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Polyomaviruses in Pediatric Renal Disease

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1. Introduction

1.0 Virology

Polyomaviruses are widely distributed in nature and have been described in humans, monkeys, cattle, rabbits, mice, hamsters, rats and parakeets. The viruses are species specific, infecting one species only or a few closely related species. They are classified as polyomaviruses according to the size of their virion (diameter 40-45 nm), non-enveloped icosahedral capsid, supercoiled, double-strand circular DNA genome, and sequence homology with other polyomaviruses. There are three members in the family that infect humans; Simian Virus 40 (SV40), BK virus (BKV) and JC virus (JCV).

SV40, JCV and BKV DNAs contain 5,243, 5,130, and 4,963 base pairs respectively (Shah, 1996). BKV, JCV and SV40 share a high degree of nucleotide sequence homology. The JCV genome shares 75% homology with the BKV genome and 69% with SV40 (Walker and Frisque, 1986). The viral genome is functionally divided into

(1) An early region, coding for large (T-ag) and small T-antigens (t-ag).

(2) A late region, coding for the viral capsid proteins VP1, VP2, VP3, and agnoprotein.

(3) A noncoding regulatory region (NCCR), containing the origin of replication (ori) and transcription control sequences (Fig 1.1).

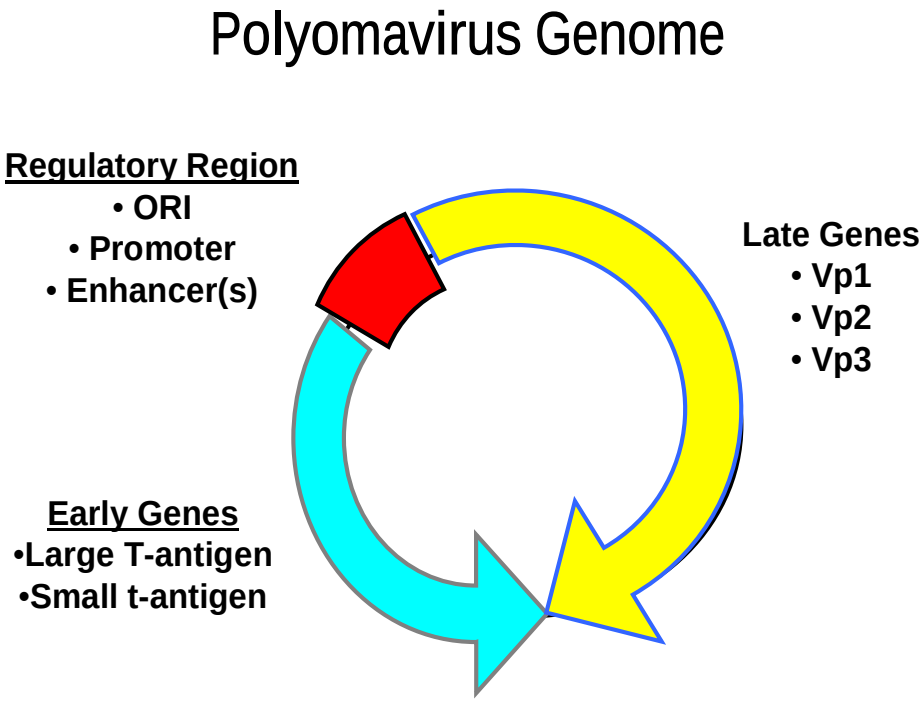

Fig (1.1) BK virus genome, showing Regulatory region, Large T-ag, small T-ag and Vp1, Vp2 and Vp3 regions.

The early region encodes the viral regulatory proteins, the tumor or T-antigens, so called because they can be detected with antisera derived from animals bearing tumors induced by these viruses (Dhar et al., 1979). Large T-ag is a 708 amino-acid protein which is expressed predominately in the cell nucleus. It has multiple functions, including direct initiation of viral DNA replication, and regulation of synthesis of both early and late viral proteins. Large T-ag is the only viral protein essential for viral DNA synthesis.

Large T-ag interacts with a number of host cell molecules including the tumor-suppressor gene retinoblastoma family (Rb) products and p53. Initially, the Large T-ag binds to products of the Rb-family (pRb, p107, and p130) thereby interfering with their activity and inducing the cell to enter the cell cycle (phase S). Subsequently and most importantly, the Large T-ag inactivation of p53 allows the re-phosphorylation of pRb through the cyclin-dependent kinase (cdk) pathway and prevents the p53-mediated cell apoptosis of infected cell. This mechanism is used to keep the cells alive during productive infection but it may lead to cell transformation (Butel and Lednicky, 1999). It is not yet proven that polyomaviruses are etiologic for human cancer. However recent investigations have associated them with the development of specific cancer types including colorectal cancer, glioblastomas, mesotheliomas, prostate cancer and possibly lymphomas

(Butel and Lednicky, 1999). VP1, VP2 and VP3 are structural proteins that make up the viral capsid. The VP1 gene displays genetic heterogeneity and this has lead to the classification of four viral genotypes; I, II, III, IV, type I being the most prevalent (Jin et al., 1993). Agnoprotein is synthesized late in productive infection and is found in the perinuclear region of the infected cell. Although it is not yet known for certain, agnoprotein is thought to play a role in several cellular processes, including cell cycle progression, DNA repair, viral capsid assembly and virion release from the cell. It is thought that it may help with intracellular migration of VP1 to the perinuclear region of the infected cell and entry of VP1 into the nucleus, with consequent viral assembly and that it could be involved with cell to cell spread of the virus (Hou Jong et al., 1987).

The genomes of JCV, BKV, and SV40 are associated with only a single serotype of virus, but extensive variation may occur in the regulatory region of each. Because of this, new terminology has appeared to indicate variant viral DNA's which differ primarily in their regulatory regions. Virus strains consistently associated with normal populations are termed archetypal strains: thought to be involved in transmission of infection. Strains differing from the archetypal strain in their regulatory region are termed variant strains. These variant or rearranged strains have been postulated to account

for their differences in ability to interact with individuals and cause disease (Chatterjee et al., 2000).

1.1 Historical Aspects

Polyomavirus research dates back to the 1950s. Gross et al., 1953, found when studying murine leukemia virus (MLV), that passage of virus through mice sometimes resulted in salivary gland, or parotid tumors, rather than leukemia. In studying this activity he attributed it to a parotid agent which he demonstrated to be different to MLV. Stewart et al., 1958 found that on inoculation of mice with this agent, the result was the formation of multiple tumors and he coined the name polyomavirus, which is derived from the Greek poly, meaning many, and the –oma, denoting cancer. SV40 which is a member of this family was discovered by Sweet & Hilleman, 1960, who were screening polio vaccines produced in monkey cell lines for the presence of contaminating viruses. They were able to isolate SV40 from the African Green monkey kidney cells used to prepare the vaccines. It became clear that a significant number of batches of the poliovirus vaccine were contaminated with the virus, and that SV40 was resistant to the formalin treatment used to inactivate the poliovirus in the vaccine. Between 1955 and 1961, millions of Americans were exposed to SV40 as a result of immunization with the contaminated poliovirus vaccines. Since this time,

SV40 has been demonstrated to be oncogenic when it was inoculated into newborn hamsters (Butel and Lednicky, 1999). In these animal models, the neoplasm's induced by SV40 included primary brain cancers, malignant mesotheliomas, bone tumors, and systemic lymphomas (Butel and Lednicky, 1999). There is mounting evidence that SV40 is an emergent human pathogen (Martini et al., 2004). Although the prevalence of SV40 infections in humans is not known, studies conducted over the last three decades indicate that SV40 infections are occurring in child and adult populations today. SV40 seroprevalence rates in the general populations of the United States and other countries range from 2 to 20% (Butel and Lednicky, 1999). During the last decade, many studies have shown the presence of SV40 large T-ag DNA or other viral markers in primary human brain and bone cancers, malignant mesotheliomas and Non Hodgkin's lymphoma (Vilchez and Butel, 2004). Therefore the major types of human malignancies associated with SV40 are the same as those induced by SV40 in animal models. These results support the conclusion of the Institute of Medicine of the National Academies that "the biological evidence is of moderate strength that SV40 exposure could lead to cancer in humans under natural conditions" and the International Agency for Research on Cancer suggests that SV40 should be included in the list of group 2A carcinogens (i.e., agents for which evidence

is indicative but not definitive for carcinogenesis in humans) (Vilchez and Butel, 2004).

Two other polyomaviruses of humans have also been described which are closely related to SV40. JC virus (JCV) and BK virus (BKV), named from the initials of the patients from whom the first isolates were made, which were isolated in 1971 from immunocompromised hosts.

JCV was isolated by Padgett et al., in 1971 by inoculating human fetal brain cells with extracts of diseased brain tissue from patients with progressive multifocal leukoencephalopathy (PML). That same year Gardner et al., 1971, isolated BKV from the urine of a Sudanese kidney transplantation recipient after inoculation into African green monkey cells.

Usually primary infections with BKV and JCV occur in childhood (Padgett and Walker, 1973), to date it is unclear as to how infection occurs. The viruses persist latently in the infected individual usually in the renal tissue (Chesters et al., 1983; Heritage et al., 1981) and only become reactivated in times of immune compromise.

The initial reason for studying these viruses was their oncogenic potential. However, these viruses became very useful model systems in molecular biology because of the small size of their genomes. In the 1970's with the advent of restriction endonucleases the polyomaviruses became some of the first DNA genomes to be completely sequenced (Fiers et al., 1978; Reddy et

al., 1978; Soeda et al., 1979 and 1980). For many years polyomaviruses were mainly studied for understanding the basic eukaryotic cell processes, such as transcription, DNA replication, RNA processing, and oncogenic transformation.

1.2 BK virus

The BKV genome is approximately 5300bp and has early, late, and regulatory regions (Reploeg et al., 2001). It has approximately 70% DNA sequence homology with SV40. BKV replicates in several human, rodent and monkey cell lines but grows slowly in conventional tube cell culture. BKV is oncogenic and can transform cells of various animal species *in vitro* including the immortalized human embryonic kidney line (Pater and Pater, 1986).

There are many different strains of BKV derived from a variety of sources and with unique features. Examples include: Gardner strain (PPPQS) derived from urine of a renal allograft patient in 1971 (Gardner et al.,1971); DDP strain derived from peripheral blood mononuclear cells (Degener et al., 1999); NCCR strain which has a rearranged DNA sequence (Moens et al., 1995); and Dunlop strain (Moens et al., 1999). It is as yet unclear what role different strains play in regards to risk of kidney damage such as; BK-associated nephropathy (BKVN), sensitivity to drug treatment or oncogenic

potential. Some authors believe that different BKV genomic variants may play a key role in the variable behavior of this viral infection in graft recipients (Randhawa et al., 2003).

JCV is very closely related to BKV. The JCV genome has approximately 5,130 bp and codes for the same regions as BKV. JCV has 69% sequence homology to SV40 and 75% homology to BKV. Sequences showing greatest divergence between these three agents lie in the regulatory region.

1.3 Epidemiology

Polyomaviruses are found throughout the world in birds, rodents, nonhuman primates and human populations. SV40 infects only Old World Rhesus monkeys, and African and Indian macaques. Most epidemiologic data has been collected on BKV and JCV because they are of human origin and cause widespread infections globally. Serological studies of populations using hemagglutination inhibition assays for the detection of antibodies indicates that seroconversion to BKV takes place early in life and conversion to JCV occurring later (Fig 1.2).

Antibody levels of Polyomaviruses in the General Population

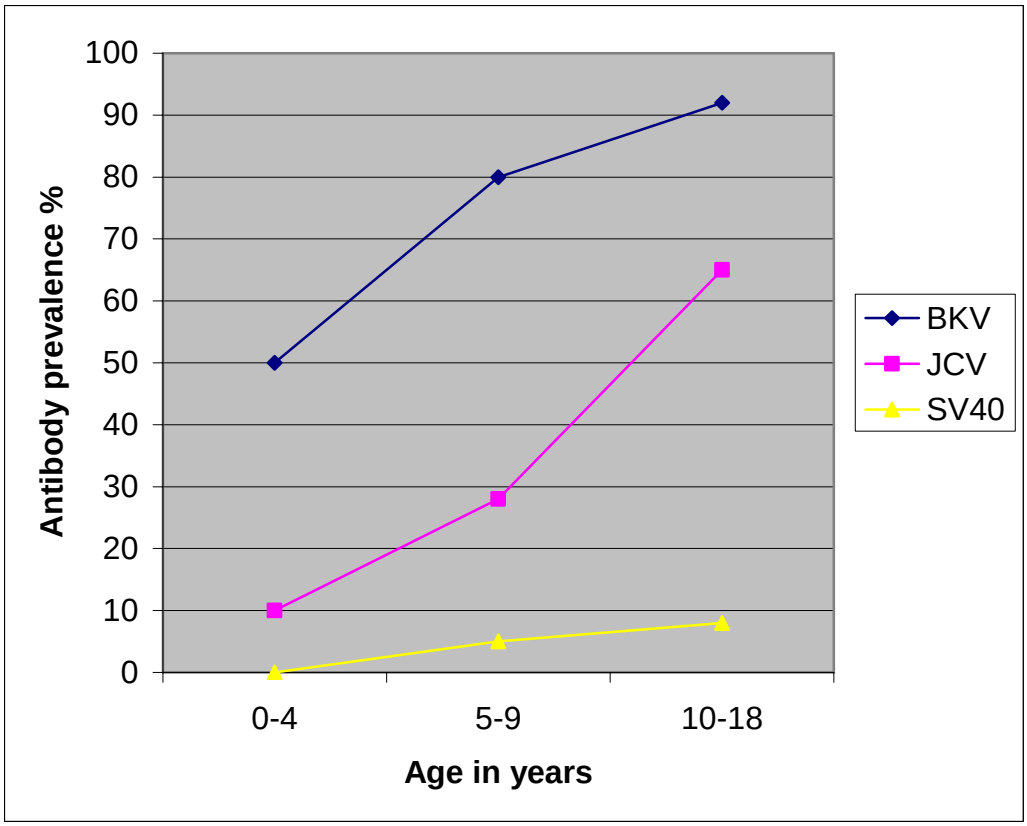

Fig (1.2). Chart showing antibody levels to the polyomaviruses (BKV, JCV and SV40) in the general population (Vanchiere et al 2005).

As shown in Fig 1.2 there is very little information known regarding prevalence of antibodies to SV40 in the worldwide population however it is known that the prevalence of antibodies to JCV is 10% in children 5 years of

age and rises to approximately 65% by adult life (Fig 1.2). Whereas the prevalence of antibodies to BKV in healthy adults is predicted to be between 60-80% and most children will have antibodies by the time they are 10 years old (Shah et al., 1973).

It is thought that primary infection of BKV is typically by the respiratory route in children between 2-5 yrs followed by a latent infection in the renal tissue, primarily in the renal tubular epithelial cells and bladder epithelium (Boldorini et al., 2005). Although it is not known for certain, it is thought that primary infection can occur by respiratory tract, oral-enteric, vertical transmission from the placental crossing and with organ transplantation. Given that BKV remains latent in the kidney, it is no surprise that in kidney transplantation the donor kidney itself appears to be an important source of infection in transplant recipients. Donor seropositivity has been implicated in development of BK viruria, viremia and BKVN in pediatric and adult recipients (Bohl et al., 2006).BKVN has emerged as an important cause of post-transplant renal allograft loss, affecting ~5% of adult renal transplant (RT) recipients. BKVN is primarily due to the reactivation of BKV in the allograft kidney, although the pathogenesis of BKVN is poorly understood. While it is clear that BKV can be pathogenic in the setting of severe immune compromise, BKV reactivation alone is insufficient to cause BKVN, suggesting that host factors play a significant role in pathogenesis.

There is conflicting information on risk factors for BKV infection in transplant recipients. Risk factors associated with transplantation include, cold-ischemia time (the time interval that begins when an organ is cooled with a cold perfusion solution after organ procurement surgery and ends when the organ is implanted), delayed graft function, immunosuppression, treatment of acute rejection with lymphocyte depleting agents or steroids, drug-toxicity, and increased number of HLA mismatches. Viral-related factors include variants in VP1 and sequence alterations in non-coding control region (NCCR). Most of these risk factors are unavoidable, however the type and degree of immunosuppression is modifiable and therefore efforts have been concentrated here to develop the best treatment for the patient.

Reactivation of BKV and JCV can also occur during pregnancy, when the mother becomes naturally immune-suppressed for the sake of the fetus (Coleman et al., 1980; Markowitz et al., 1991). This pregnancy-associated immune suppression is primarily driven by the high levels of oestrogen, progesterone and cortisol that are circulating in the mother's blood and which are necessary for the maintenance of the pregnancy.

These hormones suppress the production of Th1 cytokines (TNF-α involved in the acute phase reaction and IL-12 involved in the differentiation of naïve T cells into Th1 cells) and along with catecholamines, stimulate the

production of Th2 cytokines (IL-10 which is an anti-inflammatory cytokine which downregulates TNF-α production, IL-8 which is involved in the innate immune response and IL-6 involved in the acute phase response). Th1 cytokines are involved with cellular immunity and proliferation of cytotoxic CD8+ T cells which are needed to fight viral infections whereas Th2 cytokines are involved in humoral immunity which is less effective at dealing with a viral infection.

The resulting effect of these changes is the suppression of cell-mediated immunity and the maintenance or stimulation of humoral immunity. The immune suppression of pregnancy is a necessary but insufficient explanation for the reactivation of persistent viruses, suggesting that individual variations in the immune response and immunogenetic makeup contribute to the variability in this phenomenon (Coleman et al., 1983).

No animal model exists in which human infection can be duplicated because neither BKV nor JCV cause productive infection in non-human species. Because of this, the natural history of infection with either virus is not well understood. Early studies employing Southern hybridization detected BKV DNA and/or JCV DNA in a minority of autopsy kidney samples. BKV DNA was also found in tonsils and lymphocytes, suggesting that the virus might also infect lymphoid tissues.

In studies of immunocompromised patients, serological evidence for reactivation of BKV has been detected in 22 to 44% of individuals undergoing renal transplantation or chemotherapy for malignant disease (Shah et al., 1996). Excretion of BKV viral DNA, or virus-infected urothelial cells has been repeatedly found in immunosuppressed patients, during pregnancy and occasionally in normal children. BKV or JCV excretion in the urine of leukemic patients has been found to be 19% (Shah et al., 1996) and in the urine of renal transplant patients at 10-60% (Pang et al., 2007). BKV is shed in urine of immunocompromised or immunosuppressed patients experiencing viral reactivation in levels as high as ~1 x 10^{13} copies per daily output of urine (Leung et al., 2001). In normal healthy children and adults studies have shown that urinary excretion of BKV occurs intermittently in less than 5% of people (Knowles, 2001).

1.4 Polyomavirus associated diseases

Several uncommon but serious clinical syndromes have been associated with human polyomavirus infections. BKV and JCV are associated with persistent infection and diseases of the urogenital tract and the central nervous system. Induction of disease is usually linked to states of immunoincompetence. The most prominent underlying disorders being AIDS and lymphoproliferate disorders.

In patients with AIDs, invasion of the central nervous system by JCV causes progressive multifocal leukoencephalopathy (PML), an opportunistic demyelinating infection which before the advent of AIDS was a very rare disease (Holman et al., 1998; Greenlee, 1998; Markowitz et al., 1993). Systemic BKV-associated disease in AIDs patients, involves infection of the CNS, the lung, the eye, and the kidney (Bratt et al., 1999; Vallbracht et al., 1993; Smith et al., 1998). Along with multiple lesions in the entire nephron, an interstitial tubulonephritis is seen.

A number of patient groups have been identified that are at an increased risk of BKV infection including pregnancy, acquired immune deficiency syndrome, uncontrolled diabetes mellitus, and patients on drugs which suppress their immune systems which include corticosteroids, cytotoxic treatments, anti-metabolites and calcineurin inhibitors. Iatrogenic immunosuppression in the course of transplantation or therapy of autoimmune disorders can contribute to polyomavirus-induced disease. Hemorrhagic cystitis (HC) is a serious BKV-associated complication of bone marrow transplantation patients (Apperley et al., 1987; Vogeli et al., 1999). Prevalence of HC varies from 10% to 68% and leads to severe hemorrhage in about 25% of bone marrow recipients (Arthur et al., 1985; Azzi et al., 1994; Bedi et al., 1995).

In recent studies it has been found that interstitial tubular nephritis is the most frequent BKV-associated disease after renal transplant (Mathur et al., 1997; Pappo et al., 1996; Purighalla et al., 1995). BKVN was first described in 1996 in a needle biopsy from a renal transplant recipient suspected of having acute rejection. This heralded a new era in the study of BKV. Following this finding, many other centers worldwide reported similar findings (Hariharan. 2006; Ramos et al., 2002; Randhawa et al 1999; Nickeleit et al., 2000). The epidemic of BKVN is thought to be the result of administration of potent immunosuppressive drugs such as tacrolimus, mycophenolate mofetil and cyclosporine A. (Tacrolimus and cyclosporine A are both potent calcineurin inhibitors, calcineurin induces transcription of IL-2 which is needed for formation of efffector T cells. Mycophenolate mofetil which is also known under its trade name cellcept and myfortic is an immunosuppressive anti-rejection drug.)

Clinical features may mimic graft rejection or drug toxicity but histopathologic examination almost always shows interstitial infiltrates of plasma cells and lymphocytes. Cells of the transitional bladder epithelium were identified as target cells for BKV infection. Virus isolation, DNA detection by polymerase chain reaction, electronmicroscopy, immunohistologic staining of BKV proteins, and *in situ* hybridization suggests an etiopathologic role of the virus in about 5% of renal transplant

patients (Randhawa et al., 1999; Binet et al., 1999).With the introduction of new immunosuppressive strategies, the incidence of BKV-associated complications in renal transplants increased further (Brennan et al., 2005), thus confirming the close relationship between excessive virus growth and immunologic impairment. In contrast to renal disease under severe immunoincompetence, BKV infection in other patients is most likely in an asymptomatic state. Virus DNA is distributed in small foci throughout the cortex and medulla of the kidney (Heritage et al., 1981), infecting renal epithelial cells and lining cells of ureter and bladder as demonstrated by BKV-specific immunostaining (Fig 1.4).

BKV DNA persists in asymptomatic tissue and exfoliated cells carry intranuclear inclusions containing BKV antigen (Shinohara et al., 1993). Analysis of BKV and JCV associated diseases disclosed a variety of organs and cell types to be susceptible to virus infection; however some essential questions still remain to be answered with respect to involvement of distinct organs and cell types in polyomavirus persistence.

Normal Healthy kidney

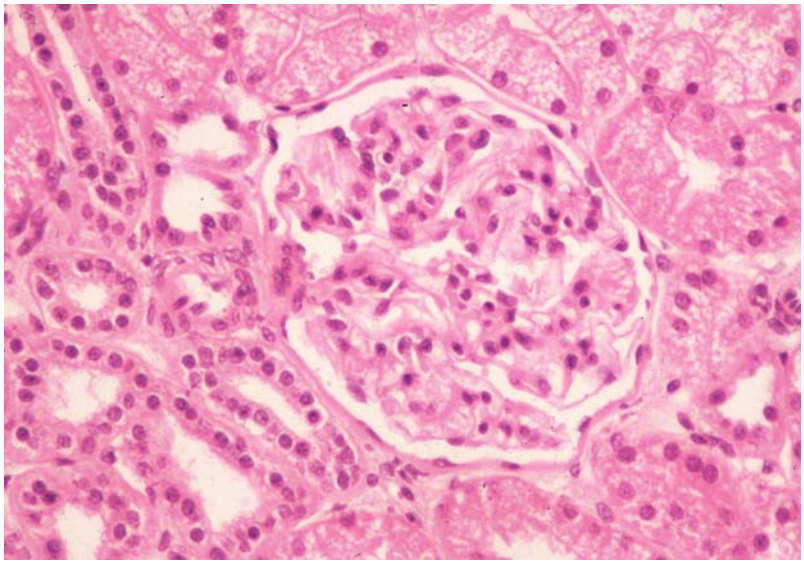

Fig 1.3 Histology of a normal healthy kidney (www.tulane.edu)

BKV nephropathy of the kidney

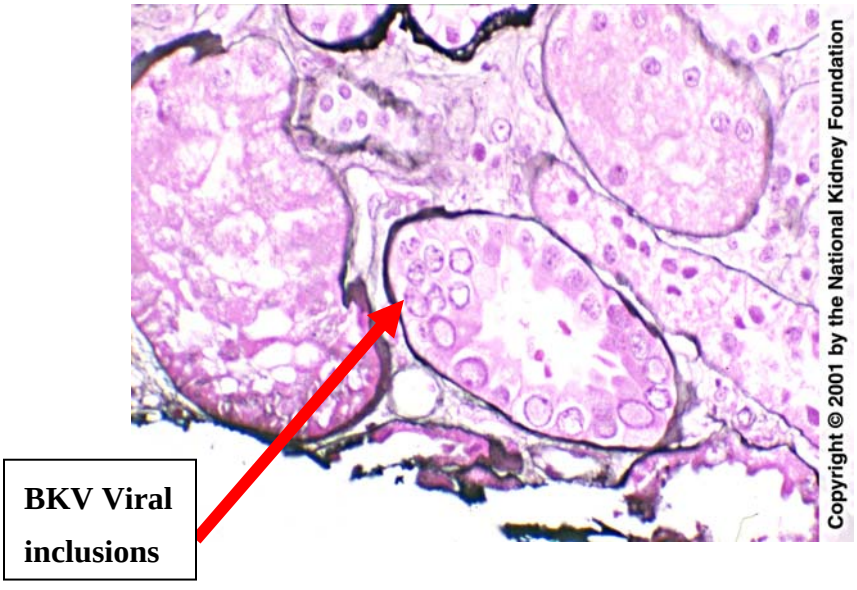

Fig 1.4 Image of viral inclusions and cellular changes typical of BK polyoma virus nephropathy are seen in the tubular epithelium of this renal transplant case. Jone's Silver Stain, original magnification X1000 (www.standford.edu).

1.5 Renal Transplants

Renal transplantation is the treatment of choice for children with end-stage renal disease with improved organ and patient survival over the past decade (Hariharan et al., 2006). An essential part of the ongoing care of renal transplant patients requires immunosuppressant drugs which have dramatically reduced the rates of acute allograft rejection over the past decade but may have exacerbated the problem of post-transplant infections. Donor viral transmission, recipient viral reactivation, and *de novo* viral infection, put the transplant recipient at a high risk of viral induced morbidity and mortality because of their immune compromised state.

BKV reactivation or BKVN in kidney transplant patients accounts for loss of allograft in 1-5% of patients. The most striking feature of BK infection in kidney transplant recipients is the lack of fever, malaise, leucopenia, anemia or other signs and symptoms typical of viral infection, despite viral loads exceeding a billion copies/ml in the urine or 100 000 copies/ml in the blood (Brennan et al., 2005).

Post-renal transplant, BKV infection will present itself in a variety of ways: asymptomatic viruria with shedding of classic ‘decoy cells’ or virus infected uro-epithelial cells in urine (Gardner et al., 1984), tubulo-interstitial nephritis and/or BKVN and ureteric obstruction (Vats et al., 2003).

The pathogenesis of tissue damage in the infected kidney is a subject of considerable interest. Transition from latent to lytic infection is likely initiated by ischemia (time without oxygenated blood), calcineurin inhibitor or rejection-associated injury.

The above reasons explain why most cases of BKVN occur in the allograft kidney, although disease in the native kidney has been recorded (Randhawa et al., 2004; Ginervi et al., 2006).

DNA microarray analysis of allograft kidney biopsies has shown that BKVN is associated with the upregulation of several major groups of mRNAs involved in the inflammatory response including; CD8, interferon-γ (IFN-γ), CXCR3 and perforin. CD8 is a marker found on cytotoxic T cells; IFN-γ has potent anti-viral properties and is the hallmark of the Th1 type T cell response. CXCR3 is a chemokine receptor which is expressed on activated Th1 cells and promotes their maturation upon ligand interaction and perforin is a cytolytic protein found in the granules of CD8+ T cells. The upregulation of all these proteins would help the patient to mount an anti-viral response to the replicating BK virus however it is notable that these molecules are also up-regulated in acute cellular rejection, thus illustrating why the differential diagnosis between viral nephropathy and acute cellular rejection is problematic (Mannon et al., 2005).

Since graft rejection usually requires increasing the immunosuppressive regimen, whereas BKV viral reactivation is often managed by decreasing immunosuppression, determining which of these entities is responsible for the pathology is critical.

The treatment of BKVN is difficult as to date there is no uniformly effective anti-viral drug. Prevention of BKVN may be a better strategy than treatment of established disease. One large study of patients was conducted using prospective monitoring of urine and blood, and preemptive withdrawal of immunosuppressive drugs upon development of viremia. This strategy resulted in clearance of viremia and viruria and prevention of progression to BKVN without increasing the risk of acute rejection (Brennan et al., 2005). There is increasing recognition of BKV infection after kidney transplantation (Hariharan 2006). However improved techniques of clinical monitoring and preemptive interventions will reduce the number of patients who develop BKV-induced graft injury.

1.6 Detection of Polyomaviruses in Human material

Laboratory monitoring strategies for BKV are still evolving. Several methods have been described for detecting BK and JC virus, including renal biopsy, urine cytology, and urine PCR (Arthur et al., 1989; Corey et al., 1997; Purighalla et al., 1995). PCR is the most sensitive of these assays and may also be quantitative (McNees et al., 2005). It is also the only reliable method for differentiating JCV and BKV viral genotypes (Boldorini et al., 2000). JCV excretion in the urine is usually insignificant, although very rare cases of JCV-associated nephropathy are on record.

Polyomavirus urinary tract infection may be suspected if epithelial cells containing intranuclear inclusions are detected in the urinary sediment. The virus may be specifically diagnosed using immunohistological staining of cytological preparations using BKV/JCV specific antibody, enzyme linked immunosorbent assay, or PCR. While urinary ‘decoy cells’ have excellent sensitivity for the detection of overt BKVN, PCR is four times more sensitive than urine cytology for monitoring asymptomatic viruria (Randhawa et al., 2004).

Detectable virus in the blood is more predictive of BKVN than viruria alone. It is thought that all patients with active nephropathy will have detectable BKV in plasma (viremia). The association of circulating virus with active nephropathy has been noted before (Hirsch et al., 2002; Randhawa et al.,

1999; Vats et al., 2003). The entry of viral DNA into the circulation likely occurs at the level of peritubular capillaries, following tubular destruction and release of free virus in the interstitial compartment. Detection of BKV in blood is highly probable of BKVN however definitive diagnosis of BKVN requires a biopsy and demonstration of BKV inclusions in tubular epithelial or Bowman's capsular epithelial cells. Viral infection is accompanied by varying degrees of inflammatory cell infiltrates, tubular atrophy and fibrosis. Confirmatory immunohistochemistry or in situ hybridization are usually preformed using antibodies against specific BKV proteins or probes complementary to viral DNA. Viral particles can also be demonstrated by electron microscopy. Ideally two biopsy cores should be examined as BKVN can be focal in distribution.

1.7 Molecular Methods of Detection

At present the preferred method for detection of polyomaviruses in the blood and urine is by real-time polymerase chain reaction (RT-PCR). RT-PCR assays are advantageous over traditional PCR protocols for the detection of polyomavirus DNA for several reasons. The sensitivity of detection of RT-PCR is increased from 10- to 100- fold over conventional PCR protocols where the lowest copy number detected by PCR agarose-gel analysis was 1000 copies (McNees et al., 2005).

RT-PCR also called quantitative real time PCR (QRT-PCR) or kinetic PCR is a technique used to simultaneously quantify and amplify a specific part of a given DNA molecule. It is used to determine whether a specific sequence is present and if so the number of copies present in a sample. The procedure is similar to conventional PCR, but the DNA is quantified after each round of amplification; this is the "real-time" aspect of it. There are two common methods of quantification; fluorescent dyes that intercalculate with ds-DNA and oligonucleotide probes that fluoresce when hybridized with complementary DNA. These fluorescent probes are the most accurate of the methods. The probe is commonly tagged with a fluorescent reporter at one end and a quencher of fluorescence at the opposite end, this probe is usually referred to as a Taq Man probe. The close proximity of the reporter to the quencher prevents fluorescence until addition of taq polymerase which has exonuclease activity (enzymes that cleave nucleotides) and breaks the reporter quencher proximity and thus allows the emission of fluorescence (Fig 1.5).

Real-time PCR Method

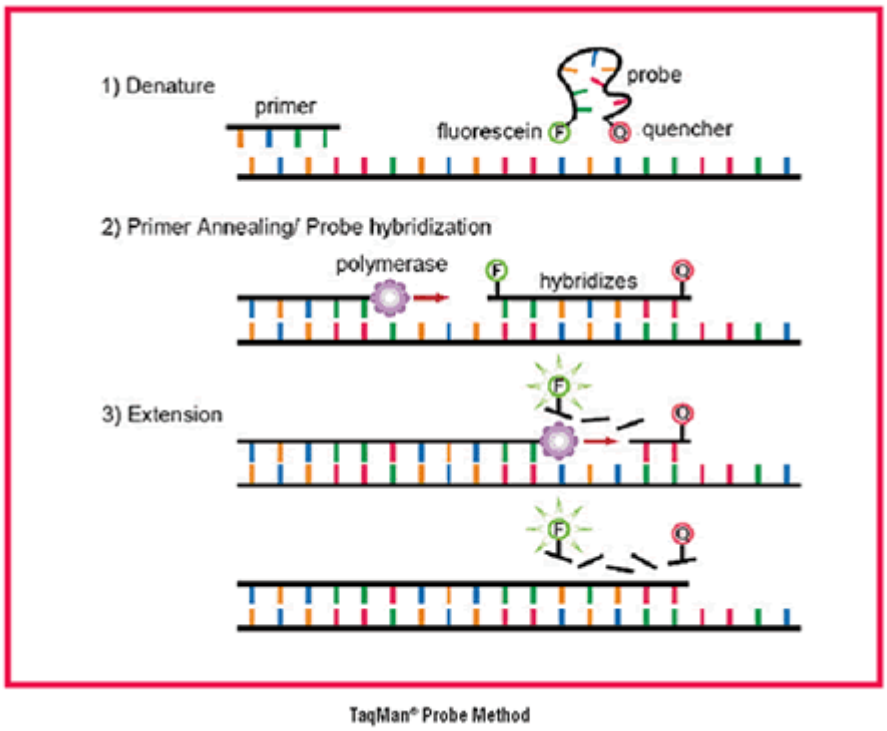

Fig 1.5 Real-time method, when probe hybridizes with target nucleotides the quencher is released allowing emission of fluorescence (www.takarabiousa.com/images/PCR_TPM.gif).

As there is an increase in the product targeted by the reporter probe after each PCR cycle there is a proportional increase in fluorescence which is recorded by the real-time thermocycler (Fig 1.6).

Real-time PCR curve

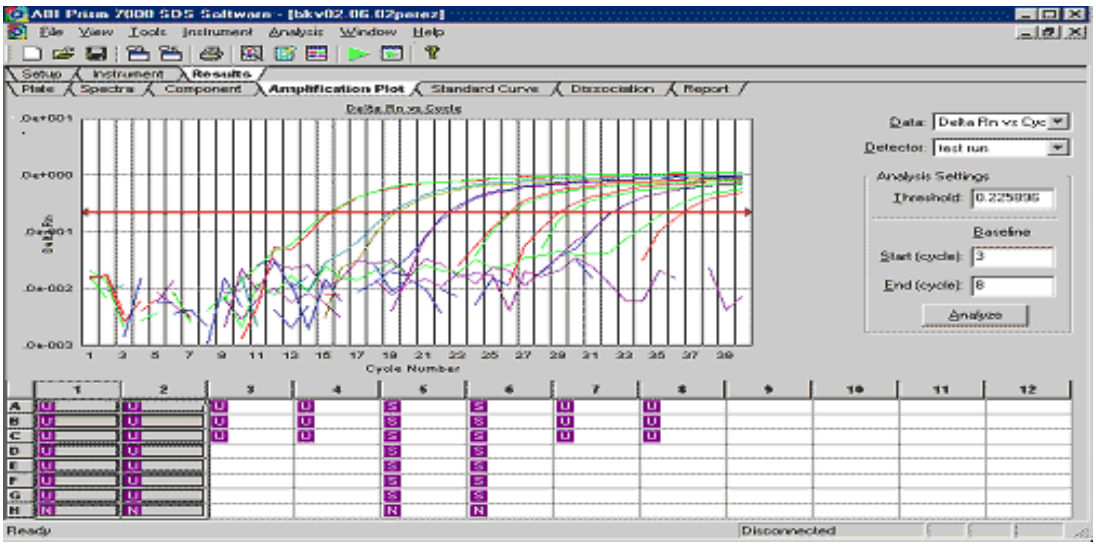

Fig 1.6 Real-time PCR curve. As there is an increase in the product targeted by the reporter probe after each PCR cycle there is a proportional increase in fluorescence after each cycle (www.viracor.com/images/Sub-Technology-Pic-PCR.gif).

The probes specific to the sequence of interest in our study, are labeled with FAM^{TM} (6-carboxyfluorescein) the reporter, at the 5'ends and with MGB (minor groove binder) the quencher, at the 3'ends.

During the extension phase of the cycle, fluorescence is generated as the reporter FAM on the 5'end of the probe is cleaved by the 5'-3' exonuclease activity of the Taq polymerase; reporter dye is released and accumulates as the reaction progresses.

ROX™ (passive reference dye) is a component of the TaqMan Universal PCR master mix and by dividing the intensity of the reporter dye by the intensity of ROX; corrections can be made for any volume fluctuations made during the preparation of the reaction. The threshold cycle number, C_T is the cycle number at which significant increase in reporter fluorescence is first detected and this threshold is set 10 standard deviations above the mean background fluorescence detected in the first 15 cycles.

When choosing primers and probes it is important to design them to mimimize the possibility of crossreactivity. The Large T-ag sequences of the BKV, JCV and SV40 viruses share considerable homology so primer and probe designs must be chosen to maximize the differences among the sequences in this region. In previous studies (McNees et al., 2005) primer express software is used to design the primers and probes and subsequently synthesized by a company i.e. Applied Biosystems.

1.8 Phylogenetic Analysis

Phylogenetic analysis is the study of the evoluntionary relationship between organisms, it can be used in virology to examine closely related viral strains in an attempt to understand viral diversity, and further elucidate the transmission and spread of disease (McCormack and Clewley, 2002).

Nucleotide sequences are compared and the evolutionary relationship is constructed graphically in a phylogenetic tree.

Phylogenetic trees can be constructed using distance or character based methods. Character based methods select trees based on the individual nucleotides at each position in the sequence alignment. The two main character based methods are maximum likelihood and maximum parsimony. Maximum Likelihood is generally the method of choice as it is character based and works on a model of evolution (McCormack and Clewley 2002). When constructing a phylogenetic tree, it is important that a representative of the common ancestor of the data set is included. This is generally done by using an outgroup. An outgroup sequence is one that is genetically related to, but not belonging to the group of interest (ingroup) (Wheeler,1990). To confirm the reliability of a phylogenetic tree, bootstrapping is performed. This is a random resampling process where the sequences are realigned repeatedly, generally 1000 times, to create the best fit tree. A value is calculated based on the frequency that a consistent branching pattern is observed. Values greater than 70% are thought to have strong bootstrap value and generally indicate that the position of the corresponding sequences on the tree is correct.

1.9 Aims

To date, there has been limited investigation of polyomavirus reactivation in pediatric patients with renal transplants or with primary renal disease. Given the association of polyomavirus reactivation with the use of immune suppressive therapies, we sought to investigate whether polyomavirus reactivation occurred in patients with pediatric kidney disease and if so how does this correlate with pediatric patients who are immunosuppressed for other reasons.

We assessed three separate pediatric patient groups who differed in the degree and type of immunosuppressive condition present.

1) Pediatric patients with nephrotic syndrome (NS)

2) Pediatric renal transplant recipients (RT)

3) Pediatric patients with other immune compromise (IC) e.g. HIV infection, acute lymphocytic leukemia or heart transplantation.

The main aims of this project were to

A) Determine the prevalence of polyomavirus viruria and viremia in pediatric patients with nephrotic syndrome, patients who have received a kidney transplant and otherwise immunocompromised patients

B) Determine presence of virus; BKV or JCV present in urine and blood samples by qualitative PCR.

C) Determine amount and type of virus; BKV or JCV present in urine and blood samples by quantitative Real-time PCR and compare this to the qualitative results.

D) Determine the type of polyomavirus present, BKV or JCV.

E) Determine the sub-type of virus present by commercial genome sequencing.

F) Determine race, gender and age association of the respective viruses.

G) Compare the patient groups for type and amount of virus present.

H) Establish mean amounts of virus present in each sample.

I) Look at trends in virus excretion in the urine of patients over time.

J) Determine if the immunotherapy a patient is being treated with has any effect on the amount of polyomavirus detectable in the urine.

K) Compare a patient's white cell count with the viral loads in their urine.

Hypothesis: Polyomavirus viremia and viruria is more common among patients after renal transplantation (RT) than in those with nephrotic syndrome (NS).

2. Materials and Methods

2.1 Study Subjects

Three categories of pediatric patients were analyzed in this study. Some patients had nephrotic syndrome (NS), some had had a renal transplant and the other group of patients were immune compromised (IC) due to another disease e.g. HIV infection, acute lymphocytic leukemia or heart transplantation. Nephrotic syndrome is a disorder where the kidneys have been damaged due to inflammation, causing them to leak protein from the blood into the urine. In children a condition called minimal change disease (MCD) accounts for 95% of nephrotic syndrome cases and the other 5% is usually due to a condition called focal segmental glomerulosclerosis (FSGS) which is a disease that involves scarring of segments of the glomerlus. Generally treatment of nephrotic syndrome involves immunosuppressive therapy and we sought to compare BKV viral excretion in these patients with those who had had renal transplantation. Our third group patients were immune suppressed but their conditions were not associated to the kidney e.g. HIV and acute lymphocytic leukemia and so it would be interesting to see if their immunosuppression was also leading to reactivation of the virus.

2.2 Study Enrollment and Sample Collection

One hundred and twenty-five patients were enrolled between February 2000 and October 2005. Enrollees were from three patient populations;

1) patients with nephrotic syndrome (NS) (n=81)
2) renal transplant recipients (RT) (n=16)
3) patients with other immune compromise (IC) e.g. HIV infection, acute lymphocytic leukemia or heart transplantation. (n=28)

Urine and blood samples were collected at the time of routine clinic visits. Blood specimens were only obtained for study purposes when blood was being drawn for clinical purposes. Five milliliters of whole blood was obtained by peripheral venipunctures, using aseptic techniques, into sodium-heparin collection tubes. Samples were allowed to settle for 2 hours and plasma was removed as was the buffy coat interphase. Urine samples were obtained in sterile collection cups and stored at 4^{o}C for < 12hrs. Aliquots of 1ml of urine and plasma were cyropreserved at -70^{o}C prior to analysis. Some urine samples were subjected to low speed (2000g) centrifugation for 10 min at room temperature, to pellet cellular material. The supernatant was separated from the pellet, both of which were stored at -70^{o}C and both were tested. Sample identification included the laboratory accession number, the date of collection, and the patient's date of birth.

For avoidance of cross-contamination we maintained strict protocol measures throughout the study, including the use of separate, plasmid-free rooms, and centrifuges for processing of clinical samples, isolation of DNA, PCR set-up and PCR analysis.

2.3 Prospective medical record review

To assess the demographic and medical factors that may be associated with polyomavirus excretion in this pediatric population, approval was obtained from the Institution Review Board for Human subjects at Baylor College of Medicine, to review the medical records of patients whose blood and urine were tested. Specific data (gender, race/ethnicity, medication, evidence of immune compromise and medical history) was sought from each chart using an electronic data-collection form. This work is ongoing at present by Dr.Vanchiere and his group.

2.4 DNA Extraction

Materials

MagNApure-LC Instrument (Roche Applied Science, Indianapolis, IN) automated nucleic acid extraction platform.

MagNa Pure LC (Roche Applied Science, Indianapolis, IN) Total Nucleic Acid Isolation Kit.

MagNA Pure LC DNA disposables (pipette tips, holders, reaction and sample cartridges, reagent tubs and lids). (Roche Applied Science, Indianapolis, IN)

Method

200µl of sample (urine or blood) was added to sample cartridge and placed on the MagNA Pure LC instrument. MagNA pure LC Total Nucleic acid isolation kit reagents and disposables were placed on instrument as per instructions on screen. The magnetic glass particle solution was vortexed prior to use and added to instrument only after all other reagents and disposables were in place. Instrument was started with an extraction time of approx 3hrs. Instrument was checked at regular intervals for faults.

Nucleic acids bound to the silica surface of the glass beads due to chaotropic salt conditions and the high ionic strength of the lysis binding buffer.

Purified nucleic acids were eluted at an elevated temperature and an elution volume of 100µl was transferred to a sterile cartridge and stored at -20ºC until needed. A negative control was included in each extraction run and consisted of 200µl of PCR grade water.

2.5 Qualitative PCR

Materials

DNA polymerase (Ampli-Taq DNA Polymerase, Applied Biosystems, Inc., Foster City, CA)

1 x PCR reaction buffer containing $MgCl_2$, final concentration 1.5mM (Applied Biosystems, Inc.)

200 µM for dNTPs (Promega, Madison, WI) assembled in a final volume of 50µl.

PYV.fr/PYV.rev primers (Appendix II). Amplify a 173-182 bp portion in the amino terminus of the T- antigen gene of BKV, JCV and SV40.

Regulatory region primers specific for BKV (Appendix II)

VP1 region primers for BKV (Appendix II).Plasmid clones of pBKV- strain Dunlop- 1 (Seif et al., 1979)

Method

Total nucleic acid was extracted from 200µl of whole urine, urine pellets, serum, plasma or buffy coats using the MagNApure-LC (Roche, Indianapolis, IN) automated nucleic acid extraction platform as directed by the manufacturer. Extracted DNA was resuspended in 100µl of elution buffer of which 10µl was used for each PCR reaction. Samples were tested by qualitative PCR with primers PYV-for and PYV rev (Bergsagel et al., 1992) that detect a 178-183bp region of the T-antigen gene in JCV, BKV and SV40 (Appendix II)

PCR reaction components were mixed to a final concentration of 0.5-1µM for primers, 0.05-0.1 U/µl for heat stable DNA polymerase (Ampli-Taq DNA Polymerase, Applied Biosystems, Inc., Foster City, CA), 1 x for PCR reaction buffer containing $MgCl_2$, final concentration 1.5mM (Applied Biosystems, Inc.), and 200 µM for dNTPs (Promega, Madison, WI) assembled in a final volume of 50µl. Conditions for PCR amplification using the PYV.fr/PYV.rev primers (Appendix II) were: 45 cycles of denaturation at 96^{o}C for 30secs, annealing at 60^{o}C for 30sec, and extension at 72^{o}C for 45secs (Bergsagel et al., 1992, Lednicky et al 1997). Plasmid clones of pBKV- strain Dunlop- 1 (Seif, 1979) were used as positive controls for PCR reactions. All plasmid stocks had been previously prepared in the lab using the EndoFree Plasmid Maxi Kit (Qiagen) (McNees et

al.,2005). A standard graph was made by amplifying serial dilutions of the BK human polyomavirus viral DNA plasmid control containing the entire BKV genome (Dunlop-strain pUC-19), (Seif et al., 1979). The plasmid concentrations plotted ranged from 1 to 10^7 genomic copies. The negative control consisted of 50µl RNase free water.

Samples that produced appropriate sized DNA amplimers with PYV.for and PYV.rev primers, as determined by direct visualization on ethidium bromide-stained agarose gels, were further tested using primers for the BKV regulatory region and VP1 region (Appendix II). These primers were previously designed in the lab using primer express software and synthesis by Applied Biosystems (Vanchiere et al., 2005).

Conditions for PCR amplification using the regulatory region primers were: 45 cycles of denaturation at 96^{o}C for 30 sec, annealing at 63^{o}C for 30 sec, and extension at 72^{o}C for 45 sec (Lednicky et al 1997).

The limit of detection for this assay is 1000 copies/ml of plasma or urine.

2.6 Agarose gel Electrophoresis

Materials

Agarose Powder, Molecular grade Promega

Combs

Pipettes and tips

5 X TBE (Tris borat EDTA)

Distilled water

Ethidium Bromide 10mg/ml Sigma

Blue loading dye Promega

100 base pair DNA ladder 0.13µg/µl Promega

Transilluminator

Power Supply

Digital camera

Microwave

Large and mini electrophoresis tanks

Method

12g/800ml (1.5g/100ml) of agarose powder was weighed on the balance. To prepare 1 X TBE running buffer, 100ml of 10X stock TBE buffer was poured into a 1 liter graduated cylinder and made up to 1 liter with deionised

water and mixed to give a homogenous solution. 800mls of TBE buffer was then added to the agarose powder in a beaker and microwaved until the solution was clear. Solution was then allowed to cool slightly before addition of ethidium bromide. 1.25µl/25ml of ethidium bromide was added in a fume cupboard and mixed to give a homogenous solution. The agarose solution was then poured slowly into a gel mould, carefully to avoid bubbles. Combs were placed at regular intervals through gel. Gel was allowed to cool for over an hour and then placed in an electrophoresis tank. TBE buffer was poured into the tank until the gel was completely covered and the combs were removed. 5µl of loading dye was added to the samples and to the 100bp DNA ladder size marker. 20µl of the mixture was added to each well and the power supply was connected to the tank with the DNA at the negative electrode (cathode). DNA is negative and will run towards the positive electrode (anode). Voltage was set to 100V and run. The gel was then visualized using an ultraviolet transilluminator.

2.7 Real-time Quantitative PCR

Materials

PRISM 7000 Sequence Detection System (Roche, Applied Biosystems)

100nM of TaqMan FAM-MGB probe

25ul of the 2 x TaqMan Universal PCR master mix (Roche, Applied Biosystems, New Jersey USA)

900nM (each) forward and reverse primers (Appendix I)

Positive displacement pipettes and barrier tips

10µl of the test DNA

Standard plasmid dilutions

Microcentrifuge

Sequence Detection System software

Method

In parallel with qualitative PCR, JCV-specific and BKV-specific quantitative PCR analysis was performed on all extracted DNA's as described previously (McNees et al., 2005). In this project the primers and probes were previously designed (Bergsagel et al., 1992) to detect sequences in the conserved N-terminal region of the Large T-ag gene for each polyomavirus. The Large T-ag sequences of the BKV, JCV and SV40 share considerable homology so primer and probe designs are chosen to maximize the differences among the sequences in this region to eliminate the

possibility of cross reactivity. The oligonucleotide sequence and corresponding nucleotide positions are shown in (Appendix I).

Quantitative real-time PCR assays were performed using the PRISM 7000 Sequence Detection System (Applied Biosystems) according to the manufacturer's recommendations. PCR amplifications were performed in a reaction volume of 50µl containing 100nM of TaqMan FAM-MGB probe, 25ul of the 2 x TaqMan Universal PCR master mix (Applied Biosystems), and 900nM (each) forward and reverse primers.

Preparation of Real Time PCR Cocktail Mix 40µl per well

	1X	100X
1) 2 X Taq Man Master Mix	(25µl)	2500µl
2) Primer 1	(1µl)	100µl
3) Primer 2	(1µl)	100µl
4) Probe	(1µl)	100µl
5) Gibco Water	(12µl)	1200µl

Table 2.1: Components of Real Time cocktail mix

PCR reactions were prepared in the PCR Clean Rooms Core Facility using positive displacement pipettes and barrier tips. 10µl of the test DNA or standard plasmid dilutions were added in duplicate outside the core facility. Thermal cycling was initiated as follows: 50^{o}C for 2 min, denaturation step of 10 min at 95^{o}C, followed by 40 cycles of denaturing at 95^{o}C for 15secs, followed by annealing and extension at 60^{o}C for 1 min at the end of which fluorescence was read. Real-time PCR amplification data were analyzed by the Sequence Detection System software provided by the manufacturer.
By amplifying serial dilutions using the BK human polyomavirus quantitated viral DNA plasmid control containing the entire BKV genome (Dunlop-strain pUC-19), (Seif et al., 1979) and JCV-Mad-1 strain (Frisque et al., 1984) the sensitivity of the quantitative PCR procedure as a minimal we established a detection level of 100 copies/ml , while for qualitative PCR it was 1000 copies/ml. Standard curves for the quantification of BKV and JCV were constructed using serial dilutions of the Dunlop-strain pUC-19 plasmid and the pJC-MAD-1 for JCV. The plasmid concentrations plotted ranged from 1 to 10^{7} genomic copies per RT-PCR. All patient samples were tested in duplicate, and the numbers of BKV viral copies were calculated from the standard curve. Data were expressed as copies of viral DNA per milliliter of urine or plasma.

2.8 Purification and sequencing of PCR Product

Materials

QIAquick® PCR Purification kit 250. (QIAGEN Sciences, Maryland 20874 USA).

Pipettes and tips

50µl storage tubes

Method

Products of the correct length for the VP1 capsid protein and amplicons of the VP1 gene (50 µl for each sample) were purified according to the manufacturer's instructions using QIAquick® PCR Purification kit 250 (QIAGEN Sciences). The eluted PCR product was stored in sterile labelled tubes before being sent for sequencing.

DNA sequencing was carried out by Lone Star Labs, Inc. (Houston, TX) utilizing the ABI Prism Automated DNA sequencer 377XL and Big Dye Terminator Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Inc) according to the manufacturers directions.

Sequences were viewed and corrected using the program. Chromas version 2.32 (http://www.technelysium.com.au/chromas.html). A maximum likelihood tree was constructed using PAUP* version 4.0 (Swofford) and

Modeltest (Posada). Bootstrap resampling was carried out for 1000 replicates of the data set.

2.9 Statistical Analysis

Statistical analysis was performed using Microsoft excel (Microsoft Corporation, Redmond, WA). Data was analyzed using the chi-squared (x^2) or student t-test. A p-value < 0.05 was considered statistically significant.

3. Results

This study aimed to examine the prevalence of polyomavirus viruria and viremia in pediatric patients with nephrotic syndrome and patients who have received a kidney transplant. To accomplish this we set about carrying out a longitudinal prospective study of one hundred and twenty five patients enrolled over a five year period between February 2000 and October 2005 from Texas children's hospital. Over this time there were 554 patient encounters in total. The median age of the patients was 11.7 years and the range was 6 months to 25 years. An average of 7.4 samples was collected per patient. A total of 388 urine specimens and 280 blood samples collected over time, were examined from the 125 patients enrolled. DNA was extracted from all samples received by MagNA pure extraction and stored at -70^{o}C in preparation for further analysis.

The first objective of this project was to determine whether there were any polyomaviruses present in the samples. To accomplish this it was decided to firstly qualitatively test the samples for the presence of polyomavirus.

Qualitative PCR (Fig 3.1) was performed using primers PYV-for/PYV-rev which amplify a 173-182 bp region in the amino terminus of the T-antigen gene of BKV, JCV and SV40 (Appendix II). 34 samples out of a total of 933 samples (blood and urines including duplicate blind samples) tested positive for a 173-182 bp product (T-antigen), however this information is

uninformative as regards which virus (BKV, JCV or SV40) was present in the sample. All 34 positive samples were from urines.

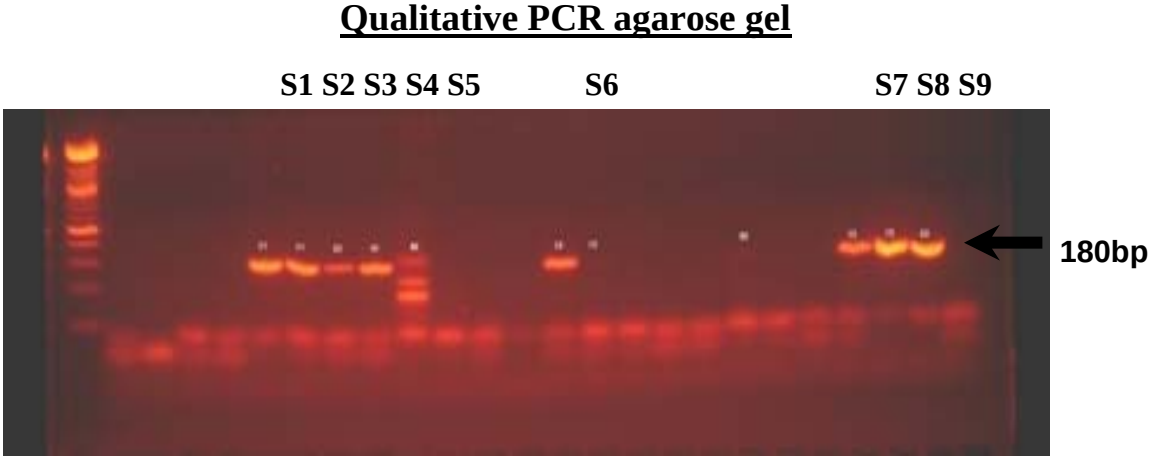

Fig 3.1. Qualitative PCR using primers PYV-for/PYV-rev which amplify a 173-182 bp region in the amino terminus of the T-antigen gene of BKV, JCV and SV40 (Appendix II). Samples S1-S9 all showing correct size product for T-antigen. S5 shows a number of different bands, however, because there is a band of the correct size present this sample is deemed positive.

Next 933 urine and blood samples were tested by real-time PCR for the presence of BKV or JCV using forward and reverse primers that were designed to amplify viral genomic regions for the large T-ag genes of BKV or JCV (Appendix I).

51 patient samples were positive by real-time PCR for BKV and 31 patient samples were positive for JCV. It was then decided to try to detect BKV by qualitative PCR using different primers, VP1 primers (Appendix II) and

regulatory region primers (Appendix II). 34 samples were positive using VP1 primers and only 7 were positive using regulatory region primers. Therefore it became clear that real-time PCR was the most informative method of detection. Since real-time PCR gave the most sensitive results it was decided only to rely on real-time results for detection of virus in samples. And all results in this section reflect these.

To assess the real-time assay for reproducibility we randomly included some samples on 2 or more occasions and tested them blindly. There were 139 samples tested in this way and 92% of the time the same result was found for the same sample tested in duplicate.

$$\% \text{ reliability of assay} = \frac{\text{True Negatives}}{\text{True Negatives + False Negatives}} = \frac{117}{117+10} = 92\%$$

117 samples were negative each time they were blindly tested for BKV, 12 samples were positive each time and 10 samples were positive sometimes and negative sometimes. For JCV 125 samples were negative each time they were tested 9 samples were positive each time and 5 samples were negative sometimes and positive sometimes. Table 3.1 shows the variability of the real-time assays.

139 Samples Tested in duplicate by Real-time PCR

Duplicates* (Total 139)	NEG/NEG	POS/POS	NEG/POS
BKV	117	12	10
JCV	125	9	5

*between 2 - 5 samples

Table 3.1 Real time assay variability found in duplicate samples. Samples were blindly tested on 2 or more occasions.

On analysis of our real-time data it became clear that almost all the positive samples for polyomavirus were in urine (Table 3.2) and it was very unlikely that we would find the virus in blood (Table 3.3). Therefore firstly we looked at virus excretion in the urine of patients in each subgroup, nephrotic syndrome (NS), renal transplant (RT) and secondary immunocompromised (IC).

Overall we found that polyomavirus DNA could be detected in the urine of 43 (42.1%) of 102 patients who gave urine samples. 28 patients (27.4%) were positive for BKV and 11 patients (10.7%) were positive for JCV. 4 patients (3.9%) were positive for both viruses (Table 3.2).

BKV and JCV Real-time results for urine samples taken from the three study populations

Urine Samples (n= 388)	Total	BKV (+)	JCV (+)	BKV & JCV (+)
Nephrotic syndrome	74	18 (24.3%)	7 (9.5%)	3 (4.0%)
Renal transplant	16	6 (37.5%)	4 (25%)	1 (6.3%)
Other Immune Compromise	12	4 (33.3%)	0	0
	102	28 (27.5%)	11 (10.7%)	4 (3.9%)

Table 3.2.Urine samples only from the three patient sub-groups.

Examining the results by patient subgroup revealed that polyomavirus excretion in general (JCV or BKV) was significantly more common among RT patients than NS patients (11/16 vs. 28/74, p=0.0005).

JCV viruria was more common among RT patients (31%) compared to NS patients (13%) (5/16 vs. 10/74, p= 0.02, x2 test), and a similar finding was observed for BKV, RT patients (43%) compared to NS patients (28%). (7/16 vs. 21/74, p=0.09, x2 test). However on analysis the result for BKV was not statistically significant between the two groups.

However it can be seen that polyomavirus reactivation in the urinary tract was more common after RT than among subjects with NS.

On examination of blood for the presence of polyomaviruses the results were very different to that of urine. We looked for the presence of virus in the 103 patients that we had blood samples on. Table 3.3 shows the amount of virus found in the 280 bloods tested. Only on two occasions was a positive result found on a real-time assay. The copy number found was < 3000 genomes/per ml of blood which was a very low copy number compared to the copy number's found in urine, 500-1.5*10^{10} copies/per ml (Table 3.4). The rare occurrence of virus to be found in blood is what was expected as none of the patients had developed BKVN, and a viremia usually precedes development of this disease. BKV viremia was observed in only 1 of the 15 RT patients we had blood samples from and 1 of the 66 NS patients that we had blood samples on. Both patients had BKV viral loads <3000 genome copies/ml of plasma.

Blood samples tested by RT-PCR in patient subgroups

Blood samples (n= 280)	Total	BKV (+)	JCV (+)	BKV & JCV(+)
Nephrotic Syndrome	66	1*	0	0
Renal Transplant	15	1*	0	0
Other Immune Compromise	22	0	0	0
Total	103	2	0	0

*Table 3.3.Blood samples only. * <3,000 ge/ml*

This study also sought to determine the viral copy numbers in the samples. When conducting the real-time PCR a standard graph was made by amplifying serial dilutions of the BK human polyomavirus viral DNA plasmid control containing the entire BKV genome (Dunlop-strain pUC-19), (Seif et al., 1979) and JCV-Mad-1 strain (Frisque et al., 1984). The plasmid concentrations plotted ranged from 1 to 10^7 genomic copies therefore we could compare the real-time results on our samples to the standard graph and determine the viral copy number in each sample.

We examined the viral copy numbers of BKV and JCV in RT patients compared to NS patients. The mean amount of virus, BKV and JCV (genome per ml of urine) found in patients with NS versus those who have had RT was not found to be statistically different (Fig 3.2). The mean BKV and JCV viral loads in urine, respectively, were $7.7x10^7$ ge/ml and $1.9x10^6$ ge/ml for subjects with NS and $1.5x10^9$ ge/ml and $8.4x10^4$ ge/ml for RT recipients ($p>0.1$ for BKV and JCV).

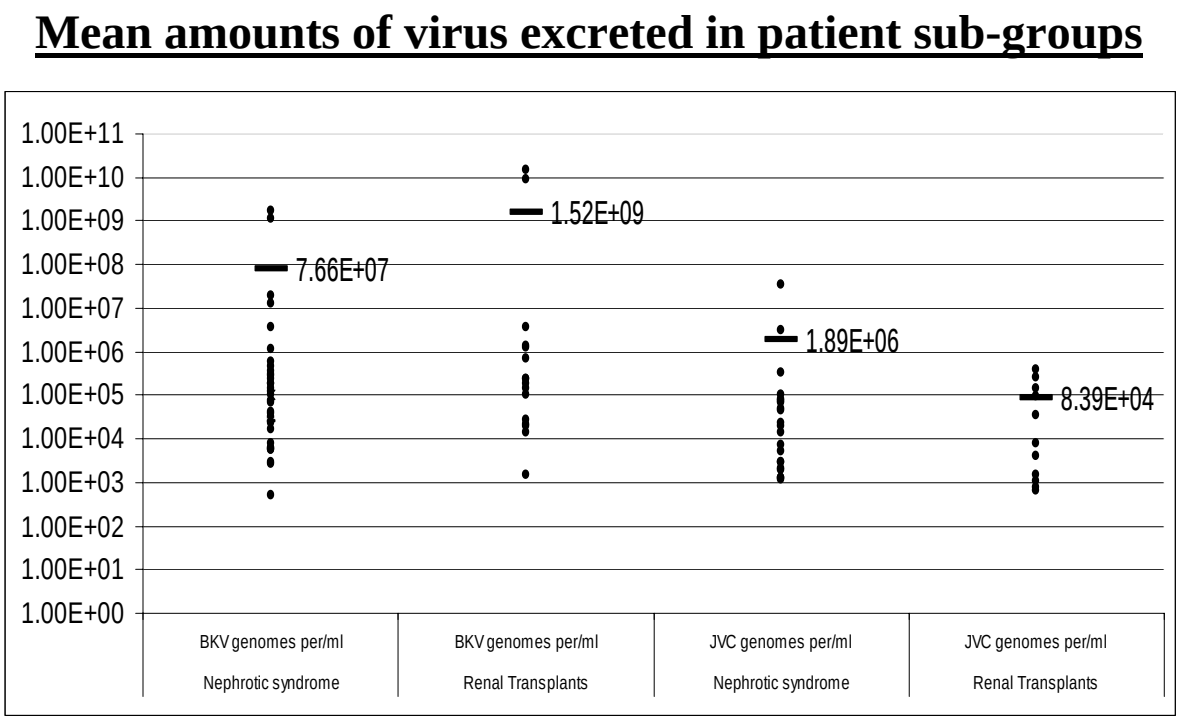

Fig 3.2.Comparison of BKV and JCV viruria in study populations. The mean amount of either virus in not statistically different between patients with nephrotic syndrome compared to those who have had renal transplants.

The majority of patients supplied us with clinical samples everytime they visited the clinic over the period of this study. Therefore the presence of virus in these patients could be studied over various time points. We sought to determine if patients who have had renal transplants were more likely to excrete BKV in their urine consistently over time compared to the nephrotic transplant patients. Table 3.6 shows the patients whom we had 5 or more consecutive urine samples on. Some patients secreted BK virus in their urine sporadically which was when BKV was found in their urine samples < 30% of the time they gave a urine sample. And other patients secreted virus more consistently >/=50% of the time they gave a urine sample. These results indicated that the renal transplant patients were more likely to be consistent shedders of BKV (7/12) compared to nephrotic syndrome patients (7/72 p= 0.0005).Again, when these results are correlated with immunosuppressive therapy they will give us more insight into the reactivation of BK virus.

Excretion of virus in patients on consecutive occasions

Positive on >1 occasion	Sporadic excretion	Consistent excretion
Nephrotic Syndrome (72 patients)	10	7
Renal Transplant (16 patients)	2	7

Sporadic	<30%
Consistent	>/=50%

Table 3.4. Patients in whom Bk virus was found to be shed in urine sporadically or consistently. Sporadic excretion was noted when patient excreted virus in less than 30% of their samples at separate clinic visits, while consistent excretion was noted in patients excreting virus in more than 50% of their samples.

Another important part of this study was to look at the influence immunosuppressive therapy has on the amount of virus replication taking part in the body and therefore being secreted in the urine. For example Fig. 3.3. shows a patient whose identification number is NS-005, viral shedding in urine over time.

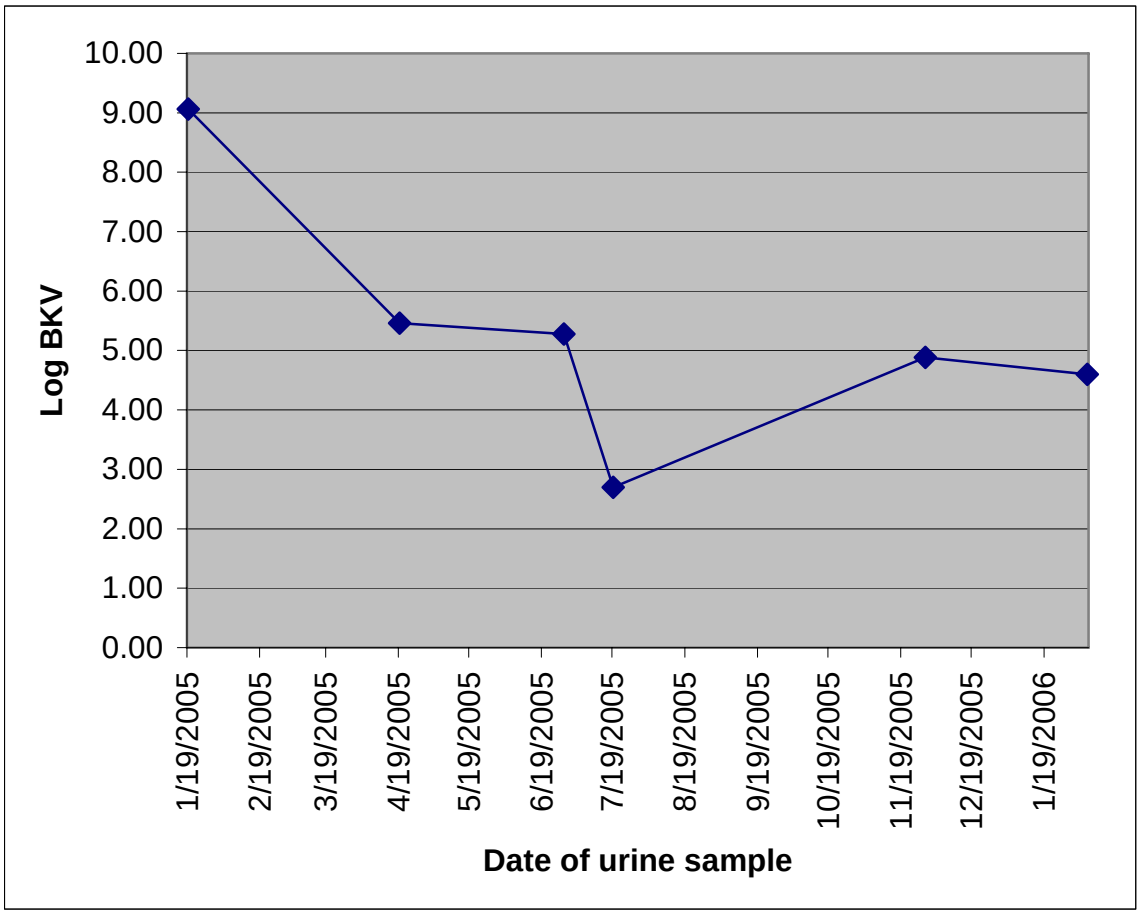

Fig 3.3. Timeline of BKV excretion in patient NS-005 (identification number). Amount of virus found in the patient's urine at different times is shown.

The first urine sample from the patient on 19th of January 2005 shows that the patient was excreting a large amount of virus in their urine, $1x10^9$ genomes/ml, this dropped to $3x10^5$ genomes/ml in July of the same year and then the amount of virus started to rise again toward the end of 2005. When we compare this to the medication the patient was receiving at the time we

can see that when the patient gave the first urine sample on 19-01-05 he/she were being medicated with cyclosporine and 30mg of steroids. This immunosuppressive therapy may be the reason the virus was able to replicate unchallenged and the viral load reached $1x10^9$ genomes per ml of urine. Then as the steroid dose reduced (19/04/05 20mg steroids) the viral load also decreased to $3x10^5$ genomes per ml and steadily decreased to $5x10^2$ genomes per ml on the 19/07/05 when the patient ceased taking cyclosporine and the steroid dose was 20mgs. Then on the 29/11/05 their viral load started to rise again to $7x10^4$ copies per ml and at this time the patient was not taking cyclosporine and their steroid dose was 10mg, therefore other host factors must be at play to keep the viral load in check in the body.

This tells us that immunosuppressive status alone does not always correlate with a rise or fall in viral load, other parameters must be looked at to see how the patient is coping with the virus. Looking at parameters such as T cell responses particularly CD8 T cells may give a clinician a better idea of how the patient is dealing with the virus. Immunological tests such as proliferation assays and functional assays of T cell subsets may be used in the future along side RT-PCR for viral load to build a picture of the bodies response to the virus and help clinicians in deciding on treatment of these patients.

Patient NS-004 also is a good example of the effect immunosuppressive therapy can have on the ability of a virus to replicate. Patient NS-004 first gave a urine sample on 13/11/00 and had no virus present in his/her consecutive urine samples until 24/11/04 when he/she began treatment with 60mg of steroids. At this time a viral load of $1.3x10^6$ copies of BKV was detected in his/her urine, indicating that as the patient became immunosuppressed, his/her ability to maintain the virus in latentcy was dimished and the virus replicated freely. When the patient's steroid dose was decreased to 20mg a day the virus was no longer detectable, indicating the patient's body regained its capacity to deal with virus. However the immunosuppressive therapy will not always have this effect on every patient. For example patient NS-032 was being treated with 60mg of steroids for 2 months and had no virus detectable in the urine, therefore as previously stated other unique host factors must come into play to allow the body to maintain the virus in latency. It is possible that patient NS-032 had a more effective T cell cytotoxic response to the virus and only by performing immunological assays can these other host parameter's be monitored.

Table 3.5 shows gives an example of patient NS-005 all other information, including immunosuppressive therapy on each patient is in Appendix IV.

I.d. Number	Date of urine sample	BKV viral Load ge/ml	Immuno-suppressive therapy	Steroids	Dose mg	WCC 10^9/ml
NS005	19/01/05	$1x10^9$	Cyclosporine	Yes	30	13.2
	19/04/05	$3x10^5$	Cyclosporine	Yes	20	7.9
	28/06/05	$1x10^5$	Nil	Yes	20	7.9
	19/07/05	$5x10^2$	Nil	Yes	20	9.3
	29/11/05	$7x10^4$	Nil	Yes	10	-----

Looking at viral load in relation to the patient's white cell counts, patient NS-055 gives a good example of when the patient's white cell count drops there is an increase in their viral load. Patient NS-055 had 11.66 WCC/10ul on the 28/10/02 and no virus in their urine. Then on 24/01/05 they had $1x10^4$ copies of JCV in their urine while their WCC had dropped to 6.71 WCC/10ul. Although decreasing white cell counts don't definitively mean that there will be a corresponding rise in viral load it can be used in conjunction with other parameters to access a patient's immuno-status and ability to fight the virus.

As the epidemiology of polyomaviruses is only beginning to be studied at the moment we sought to look at the influence if any, of virus excretion, in patients of different ages, genders and ethnicities. Patients of a variety of different ages were enrolled from 6 months to 29 years. 67 males and 58

females took part and they came from a variety of different ethnic backgrounds as outlined in (Table 3.6). 81 patients had nephrotic syndrome, 16 patients had renal transplants and 28 patients had secondary immune compromise (leukemia, heart transplant or HIV).

Demographics of the 3 patient subgroups in the study

	Nephrotic Syndrome	Renal Transplant	Immune compromised
# Enrolled	81	16	28
Median Age	10.4	18.1	8.1
Gender	44 Male 37 Female	6 Male 10 Female	17 Male 11 Female
Ethnicity:			
African-American	21	3	9
Caucasian	24	6	11
Hispanic	28	7	7
Asian	8	0	1

Table 3.6 Patient demographics. The breakdown of gender and ethnicity in each patient subgroup is similar.

As the prevalence of BKV and JCV in different age groups has previously been studied (Shah et al, 1973) we sought to compare our results with what is already known. The mean age of our patients when excreting polyomaviruses was 12.2 years (range, 3.2-31.2 years), which was not statistically different from the mean age of those not excreting polyomaviruses. Analysis of the age distribution of children with detectable polyomavirus excretion revealed the prevalence of BKV to be 20% (3/15) among patients 0-4 years old, 25% (10/40) among patients 5-9 years old, 32% (12-37) among patients 10-14 years old, 25% (7/28) among patients 15-19 years old and 14% (1/7) among patients 20-24 years old. Whereas the prevalence for JCV was 0% (0/15) among patients 0-4 years old, 10% (4/40) among patients 5-9 years old, 13% (5/37) among patients 10-14 years old, 11% (3/28) among patients 15-19 years old and 57% (4/7) among patients 20-24 years old (Fig 3.4) .

Age Distribution of Polyomavirus excretion in urine

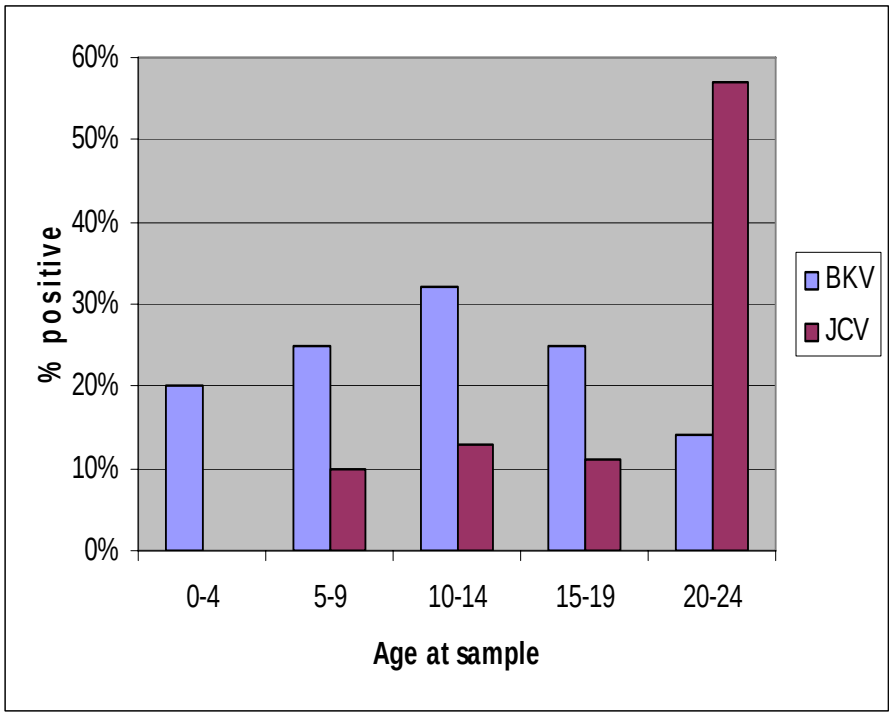

Fig 3.4. Chart showing amount of virus excreted in the urine of different age groups.

These results are consistent with previous studies on antibody prevalence (Fig 1.2), showing BKV to be more prevalent in the younger age groups and JCV more prevalent in the older (Shah et al., 1973).

We also sought to analyze our results to see if ethnicity had any influence on the excretion of BKV in urine. Evaluating BKV excretion in different ethnic categories indicated that in fact there was no statistical difference in the distribution of BKV among the different race/ethnicity categories (Fig 3.5). 36% (13/36) Caucasians, 31% (8/26) African Americans, 31.5% (10/32) Hispanics and 12.5 (1/8) Asians were found who were excreting virus in their urine. (These numbers reflect ethnicities of patients who gave urine samples only and do not contain the total no. of people with different ethnicities in the study as some patients only gave blood samples). To note, we had a low number of Asian patients in this study so comparison with this population is uninformative.

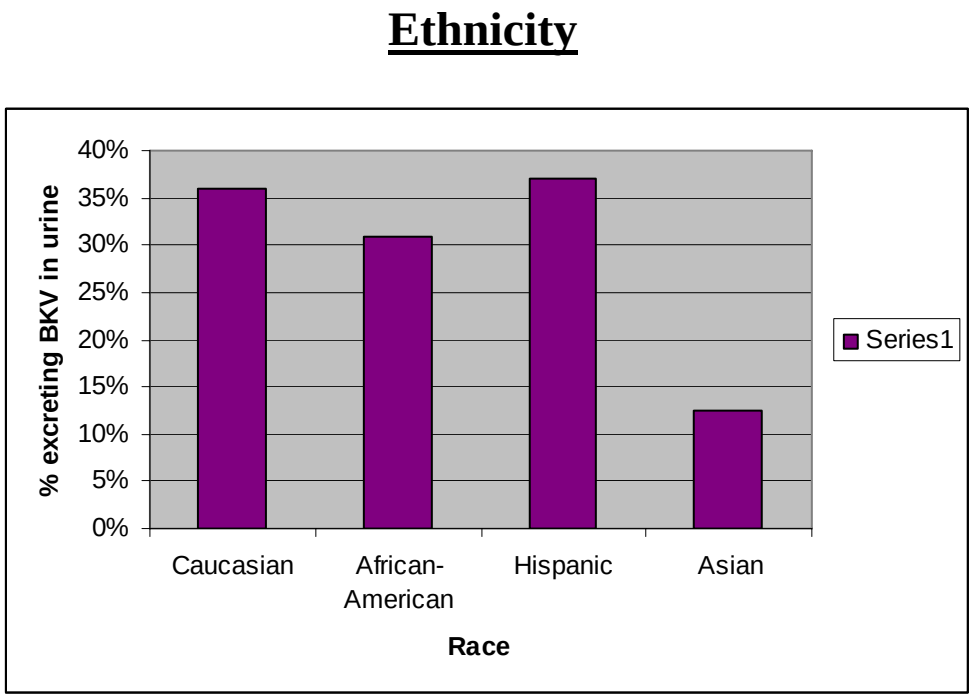

Fig 3.5.Chart showing amount of virus being excreted in the urine of different races. Results indicate no statistical difference between different races.

Regards gender, we found that neither sex has more likelihood than the other for excretion of virus in urine. BKV was not more common in male patients in either sub-group (nephrotic syndrome or renal transplant) than in female patients in either sub-group (Fig 3.6). 15/44 males with NS were excreting virus compared to 9/37 females (P>0.1) and 4/6 males with RT were excreting virus compared to 5/10 females (P>0.1).

Gender related excretion of BKV in patient sub-group

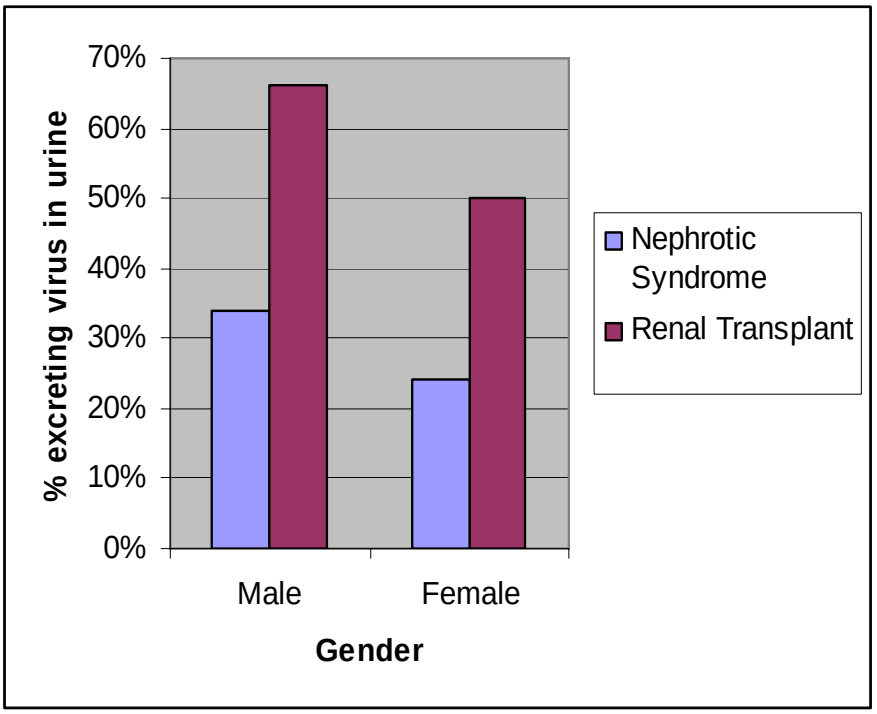

Fig 3.6.Chart showing difference in BK virus excretion in the urine of male and female nephrotic syndrome patients versus male and female Renal Transplant patients. Results show no statistical difference between male and females.

Another objective of this project was to determine what strains of virus were present in our study population and access whether one strain appears to be more prevalent than another. As previously mentioned, different BKV strains can be identified based on the VP1 region and thus divided into 4 genotypes: I, II, III, and IV.

We sought to determine what strains were present in our patient population. Qualitative PCR for the VP1 region was carried out on all samples that were BKV positive by real-time PCR. There were 74 samples in total that were BKV positive so the DNA stored from the MagNA Pure extraction was used to amplify the VP1 region. Primers for the VP1 regulatory region (Appendix II) were used and after PCR amplification the PCR product was purified using QIAquick® PCR Purification kit 250 (QIAGEN Sciences). Amplification was only successful in 34 of these samples. The eluted PCR product was sent to Lone Star Labs, Inc. (Houston, TX) for sequencing. Sequences were viewed and corrected using the program. Chromas version 2.32 (http://www.technelysium.com.au/chromas.html). A maximum likelihood tree was constructed using PAUP* version 4.0 (Swofford) and Modeltest (Posada). Bootstrap resampling was carried out for 1000 replicates of the data set.

So far DNA sequencing data reveal that 4 samples were genotype 1a, 8 were genotype 1b and 22 were not able to be determined due to poor

sequencing reactions. The significance of these different stains has yet to be established yet it is known that BKV type I is the most prevalent serotype in the population (Jin et al., 1993).

12 sequences are represented on the phylogenetic tree constructed in Fig. 3.7. all cluster as genotype I sequences. This is consistent with previous publications stating that genotype I is the most prevalent BK virus strain worldwide. In addition, our results indicate that this strain type is endemic within our patient population. We included genotype I reference strains isolated from BK positive specimens from Japan, Boston, U.S. and Manchester (Table 3.7), confirming that the strains found within our patient group are also circulating throughout the world.

Accession numbers used in phylogenetic tree

BKV Genotype	Genbank accession No.
III	M23122
II	Z19536
IV	Z19535
I	Z19534
I	AY628225
I	Z19537
I	X56913
I Boston	AY628238 Hl
I Japan	AB301099 J2B2
I Manchester	DQ457407 Man2

Table 3.7. BKV sequence accession numbers from genbank used in construction of the phylogenetic tree

BKV Phylogenetic tree

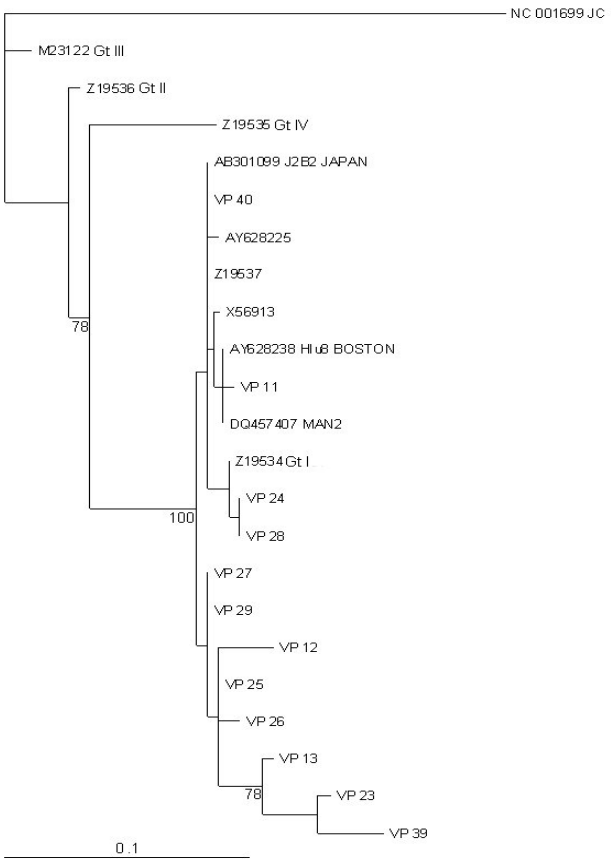

Fig 3.7. Maximum Likelihood Phylogenetic Tree of BKV-VP1 sequences detected in urine samples from 12 children in our study with Genbank representatives of the known sub-types I-IV with JC virus as the out-group. The numbers on the branches represent bootstrap values from 1,000 replicates.

All BKV genotype I strains were aligned against the reference BKV genotype I strain, Z19534. Two nonsynonymous mutations, unique to our patient sequences were detected. Valine to Leucine (T to A) and Glutamic acid to aspartic acid (G to C) (Fig 3.8) The significance of these mutations is as yet not known.

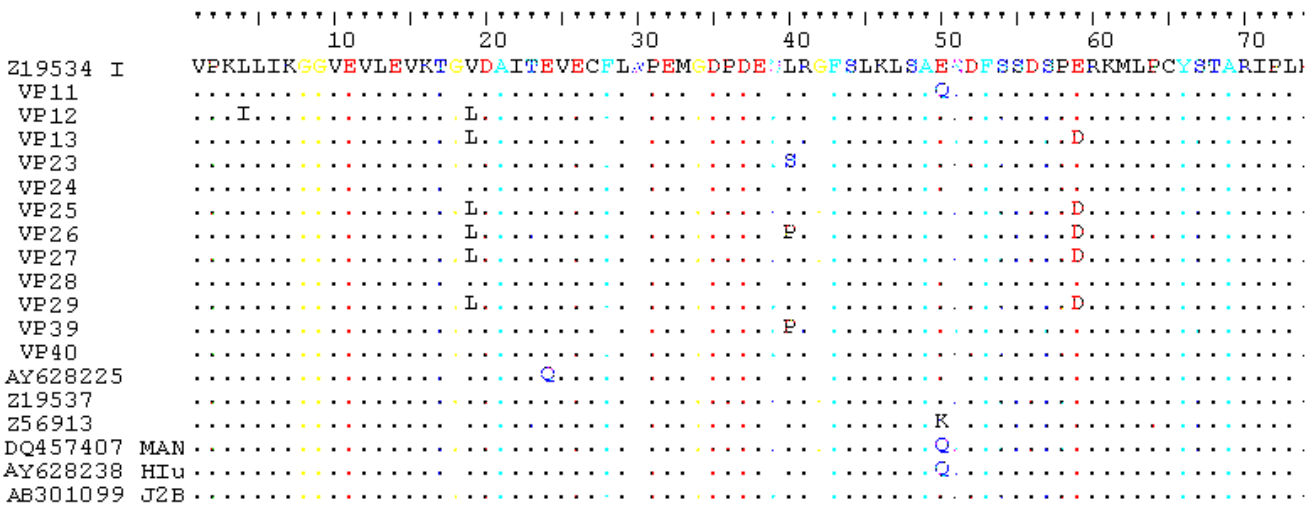

Fig 3.8. BKV genotype I strain alignments

4. Discussion

To date, there has been limited investigation of polyomavirus reactivation in pediatric patients with renal transplants or with primary renal disease. Given the association of polyomavirus reactivation with the use of immune suppressive therapies, we sought to investigate whether polyomavirus reactivation occurred in patients with pediatric kidney disease. As both BKV and JCV are acquired during childhood, children undergoing immunosuppression may be particularly at risk for the complications of this infection. When the immune system is suppressed, patients are unable to mount a successful cell-mediated cytotoxic T cell response. This leads to uncontrolled viral replication and therefore a variety of problems for the patient. As previously mentioned BKV and JCV are ubiquitous in the human population and frequently reactivate during periods of immune suppression. The emergence of polyomavirus-associated diseases among renal and hematopoietic stem cell transplant recipients has highlighted the lack of knowledge of the natural history of BKV and JCV in humans. In the pediatric population, in particular, immune suppressive therapy is commonly used as a treatment for primary renal diseases. BKVN has emerged as an important cause of post-transplant renal allograft loss, affecting ~5% of adult renal transplant (RT) recipients. BKVN is primarily due to the reactivation of BKV in the allograft kidney, although the pathogenesis of BKVN is

poorly understood. While it is clear that BKV can be pathogenic in the setting of severe immune compromise, BKV reactivation alone is insufficient to cause BKVN, suggesting that host factors play a significant role in pathogenesis.

This study sought to determine the prevalence of polyomavirus viruria and viremia in pediatric patients with nephrotic syndrome and patients who have received a kidney transplant. The hypothesis was that polyomavirus viremia and viruria is more common among patients after renal transplantation (RT) than in those with nephrotic syndrome (NS), and we found this to be true (Table 3.2). (11/16 vs. 28/74, p=0.0005).

The results we have found in this study correlate well with what is already known about polyomavirus infection in pediatric renal disease (reviewed in Knowles et al 2001). Polyomavirus reactivation in the urinary tract is common among patients with nephrotic syndrome and particularly after renal transplantation (Table 3.2) The low prevalence of BKV viremia in our study population is likely a reflection of the use of reduced-intensity immune suppression after renal transplantation. However further study is necessary to determine the significance of polyomavirus reactivation in the pre-transplant period.

One of the aims of this project was to determine whether there were any polyomaviruses present in the samples. To this end we qualitatively tested the samples for the presence of polyomavirus using conventional PCR (Section 2.2) using primers PYV-for/PYV-rev which amplify a 178-181 bp region in the amino terminus of the T-antigen gene of BKV, JCV and SV40 (Appendix II). It was found that this method was not as sensitive as the real-time PCR technique as half of the time it failed to detect the virus. Our real-time results identified BK virus in 74 patient samples and JCV in 31 patient samples and the PCR for the T-ag (PYV.fr/PYV.rev) only identified the T-ag of the polyomavirus in 34 patient samples. This may have been due to the low viral copy number found in some of these samples which the qualitative PCR would not have been sensitive enough to identify.

Our real-time results are comparable to recent studies on BKV detection by real-time PCR. In a study by Pang et al., 2007 the limit of detection of BKV in urine was 300 copies/ml of urine and in our study the limit of detection was 100 copies/ml. In this study however a 309bp region of the VP1 region (different region to the region used for VP1 RT-PCR in our study) was amplified while in our study an area of the Large T-Ag was amplified (Appendix II). This may account for the slight difference in the limit of detection in the assays.

As explained in section 3 we also attempted detection of BKV by qualitative PCR using primers for the regulatory region (Appendix II) which identified the virus in 7 samples and the VP1 region (Appendix II) which identified the virus in 34 patient samples. The inability to amplify the regulatory region and/or VP1 region from some samples that yielded sequence-proven, virus-specific PYV amplicons is consistent with findings in previous studies, and may be related to variations in viral load, the relative sensitivities of the different primer pairs, genetic differences among viral strains, and/or sizes of the amplicons produced. In a study by Zhong et al., 2007, they also identified problems when trying to amplify the noncoding control regions of BKV and found that these regions were amplified with lower efficiency then with other BKV typing regions. As the sensitivity of detection of the RT-PCR assays was increased 10-100- fold compared to conventional PCR and agarose gel protocols it was decided to use only the real-time results for detection of BKV and JCV virus.

Of note, an internal control was not included in this study. This would have been of use to control the assay for the quality of the MagNA pure extraction and also controlled for the reproducibility of the real-time reaction. Other studies have shown that spiking of samples with a virus not seen in humans and amplification of the sequence by real-time PCR can serve as an excellent internal control to show that your assay is working properly. In the

future a lot of clinical laboratories are moving towards introducing internal controls routinely into their assays.

Regarding subtype of virus in our samples, the 12 samples that we got sequenced by a commercial lab (Lone star labs, Houston, TX) were all BKV genotype I, based on their VP1 region. This was expected as genotype I is the most prevalent genotype in the general population (Jin et al., 1993). We included genotype I reference strains isolated from BK positive specimens from Japan, Boston, U.S. and Manchester (Table 3.7) to compare our samples with and confirmed they were the same genotype circulating throughout the world. We also found a mutation from Valine to Leucine (T to A) and Glutamic acid to aspartic acid (G to C) (Fig 3.8) in 6 of our samples when we aligned them to our BKV reference strain Z19534. The relevance of this mutation is not immediately apparent to us and warrants further study. Since the mutation is on the VP1 protein which is a surface protein it is possible that the mutation has arisen in order to disguise the protein from the immune system. However the reason for this mutation at present is unknown.

There were 933 samples from 125 patients processed in total for the presence of polyomavirus. All patient samples were tested using the RT-PCR protocol described in section 2.4. This number reflects samples that were tested on more than one occasion. As mentioned all samples were

blindly tested so some samples were tested on two or more occasions. We found that a sample from the same date from a patient could only be demonstrated to be reproducible 92% of the time. This result may have been in part due to the nature of the sample. As previously mentioned some urine samples were subjected to centrifugation and the supernatant removed from the pellet. Therefore blinded aliquots from an independent sample may infact represent either the pellet or the supernatant, this may have lead to the inability to always get the same result when repeatedly testing a sample. To improve reproducibility a standard sample type only be tested.

There were 388 urine samples in total and 280 blood samples and these reflect the samples that were taken from patients as they came to the clinic on different occasions throughout the time of the study. Urine proved to be the sample that was more likely to contain virus as only on two occasions was virus isolated from blood. This finding was what we expected as none of our patients went on to develop BKVN and virus is usually apparent in the blood before development of the condition.

To ensure accuracy we conducted this study blindly. We tested a total of 139 random blind samples on more than one occasion to ensure reproducibility (Table 3.1) Also it is important to note, variation in the DNA sequences of the BKV strains detected so far in this study as compared to known

laboratory strains, show that laboratory contamination is highly unlikely as an explanation for our findings.

Patients were enrolled from a variety of ethnic backgrounds. The numbers of patients enrolled from the different races were quite similar and therefore usually comparable. 33 were African-American, 41 were Caucasian and 42 were Hispanic. However there were only 9 Asian patients enrolled so comparison of this race with the others was not possible. As regards the other races, they all were found to have similar excretion of BKV in their urine (Fig 3.5).When looking at the results with regards to age at excretion of BKV, it is clear that our results reflect what is already known about the antibody prevalence in the general population (Knowles et al., 2001). Typically that children are infected with the virus at a young age (approx 5 years of age) and the incidence rises until they reach adulthood. Excretion of the virus in the urine is not indicative of a pathologic condition and occurs in many healthy individuals. In this study it was found that gender did not influence excretion of the virus in the urine and this is also consistent with previous studies (Nickeleit et al 2000).

It can be seen in fig 3.2 that the mean amount of BKV was almost double that of JCV. The reasons for this are unclear at present. Also 74 of our patients had nephrotic syndrome and 16 had renal transplant and it is known

that reactivation of BKV occurs in renal disease while it is not known whether JCV shares this relationship.

In this study we observed a higher level of polyomavirus, either BKV or JCV, viruria in renal transplant patients vs nephrotic syndrome patients (11/16 vs. 28/74, p=0.0005, x2 test). JCV viruria was more common among RT patients (31%) compared to NS patients (13%) (5/16 vs. 10/74, p= 0.02, x2 test), and a similar finding was observed for BKV, RT patients (43%) compared to NS patients (28%). (7/16 vs. 21/74, p=0.09, x2 test). However on analysis the result for BKV was not statistically significant between the two groups. Therefore it can be seen that polyomavirus reactivation in the urinary tract was more common after RT than among subjects with NS.

11 out of 16 of the renal transplant patients we tested were excreting polyomavirus in their urine and almost half of these were consistently excreting virus everytime they gave a urine sample at their clinic appointments (7 out of 16, 44%). Reactivation of the virus in these patients was likely due to the high level of immunosuppressive therapy these patients were receiving to enable them to accept the graft. Disease was not apparent in any of these patients by the end of this study so the significance of the polyomavirus viral load in these patients is unknown. It is possible that once the patients are controlling the virus and maintaining the viral load under a certain level that the virus will cause no problems.

Our study also sought to correlate the amount and type of immune suppression a patient is taking with the amount of virus being excreted in the urine. We found that immunosuppression can not always be related to a rise in viral load in urine (Appendix IV). In some patients such as NS-005 we can see that viral load did seem to be related to the immunosuppressive therapy because as the the steroid dose reduced (19/04/05 20mg steroids) the viral load also decreased to $3x10^5$ genomes per ml and steadily decreased to $5x10^2$ genomes per ml on the 19/07/05 when the patient ceased taking cyclosporine and the steroid dose was 20mgs. But then on the 29/11/05 their viral load started to rise again to $7x10^4$ copies per ml and at this time the patient was not taking cyclosporine and their steroid dose was 10mg so this does not fit in with this theory. Therefore we hypothesize that other host factors must be at play to keep the viral load in check in the body. Parameters such as T cell responses particularly CD8 T cells should be monitored to give a better picture of how the patient is dealing with the virus. Also as we saw in patient NS-055 white cell counts is another helpful parameter to access the body's ability to fight the virus. In patient NS-055 (Appendix V) as their white cell count drops there is an increase in their viral load (24/01/05 they had $1x10^4$ copies of JCV in their urine while their WCC had dropped to 6.71 WCC/10ul). However this relationship between WCC and viral loads is not apparent in all our patients (Appendix IV)

therefore immunological tests such as proliferation assays and functional assays of T cell subsets may be used in conjunction with RT- PCR, immunosuppressive therapy and white cell counts to give a more complete story for the treatment of the patient.

Some studies have shown BKV reactivation can occur in immunocompromised transplant recipients and can cause BKVN. 1% to 10% of kidney recipients develop BKVN (Hirsch et al., 2005), characterized by persistent graft dysfunction; of which about half will go on to lose their allografts (Nickeleit at al., 2000). Therefore it is imperative that the clinician stops BKVN developing by consistently monitoring their patients BKV viral loads.

Although little is known about the clinical relevance of polyomavirus infection in children, some case reports do exist documenting BKV nephropathy in pediatric kidney recipients (Vats et al., 2003) and in children with immunodeficiency (De Silva et al.,1995), (Rosen et al., 1983), (Cubukcu-Dimopulo et al., 2000). An example of a child who had an immunodeficiency that resulted in death by BKV was shown in a case report by Cubukcu-Dimopulo et al., 2000. He documented a rare case of a 14 year old boy with AIDS who developed a BKV infection in the lung and kidney and progressed to diffuse alveolar damage and death. The tubular epithelial cells in the kidney showed large, homogenous purple intranuclear inclusions

and in situ hybridization confirmed BKV as the pathogen. Therefore BKV infection should be considered as a differential diagnosis of opportunistic infections in patients with immunodefiencies also.

There is limited knowledge available about the prevalence and effect of BKV in pediatric renal transplantation. However a prospective case controlled study of 18 Australian pediatric renal transplant patients showed an increase in BKV viruria in children with immunosuppression; 56% of patients were BKV IgG antibody sero-positive with a viruria rate of 33% by PCR compared with 39% sero-positivity and no viruria in the age matched controls (Haysom et al., 2004).

The only other pediatric study to date to prospectively monitor both JCV and BKV polyomavirus in blood and urine was done in 46 pediatric renal transplant patients (Herman et al., 2004). In this study there were 2 patients with BKVN, 31% had BKV viruria, 17% had JCV viruria, and viruria was detectable prior to viremia. Our study in comparison found no renal transplant patients with BKVN, 37.5% had BKV viruria, and 25% had JCV viruria.

BKVN is an important cause of progressive graft dysfunction in adult renal transplant recipients but very limited information has been published regarding BKVN specifically in the pediatric population (Hirsch et al., 2002). In a study of adult renal transplants (Smith et al., 2004) they found an

incidence of 3.5% of patients developing BKVN which is similar to what has previously been reported in adults (Nickeleit et al., 2000). The incidence of BKVN in pediatric kidney transplants on the other hand has not been well defined. In a previous study an incidence of 3% of BKVN was found in a pediatric cohort where three cases of biopsy confirmed BKVN was presented among a cohort of 100 patients. This incidence of 3% correlated with the incidence previously reported in adult kidney transplant recipients (Nickeleit et al., 2000). At the time of completion of this study none of the patients had gone on to develop BKVN.

More prospective studies will need to be carried out to elucidate the relationship between polyomavirus reactivation in pediatric renal transplants.

There are several strengths and limitations to this study. The strengths include the large number of samples studied, the diversity in demographic background of the patients and the development of a PCR that detects all three human polyomaviruses in a single test. Limitations of this study include; silent infections in the children with different polyomaviruses and the fact that we cannot ascertain whether the observed excretions were associated with primary infection or viral reactivation.

Also it is thought that another major cause of BKV nephropathy may be the transplantation of kidneys from BKV-seropositive donors into BKV-

seronegative recipients. In our study the serological status of the patient pre-transplantation is unknown so we can not hypothesis as to whether the BKV nephropathy constituted a primary or secondary infection.

Currently, renal biopsy is the gold standard, for the diagnosis of polyomavirus nephropathy. For the pathologist this can present a problem as distinguishing BKV nephropathy from acute cellular rejection is inherently difficult, also considering that both conditions can coexist (Nickeleit et al., 1999). RT-PCR to quantify BKV in urine offers a less invasive diagnostic tool for monitoring the possible development of BKVN compared to renal biopsy. Importantly to note the viral load can only be used as an indicator as a renal biopsy is always needed for the definitive diagnosis of BKVN.

Several studies have evaluated the feasibility of using quantiatative PCR to monitor BKV-associated disease and suggest that correlations exist between viral shedding and disease states (Biel et al., 2000; Randhawa et al., 2004; Vats et al., 2003). Another advantage of RT-PCR is the ability to monitor the efficacy of antiviral drug treatments such as cidofovir (Vats et al., 2003).

With the introduction of more potent immunosuppressive agents such as tacrolimus and mycophenolate mofetil, more BKV nephropathy in kidney transplant recipients is being documented (Binet et al., 1999).

Management of BKV nephropathy is particularly difficult because the first therapeutic approach, is to reduce the amount of immunosuppression in the

patient to allow his/her body to mount an immune response to the infection. However this reduction in immunosuppression can lead to acute rejection of the kidney allograft, whose treatment in turn leads to reactivation of the virus. Therefore the solution to this problem must come in the form of an antiviral agent. To date there is no universally accepted therapy however in a recent study by Vats et al, treatment with a low dose of cidofovir was seen to rapidly clear virus from the plasma and urine (Vats et al., 2003). However there is a need for controlled trials to be carried out before this will become an acceptable treatment.

5.Conclusion

Our study shows that monitoring the polyomavirus viral load in the urine of patients by a quantitative PCR technique is a useful tool to help to monitor polyomavirus infection and therefore its subsequent treatment. BKV viruria was detected in 37.5 % of renal transplant patients, 24.3% of patients with nephrotic syndrome and 33.3% of patients who had a secondary immunocompromise. Thus showing us that BKV viruria is quite similar between our patient groups. The use of immunosuppressive therapy does not always contribute to BKV viruria, nor does the type or amount of immunosuppressant being administered. Therefore it can be hypothesized that a range of host factors must come into play when dealing with the virus in each individual patient.

Analysis of viral DNA sequence data and clinical data on the patients in this study is ongoing in Dr Vanchiere's group in Houston. Identification of different BKV strains by DNA sequencing will help in understanding their relative contribution to disease.

Understanding the virologic and host factors that maintain BKV latency in immune competent subjects will pave the way for understanding the pathogenesis of BKV related diseases and allow for development of clinical strategies to prevent and treat BKV disease.

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Publications

The findings of this project are being presented at the American Society of Nephrology meeting in San Francisco and will be submitted to International Journal of Nephrology, Dialysis and Transplantation. Submission will also be made to the Journal of Pediatric Nephrology.

Appendix

I: Primers and TaqMan probes for polyomaviruses

BKV 4400 gggtcttcta ccttttcttttt ttttttgggt ggtgttgagt gttgagaatc

JCV 4263 gggtcttcta ccttttttttt ctttttaggt ggggtagagt gttgggatcc

BKV tgctgttgct tcttcatcac tggcaaacat atcttcatgg caaaataaat

JCV tgtgttttca tc---atcac tggcaaacat ttcttcatgg caaaacaggt

BKV cttcatccca tttttcatta aaggaactcc accaggactc ccactcttct **4549**

JCV cttcatccca cttctcatta aatgtattcc accaggattc ccattcatct **4409**

Appendix I: Primers and TaqMan probes for polyomaviruses. These were designed to amplify viral genomic regions shown for the large T-ag genes of BKV, and JCV. Regions used for primer sequences are highlighted by boxed text and the probe sequences are underlined. Nucleotide numbers are shown and sequences are from p- BKV strain Dunlop-1(Seif et al., 1979) and JCV strain MAD-1 (Frisque et al., 1984).

II : Primers used in qualitative PCR analysis

Primer	Position	Amplicon (bp)
Polyomavirus conserved sequence (T-antigens)	virus-specific	173-182
PYV.for 5'-TAG TGC CAA CCT ATG GAA CAG A-3'		
PYV.rev 5'-GAA AGT CTT TAG GGT CTT CTA CC-3'		
BK virus (BKV) regulatory region		
BK1 5'-GGC CTC AGA AAA AGC TTC CAC ACC CTT		
ACT ACT TGA-3'	nt 50-85	
BK2 5'-CTT GTC GTG ACA GCT GGC GCA GAA C-3'	nt 415-391	355
VP1 region		
327-1 5'-CAA GTG CCA AAA CTA CTA AT-3'	nt 1630-1650	
327-2 5'TGC ATG AAG GTT AAG CAT GC-3'	nt 1956-1936	327

III Clinical characteristics of 102 children evaluated for BKV and JCV in urine samples

	BKV viruria	JCV viruria	Both
Number of patients (n = 102)	28	11	4
Age, mean (range), years	17.73 (3.2-31.2)	15.19 (8.5- 23.6)	17.38 (12.65- 21.5)
Race/ethnicity			
White (n = 36)	12	2	1
African American (n = 26)	7	3	1
Hispanic (n = 32)	8	4	2
Asian (n = 8)	1	2	0
Sex			
Female (n = 45)	12	7	3
Male (n = 57)	18	4	1
Immune compromise			
Nephrotic syndrome (n = 74)	18	7	3
Renal Transplant (n = 16)	6	4	1
2ndary Immune Compromise (12)	4	0	0
Viruria (copies/ml urine)			
Median	$1.0*10^5$	$1.0*10^4$	
Range	$500\sim1.5*10^{10}$	$650\sim3.1*10^6$	

IV Table of Patient immunosuppressive therapy

I.D. Number	Sub-Arm	visit date	IS	Cellcept	Cyclosporine A	steroids	steroid dose
IC-001	ALL	18/02/2000	yes	None	None	yes	
IC-001	ALL	08/12/2000	no	None	None	None	
IC-002	OHT	24/02/2000	no	no	no	no	
IC-003	OHT	24/04/2000	no	None	None	None	
IC-003	OHT	?	no	None	None	None	
IC-004	OHT	14/04/2000	no	no	no	no	
IC-005	OHT	20/04/2000	no	no	no	no	
IC-005	OHT	06/12/2000	no	no	no	None	
IC-006	OHT	20/04/2000	no	no	no	no	
IC-006	OHT	21/12/2000	no	no	no	None	
IC-006	OHT	21/09/2004	yes	yes	yes	None	
IC-006	OHT	?	no	None	None	None	
IC-007	ALL	12/05/2000	no	None	None	None	
IC-007	ALL	13/07/2001	no	None	None	None	
IC-007	ALL	?	no	None	None	None	
IC-011	HIV	08/08/2000	no	None	None	None	
IC-011	HIV	09/02/2001	no	None	None	None	
IC-011	HIV	?	no	None	None	None	
IC-012	HIV	08/08/2000	no	no	no	no	
IC-013	HIV	08/08/2000	no	None	None	None	
IC-013	HIV	?	no	None	None	None	
IC-014	HIV	10/08/2000	no	None	None	None	
IC-014	HIV	15/03/2001	no	None	None	None	
IC-014	HIV	21/08/2001	no	None	None	None	
IC-015	HIV	29/08/2000	no	no	no	no	
IC-015	HIV	15/03/2001	no	None	None	None	
IC-015	HIV	13/07/2001	no	None	None	None	
IC-015	HIV	?	no	None	None	None	
IC-016	HIV	29/08/2000	no	None	None	None	
IC-016	HIV	21/02/2001	no	no	no	no	
IC-017	HIV	30/08/2000	no	None	None	None	
IC-018	HIV	30/08/2000	no	no	no	no	
IC-018	HIV	21/02/2001	no	no	no	no	
IC-018	HIV	?	no	None	None	None	
IC-019	HIV	06/09/2000	no	no	no	None	
IC-020	ALL	11/09/2000	no	no	no	no	
IC-020	ALL	05/03/2001	no	None	None	None	
IC-020	ALL	?	no	None	None	None	
IC-020	ALL	?	no	None	None	None	
IC-021	HIV	19/09/2000	no	no	no	None	
IC-021	HIV	13/07/2001	no	no	no	None	

I.D. Number	Sub-Arm	visit date	IS	Cellcept	Cyclosporine A	steroids	steroid dose
IC-022	HIV	19/09/2000	no	no	no	None	
IC-022	HIV	13/07/2001	no	no	no	None	
IC-022	HIV	06/09/2001	no	None	None	None	
IC-022	HIV	19/02/2003	no	None	None	None	
IC-023	HIV	19/09/2000	no	None	None	None	
IC-023	HIV	15/03/2001	no	None	None	None	
IC-023	HIV	06/09/2001	no	None	None	None	
IC-025	OHT	27/09/2000	no	no	no	no	
IC-025	OHT	13/12/2000	no	None	None	None	
IC-026	HIV	?	no	None	None	None	
IC-028	OHT	29/09/2000	no	None	None	None	
IC-028	OHT	?	no	None	None	None	
IC-030	OHT	09/02/2001	no	no	no	no	
IC-031	HIV	16/02/2001	no	no	no	no	
IC-034	ALL	12/04/2001	yes	None	None	yes	2.7
IC-034	ALL	11/07/2001	no	None	None	None	
IC-036	ALL	16/04/2001	yes	None	None	yes	20
IC-036	ALL	16/04/2001	yes	None	None	yes	20
IC-046	OHT	?	no	None	None	None	
NS-001	NS	06/09/2000	no	None	None	None	
NS-002	NS	13/11/2000	yes	no	yes	no	
NS-002	NS	12/04/2001	no	None	None	None	
NS-002	NS	18/07/2001	no	None	None	None	
NS-002	NS	25/09/2002	yes	no	yes	yes	20
NS-002	NS	07/04/2004	yes	no	yes	yes	20
NS-002	NS	28/07/2004	yes	no	yes	yes	5
NS-002	NS	28/12/2004	yes	no	yes	yes	10
NS-002	NS	02/01/2005	yes	no	yes	yes	
NS-002	NS	19/04/2005	yes	no	yes	yes	
NS-002	NS	07/11/2005	yes	no	yes	yes	30
NS-002	NS	28/02/2006	yes	no	yes	yes	5
NS-003	NS	13/11/2000	yes	no	no	yes	
NS-003	NS	27/08/2001	no	None	None	None	
NS-003	NS	29/08/2001	no	None	None	None	
NS-003	NS	10/09/2001	no	None	None	None	
NS-003	NS	25/09/2002	no	None	None	None	
NS-003	NS	10/03/2004	yes	None	yes	None	
NS-003	NS	19/01/2005	yes	None	yes	None	
NS-004	NS	13/11/2000	yes	no	yes	yes	
NS-004	NS	23/04/2001	no	None	None	None	
NS-004	NS	21/05/2001	no	None	None	None	
NS-004	NS	18/07/2001	no	None	None	None	
NS-004	NS	14/08/2001	no	None	None	None	
NS-004	NS	24/11/2004	yes	no	yes	yes	60
NS-004	NS	04/01/2005	yes	no	yes	yes	60
NS-004	NS	09/02/2005	yes	None	None	yes	20
NS-004	NS	06/04/2005	yes	no	yes	None	

I.D. Number	Sub-Arm	visit date	IS	Cellcept	Cyclosporine A	steroids	steroid dose
NS-004	NS	28/06/2005	yes	no	yes	None	
NS-004	NS	20/07/2005	yes	no	yes	None	
NS-004	NS	27/09/2005	yes	no	yes	None	
NS-004	NS	07/11/2005	yes	no	yes	yes	60
NS-005	NS	13/11/2000	no	no	no	no	
NS-005	NS	07/05/2001	no	None	None	None	
NS-005	NS	09/07/2001	no	None	None	None	
NS-005	NS	10/09/2001	no	None	None	None	
NS-005	NS	14/01/2002	no	None	None	None	
NS-005	NS	19/01/2005	yes	None	yes	yes	30
NS-005	NS	19/04/2005	yes	None	yes	yes	20
NS-005	NS	28/06/2005	yes	None	None	yes	20
NS-005	NS	19/07/2005	yes	None	None	yes	20
NS-005	NS	29/11/2005	yes	None	None	yes	10
NS-006	NS	13/11/2000	yes	no	yes	yes	80
NS-006	NS	14/01/2002	no	None	None	None	
NS-006	NS	25/10/2004	no	no	no	None	
NS-006	NS	31/01/2005	yes	no	yes	no	
NS-006	NS	22/08/2005	no	None	None	None	
NS-007	NS	02/12/2000	yes	no	yes	yes	
NS-007	NS	31/01/2005	yes	no	yes	yes	
NS-007	NS	?	no	None	None	None	
NS-007	NS	?	no	None	None	None	
NS-007	NS	?	no	None	None	None	
NS-007	NS	?	no	None	None	None	
NS-007	NS	?	no	None	None	None	
NS-007	NS	?	no	None	None	None	
NS-007	NS	?	no	None	None	None	
NS-007	NS	?	no	None	None	None	
NS-007	NS	?	no	None	None	None	
NS-008	NS	14/11/2000	no	None	None	None	
NS-008	NS	27/08/2001	no	None	None	None	
NS-008	NS	23/07/2002	no	None	None	None	
NS-008	NS	02/12/2002	no	None	None	None	
NS-009	NS	16/11/2000	no	None	None	None	
NS-009	NS	14/05/2001	no	None	None	None	
NS-009	NS	10/09/2002	no	None	None	None	
NS-010	NS	04/12/2000	yes	no	yes	yes	
NS-010	NS	28/02/2001	no	None	None	None	
NS-010	NS	17/09/2001	no	None	None	None	
NS-010	NS	04/09/2002	no	None	None	None	
NS-011	NS	04/12/2000	yes	no	yes	yes	
NS-011	NS	05/03/2001	no	None	None	None	
NS-011	NS	14/01/2002	no	None	None	None	
NS-011	NS	29/07/2002	no	None	None	None	
NS-011	NS	24/02/2003	no	None	None	None	
NS-011	NS	22/03/2004	no	None	None	None	

I.D. Number	Sub-Arm	visit date	IS	Cellcept	Cyclosporine A	steroids	steroid dose
NS-011	NS	24/11/2004	no	None	None	None	
NS-011	NS	21/05/2201	no	None	None	None	
NS-012	NS	05/12/2000	no	None	None	None	
NS-013	NS	05/12/2000	yes	no	no	yes	
NS-014	NS	01/12/2000	yes	no	no	yes	
NS-015	NS	07/12/2000	yes	no	no	yes	
NS-015	NS	16/10/2002	no	None	None	None	
NS-015	NS	24/01/2005	no	None	None	None	
NS-016	NS	18/09/2001	yes	no	yes	no	
NS-017	NS	18/12/2000	yes	no	yes	yes	
NS-017	NS	17/09/2001	no	None	None	None	
NS-017	NS	16/01/2002	no	None	None	None	
NS-017	NS	31/07/2002	no	None	None	None	
NS-017	NS	10/01/2005	yes	yes	no	no	
NS-017	NS	11/04/2005	yes	yes	no	no	
NS-017	NS	15/08/2005	yes	yes	no	None	
NS-018	NS	18/12/2000	yes	no	yes	no	
NS-018	NS	27/08/2001	no	None	None	None	
NS-018	NS	06/01/2004	yes	None	yes	None	
NS-018	NS	05/07/2005	yes	None	yes	unknown	10
NS-018	NS	28/02/2006	yes	None	yes	yes	15
NS-019	NS	?	no	None	None	None	
NS-019	NS	?	no	None	None	None	
NS-020	NS	19/02/2001	no	None	None	None	
NS-020	NS	05/03/2001	no	None	None	None	
NS-021	NS	26/02/2001	yes	no	yes	no	
NS-021	NS	22/07/2002	no	None	None	None	
NS-022	NS	26/02/2001	no	no	no	no	
NS-023	NS	28/02/2001	yes	no	no	yes	
NS-023	NS	23/04/2001	no	None	None	None	
NS-023	NS	01/08/2002	no	None	None	None	
NS-023	NS	26/08/2002	no	None	None	None	
NS-023	NS	03/02/2003	yes	None	None	yes	
NS-023	NS	10/11/2004	yes	None	None	yes	25
NS-023	NS	12/01/2005	yes	None	None	yes	20
NS-023	NS	23/03/2005	yes	None	None	yes	30
NS-023	NS	13/07/2005	yes	None	None	yes	15
NS-023	NS	26/04/2006	yes	None	None	yes	2
NS-024	NS	05/03/2001	yes	no	yes	no	
NS-024	NS	08/10/2001	no	None	None	None	
NS-024	NS	12/10/2004	no	None	None	None	
NS-024	NS	18/01/2005	no	None	None	None	
NS-024	NS	05/07/2005	no	None	None	None	
NS-025	NS	21/01/2003	no	None	None	None	
NS-025	NS	26/02/2003	no	None	None	None	
NS-025	NS	22/03/2004	no	None	None	None	
NS-025	NS	20/12/2004	no	None	None	None	

I.D. Number	Sub-Arm	visit date	IS	Cellcept	Cyclosporine A	steroids	steroid dose
NS-025	NS	05/07/2005	yes	None	None	yes	60
NS-025	NS	?	yes	no	no	yes	
NS-026	NS	12/03/2001	yes	no	no	yes	
NS-026	NS	23/04/2001	no	no	no	None	
NS-027	NS	13/03/2001	no	no	no	no	
NS-028	NS	27/03/2001	no	no	no	no	
NS-028	NS	07/05/2001	no	None	None	None	
NS-028	NS	01/10/2002	no	None	None	None	
NS-028	NS	10/11/2004	yes	no	yes	yes	33
NS-028	NS	01/02/2005	yes	no	yes	yes	
NS-028	NS	29/03/2005	yes	no	yes	yes	45
NS-028	NS	20/09/2005	yes	no	yes	yes	45
NS-029	NS	16/04/2001	no	None	None	None	
NS-029	NS	07/05/2001	no	None	None	None	
NS-030	NS	16/04/2001	yes	no	no	yes	
NS-030	NS	10/09/2001	no	None	None	None	
NS-030	NS	28/10/2002	no	None	None	None	
NS-030	NS	15/11/2004	yes	None	yes	None	
NS-030	NS	06/03/2006	yes	None	yes	None	
NS-031	NS	07/05/2001	yes	no	yes	yes	
NS-031	NS	28/08/2002	no	None	None	None	
NS-031	NS	01/03/2004	no	None	None	None	
NS-031	NS	24/08/2005	no	None	None	None	
NS-032	NS	07/05/2001	yes	no	yes	no	
NS-032	NS	09/07/2001	no	None	None	None	
NS-032	NS	30/03/2004	yes	no	yes	None	
NS-032	NS	22/12/2004	yes	no	yes	None	
NS-032	NS	26/01/2005	yes	no	yes	no	
NS-032	NS	29/03/2005	yes	no	yes	None	
NS-032	NS	21/09/2005	yes	no	yes	yes	60
NS-032	NS	19/10/2005	yes	no	yes	yes	60
NS-032	NS	15/11/2005	yes	no	yes	yes	60
NS-032	NS	15/03/2006	yes	no	yes	None	
NS-032	NS	25/04/2006	yes	no	yes	None	
NS-034	NS	14/05/2001	yes	no	no	yes	
NS-034	NS	22/03/2004	yes	None	None	yes	25
NS-034	NS	30/03/2004	yes	None	None	yes	45
NS-034	NS	29/09/2004	yes	None	None	yes	20
NS-035	NS	21/05/2001	yes	no	yes	no	
NS-035	NS	01/10/2002	no	no	no	None	
NS-035	NS	10/12/2002	no	no	no	None	
NS-035	NS	14/03/2006	no	None	None	None	
NS-036	NS	21/05/2001	yes	no	yes	yes	
NS-036	NS	14/01/2002	no	None	None	None	
NS-036	NS	19/08/2002	no	None	None	None	
NS-036	NS	04/02/2003	no	None	None	None	
NS-036	NS	19/07/2004	yes	no	yes	yes	80

I.D. Number	Sub-Arm	visit date	IS	Cellcept	Cyclosporine A	steroids	steroid dose
NS-036	NS	05/11/2004	yes	no	yes	yes	80
NS-036	NS	18/07/2005	yes	no	yes	yes	80
NS-037	NS	?	no	None	None	None	
NS-037	NS	?	no	None	None	None	
NS-037	NS	07/06/2001	no	None	None	None	
NS-037	NS	09/07/2001	no	None	None	None	
NS-037	NS	10/09/2001	no	None	None	None	
NS-037	NS	22/07/2002	no	None	None	None	
NS-037	NS	24/02/2003	no	None	None	None	
NS-037	NS	14/12/2004	no	None	None	None	
NS-038	NS	06/06/2001	yes	no	no	yes	
NS-038	NS	07/06/2001	no	None	None	None	
NS-038	NS	06/07/2001	no	None	None	None	
NS-038	NS	17/09/2001	no	None	None	None	
NS-038	NS	10/12/2002	no	None	None	None	
NS-038	NS	13/01/2003	no	None	None	None	
NS-039	NS	16/07/2001	yes	no	yes	yes	
NS-039	NS	10/09/2001	no	None	None	None	
NS-039	NS	14/03/2003	no	None	None	None	
NS-039	NS	19/03/2004	yes	yes	no	yes	10
NS-039	NS	01/02/2005	yes	yes	no	yes	40
NS-039	NS	10/08/2005	yes	yes	no	yes	20
NS-039	NS	19/09/2005	yes	yes	no	no	
NS-039	NS	10/10/2005	yes	yes	no	None	
NS-040	NS	16/07/2001	no	None	None	None	
NS-040	NS	18/07/2001	no	None	None	None	
NS-041	NS	13/08/2001	no	None	None	None	
NS-041	NS	21/08/2002	no	None	None	None	
NS-042	NS	14/08/2001	no	None	None	None	
NS-042	NS	17/09/2001	no	None	None	None	
NS-043	NS	14/08/2001	yes	no	yes	yes	
NS-043	NS	01/08/2002	no	no	no	None	
NS-043	NS	02/12/2002	no	no	no	None	
NS-043	NS	01/12/2004	yes	None	yes	yes	80
NS-043	NS	14/02/2005	yes	None	yes	yes	40
NS-043	NS	28/03/2005	yes	None	yes	yes	40
NS-043	NS	05/07/2005	yes	None	yes	None	
NS-043	NS	19/09/2005	yes	None	yes	None	
NS-043	NS	05/12/2005	yes	None	yes	None	
NS-044	NS	20/08/2001	yes	no	yes	yes	
NS-044	NS	26/08/2002	yes	None	yes	None	
NS-044	NS	28/10/2002	yes	None	yes	None	
NS-044	NS	20/12/2004	yes	None	yes	yes	20
NS-044	NS	17/10/2005	yes	None	yes	yes	20
NS-044	NS	07/11/2005	yes	None	yes	yes	20
NS-045	NS	10/09/2001	yes	no	yes	yes	
NS-046	NS	18/09/2001	no	None	None	None	

I.D. Number	Sub-Arm	visit date	IS	Cellcept	Cyclosporine A	steroids	steroid dose
NS-047	NS	18/09/2001	yes	no	no	yes	
NS-047	NS	?	no	None	None	None	
NS-047	NS	?	no	None	None	None	
NS-048	NS	18/09/2001	yes	no	no	yes	
NS-050	NS	08/10/2001	yes	no	yes	no	
NS-051	NS	07/10/2002	yes	no	yes	yes	
NS-051	NS	?	no	None	None	None	
NS-051	NS	?	no	None	None	None	
NS-051	NS	?	no	None	None	None	
NS-051	NS	?	no	None	None	None	
NS-051	NS	?	no	None	None	None	
NS-051	NS	?	no	None	None	None	
NS-052	NS	14/10/2002	yes	no	yes	yes	
NS-052	NS	?	no	None	None	None	
NS-052	NS	?	no	None	None	None	
NS-053	NS	15/10/2002	yes	no	no	yes	
NS-053	NS	11/02/2005	yes	no	yes	None	
NS-053	NS	12/04/2005	yes	no	yes	None	
NS-053	NS	29/11/2005	yes	no	yes	no	
NS-054	NS	23/10/2002	yes	no	yes	yes	
NS-054	NS	?	no	None	None	None	
NS-055	NS	28/10/2002	yes	no	yes	yes	
NS-055	NS	24/02/2003	no	None	None	None	
NS-055	NS	08/11/2004	yes	no	yes	yes	20
NS-055	NS	24/01/2005	yes	no	yes	None	
NS-055	NS	12/07/2005	yes	no	yes	yes	10
NS-055	NS	17/10/2005	yes	no	yes	yes	50
NS-056	NS	14/01/2003	yes	no	yes	no	
NS-057	NS	14/01/2003	no	no	no	no	
NS-058	NS	22/01/2003	yes	no	no	yes	
NS-061	NS	?	no	None	None	None	
NS-061	NS	?	no	None	None	None	
NS-062	NS	?	no	None	None	None	
NS-063	NS	04/02/2003	yes	no	yes	yes	
NS-064	NS	11/02/2003	yes	no	yes	yes	
NS-064	NS	?	no	None	None	None	
NS-064	NS	?	no	None	None	None	
NS-065	NS	?	no	None	None	None	
NS-065	NS	?	no	None	None	None	
NS-065	NS	?	no	None	None	None	
NS-066	NS	?	no	None	None	None	
NS-068	NS	26/02/2003	yes	yes	None	yes	20
NS-069	NS	02/03/2004	yes	None	None	yes	60
NS-069	NS	14/12/2004	no	None	None	None	
NS-070	NS	03/03/2004	yes	no	yes	no	
NS-070	NS	?	no	None	None	None	
NS-070	NS	?	no	None	None	None	

I.D. Number	Sub-Arm	visit date	IS	Cellcept	Cyclosporine A	steroids	steroid dose
NS-070	NS	?	no	None	None	None	
NS-071	NS	?	no	None	None	None	
NS-071	NS	?	no	None	None	None	
NS-071	NS	?	no	None	None	None	
NS-071	NS	?	no	None	None	None	
NS-071	NS	?	no	None	None	None	
NS-071	NS	?	no	None	None	None	
NS-072	NS	?	no	None	None	None	
NS-072	NS	?	no	None	None	None	
NS-074	NS	?	no	None	None	None	
NS-074	NS	?	no	None	None	None	
NS-075	NS	15/03/2004	no	None	None	None	
NS-075	NS	19/07/2004	no	None	None	None	
NS-076	NS	?	no	None	None	None	
NS-077	NS	?	no	None	None	None	
NS-077	NS	?	no	None	None	None	
NS-077	NS	?	no	None	None	None	
NS-077	NS	?	no	None	None	None	
NS-077	NS	?	no	None	None	None	
NS-078	NS	?	no	None	None	None	
NS-078	NS	?	no	None	None	None	
NS-078	NS	?	no	None	None	None	
NS-079	NS	?	no	None	None	None	
NS-079	NS	?	no	None	None	None	
NS-080	NS	?	no	None	None	None	
NS-080	NS	?	no	None	None	None	
NS-080	NS	?	no	None	None	None	
NS-080	NS	?	no	None	None	None	
NS-080	NS	?	no	None	None	None	
NS-081	NS	?	no	None	None	None	
NS-081	NS	?	no	None	None	None	
NS-082	NS	22/03/2005	yes	None	None	yes	36
NS-082	NS	05/04/2005	yes	None	None	yes	36
NS-082	NS	19/04/2005	yes	None	None	yes	36
NS-082	NS	28/06/2005	yes	None	yes	yes	30
NS-082	NS	13/07/2005	yes	None	yes	yes	24
NS-082	NS	07/09/2005	yes	None	yes	yes	3
NS-083	NS	?	no	None	None	None	
NS-085	NS	?	no	None	None	None	
NS-086	NS	?	no	None	None	None	
NS-086	NS	?	no	None	None	None	
NS-087	NS	25/10/2005	no	None	None	None	
RT-044	ORT	21/09/2004	yes	None	yes	yes	5
RT-044	ORT	14/12/2004	yes	None	yes	yes	5
RT-044	ORT	15/03/2005	yes	None	yes	yes	5
RT-008	ORT	12/04/2001	no	None	None	None	
RT-008	ORT	02/11/2004	yes	yes	yes	yes	10

I.D. Number	Sub-Arm	visit date	IS	Cellcept	Cyclosporine A	steroids	steroid dose
RT-008	ORT	?	no	None	None	None	
RT-008	ORT	?	no	None	None	None	
RT-008	ORT	?	no	None	None	None	
RT-008	ORT	?	no	None	None	None	
RT-008	ORT	?	no	None	None	None	
RT-008	ORT	?	no	None	None	None	
RT-008	ORT	?	no	None	None	None	
RT-008	ORT	?	no	None	None	None	
RT-008	ORT	?	no	None	None	None	
RT-008	ORT	?	no	no	no	no	
RT-008	ORT	?	no	None	None	None	
RT-008	ORT	?	no	None	None	None	
RT-009	ORT	28/07/2000	no	None	None	None	
RT-009	ORT	05/12/2000	yes	None	None	yes	14
RT-009	ORT	20/09/2001	yes	None	None	yes	8
RT-009	ORT	13/03/2006	yes	no	yes	yes	4
RT-009	ORT	?	no	None	None	None	
RT-009	ORT	?	no	None	None	None	
RT-009	ORT	?	no	None	None	None	
RT-009	ORT	?	no	None	None	None	
RT-009	ORT	?	no	None	None	None	
RT-009	ORT	?	no	None	None	None	
RT-009	ORT	?	no	None	None	None	
RT-009	ORT	?	no	None	None	None	
RT-009	ORT	?	no	None	None	None	
RT-009	ORT	?	no	None	None	None	
RT-009	ORT	?	no	None	None	None	
RT-009	ORT	?	no	None	None	None	
RT-009	ORT	?	no	None	None	None	
RT-009	ORT	?	no	None	None	None	
RT-009	ORT	?	no	None	None	None	
RT-009	ORT	?	no	None	None	None	
RT-009	ORT	?	no	None	None	None	
RT-009	ORT	?	no	None	None	None	
RT-010	ORT	28/07/2000	no	no	no	no	
RT-010	ORT	07/05/2001	no	no	no	None	
RT-010	ORT	?	no	None	None	None	
RT-010	ORT	?	no	None	None	None	
RT-024	ORT	20/09/2000	no	no	no	None	
RT-024	ORT	16/04/2001	no	no	no	None	
RT-024	ORT	11/07/2001	no	no	no	None	
RT-024	ORT	22/01/2003	no	None	None	unknown	12
RT-024	ORT	?	no	None	None	None	
RT-024	ORT	?	no	None	None	None	
RT-024	ORT	?	no	None	None	None	
RT-024	ORT	?	no	None	None	None	
RT-027	ORT	02/03/2004	yes	yes	yes	yes	5

I.D. Number	Sub-Arm	visit date	IS	Cellcept	Cyclosporine A	steroids	steroid dose
RT-027	ORT	20/09/2000	no	None	None	None	
RT-027	ORT	16/04/2001	no	None	None	None	
RT-027	ORT	11/07/2001	no	None	None	None	
RT-027	ORT	04/12/2001	no	None	None	None	
RT-035	ORT	12/04/2001	no	None	None	None	
RT-035	ORT	11/07/2001	no	None	None	None	
RT-035	ORT	?	no	None	None	None	
RT-035	ORT	?	no	None	None	None	
RT-035	ORT	?	no	None	None	None	
RT-035	ORT	?	no	None	None	None	
RT-038	ORT	?	no	None	None	None	
RT-038	ORT	?	no	None	None	None	
RT-038	ORT	?	no	None	None	None	
RT-038	ORT	?	no	None	None	None	
RT-040	ORT	?	no	None	None	None	
RT-040	ORT	?	no	None	None	None	
RT-040	ORT	?	no	None	None	None	
RT-040	ORT	?	no	None	None	None	
RT-040	ORT	?	no	None	None	None	
RT-041	ORT	?	no	None	None	None	
RT-041	ORT	?	no	None	None	None	
RT-041	ORT	?	no	None	None	None	
RT-041	ORT	?	no	None	None	None	
RT-041	ORT	?	no	None	None	None	
RT-041	ORT	?	no	None	None	None	
RT-043	ORT	?	no	None	None	None	
RT-043	ORT	?	no	None	None	None	
RT-043	ORT	?	no	None	None	None	
RT-043	ORT	?	no	None	None	None	
RT-045	ORT	05/07/2005	yes	no	yes	yes	5
RT-045	ORT	?	no	None	None	None	
RT-045	ORT	?	no	None	None	None	
RT-050	ORT	22/03/2005	yes	yes	yes	yes	10
RT-050	ORT	27/09/2005	yes	yes	yes	yes	10
RT-050	ORT	18/10/2005	yes	yes	yes	yes	10
RT-050	ORT	07/03/2006	yes	yes	yes	yes	10
RT-050	ORT	18/04/2006	yes	yes	yes	yes	10
RT-051	ORT	?	no	None	None	None	
RT-051	ORT	?	no	None	None	None	
RT-051	ORT	?	no	None	None	None	
RT-051	ORT	?	no	None	None	None	
RT-051	ORT	?	no	None	None	None	
RT-051	ORT	?	no	None	None	None	
RT-051	ORT	?	no	None	None	None	

Legend for appendix IV and V

I.D. number = identification number given to each individual patient

Sub-arm= type of condition of patient

Visit-date= date of patient visit to clinic when blood or urine was taken.

Cellcept= immunosuppressive drug also known as myfortic and mycophenolate.

Cyclosporine A= anti-rejection drug, calcineurin inhibitor.

IC= immunocompromise

NS=nephrotic syndrome

ORT=orthotopic renal transplant

IS=immune-suppression

ALL=acute lymphocytic leukemia.

OHT= orthotopic heart transplant

HIV=human immuno-defiency virus

Steroid dose= milligrams

V Table of Patients viral loads

I.D. Number	Sub-Arm	visit date	Real-time BKV copies/ml	Real-time JCV copies/ml
IC-001	ALL	18/02/2000	0	0
IC-001	ALL	08/12/2000	0	0
IC-002	OHT	24/02/2000	0	0
IC-003	OHT	24/04/2000	0	0
IC-003	OHT	?	317723.5	0
IC-004	OHT	14/04/2000	0	0
IC-005	OHT	20/04/2000	0	0
IC-005	OHT	06/12/2000	0	0
IC-006	OHT	20/04/2000	0	0
IC-006	OHT	21/12/2000	0	0
IC-006	OHT	21/09/2004	0	0
IC-006	OHT	?	0	0
IC-007	ALL	12/05/2000	0	0
IC-007	ALL	13/07/2001	0	0
IC-007	ALL	?	0	0
IC-011	HIV	08/08/2000	0	0
IC-011	HIV	09/02/2001	0	0
IC-011	HIV	?	0	0
IC-012	HIV	08/08/2000	0	0
IC-013	HIV	08/08/2000	0	0
IC-013	HIV	?	0	0
IC-014	HIV	10/08/2000	0	0
IC-014	HIV	15/03/2001	0	0
IC-014	HIV	21/08/2001	0	0
IC-015	HIV	29/08/2000	0	0
IC-015	HIV	15/03/2001	0	0
IC-015	HIV	13/07/2001	0	0
IC-015	HIV	?	0	0
IC-016	HIV	29/08/2000	0	0
IC-016	HIV	21/02/2001	0	0
IC-017	HIV	30/08/2000	0	0
IC-018	HIV	30/08/2000	0	0
IC-018	HIV	21/02/2001	150	0
IC-018	HIV	?	0	0
IC-019	HIV	06/09/2000	0	0
IC-020	ALL	11/09/2000	0	0
IC-020	ALL	05/03/2001	0	0
IC-020	ALL	?	0	0
IC-020	ALL	?	0	0
IC-021	HIV	19/09/2000	0	0
IC-021	HIV	13/07/2001	0	0
IC-022	HIV	19/09/2000	0	0
IC-022	HIV	13/07/2001	0	0
IC-022	HIV	06/09/2001	0	0

I.D. Number	Sub-Arm	visit date	Real-time BKV copies/ml	Real-time JCV copies/ml
IC-023	HIV	19/09/2000	0	0
IC-023	HIV	15/03/2001	0	0
IC-023	HIV	06/09/2001	0	0
IC-025	OHT	27/09/2000	0	0
IC-025	OHT	13/12/2000	0	0
IC-026	HIV	?	150	0
IC-028	OHT	29/09/2000	0	0
IC-028	OHT	?	0	0
IC-030	OHT	09/02/2001	0	0
IC-031	HIV	16/02/2001	3619250	0
IC-034	ALL	12/04/2001	0	0
IC-034	ALL	11/07/2001	0	0
IC-036	ALL	16/04/2001	0	0
IC-036	ALL	16/04/2001	0	0
IC-046	OHT	?	0	0
NS-001	NS	06/09/2000	0	0
NS-002	NS	13/11/2000	0	0
NS-002	NS	12/04/2001	0	0
NS-002	NS	18/07/2001	0	0
NS-002	NS	25/09/2002	0	0
NS-002	NS	07/04/2004	0	0
NS-002	NS	28/07/2004	0	0
NS-002	NS	28/12/2004	0	0
NS-002	NS	02/01/2005	0	0
NS-002	NS	19/04/2005	0	0
NS-002	NS	07/11/2005	0	0
NS-002	NS	28/02/2006	0	0
NS-003	NS	13/11/2000	0	0
NS-003	NS	27/08/2001	0	0
NS-003	NS	29/08/2001	343898	0
NS-003	NS	10/09/2001	0	0
NS-003	NS	25/09/2002	120800	0
NS-003	NS	10/03/2004	0	0
NS-003	NS	19/01/2005	7650	0
NS-004	NS	13/11/2000	0	0
NS-004	NS	23/04/2001	0	0
NS-004	NS	21/05/2001	0	0
NS-004	NS	18/07/2001	0	0
NS-004	NS	14/08/2001	0	0
NS-004	NS	24/11/2004	13179750	0
NS-004	NS	04/01/2005	19763350	1950
NS-004	NS	09/02/2005	0	0
NS-004	NS	06/04/2005	0	0
NS-004	NS	28/06/2005	0	0
NS-004	NS	20/07/2005	0	0
NS-004	NS	27/09/2005	0	0
NS-004	NS	07/11/2005	0	0

I.D. Number	Sub-Arm	visit date	Real-time BKV copies/ml	Real-time JCV copies/ml
NS-005	NS	13/11/2000	0	0
NS-005	NS	07/05/2001	0	0
NS-005	NS	09/07/2001	0	0
NS-005	NS	10/09/2001	0	0
NS-005	NS	14/01/2002	0	0
NS-005	NS	19/01/2005	1150125000	0
NS-005	NS	19/04/2005	287573.5	0
NS-005	NS	28/06/2005	190111.5	0
NS-005	NS	19/07/2005	500	0
NS-005	NS	29/11/2005	77000	0
NS-006	NS	13/11/2000	100	0
NS-006	NS	14/01/2002	0	0
NS-006	NS	25/10/2004	0	0
NS-006	NS	31/01/2005	0	0
NS-006	NS	22/08/2005	24600	0
NS-007	NS	02/12/2000	0	0
NS-007	NS	31/01/2005	0	0
NS-007	NS	?	0	0
NS-007	NS	?	0	0
NS-007	NS	?	0	0
NS-007	NS	?	0	0
NS-007	NS	?	0	0
NS-007	NS	?	0	0
NS-007	NS	?	0	0
NS-007	NS	?	0	0
NS-007	NS	?	0	0
NS-008	NS	14/11/2000	0	0
NS-008	NS	27/08/2001	0	0
NS-008	NS	23/07/2002	0	0
NS-008	NS	02/12/2002	0	0
NS-009	NS	16/11/2000	0	0
NS-009	NS	14/05/2001	0	0
NS-009	NS	10/09/2002	0	0
NS-010	NS	04/12/2000	0	0
NS-010	NS	28/02/2001	0	0
NS-010	NS	17/09/2001	0	0
NS-010	NS	04/09/2002	0	0
NS-011	NS	04/12/2000	0	0
NS-011	NS	05/03/2001	0	0
NS-011	NS	14/01/2002	0	0
NS-011	NS	29/07/2002	67500	0
NS-011	NS	24/02/2003	0	7150
NS-011	NS	22/03/2004	0	5250
NS-011	NS	24/11/2004	0	1250
NS-011	NS	21/05/2201	0	0
NS-012	NS	05/12/2000	0	0
NS-013	NS	05/12/2000	0	0

I.D. Number	Sub-Arm	visit date	Real-time BKV copies/ml	Real-time JCV copies/ml
NS-014	NS	01/12/2000	0	0
NS-015	NS	07/12/2000	0	0
NS-015	NS	16/10/2002	0	0
NS-015	NS	24/01/2005	0	0
NS-016	NS	18/09/2001	0	0
NS-017	NS	18/12/2000	0	23100
NS-017	NS	17/09/2001	0	101750
NS-017	NS	16/01/2002	0	0
NS-017	NS	31/07/2002	298400	78500
NS-017	NS	10/01/2005	0	0
NS-017	NS	11/04/2005	0	0
NS-017	NS	15/08/2005	400	67850
NS-018	NS	18/12/2000	0	0
NS-018	NS	27/08/2001	0	0
NS-018	NS	06/01/2004	0	0
NS-018	NS	05/07/2005	0	0
NS-018	NS	28/02/2006	0	0
NS-019	NS	?	0	0
NS-019	NS	?	0	0
NS-020	NS	19/02/2001	0	0
NS-020	NS	05/03/2001	0	0
NS-021	NS	26/02/2001	0	0
NS-021	NS	22/07/2002	0	0
NS-022	NS	26/02/2001	0	0
NS-023	NS	28/02/2001	0	0
NS-023	NS	23/04/2001	0	0
NS-023	NS	01/08/2002	187600	0
NS-023	NS	26/08/2002	2900	0
NS-023	NS	03/02/2003	0	0
NS-023	NS	10/11/2004	0	0
NS-023	NS	12/01/2005	0	0
NS-023	NS	23/03/2005	0	0
NS-023	NS	13/07/2005	0	0
NS-023	NS	26/04/2006	0	0
NS-024	NS	05/03/2001	0	0
NS-024	NS	08/10/2001	0	0
NS-024	NS	12/10/2004	0	0
NS-024	NS	18/01/2005	0	0
NS-024	NS	05/07/2005	0	0
NS-025	NS	21/01/2003	0	0
NS-025	NS	26/02/2003	0	0
NS-025	NS	22/03/2004	0	0
NS-025	NS	20/12/2004	0	0
NS-025	NS	05/07/2005	0	0
NS-025	NS	?	0	0
NS-026	NS	12/03/2001	0	400
NS-026	NS	23/04/2001	0	1200

I.D. Number	Sub-Arm	visit date	Real-time BKV copies/ml	Real-time JCV copies/ml
NS-027	NS	13/03/2001	0	0
NS-028	NS	27/03/2001	0	0
NS-028	NS	07/05/2001	0	0
NS-028	NS	01/10/2002	0	0
NS-028	NS	10/11/2004	0	0
NS-028	NS	01/02/2005	0	0
NS-028	NS	29/03/2005	39150	0
NS-028	NS	20/09/2005	6000	0
NS-029	NS	16/04/2001	0	0
NS-029	NS	07/05/2001	1101350	0
NS-030	NS	16/04/2001	0	0
NS-030	NS	10/09/2001	0	0
NS-030	NS	28/10/2002	0	0
NS-030	NS	15/11/2004	0	0
NS-030	NS	06/03/2006	0	0
NS-031	NS	07/05/2001	0	0
NS-031	NS	28/08/2002	102750	0
NS-031	NS	01/03/2004	0	0
NS-031	NS	24/08/2005	0	0
NS-032	NS	07/05/2001	0	0
NS-032	NS	09/07/2001	0	0
NS-032	NS	30/03/2004	0	0
NS-032	NS	22/12/2004	0	0
NS-032	NS	26/01/2005	0	0
NS-032	NS	29/03/2005	0	0
NS-032	NS	21/09/2005	0	0
NS-032	NS	19/10/2005	0	0
NS-032	NS	15/11/2005	0	0
NS-032	NS	15/03/2006	0	0
NS-032	NS	25/04/2006	0	0
NS-034	NS	14/05/2001	0	0
NS-034	NS	22/03/2004	0	0
NS-034	NS	30/03/2004	0	0
NS-034	NS	29/09/2004	0	0
NS-035	NS	21/05/2001	0	0
NS-035	NS	01/10/2002	26200	0
NS-035	NS	10/12/2002	0	0
NS-035	NS	14/03/2006	0	0
NS-036	NS	21/05/2001	0	0
NS-036	NS	14/01/2002	0	0
NS-036	NS	19/08/2002	0	0
NS-036	NS	04/02/2003	0	0
NS-036	NS	19/07/2004	0	0
NS-036	NS	05/11/2004	0	0
NS-036	NS	18/07/2 005	0	0
NS-037	NS	?	0	0
NS-037	NS	?	0	0

I.D. Number	Sub-Arm	visit date	Real-time BKV copies/ml	Real-time JCV copies/ml
NS-037	NS	07/06/2001	0	0
NS-037	NS	09/07/2001	0	0
NS-037	NS	10/09/2001	0	0
NS-037	NS	22/07/2002	0	0
NS-037	NS	24/02/2003	0	0
NS-037	NS	14/12/2004	0	0
NS-038	NS	06/06/2001	0	0
NS-038	NS	07/06/2001	0	0
NS-038	NS	06/07/2001	0	0
NS-038	NS	17/09/2001	106400	0
NS-038	NS	10/12/2002	0	0
NS-038	NS	13/01/2003	0	0
NS-039	NS	16/07/2001	0	0
NS-039	NS	10/09/2001	0	0
NS-039	NS	14/03/2003	0	0
NS-039	NS	19/03/2004	0	0
NS-039	NS	01/02/2005	0	0
NS-039	NS	10/08/2005	0	0
NS-039	NS	19/09/2005	0	0
NS-039	NS	10/10/2005	0	0
NS-040	NS	16/07/2001	0	0
NS-040	NS	18/07/2001	0	0
NS-041	NS	13/08/2001	0	0
NS-041	NS	21/08/2002	5850	0
NS-042	NS	14/08/2001	0	0
NS-042	NS	17/09/2001	0	0
NS-043	NS	14/08/2001	0	0
NS-043	NS	01/08/2002	25250	0
NS-043	NS	02/12/2002	0	0
NS-043	NS	01/12/2004	0	0
NS-043	NS	14/02/2005	0	0
NS-043	NS	28/03/2005	0	0
NS-043	NS	05/07/2005	0	0
NS-043	NS	19/09/2005	0	0
NS-043	NS	05/12/2005	0	0
NS-044	NS	20/08/2001	0	0
NS-044	NS	26/08/2002	22400	0
NS-044	NS	28/10/2002	0	0
NS-044	NS	20/12/2004	0	0
NS-044	NS	17/10/2005	0	0
NS-044	NS	07/11/2005	0	0
NS-045	NS	10/09/2001	0	0
NS-046	NS	18/09/2001	0	0
NS-047	NS	18/09/2001	0	0
NS-047	NS	?	0	2050
NS-047	NS	?	0	44500
NS-048	NS	18/09/2001	3539750	0

I.D. Number	Sub-Arm	visit date	Real-time BKV copies/ml	Real-time JCV copies/ml
NS-050	NS	08/10/2001	0	0
NS-051	NS	07/10/2002	0	0
NS-051	NS	?	0	0
NS-051	NS	?	0	0
NS-051	NS	?	0	0
NS-051	NS	?	0	0
NS-051	NS	?	0	0
NS-051	NS	?	0	0
NS-052	NS	14/10/2002	0	0
NS-052	NS	?	0	0
NS-052	NS	?	0	0
NS-053	NS	15/10/2002	570350	0
NS-053	NS	11/02/2005	461850	0
NS-053	NS	12/04/2005	0	0
NS-053	NS	29/11/2005	0	0
NS-054	NS	23/10/2002	0	0
NS-054	NS	?	0	0
NS-055	NS	28/10/2002	0	0
NS-055	NS	24/02/2003	0	290
NS-055	NS	08/11/2004	0	63.5
NS-055	NS	24/01/2005	0	13500
NS-055	NS	12/07/2005	0	0
NS-055	NS	17/10/2005	0	0
NS-056	NS	14/01/2003	0	0
NS-057	NS	14/01/2003	0	0
NS-058	NS	22/01/2003	0	0
NS-061	NS	?	0	0
NS-061	NS	?	0	0
NS-062	NS	?	0	0
NS-063	NS	04/02/2003	0	0
NS-064	NS	11/02/2003	0	0
NS-064	NS	?	0	0
NS-064	NS	?	0	48050
NS-065	NS	?	1644500000	3108243.5
NS-065	NS	?	0	0
NS-065	NS	?	0	0
NS-066	NS	?	0	0
NS-068	NS	26/02/2003	0	0
NS-069	NS	02/03/2004	0	0
NS-069	NS	14/12/2004	0	0
NS-070	NS	03/03/2004	0	0
NS-070	NS	?	0	0
NS-070	NS	?	0	0
NS-070	NS	?	0	0
NS-071	NS	?	0	0
NS-071	NS	?	0	0
NS-071	NS	?	0	0

I.D. Number	Sub-Arm	visit date	Real-time BKV copies/ml	Real-time JCV copies/ml
NS-071	NS	?	0	0
NS-071	NS	?	0	0
NS-071	NS	?	0	0
NS-072	NS	?	137950	0
NS-072	NS	?	0	0
NS-074	NS	?	236150	0
NS-074	NS	?	16750	0
NS-075	NS	15/03/2004	0	0
NS-075	NS	19/07/2004	32500	0
NS-076	NS	?	0	0
NS-077	NS	?	0	0
NS-077	NS	?	0	0
NS-077	NS	?	0	0
NS-077	NS	?	0	0
NS-077	NS	?	0	0
NS-078	NS	?	0	0
NS-078	NS	?	0	0
NS-078	NS	?	0	0
NS-079	NS	?	0	0
NS-079	NS	?	0	0
NS-080	NS	?	0	0
NS-080	NS	?	0	0
NS-080	NS	?	0	0
NS-080	NS	?	0	0
NS-080	NS	?	0	0
NS-081	NS	?	0	0
NS-081	NS	?	0	0
NS-082	NS	22/03/2005	0	0
NS-082	NS	05/04/2005	0	0
NS-082	NS	19/04/2005	0	0
NS-082	NS	28/06/2005	0	0
NS-082	NS	13/07/2005	0	0
NS-082	NS	07/09/2005	0	0
NS-083	NS	?	0	330400
NS-085	NS	?	0	0
NS-086	NS	?	0	0
NS-086	NS	?	0	0
NS-087	NS	25/10/2005	0	0
R-044	ORT	21/09/2004	19702.5	0
R-044	ORT	14/12/2004	76100	0
R-044	ORT	15/03/2005	0	0
RT-008	ORT	12/04/2001	0	0
RT-008	ORT	02/11/2004	0	0
RT-008	ORT	?	0	0
RT-008	ORT	?	0	0
RT-008	ORT	?	0	0
RT-008	ORT	?	0	0

I.D. Number	Sub-Arm	visit date	Real-time BKV copies/ml	Real-time JCV copies/ml
RT-008	ORT	?	0	0
RT-008	ORT	?	0	0
RT-008	ORT	?	0	0
RT-008	ORT	?	0	0
RT-008	ORT	?	0	0
RT-008	ORT	?	0	0
RT-008	ORT	?	0	3900
RT-008	ORT	?	0	92550
RT-009	ORT	28/07/2000	0	0
RT-009	ORT	05/12/2000	0	0
RT-009	ORT	20/09/2001	0	0
RT-009	ORT	13/03/2006	0	0
RT-009	ORT	?	1239715	0
RT-009	ORT	?	27500	0
RT-009	ORT	?	0	0
RT-009	ORT	?	0	0
RT-009	ORT	?	0	0
RT-009	ORT	?	0	0
RT-009	ORT	?	0	0
RT-009	ORT	?	0	0
RT-009	ORT	?	0	0
RT-009	ORT	?	0	0
RT-009	ORT	?	0	0
RT-009	ORT	?	0	0
RT-009	ORT	?	0	0
RT-009	ORT	?	0	0
RT-009	ORT	?	0	0
RT-009	ORT	?	0	0
RT-009	ORT	?	0	0
RT-009	ORT	?	0	0
RT-010	ORT	28/07/2000	0	0
RT-010	ORT	07/05/2001	0	0
RT-010	ORT	?	0	0
RT-010	ORT	?	0	147750
RT-024	ORT	20/09/2000	0	0
RT-024	ORT	16/04/2001	0	0
RT-024	ORT	11/07/2001	0	0
RT-024	ORT	22/01/2003	0	0
RT-024	ORT	?	15250000000	0
RT-024	ORT	?	9000000000	0
RT-024	ORT	?	1329950	0
RT-024	ORT	?	188400	0
RT-027	ORT	02/03/2004	20788.5	0
RT-027	ORT	20/09/2000	0	0
RT-027	ORT	16/04/2001	0	0
RT-027	ORT	11/07/2001	0	0
RT-027	ORT	04/12/2001	0	0

I.D. Number	Sub-Arm	visit date	Real-time BKV copies/ml	Real-time JCV copies/ml
RT-035	ORT	12/04/2001	0	0
RT-035	ORT	11/07/2001	0	0
RT-035	ORT	?	234402.5	0
RT-035	ORT	?	0	0
RT-035	ORT	?	0	0
RT-035	ORT	?	0	0
RT-038	ORT	?	0	0
RT-038	ORT	?	0	0
RT-038	ORT	?	0	0
RT-038	ORT	?	0	0
RT-040	ORT	?	0	0
RT-040	ORT	?	0	800
RT-040	ORT	?	0	35159.5
RT-040	ORT	?	0	1449.5
RT-040	ORT	?	0	247000
RT-041	ORT	?	0	0
RT-041	ORT	?	0	0
RT-041	ORT	?	0	0
RT-041	ORT	?	0	0
RT-041	ORT	?	0	100
RT-041	ORT	?	0	1100
RT-043	ORT	?	703400	650
RT-043	ORT	?	240200	384750
RT-043	ORT	?	148650	0
RT-043	ORT	?	100280	7550
RT-045	ORT	05/07/2005	0	0
RT-045	ORT	?	1300	0
RT-045	ORT	?	0	0
RT-050	ORT	22/03/2005	0	0
RT-050	ORT	27/09/2005	0	0
RT-050	ORT	18/10/2005	0	0
RT-050	ORT	07/03/2006	0	0
RT-050	ORT	18/04/2006	0	0
RT-051	ORT	?	0	0
RT-051	ORT	?	0	0
RT-051	ORT	?	0	0
RT-051	ORT	?	0	0
RT-051	ORT	?	0	0
RT-051	ORT	?	0	0
RT-051	ORT	?	0	0

VI VP1 Sequences.

VP_11

GTGCCAAAACTACTAATAAAAGGAGGAGTAGAAGTTCTAGAAGTTAAAACTGGGGTAGA
TGCTATAACAGAGGTAGAATGCTTCCTAAACCCAGAAATGGGGGATCCAGATGAAAACC
TTAGGGGCTTTAGTCTAAAGCTAAGTGCTCAAAATGACTTTAGCAGTGATAGCCCAGAA
AGAAAAATGCTTCCTTGTTACAGCACAGCAAGAATTCCCCTCCCCAATTTAAATGAGGA

VP_12

GTGCCAAAAATACTAATAAAAGGAGGAGTAGAAGTTTTAGAAGTTAAAACTGGGCTAGA
TGCTATAACAGAGGTAGAATGCTTCCTAAACCCAGAAATGGGGGATCCAGATGAAAACC
TTAGGGGCTTTAGTCTAAAGCTAAGTGCTGAAAATGACTTTAGCAGTGATAGCCCCGAA
AGAAAAATGCTTCCCTGTTACAGCACAGCAAGAATTCCCCTCCCCAGTTTAAATGAGGT

VP_13

GTGCCAAAACTACTAATAAAAGGAGGAGTAGAAGTTCTAGAAGTTAAAACTGGGCTAGA
TGCTATAACAGAGGTAGAATGCTTCCTAAACCCAGAAATGGGGGATCCAGATGAAAACC
TTAGGGGCTTTAGTCTAAAGCTAAGTGCTGAAAATGACTTTAGCAGTGATAGCCCAGAC
AGAAAAATGCTTCCCTGTTACAGCACAGCAAGAATTCCCCTCCCCAATTAAAAGTGGAT

VP_23

GTGCCAAAACTACTAATAAAAGGAGGAGTAGAAGTTCTAGAAGTTAAAACTGGGGTAGA
TGCTATAACAGAGGTAGAATGCTTCCTAAACCCAGAAATGGGGGATCCAGATGAAAACT
CTAGGGGCTTTAGTCTAAAGTTAAGTGCTGAAAATGACTTTAGCAGTGATAGCCCAGAA
AGAAAAATGCTTCCCTGTTACAGCACAGCAAGAATTCCCCTCCCCAATTTCAAGTGGAT

VP_24

GTGCCAAAACTACTAATAAAAGGAGGAGTAGAAGTTCTAGAAGTTAAAACTGGGGTAGA
TGCTATTACAGAGGTAGAATGCTTCCTAAACCCAGAAATGGGGGATCCAGATGAAAACC
TTAGGGGCTTTAGTCTAAAGCTAAGTGCTGAAAATGACTTTAGCAGTGATAGCCCAGAG
AGAAAAATGCTTCCCTGTTACAGCACAGCAAGAATTCCCCTGCCCAATTTAAATGAGGA

VP_25

GTGCCAAAACTACTAATAAAAGGAGGAGTAGAAGTTCTAGAAGTTAAAACTGGGCTAGA
TGCTATAACAGAGGTAGAATGCTTCCTAAACCCAGAAATGGGGGATCCAGATGAAAACC
TTAGGGGCTTTAGTCTAAAGCTAAGTGCTGAAAATGACTTTAGCAGTGATAGCCCAGAC
AGAAAAATGCTTCCCTGTTACAGCACAGCAAGAATTCCCCTCCCCAATTTAAATGAGGT

VP_26

GTGCCAAAACTACTAATAAAAGGAGGAGTAGAAGTTCTAGAAGTTAAAACTGGGCTAGA
TGCTATAACAGAGGTAGAATGCTTCCTAAACCCAGAAATGGGGGATCCAGATGAAAACC
CTAGGGGCTTTAGTCTAAAGCTAAGTGCTGAAAATGACTTTAGCAGTGATAGCCCAGAC
AGAAAAATGCTTCCCTGTTACAGCACAGCAAGAATTCCCCTCCCCAATTTTAATGAGGT

VP_27

GTGCCAAAACTACTAATAAAAGGAGGAGTAGAAGTTCTAGAAGTTAAAACTGGGCTAGA
TGCTATAACAGAGGTAGAATGCTTCCTAAACCCAGAAATGGGGGATCCAGATGAAAACC
TTAGGGGCTTTAGTCTAAAGCTAAGTGCTGAAAATGACTTTAGCAGTGATAGCCCAGAC
AGAAAAATGCTTCCCTGTTACAGCACAGCAAGAATTCCCCTCCCCAATTTAAATGAGGA

VP_28

GTGCCAAAACTACTAATAAAAGGAGGAGTAGAAGTTCTAGAAGTTAAAACTGGGGTAGA
TGCTATTACAGAGGTAGAATGCTTCCTAAACCCAGAAATGGGGGATCCAGATGAAAACC
TTAGGGGCTTTAGTCTAAAGCTAAGTGCTGAAAATGACTTTAGCAGTGATAGCCCAGAG
AGAAAAATGCTTCCCTGTTACAGCACAGCAAGAATTCCCCTGCCCAATTTAAATGAGGA

VP_29

GTGCCAAAACTACTAATAAAAGGAGGAGTAGAAGTTCTAGAAGTTAAAACTGGGCTAGA
TGCTATAACAGAGGTAGAATGCTTCCTAAACCCAGAAATGGGGGATCCAGATGAAAACC
TTAGGGGCTTTAGTCTAAAGCTAAGTGCTGAAAATGACTTTAGCAGTGATAGCCCAGAC
AGAAAAATGCTTCCCTGTTACAGCACAGCAAGAATTCCCCTCCCCAATTTAAATGAGGA

VP_39

GTGCCAAAACTACTAATAAAAGGAGGAGTAGAAGTTCTAGAAGTTAAAACTGGGGTAGA
TGCTATTACAGAGGTAGAATGCTTCCTAAACCCAGAAATGGGGGATCCAGATGAAAACC
CTAGGGGCTTTAGTCTAAAGTTAAGTGCTGAAAATGACTTTAGCAGTGATAGCCCAGAG
AGAAAAATGCTTCCCTGTTACAGCACCGCAAGAATTCCCCTGCCCAATTTCATGTCGAT

VP_40

GTGCCAAAACTACTAATAAAAGGAGGAGTAGAAGTTCTAGAAGTTAAAACTGGGGTAGA
TGCTATAACAGAGGTAGAATGCTTCCTAAACCCAGAAATGGGGGATCCAGATGAAAACC
TTAGGGGCTTTAGTCTAAAGCTAAGTGCTGAAAATGACTTTAGCAGTGATAGCCCAGAA
AGAAAAATGCTTCCCTGTTACAGCACAGCAAGAATTCCCCTCCCCAATTTAAATGAGGA