific primers to produce PCR products that differ in size depending on whether the variant was A or B (Aubin *et al.,* 1994) and the use of restriction endonuclease analysis of PCR products as described in this thesis.

Drobyski *et al.* (1993) used 20 bp primers selected from the first open reading frame of the sequence of the U1102 strain (variant A) to amplify a 385 bp PCR product from HHV-6 variants A and B-infected culture. The amplified DNA products were digested with *Alu*I which has 2 cut sites on variant B (strain Z29) but none on variant A (strains U1102 and GS). The fact that the enzyme used, cuts only one of the 2 variants is disadvantageous for the reasons mentioned above. Moreover, although the specificity of the primers used was assessed, their sensitivity was not mentioned and therefore is very difficult to judge whether these primers would by appropriate for use in clinical specimens. Similarly, a recent study be Kidd *et al.,* (1998) combined the primer pair utilised in this study (H6-6/H6-7) with that for HHV-7 in a multiplex PCR for the detection of HHV-6 and HHV-7. The 223 bp PCR product generated by the HHV-6

primer pair was subjected to *Ava*II which cuts variant A but not B. Indeed, the nucleotide sequence analysis performed in this thesis confirmed the accuracy of this identification method and proved satisfactory for typing of both variants.

Another PCR-based typing method utilised nested variant-specific HHV-6 primers (Yalcin *et al.,* 1994). The primers covered the area deleted in U1102 strain and variants could be distinguished by the size differences of amplified products (195 bp for variant A and 423 bp for variant B) and confirmation of typing was achieved by variant-specific probe hybridisation. Although the methodology described circumvents the need for restriction endonuclease analysis, nested PCR strategy is known to increase the risk of false positives due to contamination and complicate the PCR diagnostics in routine settings.

4.8. Implication of HHV-6 as a causative antigen in psoriasis

A total of 20 skin biopsies including the involved and uninvolved skin from 10 patients with chronic plaque psoriasis were collected and tested with the HHV-6, HHV-7 and HCMV PCRs. HHV-6 DNA was detected in 2 skin biopsies representing the involved and uninvolved skin of one patient. HHV-7 and HCMV were not detected in either involved or uninvolved skin in any of the patients.

Psoriasis is a common chronic inflammatory disorder of the skin and affects more than 2% of people with European ancestry (Stern and Wu, 1996). The disease is characterised by inflammation, hyperproliferation of the epidermis, altered maturation of the epidermis resulting in scarring and vascular alterations intensifying the redness observed (Stern, 1997).

The pathogenesis of psoriasis remains to be fully elucidated but an immunopathogenic origin has been suggested (Valdimarsson *et al.,* 1995). This is evident from the predominant dermal infiltration of CD4+ T cells and intraepidermal CD8+ T cells (Chang *et al.,* 1994; Camp and Vekony, 1998). The cloning of certain subsets of CD8+ cells within plaques of psoriasis is similar to that observed in other T cell-mediated diseases including multiple sclerosis and diabetes (Muraro *et al.,* 1997; Simone *et al.,*1996) and has been linked with the presence of local stimulating auto-antigens (Menssen *et al.,* 1995).

Viral antigens are important in the immunopathogensis of both diabetes and multiple sclerosis which both share similar immunological features of psoriasis. In addition, the involvement of viral antigens in the pathogenesis of psoriasis may be drawn from the effective treatment for psoriasis using the antiviral agent zidovudine (Townsend *et al.,* 1995), the efficiency of hydroxyurea, which has antiretroviral properties, as an effective treatment for psoriasis (Lisziewcz *et al.,* 1998) and the detection of HIV, hepatitis C virus and human papilloma virus in plaques of psoriasis (Mahoney *et al.,* 1991; Yamamoto *et al.,* 1995; Favre *et al.,* 1998)

Although strong association between HHV-6 and psoriasis could not be demonstrated in this study as only 1 of the 10 patients studied demonstrated HHV-6 DNA which was detected both in the involved and uninvolved skin, it is intriguing to speculate that the virus may be responsible for some cases of chronic psoriasis and that HHV-7 and HCMV is unlikely to be the cause.

4.9. Conclusions and Future work

Both HCMV and HHV-6 are common pathogens in renal and bone marrow transplant recipients. Considering the numerous reports on clinical findings in these patients associated with either virus, screening of both viruses in either group before and after transplantation would be of great clinical usefulness. In addition, the advantage of DNA quantitation using for example quantitative PCRs for viral load should be considered. However, controlled studies for understanding the role of these viruses especially HHV-6 would be of great advantage. This would help in more accurate association of the virus with any clinical manifestation in these group of patients.

No association of HHV-7 with any group studied in this thesis was demonstrated and this virus may not be as pathogenic as HHV-6. Understanding the biological and pathogenic difference between HHV-6 and HHV-7 may help to elucidate this finding and may help to increase our knowledge concerning the epidemiology and pathogenesis of HHV-6.

In this study, the first association of HHV-6 with *hydrops fetalis* has been confirmed. This should lead to future research that could be directed towards understanding the incidence and control of HHV-6 in this clinical entity. However, although the virus was detected in one case with chronic psoriasis, further work is required to confirm this finding and to screen for other potential viruses in this dermatological manifestation.

Finally, the study detailed in this thesis has demonstrated the occurrence of HHV-6 variant A in all the positive HHV-6 samples. This finding, although uncommon, should highlight the possibility that both variants of the virus are equally pathogenic and that the typing protocol utilised in this study is of great advantage.

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HCMV, HHV6 and HHV7 DNA detection by PCR in sera from renal transplant patients

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Introduction

Two new herpesviruses, HHV6 and HHV7 appear to be closely related to HCMV and can be found in similar clinical situations. All three viruses may be reactivated in the immunocompromised including transplant and AIDS patients. HHV6 may act as an opportunistic agent in these patients and has been implicated in interstitial pneumonitis,[2] encephalitis,[3] and bone marrow graft failure[4]. Two major subgroups of HHV6 have been identified, HHV6 subtype B is the most commonly detected variant and is the aetiological agent of exanthem subitum. It has been found in renal transplant patients[5] and bone marrow transplant patients[6] in circulating leukocytes or bone marrow. HHV6 subtype A has mainly been found in AIDS patients and has also been found in the serum of solid organ transplant recipients.[6]

In this study, PCR was used to detect the DNA of all three viruses in serial serum specimens from renal transplant recipients.The presence of viral DNA in serum rather than PBMC is more likely to represent active infection than latency.

Methods

1) *Specimens*: 425 serum samples collected from 89 renal transplant recipients (24 female and 65 male, age range 23 to 73 years) were examined. The sera were collected over a period of 4 years and had been stored at -40°C.

2) *Extraction of DNA*: DNA was extracted from the sera by guanidinium thiocyanate/isopropanol precipitation.[7]

3) *PCR*: Specific primers for HCMV[8], HHV6[9] and HHV7 (HV7 & HV8)[1] were used in single target PCRs using Amplitaq Gold™ (Perkin-Elmer). One cycle of 94°C/9.9min, 55°C/1.0min and 72°C/1.0min was followed by 40 cycles at the same temperatures for 1.0min each. Each PCR run included a contamination control of sterile distilled water instead of sample and an extraction control where foetal calf serum was extracted at the same time as the specimens.

4) *Control DNA*: The *Hin*dIII restriction fragment J of HCMV (AD169) DNA cloned in pAT153[10] containing the viral phospho-protein gene, and the *Hin*dIII restriction fragment pHD5 of HHV6 DNA[11] were propagated and purified. The plasmid concentrations were estimated spectrophotometrically. HHV7 infected cell DNA was purchased from Advanced Biotechnologies Inc.

5) *HHV6 Typing*: HHV6 was typed with *Ava*II restriction endonuclease digestion of the PCR product. Type A is cut to give two fragments of 138 and 85 base pairs whereas type B is uncut.[12]

Results

1) *Sensitivity of the PCR*: - 10 copies of the HHV6 plasmid, 10 copies of the HCMV plasmid and a dilution of $10^{-6.5}$ for HHV7-infected cell DNA.

2) *PCR of serum samples*. A typical gel for HHV6 is shown in Figure 1. HCMV was found in a total of 65 of 425 samples(15.0%) and HHV6 in 111 samples (26.0%), but HHV7 was not detected in any of the samples (0%). Thirty-three (8.0%) - from 16 patients - contained both HCMV and HHV6 DNA. A further 4 patients showed evidence of both HHV6 and HCMV infection but not in the same serum samples. Eleven patients had HCMV infection alone and 9 only HHV6, 49 patients showed no evidence of infection as judged by PCR (Table 1).

3) *Typing of HHV6 PCR product*. Of 111 serum samples positive for HHV6, 88 have so far been typed using *Ava*II. Figure 2 shows examples of three sera from different patients. All 88 samples tested typed as type A.

	HCMV	HHV6	SAMPLES	PATIENTS
Dual[1]	+	+	33	16
Single[2]	+	+	n/a	4
	+	-	32	11
	-	+	78	9
	-	-	282	49

Table 1

[1] HCMV and HHV6 DNA present in the same sample. Other samples from these patients may also have had either HHV6 or HCMV DNA detected.
[2] HCMV and HHV6 DNA detected in the same patient but in different samples.

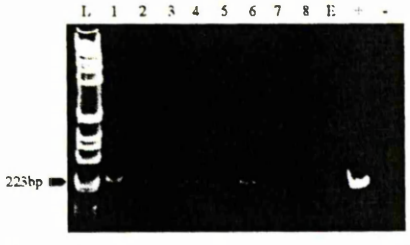

Figure 1. PCR of serum samples from renal transplant patients. Lane L, DNA Ladder; Lanes 1-8, serum samples; E, Extraction control; +, positive control; -, Contamination control. Positive serum samples are in lanes 1, 2, 4, 6 and 8.

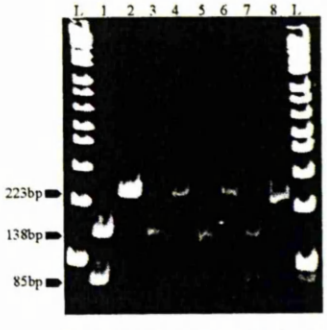

Figure 2. *Ava*II cleavage of HHV6 PCR products. Lanes L, DNA Ladder; Lane 1, Product from HHV6 type A-infected HSB-2 cells cut with *Ava*II; Lane 2, Uncut product from HHV6 type A-infected HSB-2 cells; Lanes 4, 6 and 8, Uncut product from patients' sera; Lanes 3, 5 and 7, *Ava*II-cut product from patients' sera. All products are from HHV6 type A.

Conclusion

Long term detection of HHV6 was observed in four patients; two for 12 months, one for 15 months and one for 27 months. In each case the renal graft faired badly but episodes of rejection could not be conclusively linked to virus reactivation. Further prospective studies will be required to properly evaluate the possible role of HHV6 reactivation in renal allograft failure. Remarkably all 88 HHV6 PCR products typed so far are type A. This may reflect that all patients are immunocompromised or the use of serum rather than PBMC.[6]

An intriguing finding was the failure to identify HHV7 DNA in any of the 425 samples examined. These results are not believed to reflect relative insensitivity of the HHV7 PCR technique (whilst quantitation of HHV7 PCR sensitivity using plasmid derived DNA remains to be done, positive PCR results have been obtained with children's sera (data not shown)). As HCMV, HHV6 and HHV7 are believed to share similar sites of latency, reactivation of HHV7 during renal transplantation was expected to occur. These results suggest that reactivation of HHV7 in these patients is in fact uncommon when compared to the other two viruses (i.e.HHV6 > HCMV >>HHV7). A possible reason for this phenomenon is a difference in the triggers for reactivation of the three viruses. If this can be confirmed there appears to be a fascinating possibility that a comparison between these viruses may allow an elucidation of the triggers for herpesvirus reactivation in renal transplantation.

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Investigation of cytomegalovirus and human herpes viruses-6 and 7 as possible causative antigens in psoriasis.

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To the Editor,

Although the ultimate pathogenesis of psoriasis remains unclear, there is strong evidence suggesting it to be a T-cell-mediated disease[1]. Immunohistochemically, plaques of psoriasis are characterised by a predominantly dermal infiltration of CD4+ T cells and intraepidermal CD8+ T cells[2,3]. These T cells produce multiple cytokines in a predominantly Th1 (interferon-γ and interleukin-2) profile.[4] Classically the stimulation of CD8+ T cells with production of Th1 cytokines occurs in response to intracellular bacterial and viral antigens. Recently it has been shown that there is cloning of certain subsets of CD8+T cells within plaques of psoriasis [2,3] and that such cloning (V-β restriction) is similar to that observed in other T-cell-mediated diseases including multiple sclerosis[5] and diabetes[6]. V-β restriction of T cells within the plaques may suggest the presence of local stimulating (auto)antigens[7]. It has been proposed that viral antigens are important in the immunopathogenesis of both diabetes and multiple sclerosis, diseases that share similar immunological features of psoriasis. Also the anti-viral agent zidovudine has been reported as effective in the treatment of psoriasis in non-HIV infected patients[8]. Similarly hydroxyurea an effective treatment for psoriasis has anti-retroviral properties[9]. The presence of DNA for human immunodeficiency virus (HIV)[10], hepatitis C virus[11] and human papilloma virus,[12] in plaques of psoriasis has been previously described. We hypothesise that the psoriatic phenotype occurs as a result of an immune reaction to normally asymptomatic local viral, or similar, infection in genetically pre-disposed individuals.

Human herpes viruses 6 and 7 and cytomegalovirus (CMV) commonly produce chronic asymptomatic infections and can cause local immune-mediated reactions[13]. CMV can stimulate local increases in tumour necrosis factor-α and interleukin-2

production. Indeed both cytokines are increased in psoriatic plaques. Asymptomatic infection with human herpes viruses (HHV) 6 and 7 is ubiquitous[15] and these are implicated in the pathogensis of multiple sclerosis[14], which like psoriasis demonstrates Vβ restriction of T-cells in active plaques. We have therefore investigated whether patients with chronic plaque psoriasis exhibit evidence of infection with CMV, HHV-6 and 7.

Patients aged 18 or over, with chronic plaque psoriasis were recruited from our psoriasis clinic. After written informed consent (approved by the Salford and Trafford local research Ethics committee) blood was taken from 29 patients. Ten other patients had 4 mm diameter punch skin biopsies (using lidocaine and epinephrine as local anaesthetic) taken from involved and uninvolved skin. All patients had chronic plaque psoriasis and were not on systemic therapy or phototherapy for psoriasis. Serum antibodies to CMV (IgG) were measured by ELISA. Infection with HHV-6 and 7 is ubiquitous by adulthood and therefore serological tests for antibodies to these organisms were not performed. Biopsies from involved and uninvolved skin were examined for DNA of CMV, HHV-6 and 7.

Skin biopsies were initially snap frozen in liquid nitrogen and stored at -80^{0}C. The skin biopsies were disrupted during thawing by using a glass homogeniser. DNA was extracted from this material with guanidine thiocyanate and isopropanol precipitation[16]. The sequences of the human CMV primers were 5′-TGCAGTTTGGTCCCTTAAAG-3′ (233C) and 5′AAGAATCCTCACCTGGCTTA-3′ (724C)[17] which were derived from the DNA sequence of the CMV phosphoprotein gene and give a product size of 171bp. The HHV-6 primer sequences were 5′-

AAGCTTGCACAATGCCAAAAAACAG-3′ (H6-6)[18] and

5′CTCGAGTATGCCGAGACCCCTAATC-3′ (H6-7) and give a product size of 223 bp. HHV-7 primers were 5′-CAGAAATGATAGACAGATGTTGG-3′ (HV-10) and 5′-TAGATTTTTTGAAAAAGATTTAATAAC-3′ (HV-11)[19] with a product size of 123bp. The β-globin primers were 5′-GAAGAGCCAAGGACAGGTAC-3′ (GH20) and 5′-CAACTTCATCCACGTTCACC-3′ (PC04)[20] with a product size of 268bp.

The PCR mixture comprised 10mM Tris/HCL (pH 8.3), 1.5mM $MgCl_2$, 50 mM KCl, 200 μM each deoxynucleoside triphosphate, 0.2μM each of the appropriate primers, 1.25U of Amplitaq Gold polymerase (Perkin-Elmer) and 5μl of appropriate DNA sample to a final volume of 50μl. Every PCR run included a contamination control where sterilised distilled water replaced the DNA sample and an extraction control where sterilised distilled water was extracted alongside the specimens and added to a PCR mix. Each reaction mixture was overlaid with one drop of mineral oil to prevent evaporation. An initial denaturing step at 94^0C for 9.9min was followed by 40 cycles of 94^0C (1min), 55^0C (1min) and 72^0C (1min) on a PHC-1 thermal cycler (Techne). The amplification products were analysed by ethidium bromide staining after electrophoresis in 6% polyacrylamide gels and the anti-contamination measures were as previously described.[21] They included separate rooms for preparation of the reaction mixtures, preparation and addition of DNA extracts and a third room for product analysis. Plugged pipette tips were used throughout.

Positive control DNA comprised; (a) human genomic DNA (Boehringer); (b) the HindIII restriction fragment J of HCMV (AD169) DNA cloned into plasmid

pAT153 [22]; and (c) the HindIII restriction fragment pHD5X of HHV6 DNA[23] and HHV7-infected cell DNA (Advanced Biotechnologies). Using these control DNAs, the β-globin and HHV6 PCRs had sensitivities of less than 10 copies per reaction mixture, the CMV PCR could detect 10-100 copies and the HHV7 could detect a $10^{-6.5}$ dilution of HHV-7 infected cell DNA. For HHV-6-positive samples, 5μl of reaction product was digested overnight with 5 units of *AVA*II (Boehringer) according to the manufacturer's instructions [24]. The products were analysed by polyacrylamide gel electrophoresis.

Twenty-one of 29 (72%) patients had IgG antibodies to CMV compared to 50% in our general control population[25]. Antenatal screening testing has suggested that the incidence of CMV infection increased by 1% per year after adolescence[26]. As the mean age of our population was 44.6 ± 10.8 years (range 23-69), there was thus no significant increase in CMV infection in our patients with psoriasis. There was no correlation between infection with CMV, clinical severity of psoriasis and previous treatment with systemic therapy. CMV and HHV-7 DNA was not detected in either involved or uninvolved skin in any patient. DNA for HHV-6 subgroup A was detected in both the involved and uninvolved skin in one of the 10 patients. We feel this may be of significance in a minority of patients and like HIV and hepatitis C this virus may have a role in non-specific local immune modulation and psoriasis pathogenesis when present in the skin of some patients.

In conclusion, there is strong circumstantial evidence that local viral or intracellular bacteria may be responsible for the immunological events seen in chronic plaque

psoriasis. However, we have shown that infection with CMV and HHV-7 is unlikely to be the cause although HHV-6 infection may play a role in a minority of patients.

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