BK polyomavirus infection after kidney transplantation: risk and prevention

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Colophon

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Strength grows in the moments you think you can't go on,
but you keep going anyway

Karen Salmonsohn (7 July 2017)

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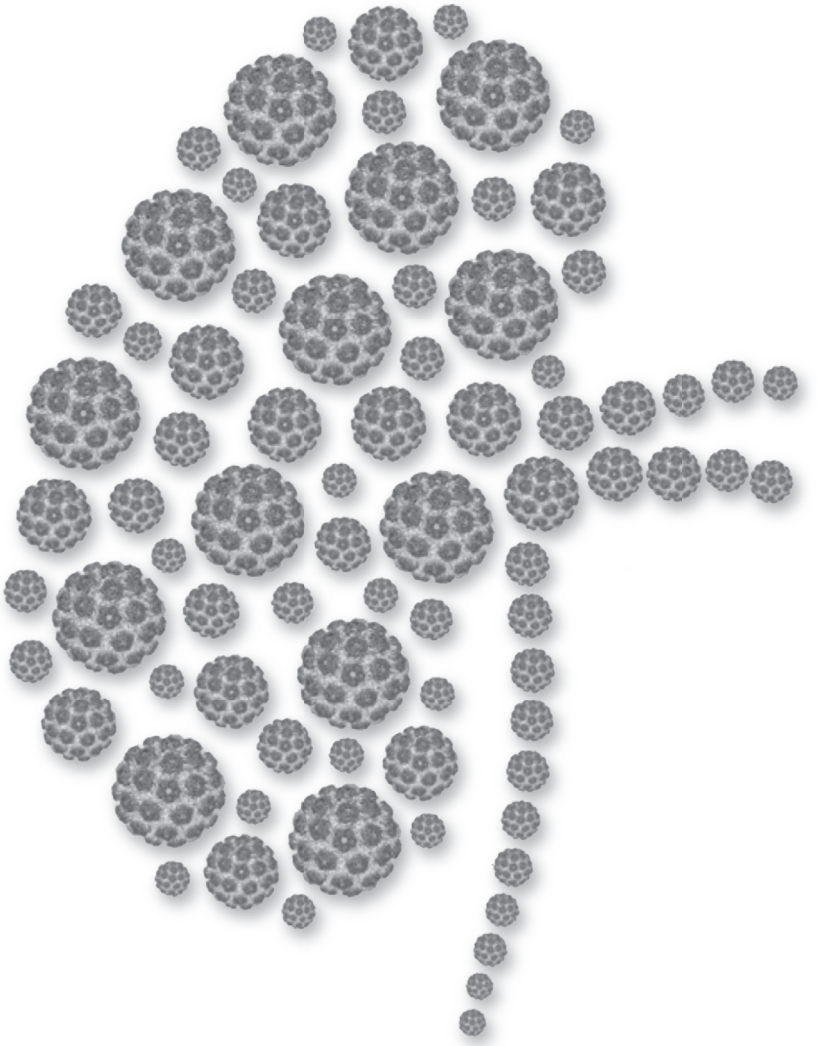


Chapter 1

General introduction

The family of *Polyomaviridae*

Ludwig Gross discovered the first polyomavirus (PyV) in 1953, while he was studying murine leukemia virus (MLV). He observed that newborn mice inoculated with a contaminated preparation of MLV developed not only leukemia but also tumours of the parotid gland (1). Due to its ability to induce various ("poly") tumours ("oma") in mice, the virus was later named mouse PyV (2, 3). Since 1999, PyVs have been recognized as a separate virus family, the *Polyomaviridae* (4). Before that time they belonged to the genus *Polyomavirus* in the family *Papovaviridae* that contained the papillomaviruses, PyVs and the simian vacuolating agent 40 (SV40).

SV40, the prototype of the *Polyomaviridae* family (5), was first identified as a contaminant of rhesus monkey kidney cell cultures used for poliovirus and adenovirus vaccine production between 1955 and 1963 (6, 7). This caused serious concerns, but so far SV40 has never been shown to be harmful in humans (8). In general, SV40 only leads to a persistent asymptomatic infection of its natural host, the rhesus macaque (9).

Advanced techniques such as polymerase chain reaction (PCR) with degenerated primers, rolling circle amplification (RCA), and next generation sequencing (NGS) have led to a fast increase in identification of novel PyVs during the last decade (4, 10, 11). Of the approximately 100 PyVs currently known, 80 have been recently classified as species (4, 11).

PyVs are small, non-enveloped viruses with circular dsDNA genomes of approximately 5 kbp (Table 1). They have a restricted host range and besides mammalian viruses, PyVs have also been isolated from birds, reptiles, amphibians, fish and invertebrates (12, 13).

Table 1. Characteristics of the family Polyomaviridae

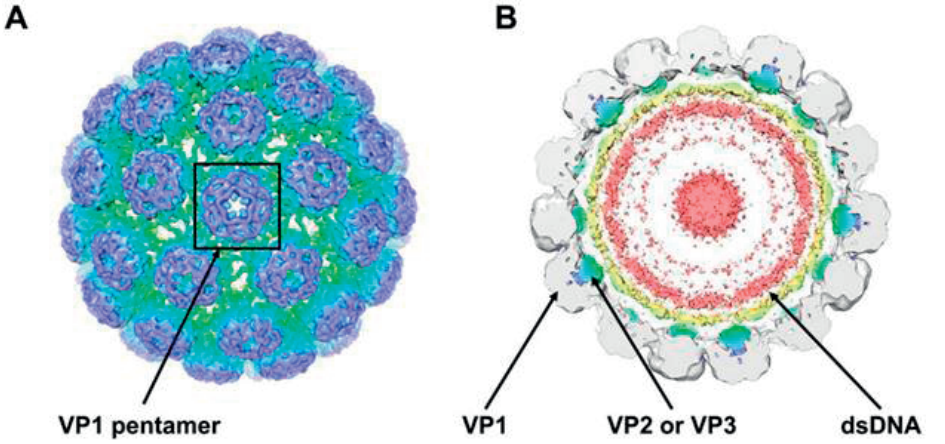
Virion	Non-enveloped, 40-45 nm, icosahedral
Genome	Approximately 5 kbp circular dsDNA
Replication	Bidirectional from a unique origin of DNA replication
Translation	Early and late transcripts, alternative splicing, alternative open reading frames
Host range	Mammals, birds, fish, reptiles, amphibians, invertebrates
Taxonomy	Four genera including more than 80 species

Virion structure and viral genome

Members of the *Polyomaviridae* family demonstrate structural similarity, with similar capsid sizes, high levels of genetic homology, and comparable genome

sizes. PyV particles are built up of an icosahedral capsid, enclosing a single copy of the viral genome. The capsid is comprised of the virus-encoded capsid proteins VP1, VP2 and VP3, and consists of 72 pentameric capsomers in a skewed lattice arrangement (T = 7) (Figure 1). VP1 is the major virion protein accounting for 80% of the total protein content (14). The protruding pentamers are composed of five copies of VP1, stabilized by intra- and inter-pentameric disulphide bonds and Ca^{2+} cations. In addition to these pentamers residing at the outer surface of the viral capsid, the other viral proteins VP2 and VP3, reside in the inner part of the viral particle, where a single copy of one of these two proteins binds into the cavity on the internal face of each pentamer in a hair-pin like manner and links the genome to the capsid (Figure 1) (10, 11, 15).

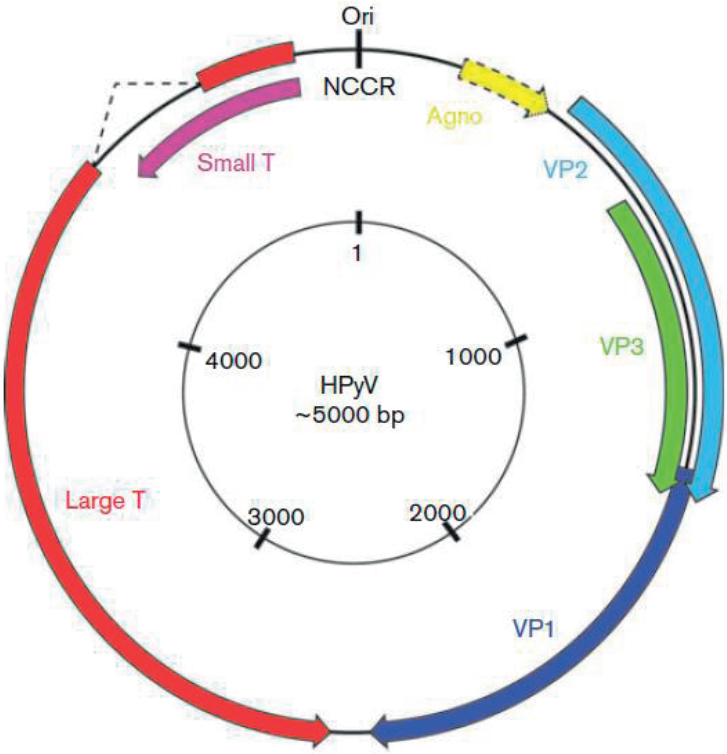
Figure 1. Cryo-electron microscopy structure of BK polyomavirus (BKPyV) viral particles (adopted from (14)).



A External view of the BKPyV virion shown at a contour level of 0.022. A viral protein VP1 pentamer is highlighted. **B** View of a 40-Å-thick slab through the unsharpened/unmasked virion map shown at a contour level of 0.0034. Pyramidal density below each VP1 penton and two shells of electron density adjacent to the inner capsid layer can be seen. The density within 6 Å of the fitted coordinates for SV40 VP1 is coloured grey. Density for VP2 and VP3 is coloured blue/green and for packaged dsDNA yellow/pink.

All PyVs have a circular dsDNA genome that is packed with cellular histones and divided in three functional domains; the early region encoding the regulatory proteins, called tumor (T) antigens, the late region encoding capsid proteins (VP1, VP2 and VP3), and the non-coding control region (NCCR), which contains the origin of DNA replication (ori) and the promoter/enhancer elements directing transcription of the viral genes (Figure 2).

Figure 2. Schematic representation of the BK polyomavirus genome made up of circular dsDNA (adopted from (4)). Regions that code for the indicated gene products are shown in colour. The agnoprotein-encoding gene is not found in every human polyomavirus genome.



The early region on the proximal side of the ori is transcribed before genome replication starts and encodes amongst others the large (LT) and small tumor (ST) antigen, which are expressed from two different mRNAs derived by alternative splicing of a single primary transcript. LT and ST accumulate in the nucleus and help in the replication of viral DNA.

LT is the major regulatory protein and is indispensable for PyV replication. During the viral DNA replication process, LT forms a multimeric complex which binds to the ori and acts like a helicase to facilitate the transcription of the late coding region. LT is also a key regulatory molecule driving the host cell to S phase of the cell cycle by binding to the tumor suppressor proteins Rb, p107, p130, and p53 (16). ST is involved in viral replication, cell cycle progression, and transformation (15, 16).

The late region on the distal side of the ori encodes the three structural or viral capsid proteins, (VP1, VP2, and VP3) involved in viral packaging and the non-structural agnoprotein. The late region is expressed after the onset of viral DNA replication. The capsid proteins are produced in the cytoplasm and are recruited into the nucleus by use of nuclear localization signals attached to them. Once the capsid proteins enter the nucleus, viral assembly occurs and the viral progenies accumulate in the nucleus. The agnoprotein is involved in virus release from host cells (17-19).

The NCCR contains the ori and a bidirectional promoter-enhancer region containing several transcription factor binding sites. As such, this region directs early and late transcription and replication of the genome. The NCCR has been arbitrarily divided into five sequence blocks denoted O143, P68, Q39, R63 and S63 where the numbers indicate the number of base pairs. The NCCR may have deletions, insertions or duplications of complete or partial blocks and is than generally referred to as a rearranged NCCR (re-NCCR). The nonrearranged NCCR, or NCCR from the archetype strain, is most frequently found in urine and the archetype strain is regarded as the transmissible virus. In contrast to the archetype, the re-NCCR have been detected after serial passage in tissue, culture and by direct-PCR amplification and sequencing, typically from nonurinary specimens (20). It is believed that these re-NCCR increase LT transcription and enhance the virus replication rate or 'viral fitness' in the respective host-cell environment (21-23). The full PyV replication cycle is shown in Figure 3.

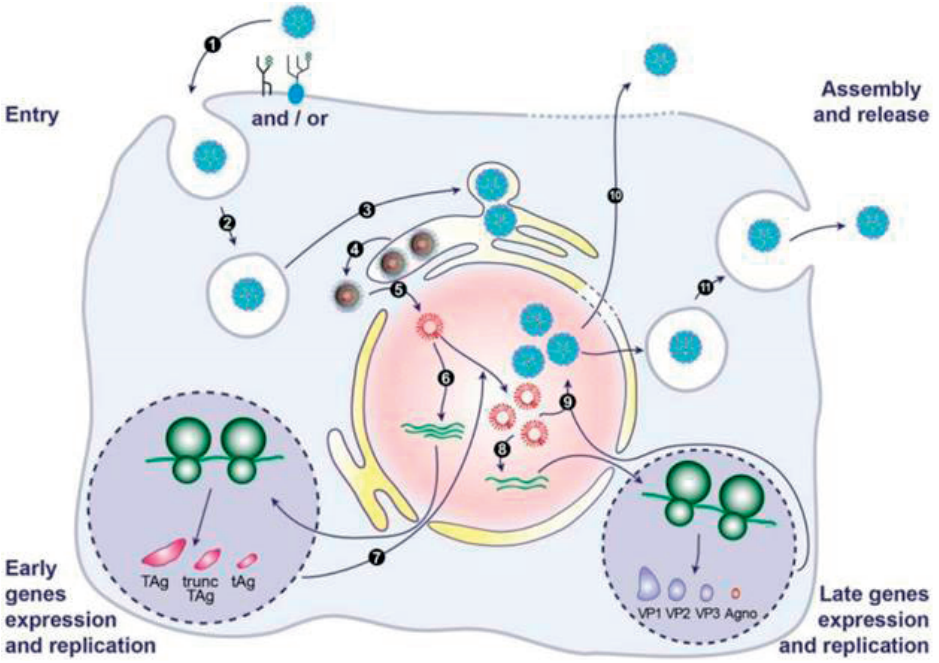
Taxonomy of the *Polyomaviridae*

From 2011 to 2016 the Polyomavirus genus was divided into three genera, *Orthopolyomavirus*, *Wukipolyomavirus* and *Avipolyomavirus* (7), with the first two genera containing mammalian species and the latter only avian species. The Polyomaviridae Study Group of the International Committee on Taxonomy of Viruses (ICTV) in addition proposed a demarcation criterion for new polyomavirus species, with a whole genome sequence identity defined to be less than 81% compared to members of known species.

After the identification of dozens of new PyVs our group assembled and published a new tentative PyV phylogenetic tree based on the alignment of concatenated VP1, VP2 and LT (4). An updated version of this tree is shown in Figure 4. This tree proposed to subdivide the *Orthopolyomavirus* genus in two lineages, *Orthopolyomavirus-I* and *–II*, and add a fifth genus called *Malawipolyomavirus*. The human PyVs (HPyVs) in Figure 4 are shown in red and do not form a monophyletic cluster,

but are unevenly distributed among four of the suggested five genera. Whether the HPyV distribution among the different genera should be considered as the result of crossing the species barrier by zoonotic viruses instead of virus-host coevolution, the most popular model of PyV evolution until a few years ago (24, 25), is unknown.

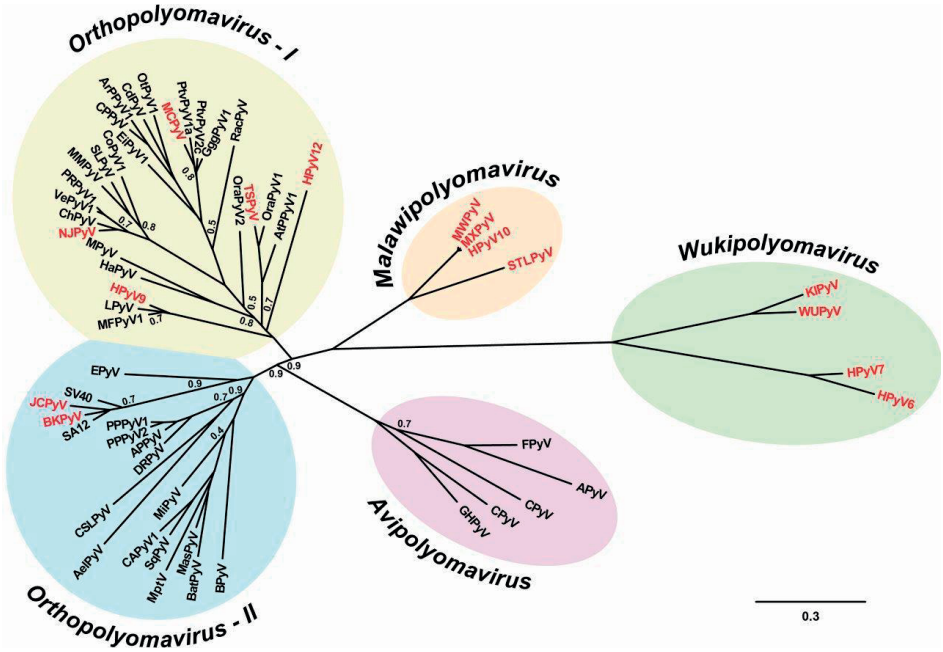
Figure 3. Model of the PyV life cycle.



PyV infection begins with binding of virions to the specific receptors and/or N-linked glycoproteins containing α(2,3)-linked sialic acid, at the cell surface [1]. This is followed by internalization potentially through a caveola-mediated endocytosis step within the first 4 h after adsorption [2]. The virus subsequently traffics from the late endosomes to the endoplasmic reticulum (ER), where it arrives approximately 10 h post-infection [3]. In the ER, virions benefit from chaperones, disulphide isomerases and reductases to facilitate the partial capsid uncoating. This creates a hydrophobic surface exposing VP2/VP3 that binds to and integrates into the ER membrane, leading to the release of partially uncoated viruses into the cytosol [4]. The viral genome is then transported into the nucleus via the nuclear pore complex [5]. Expression of early genes occurs approximately 24 h post-infection [6]. Early proteins are translocated into the nucleus where they serve to initiate viral DNA replication [7]. Late genes are then expressed [8]. VP1, VP2 and VP3 are translocated into the nucleus where they self-assemble to form capsids into which newly synthetized double stranded viral DNA is packaged [9]. Progeny virions are mainly released from infected cells after cell lysis [10]. However, a small fraction of progeny virions may also be released into the extracellular environment through a non-lytic egress that depends on the cellular secretion pathway [11].

In 2016 and 2017, in the 9th and 10th report of the *Polyomaviridae* Study Group, the international Committee on Taxonomy of Viruses (ICTV) updated the phylogenetic

Figure 4. The unrooted phylogenetic tree consists of all (putative) polyomavirus species known until June 2014 and is based the alignment of concatenated VP1, VP2, and LT amino acid sequences. The obtained branching pattern (topology) of basal nodes in the tree matches that proposed by Johne and colleagues (7). One distinct clade designated the *Avipolyomavirus* contains only the bird PyV types. The other four clades, *Orthopolyomavirus-I* and *–II*, *Wukipolyomavirus* and *Malawipolyomavirus* consist of mammalian species. HPyVs are shown in red. The bar indicates the number of substitutions per site. Numbers at branching events represent probability support values ranging from 0 (no support) to 1 (best support). Only probability support values lower than 1 are shown.



relationships among polyomaviruses, based on the amino acid sequence of LT (5). This resulted in the delineation of four genera: *Alphapolyomavirus*, *Betapolyomavirus*, *Gammapolyomavirus* and *Deltapolyomavirus* (Figure 5) (5, 13). Which coincide with the Orthopolyomavirus-I, -II, Avipolyomavirus and Malawipolyomavirus clades shown in Figure 4. LT amino acid sequences were used instead of whole genome analyses, in order to avoid the confounding effect of recombination that has occurred between early and late regions of the genome in some mammalian lineages.

Seroprevalence and antigenicity of human polyomaviruses

HPyVs are ubiquitous with varying seroprevalence rates (Table 2).Typically, primary infection occurs during early childhood and seroprevalence increases with age (26-30). Most seroepidemiological studies have been performed using VP1-directed IgG seroresponses, as VP1 is the immunodominant major capsid protein

Figure 5. Phylogenetic relationships of polyomaviruses based on conserved amino acid blocks of the LTAg coding sequence. PyVs are denoted by species names and Genbank accession numbers, and their genome sizes are given on the right-hand side of the figure. They are grouped into genera by colouring. For phylogenetic analyses, the recommendations were followed that have been published previously (13). Bayesian Monte Carlo Markov chain analyses were performed to generate a maximum clade credibility tree whose topology was essentially similar to the topology of the maximum likelihood tree presented in this figure. Grey branches are relatively weakly supported, with Shimodaira-Hasegawa-like approximate likelihood ratio test (SH-like aLRT) values <0.95 and/or posterior probability <0.95.

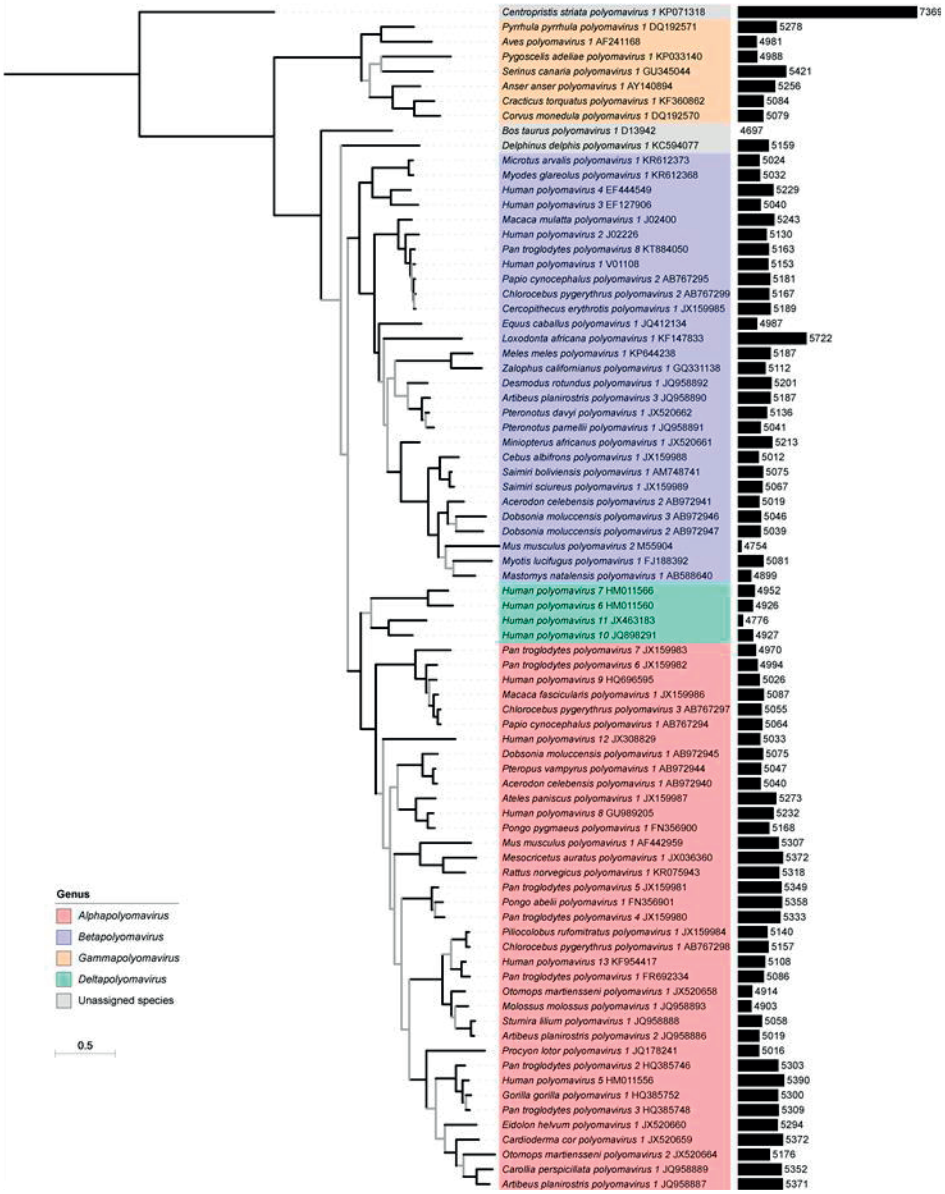


Table 2. Human polyomaviruses summary information with proven and possible disease associations

Name	Abbreviation	Year identified	Polyomavirus genus*	Seroprevalence	Associated disease
BK polyomavirus	BKPyV	1971[40]	Ortho-II / Betapolyomavirus	80 – 100% [26, 27, 32, 37, 50-52]	BKPyV-associated nephropathy, hemorrhagic cystitis, urethral stenosis, progressive multifocal leukoencephalopathy, meningitis / encephalitis, retinitis, pneumonitis, prostate cancer, HIV-associated salivary gland disease, renal carcinoma [20, 28, 44, 47, 53-59]
JC polyomavirus	JCPyV	1971[41]	Ortho-II / Betapolyomavirus	40 – 80% [26, 27, 32, 37, 50-52]	Progressive multifocal leukoencephalopathy, JC-associated nephropathy [42, 43, 45, 60-63]
Karolinska Institute polyomavirus	KIPyV	2007 [64]	Wuki / Betapolyomavirus	60 – 90% [26, 27, 37, 65, 66]	Respiratory disease [67-69]
Washington University polyomavirus	WUPyV	2007[70]	Wuki / Betapolyomavirus	40 – 95% [26, 27, 37, 65, 66]	Respiratory disease [67, 68, 71]
Merkel cell polyomavirus	MCPyV	2008 [72]	Ortho-1 / Alphapolyomavirus	40 – 80% [26, 27, 29, 30, 51, 65, 73, 74]	Merkel cell carcinoma [72]
Human polyomavirus 6	HPyV6	2010 [75]	Wuki / Deltapolyomavirus	70 – 85% [26, 29, 30, 75]	Keratoacanthoma, Kimura disease, pruritic and dyskeratotic dermatosis [76-79]
Human polyomavirus 7	HPyV7	2010 [75]	Wuki / Deltapolyomavirus	35 – 65% [26, 29, 30, 75]	Pruritic and dyskeratotic dermatosis, thymomas [77, 80, 81]
Trichodysplasia spinulosa-associated polyomavirus	TSPyV	2010 [82]	Ortho-1 / Alphapolyomavirus	70 – 85% [26, 29, 30, 83, 84]	Trichodysplasia spinulosa [82, 85]
Human polyomavirus 9	HPyV9	2011 [86, 87]	Ortho-1 / Alphapolyomavirus	20 – 50% [26, 29, 30, 88, 89]	Unknown
Malawi polyomavirus, Human polyomavirus 10, Mexican polyomavirus	MWPyV. HPyV10, MXPyV	2012 [90-92]	Malawi / Deltapolyomavirus	40 – 99% [26, 93, 94]	Unknown
St Louis polyomavirus	STLPyV	2012 [95]	Malawi / Deltapolyomavirus	70-93% [96, 97]	Unknown
Human polyomavirus 12	HPyV12	2013 [98]	Ortho-1 / Alphapolyomavirus	20-97% [96, 98, 99]	Unknown
New Jersey polyomavirus	NJPyV	2014 [100]	Ortho-1 / Alphapolyomavirus	0 – 58% [31, 96]	Retinal blindness, vasculitic myopathy [100]

*Genus delineation according to https://www.ncbi.nlm.nih.gov/pubmed/23255626, https://www.ncbi.nlm.nih.gov/pubmed/26923930

(14). VP1 is the main viral protein exposed on the outside of the virion. Therefore it determines the antigenicity and is responsible for attachment of the virus to host cell receptors that aid in entry into the host cell (Figure 3).

Coinfection with multiple HPyVs is common (26). For example, a healthy Dutch blood donor is persistently infected with an average of nine HPyVs (31). Cross-reactivity between HPyV capsid and nonstructural proteins seems likely due to the high amino acid sequence similarity between the different HPyVs, but only occurs between HpyV6 and HpyV7 (27, 30-33). VP1 shows extended and structurally variable surface loops that emanate from a conserved b-sheet core structure. These surfaces loops are referred to as the BC-, DE-, EF-, GH and HI-loop (34). The surface-exposed BC-loop is highly antigenic, and it is markedly divergent in the VP1 proteins of PyVs, which may explain why little or no serological cross-reactivity is observed.

Most seroepidemiological studies have been conducted with recombinant VP1 expressed as (GST-fusion) protein forming monomers, pentamers or virus-like particles (VLPs). Two of the most commonly used methods to express VP1 antigens are based on generation of glutathione-S-transferase (GST)-HPyV VP1 fusion proteins or VP1 VLPs (4, 32, 35). Both antigen preparations can be bound to Luminex beads or ELISA plates (26, 27, 29, 30, 36). The assays may also be used to discriminate genotypes of a particular HPyV species, as has been shown for BK polyomavirus (BKPyV) and Merkel cell polyomavirus (MCPyV) antigenic variants that are also designated serotypes (27, 37-39).

Human polyomaviruses and disease

It is remarkable that the first isolations of the two HPyVs were reported simultaneously in *The Lancet* with the work of each group, unknown to the other (40, 41). Both viruses were discovered in 1971, and the names of the viruses were derived from the initials of the patients were they originated from, B.K. and J.C. respectively. BKPyV and JCPyV are both associated with severe disease in immunocompromised patients. The role of BKPyV in BKPyV-associated nephropathy (BKPyVAN) in patients after kidney transplantation (KTx) and hemorrhagic cystitis in patients after hematopoietic stem cell transplantation, and the role of JCPyV in progressive multifocal leukoencephalopathy in AIDS patients and in patients treated with the monoclonal antibody nataluzimab for multiple sclerosis, is well documented (20, 28, 42-47). BKPyV has also been suggested to drive tumorigenesis in a small subset of bladder cancers (48). The other disease associations of BKPyV and JCPyV (Table 2) are less common and lack convincing evidence (42, 47, 49).

BKPyV and JCPyV were the only two known HPyVs for a few decades, but since 2007 large scale molecular screening techniques have led to the identification of eleven new HPyVs (4, 11, 31).

In 2007, two new HPyVs were reported that were both recovered from respiratory tract samples from subjects with (acute) respiratory tract disease (64, 70). They were named after the institute were they were isolated, namely Karolinska Institute polyomavirus (KIPyV) and Washington University polyomavirus (WUPyV). Their pathogenicity remains unclear, although some reports showed evidence for KIPyV and WUPyV as causative agents of respiratory infections (64, 66, 68, 70). After 2017 more HPyVs were discovered and implicated with disease, such as the trichodysplasia spinulosa polyomavirus (TSPyV) with a dysplastic hair follicle disorder; and Merkel cell polyomavirus (MCPyV) with Merkel cell carcinoma, a highly aggressive neuroendocrine skin tumour (30, 72, 101, 102). HPyV6 and HPyV7 have been associated with pruritic and dyskeratotic dermatosis (77), and HPyV7 might be involved in thymomagenesis (81, 103). Other HPyVs like HPyV9, Malawi polyomavirus (MWPyV), St. Louis polyomavirus (STLPyV) and HPyV12 have not yet been associated with disease (87, 91, 95, 98). The thirteenth HPyV described in 2014 in a pancreatic transplant recipient who had been evacuated through floodwaters during superstorm Sandy, was New Jersey polyomavirus (NJPyV) that has been associated with vasculitic myositis and retinal blindness (100). However, it is questionable if NJPyV really represents a HPyV or is rather a zoonotic PyV that was introduced into man under exceptional conditions, as indicated by the lack of detectable human seroresponses (31).

Despite their name, indicating a role in tumorigenesis, only one other (nonhuman) polyomavirus than MCPyV, raccoon PyV, causes cancer in its natural host (104). However some HPyVs, like BKPyV and JCPyV have been suggested to be involved in development of human cancer (48, 56, 58, 60, 105-107).

Although HPyV infection is widespread and the associated pathology diverse, symptomatic or manifest HPyV infections are usually limited to the immunocompromised and the elderly. The following thesis will focus on BKPyV-associated disease in KTx recipients (KTRs).

Kidney transplantation

The history of KTx began in Vienna in 1902, when the Austrian surgeon Emerich Ullman, successfully performed an experimental auto-transplantation of a dog kidney from its normal position to the vessels of the neck (108, 109). The organ

produced urine for a couple of days until the dog died. In 1933, after several years of experimentation, the Ukrainian surgeon Yurii Voronoy performed the first human deceased donor KTx in a young woman who had acute renal failure due to mercury poisoning, by anastomosing the renal vessels to the right femoral vessels. However, due to a blood group mismatch the kidney never functioned, and the recipient died after two days (108, 109).

The first temporarily successful human KTx was performed in 1953 in Paris by surgeon Jean Hamburger, where a 16-year old boy received a living donor transplantation from his mother that was rejected after three weeks. The real milestone of KTx history however, took place at the Peter Bent Brigham Hospital in Boston, Massachusetts, in 1954. Joseph Murray (Figure 6) transplanted a kidney between two monozygotic twin brothers, and the recipient and its allograft survived for eight years (108, 109). In 1990, Murray was honored with the Nobel Prize for this important contribution to the field of organ transplantation.

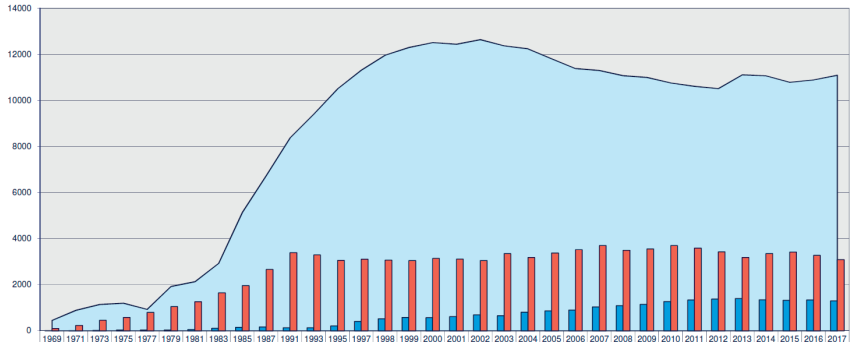
In the Netherlands, the first successful KTx was performed in Leiden in 1966 by surgeon Hans Terpstra together with the help of immunologist Jon van Rood (Figure 6) (110). Jon van Rood later established Eurotransplant, a nonprofit organization that facilitates patient-orientated allocation and cross-border exchange of deceased donor-organs between eight European countries (110). The process of KTx has been revolutionized since then, and the introduction of immunosuppressive medication, among other things, has made renal allografts a viable clinical option (111).

Figure 6. Joseph Murray (*left*), Hans Terpstra (*middle*), and Jon van Rood (*right*).

However, despite the progress made during the last decades, the KTx process still has many challenges. For instance, the KTR population continues to increase and a growing number of patients with kidney failure are on the waiting list to receive a donor kidney (Figure 7) (109, 112-114).

For patients with end-stage renal disease, KTx is often the best treatment option because it improves quality of life, prolongs survival and is cost-effective (113). However, the shortage of (registered) organ donors combined with the exponential increase in the number of patients with kidney failure in need of a donated organ (Figure 7), has led to deaths of patients on the KTx waiting list (109). To address the shortage of (deceased) organ donations in the Netherlands a new law is expected to be implemented in 2020 that would make all adults in the Netherlands organ donors, unless they opt out of the system.

Figure 7. Dynamics of the Eurotransplant kidney waiting list and transplants between 1969 and 2017. The eight countries cooperating with Eurotransplant are Austria, Belgium, Croatia, Germany, Hungary, Luxembourg, the Netherlands and Slovenia.



	1969	1971	1973	1975	1977	1979	1981	1983	1985	1987	1991	1993	1995	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017
Kidney waiting list	450	892	1147	1200	934	1929	2135	2928	5150	6740	8376	9418	10510	11324	11975	12313	12524	12450	12653	12382	12251	11814	11393	11081	10821	11010	10768	10622	10525	11120	11080	10797	10901	11105
Living donor transplants	9	5	11	33	33	33	53	108	150	161	129	127	212	411	526	579	569	617	697	655	803	867	901	1032	1091	1150	1266	1339	1381	1403	1347	1323	1339	1293
Deceased donor transplants	102	228	454	583	800	1050	1263	1645	1965	2665	3395	3293	3064	3110	3068	3050	3145	3121	3047	3352	3183	3383	3518	3703	3492	3561	3705	3585	3432	3183	3348	3423	3278	3093

Currently, ~75.000 KTx are performed per year worldwide. In the Netherlands during the past 25 years, the number of kidney transplantations increased from about 400 to around 1000 per year. During this period the number of KTx from deceased donors remained stable and therefore the increase is primarily due to an increase in KTx from living donors. Currently in the Netherlands, in contrast to most other European countries, >50% of KTx are from living donors. This is important as the source of a KTx greatly affects long-term graft survival (115). The outcome of a living KTx is superior with a life expectancy of the allograft of 20-25 year versus around 10 years in the case of a deceased donor KTx (115). Another

important advantage of KTx is that the quality of life of a patient after KTx is better than during dialysis (115-117).

However, when the kidney allograft stops functioning, a patient will be dependent from dialysis again and needs to be replaced on the KTx waiting list. Therefore, besides the problem of a shortage in donated kidney organs, it is of utmost importance to improve the life expectancy of the available donated kidneys that are essential for the KTRs quality of life and their life expectancy.

The main challenges for extending the life expectancy of the kidney allograft and its recipient are the management of chronic and acute immune-mediated rejection, nephrotoxicity from immunosuppressants and other drugs, the elevated risk for malignancies and heart and vascular disease after KTx, and the control of opportunistic infections. Currently, the most challenging opportunistic infection in the KTR population is caused by BKPyV, and BKPyVAN represents one of the major causes of graft dysfunction and loss in KTRs (10, 15, 20, 28, 46, 47, 118-120).

BK polyomavirus; discovery and epidemiology

BKPyV was first isolated in 1971 from a Sudanese KTR who had received a KTx from his brother (40). The patient urine was investigated due to an ureteric obstruction and the containment of many inclusion-bearing cells. Electron microscopic examination from the high speed urine pellet showed very large numbers of papovavirus-like particles. Ultrathin sectioning of the cells in a subsequent sample showed many virus particles within enlarged nuclei (121). The virus was named after the initials of the patient (B.K.) and the ICTV later designated the virus as BK polyomavirus, abbreviated as BKPyV (5, 40).

Many aspects of the epidemiology of BKPyV in the general population, such as the source of infectious virus, the route of natural transmission, and the site of initial virus replication are still unknown (20, 122). The tonsils have been indicated as site of initial replication due to the presence of BKPyV DNA in tonsillar tissue, but there have been conflicting reports and other routes of transmission have also been suggested (10, 20, 123-126).

BKPyV causes asymptomatic infection early in life (15, 20), reaching a seroprevalence of ~90% in adults. During primary infection a period of viremia has to occur as the virus thereafter persists in the urothelium and renal tubular cells for life (127, 128). After primary infection, small amounts of viral progeny can be

temporarily detected in urine of 7-55% of healthy individuals, depending on the sampling frequency (50, 129, 130).

BK polyomavirus infection after kidney transplantation

In 1995, 24 years after the discovery of the virus, BKPyV was described as cause of nephropathy in a kidney allograft for the first time (102). BKPyV-DNA is detected in urine (viruria) or blood (viremia) in approximately 50% and 20-30% of all KTRs in the months following transplantation (10, 46, 120, 131-133). BKPyVAN generally develops in 1-10% of KTRs, usually in those with sustained viremia and viral DNA-loads above 10^4 genome copies/ml (15, 20, 46, 133, 134).

The kidney allograft has been suggested as the potential source of BKPyV infection as recipients of other solid organ transplants, HIV patients and hematopoietic stem cell transplantation recipients that often receive even higher immunosuppression hardly ever develop BKPyVAN (135, 136). Furthermore, in KTRs with BKPyVAN that undergone allograft nephrectomy, plasma BKPyV loads dropped rapidly indicating the allograft as the origin of viral replication (137).

BKPyVAN is now one of the major causes of graft dysfunction and loss in KTRs, and due to the continuous increase of the KTR population worldwide it is a serious problem in transplantation medicine. Despite the clinical need, BKPyV-specific antiviral drugs are not available, and reduction of immunosuppression is the only effective evidence-based treatment so far (133, 138-140).

To prevent BKPyVAN or progression of BKPyVAN, current guidelines recommend regular screening to detect BKPyV viremia of KTRs at least monthly for the first 3-6 months after transplantation, then every 3 months until the end of the first post-transplant year, whenever there is an unexplained rise in serum creatinine, and after treatment for acute rejection (141). This regular screening for BKPyV viremia in KTRs is performed to guide timely reduction of immunosuppression if the BKPyV plasma load is greater than 10.000 copies/ml (46, 120, 133, 139, 141), which improves BKPyV immunity, but at the same time increases the risk of acute rejection (133, 139, 140). This paradox makes management of BKPyV infection challenging for transplantation physicians. Clinical guidelines for the treatment of KTRs (KDIGO) suggest the use of intensive immunosuppression during the initial stages of the process, followed by a diminished dose of immunosuppressants by 2-4 months after transplantation, if there are no signs of acute rejection (38). However, due to the delayed nature of the current pre-emptive BKPyV screening strategy in KTRs, which does not fully eliminate the risk of BKPyVAN and can

increase the risk of donor-specific antibodies (DSA), graft rejection, and death, there is an urgent need for reliable pretransplantation predictive markers that can identify KTRs at risk. Such a predictive marker could ideally be used to clinically stratify and tailor the currently employed BKPyV screening and treatment strategies.

Risk factors for BK polyomavirus infection after kidney transplantation

The overall degree of immunosuppression is thought to be the largest factor promoting BKPyV infection after KTx. Immunosuppressive treatment with tacrolimus and rejection-treatment with prednisolone have been shown to increase the risk of BKPyVAN (20, 46, 120, 142). Despite intensive study, pretransplantation risk factors for BKPyV viremia and BKPyVAN including age, gender, ethnicity, retransplantation, immunosuppressive regimen, ischemia-reperfusion injury, prior acute rejection episodes, corticosteroid therapy, percentage of panel reactive antibodies (PRA), HLA mismatches, blood group incompatibility, underlying conditions and comorbidities have not been identified (20, 46, 120, 131, 132, 135, 143). Also the role that individual BKPyV genotypes play in the risk and course of BKPyV infection and BKPyVAN after KTx is unknown. A number of studies, however, reported associations between recipient BKPyV infection and pretransplantation BKPyV-serostatus (seropositive or seronegative) of kidney transplant donors and recipients (135, 144, 145).

Thus, it is evident that the degree of immunosuppression plays an important role in the pathogenesis of BKPyV infection after KTx. However, although several studies suggest a correlation between donor and/or recipient serostatus and the development of BKPyV viremia, other specific risk factors for BKPyV related complications, either viral, donor, recipient or transplantation related, remain to be established.

Outline of this thesis

The aim of the research described in this thesis was to obtain more insight in the risk factors of BKPyV infection after KTx, with special emphasis on pretransplantation related risk factors. Both donor and recipient factors, such as BKPyV serostatus, BKPyV seroreactivity or HLA composition, as well as viral factors, including BKPyV genotype were investigated. The ultimate goal was to identify reliable predictive markers of BKPyV infection after KTx, thereby providing opportunities to optimize and ideally personalize the currently recommended suboptimal BKPyV screening strategy.

In **Chapter Two** the correlation between pretransplantation donor-recipient pair seroreactivity against BKPyV and development of BKPyV viremia and BKPyVAN after KTx is described.

In **Chapter Three** the stability of BKPyV seroreactivity in KTRs and healthy blood donors, and the correlation of BKPyV seroreactivity with preceding viremia in KTRs is described.

In **Chapter Four** the reduced risk of BKPyV infection in HLA-B51 positive recipients after KTx is described.

Chapter Five describes the development and evaluation of a Luminex bead-based multiplex immunoassay for BKPyV serotyping.

In **Chapter Six** the application of the Luminex bead-based multiplex immunoassay for BKPyV serotyping is described in a cohort of KTx donor-recipient pairs.

In the **General Discussion** implications for prediction of BKPyV infections in recipients after KTx, as well as suggestions for further research are described.

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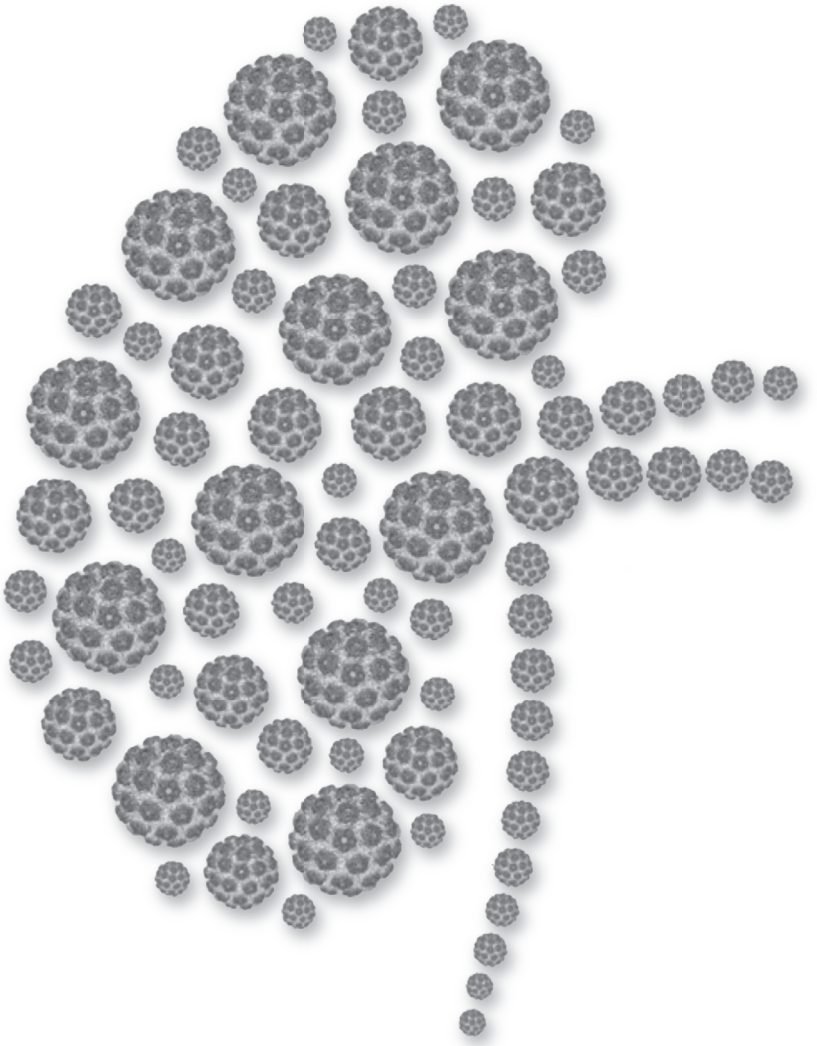

Chapter 2

Pretransplantation donor-recipient pair seroreactivity against BK polyomavirus predicts viremia and nephropathy after kidney transplantation

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Abstract

Kidney transplant donors are currently not implicated in predicting BK polyomavirus (BKPyV) infection in kidney transplant recipients. It has been postulated, however, that BKPyV infection originates from the kidney allograft. Because BKPyV seroreactivity correlates with BKPyV replication and, therefore, might mirror the infectious load, we investigated whether BKPyV seroreactivity of the donor predicts viremia and BKPyV-associated nephropathy (BKPyVAN) in the recipient. In a retrospective cohort of 407 living kidney donor-recipient pairs pretransplantation donor and recipient sera were tested for BKPyV IgG-levels and correlated with the occurrence of recipient BKPyV viremia and BKPyVAN within one year posttransplantation. Donor BKPyV IgG-level was strongly associated with BKPyV viremia and BKPyVAN ($p < 0.001$), while recipient BKPyV seroreactivity showed a nonsignificant inverse trend. Pairing of high-BKPyV-seroreactive donors with low-seroreactive recipients resulted in a 10-fold increased risk of BKPyV viremia (hazard ratio 10.1, 95% CI 3.5-29.0, $p < 0.001$). In multivariate analysis, donor BKPyV seroreactivity was the strongest pretransplantation factor associated with viremia ($p < 0.001$) and BKPyVAN ($p = 0.007$). The proportional relation between donor BKPyV seroreactivity and recipient infection suggests that donor BKPyV seroreactivity reflects the infectious load of the kidney allograft, and calls for the use of pretransplantation BKPyV serological testing of (potential) donors and recipients.

Introduction

Solid organ transplant recipients require immunosuppression to prevent allograft rejection. This renders them vulnerable to exogenous and endogenous viral infections (reactivation). In the latter, particularly the ubiquitous herpes- and polyomaviruses are involved. Currently, no method exists to reliably predict these infectious complications, and therefore general and frequent blood viral load monitoring in recipients after transplantation is recommended.

BK polyomavirus (BKPyV) causes asymptomatic infection early in life (1,2), reaching a seroprevalence of ~90% in adults (3,4). After primary infection, BKPyV latently persists in the urothelium and renal tubular cells (5,6), and small amounts of viral progeny can be temporarily detected in urine of 7-55% of healthy individuals, depending on the sampling frequency (7-9).

In immunocompromised patients, BKPyV infections can cause manifest disease, such as hemorrhagic cystitis in hematopoietic stem cell transplant recipients and BKPyV-associated nephropathy (BKPyVAN) in kidney transplant recipients (1,2,10). Reduction of immunosuppressive therapy is the only effective evidence-based treatment so far (11,12).

BKPyV infection is observed in approximately half of kidney transplant recipients by detection of BKPyV-DNA in urine (viruria) (10,12-15). In a subset of viruric recipients, 15-30% of the total number of recipients, viral DNA is detected in the circulation (viremia), of which a small proportion, 1-10% of total, develops BKPyVAN ultimately causing allograft failure (10,12-16). Sustained viremia and BKPyV-loads above 10^4 genome copies/ml (c/ml) are associated with BKPyVAN development (1,2,10). In order to identify this subgroup of recipients at risk that require tapering of immunosuppression (14,17), currently in most kidney transplantation centers recipients are regularly evaluated for detectable BKPyV-DNA in blood (10-12,16).

Immunosuppressive treatment with tacrolimus and rejection-treatment with prednisolone have been shown to increase the risk of BKPyVAN (2,10,14,18). Despite intensive study, pretransplantation risk factors for BKPyV viremia and BKPyVAN including age, gender, ethnicity, retransplantation, immunosuppressive regimen, ischemia-reperfusion injury, prior acute rejection episodes, corticosteroid therapy, percentage of panel reactive antibodies (PRA), HLA mismatches, blood group incompatibility, underlying conditions and comorbidities have not been

identified (2,10,13-15,19,20). A number of studies, however, reported associations between recipient BKPyV infection and pretransplantation BKPyV-serostatus (seropositive or seronegative) of kidney transplant donors and recipients (19,21,22). We considered the donor of particular interest in this regard, since BKPyV infection in recipients is thought to originate from the kidney allograft (19,23).

Based on previous studies suggesting that BKPyV seroreactivity is associated with BKPyV-replication (21,24,25), we hypothesized that the level of donor BKPyV seroreactivity reflects the BKPyV infectious load of the allograft, and thereby predicts BKPyV viremia and BKPyVAN in the recipient. To investigate this hypothesis, living kidney allograft donor-recipient pairs were analyzed for BKPyV seroreactivity pretransplantation. Measured pretransplantation levels of BKPyV IgG of donors and recipients were correlated with the incidence of BKPyV viremia and BKPyVAN, and compared with other potentially relevant baseline donor, recipient and transplant-related characteristics.

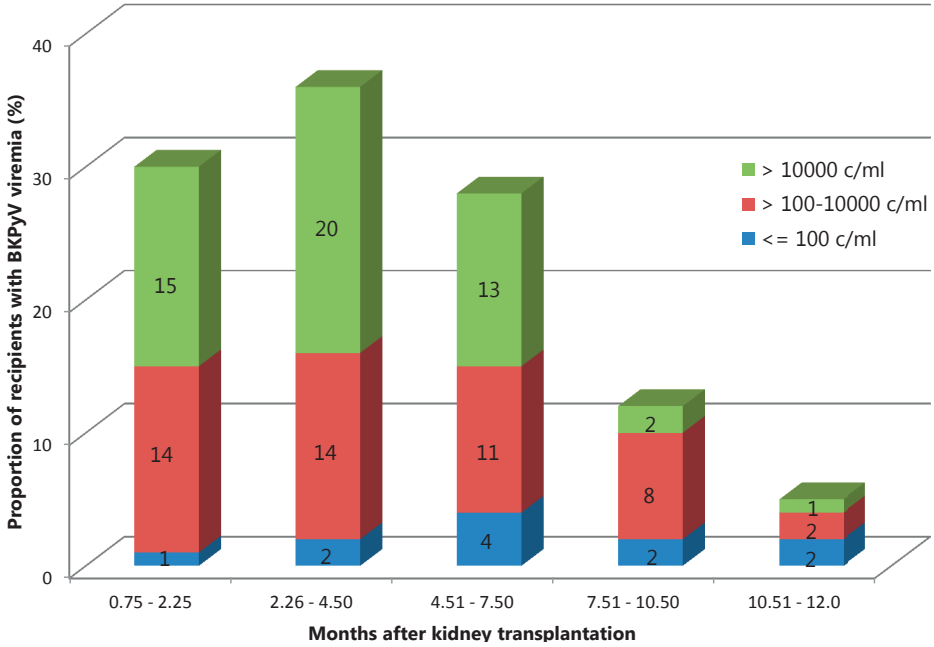
Materials and Methods

Study population and sample collection

To ensure availability of pretransplantation donor and recipient sera, only living kidney allograft donor-recipient pairs were included. All adult (> 18 years of age) living donor-recipient pairs transplanted at the Leiden University Medical Center (LUMC) between 2003 and 2013 were eligible for this retrospective cohort study. In total, 519 living donor-recipient pairs were identified. Fifty-three pairs were excluded because no baseline serum sample was available from either donor or recipient; another 59 because less than two recipient plasma samples collected after transplantation were available for analysis (Figure S1). The remaining 407 donor-recipient pairs were included in the study.

Baseline donor and recipient sera were collected at a mean period of 5.5 months (range 0.7-26.8) and 0.2 months (range 0-3.7) pretransplantation, respectively. Recipient plasmas screened for BKPyV-DNA were collected at five regular time-points posttransplantation (Figure 1). The mean follow-up was 9.1 months and 80%, 95%, 87%, 63% and 36% of the recipient serum samples were available at time point 1, 2, 3, 4 and 5, respectively. The median number of time-points analyzed per recipient was 3.6. All samples were originally collected for routine serological and molecular virus-screening and stored at -20˚C. The study protocol was submitted to the Medical Ethical Committee of the LUMC that decided formal approval

Figure 1. Characteristics of BKPyV viremia in viremic recipients (n = 111).



The time-points (ranges) of first detection of BKPyV viremia after kidney transplantation are indicated in months after transplantation, as well as the height of the measured BKPyV-loads in copies/ml. BKPyV, BK polyomavirus; c/ml, copies/ml.

was not needed, due to the retrospective study design and the use of previously collected and anonymized samples.

Detection of BKPyV viremia and assessment of BKPyVAN

To measure the presence of BKPyV-DNA in blood, blood plasma was analyzed by quantitative BKPyV real-time polymerase chain reaction (PCR). Using the primers 440BKVs 5'-GAAAAGGAGAGTGTCCAGGG-3' and 441BKVas 5'-GAACTTC-TACTCCTCCTTTTATTAGT-3' and a Taqman (Thermo Fisher Scientific, Waltham, MA) probe 576BKV-TQ-FAM FAM 5'-CCAAAAAGCCAAAGGAACCC-3'-BHQ1, a 90-bp fragment within the BKPyV VP1 gene is amplified. Simultaneous isolation, amplification, and detection of a standard amount of phocid herpesvirus were used for internal control of inhibition (26).

Routine recipient BKPyV-load screening at 1.5, 3 and 6 months posttransplantation was implemented in May 2007. In case of clinical suspicion of BKPyV infection, BKPyV-loads were also determined later than 6 months posttransplantation.

In samples obtained before 2007 and in samples obtained after 2007 that had not been routinely analyzed, BKPyV-loads were determined in retrospect.

Sustained BKPyV viremia was defined as ≥ 2 consecutive BKPyV-positive samples spanning ≥ 3 weeks. Peak viral load was defined as the highest BKPyV-DNA plasma load measured in a viremic subject during follow-up.

A kidney biopsy was performed if clinically indicated in the view of the treating physician. BKPyVAN was diagnosed based on immunohistological examination of allograft biopsy specimens showing characteristic pathological features, such as intranuclear viral inclusions in tubular epithelial cells, cell enlargement with polymorphic nuclei, interstitial inflammation and tubular atrophy or fibrosis. BKPyVAN diagnosis was confirmed by immunohistochemical staining with a PyV-cross-reacting mouse monoclonal antibody (PAb416, Calbiochem) raised against large T antigen of SV40 polyomavirus (SV40).

BKPyV serology

Pretransplantation serum samples obtained from 407 donor-recipient pairs, 814 in total, were analyzed by an in-house Luminex immunoassay detecting IgG-reactivity against the BKPyV-genotype Ib1 major viral capsid protein 1 (VP1), according to a published protocol (4,27). This protocol has been used to analyze seroresponses against various human polyomaviruses (4). In brief, 1:100 diluted serum samples were mixed with affinity purified glutathione S-transferase (GST) BKPyV VP1 fusion protein or with GST alone coupled with fluorescent, unique colored polystyrene beads (Bio-Rad). VP1-bound antibodies were detected with biotinylated goat antihuman IgG (H+L; Jackson Immuno Research, West Grove, PA, USA) and streptavidin-R-phycoerythrin (Invitrogen). The bead colors and the phycoerythrin signal were analyzed in a Bio-plex 100 Analyzer (Bio-Rad) and expressed as median fluorescent intensity (MFI). MFI values obtained with GST alone were subtracted to obtain BKPyV VP1 specific signals. The cut-off value to determine BKPyV-seropositivity was based on sera of healthy children aged 10-15 months old, as described (4,27).

A serially diluted control serum was included on each plate to control for inter-plate ($n = 10$) test variance. A high agreement was observed between the test plates (r 0.963 – 0.999, $p < 0.001$). Good intertest reproducibility of the assay was previously shown for trichodysplasia spinulosa-associated polyomavirus in a group of 80 kidney transplant recipients (28), and was also calculated in these recipients for BKPyV (r 0.891, $p < 0.001$).

Of the 407 donor samples included in the current study, 396 (97.3%) were independently reanalyzed for serological confirmation with BKPyV VP1 virus-like particles (VLP) by enzyme-linked immunosorbent assay (ELISA), as described (29-31). The VP1 antigen of this assay was obtained from BKPyV-genotype Ib2 (29-31), that differs by five amino acids from Ib1 (data not shown).

Immunosuppression, rejection treatment and management of BKPyV infection

Induction treatment consisted of basiliximab (93%) or alemtuzumab (7%), and the standard maintenance immunosuppressive regimen included a calcineurin-inhibitor (CNI), tacrolimus (76%) or cyclosporin A (24%), combined with corticosteroids (100%) and mycophenolate mofetil (MMF) (99.5%), azathioprine (0.25%), or everolimus (0.25%).

The targeted 12-hour area under the curve (AUC) of the CNI in the first weeks after transplantation was 160-200 µg*h/l for tacrolimus and 4500-5500 µg*h/l for cyclosporin A. The dose of the CNI was tapered 6 weeks after transplantation to targeted 12-hours AUCs of 80-100 µg*h/l and 3000-3500 µg*h/l, respectively. Rejection treatment consisted of methylprednisolone 1000 mg intravenously once daily for 3 days.

In case of a positive BKPyV-load, since 2007, a monthly screening interval was implemented until the BKPyV-PCR was negative. In case of a BKPyV-load $< 10^4$ c/ml, MMF was reduced by 50% and CNI serum levels were evaluated and if needed adjusted accordingly. If tacrolimus was used, prednisolone was lowered to 5 mg/day and in the case of cyclosporin A, prednisolone was lowered to 7.5 mg/day. Detection of a BKPyV-load $\geq 10^4$ c/ml prompted adjustment of the immunosuppressive regimen by 50% reduction of the CNI, reduction of m-TOR inhibitor, and 50% reduction or cessation of MMF.

Statistical analyses

Data were analyzed with IBM SPSS Statistics software version 20. Descriptive analyses were used to report cohort characteristics. Differences between viremic and non-viremic recipients and viremic recipients with or without BKPyVAN were assessed using the Chi-Square test, Fisher's exact test, Student's *t*-test or Mann-Whitney U test as appropriate. To indicate onset of recipient BKPyV viremia, separate Kaplan-Meier curves were generated according to donor and recipient

BKPyV seroreactivity groups, measured pretransplantation. Association between baseline donor and recipient BKPyV seroreactivity groups and the combination of both with onset of posttransplantation recipient BKPyV viremia was determined by Cox regression. Uni- and multivariate Cox regressions were performed to determine which additional baseline covariates affected development of BKPyV viremia and BKPyVAN. Chi-Square test, Fisher's Exact test or Mann-Whitney U test were used for evaluating differences of BKPyV viremia characteristics between viremic recipients with and without BKPyVAN. For all performed tests a p-value < 0·05 in a two-sided test was considered statistically significant.

Results

In total, 111 of 407 recipients (27%) became viremic during follow-up (Table 1, Figure S1), the majority within 6 months posttransplantation (Figure 1), 87 of them (79%) with sustained viremia (Figure S1). The median peak viral load was 6.9×10^3 c/ml (interquartile range 8.8×10^2 - 4.2×10^5 c/ml). Peak viral loads > 10^4 c/ml were particularly prevalent among recipients that developed viremia within the first 6 months after transplantation (Figure 1).

BKPyVAN was diagnosed in only 12 subjects (3%) (Table 1, Figure S1), probably because tapering of immunosuppression was installed upon detection of viremia. All recipients diagnosed with BKPyVAN had peak BKPyV-loads ≥ 10^4 c/ml (Table S1), and both peak BKPyV-load and AUC of BKPyV-load during follow-up were significantly associated with development of BKPyVAN ($p < 0.001$) (Table S1).

The incidence of BKPyV viremia and BKPyVAN during follow-up was compared with specific donor, recipient, and transplantation characteristics (Table 1). No significant differences were observed between viremic and non-viremic recipients with regard to any of the listed donor or recipient baseline characteristics, including underlying condition, immunosuppressive regime and PRA immunization. With respect to type of transplantation, BKPyV viremia was more common among recipients from unrelated donors (60% vs. 49%, $p = 0.035$). Blood group compatibility and HLA matching were not significantly different between viremic and non-viremic recipients. As anticipated, the use of tacrolimus (Table 1) and rejection treatment with prednisolone (Tables 1 and 5) were associated with development of BKPyVAN in our cohort.

Table 1. Donor, recipient and transplantation characteristics sorted for BKPyV viremia and BKPyVAN among 407 kidney transplantation recipients in the first year after kidney transplantation.

	All recipients (n = 407)			Viremic recipients (n = 111)		
	No BKPyV viremia (n = 296)	BKPyV viremia (n = 111)	p-value[1]	No BKPyVAN (n = 99)	BKPyVAN (n = 12)	p-value[1]
Donor						
Age (years)	53 (11.7)	54 (11.5)	0.354	54 (11.7)	57 (9.6)	0.386
Gender						
Male	119 (40%)	42 (38%)	0.664	37 (37%)	5 (42%)	0.763
Recipient						
Age (years)	50 (13.5)	53 (14.2)	0.080	53 (14.1)	53 (16.1)	0.790
Gender						
Male	177 (60%)	73 (66%)	0.271	65 (66%)	8 (67%)	1.000
Underlying condition[2]						
Inherited	72 (24%)	26 (23%)	0.239	22 (22%)	4 (33%)	0.411
Glomerular	80 (27%)	26 (23%)		23 (23%)	3 (25%)	
Vascular	55 (19%)	32 (29%)		31 (31%)	1 (8%)	
Obstructive	27 (9%)	7 (6%)		6 (6%)	1 (8%)	
Other	62 (21%)	20 (18%)		17 (17%)	3 (25%)	
Dialysis pretransplantation	182 (62%)	64 (58%)	0.482	57 (58%)	7 (58%)	1.000
Duration dialysis (months)	12 (18.4)	9 (12.1)	0.106	9 (11.6)	12 (15.9)	0.730
PRA pretransplantation						
Non-immunized[3]	284 (96%)	108 (97%)	0.768	97 (98%)	11 (92%)	0.293
Monoclonal antibody						
Basiliximab	277 (94%)	103 (93%)	0.776	92 (93%)	11 (92%)	1.000
Alemtuzumab	19 (6%)	8 (7%)		7 (7%)	1 (8%)	
Calcineurin inhibitor						
Cyclosporin A	70 (24%)	27 (24%)	0.887	27 (27%)	0 (0%)	0.037
Tacrolimus	226 (76%)	84 (76%)		72 (73%)	12 (100%)	
Proliferation inhibitor						
Azathioprine	0 (0%)	1 (<1%)	0.273	1 (1%)		1.000
Everolimus	1 (<1%)	0 (0%)	1.000	0 (0%)	0 (0|%)	n.p.
Mycophenolate mofetil	295 (100%)	110 (99%)	0.472	98 (99%)	0 (0%)	1.000
Corticosteroids	296 (100%)	111 (100%)	n.p.	99 (100%)	12 (100%)	n.p.
Rejection treatment[4]	61 (21%)	31 (28%)	0.116	22 (22%)	9 (75%)	<0.001
Transplantation						
Retransplantation	25 (8%)	11 (10%)	0.650	9 (9%)	2 (17%)	0.339
Year of transplantation						
Before 2007	43 (15%)	18 (16%)	0.671	18 (18%)	0 (0%)	0.209
2007 to 2013	253 (85%)	93 (84%)		81 (82%)	12 (100%)	
Unrelated donor	144 (49%)	67 (60%)	0.035	58 (59%)	9 (75%)	0.357

Table 1. (continued)

	All recipients (n = 407)			Viremic recipients (n = 111)		
	No BKPyV viremia (n = 296)	BKPyV viremia (n = 111)	p-value[1]	No BKPyVAN (n = 99)	BKPyVAN (n = 12)	p-value[1]
Blood group						
Compatible[5]	283 (96%)	104 (94%)	0.341	92 (93%)	12 (100%)	1.000
HLA mismatched						
A, B and DR loci[6]						
0	17 (6%)	6 (5%)	0.888	6 (6%)	0 (0%)	0.437
1-3	143 (48%)	51 (46%)		47 (48%)	4 (33%)	
4-6	136 (46%)	54 (49%)		46 (46%)	8 (67%)	

Data are shown as mean (SD) or n (%).
BKPyV, BK polyomavirus; BKPyVAN, BK polyomavirus-associated nephropathy; n.p; not possible; PRA, panel reactive antibody.
[1]The p-values were calculated using the Chi-Square test, Fisher's exact test or Student's *t*-test. A p-value <0.05 was considered statistically significant.
[2]Inherited diseases include autosomal dominant polycystic kidney disease, medullary cystic disease, cystic kidney disease not otherwise specified, arteriovenous malformation due to Klippel-Trénaunay-Weber syndrome, familiar erythrocyturia, Alport syndrome, familiar focal segmental glomerulosclerosis by NPHS2-mutation, familiar haemolytic uremic syndrome, and kidney dys- and agenesis; Glomerular diseases include membranous nephropathy, IgA nephropathy, systemic lupus erythematosus, proliferative glomerulonephritis, membranoproliferative glomerulonephritis, focal segmental glomerulosclerosis, pauci-immune crescentic glomerulonephritis, Morbus Wegener, ANCA-associated vasculitis, anti-glomerular basement membrane nephritis, global glomerulosclerosis, and immunotactoid glomerulonephritis; Vascular diseases include diabetes mellitus type I and II, hypertension, nephrosclerosis, haemolytic uremic syndrome, arteria renalis stenosis, and thrombotic microangiopathy; Obstructive diseases include reflux nephropathy, urethral valves, nephrolithiasis, obstructive uropathy, and prostate hypertrophy; Other include chronic pyelonephritis, acute tubular necrosis, tubulointerstitial nephritis, lithium nephropathy, urate and analgesic nephropathy, iatrogenic, and unknown underlying condition.
[3]Panel reactive antibody (PRA) immunization: non-immunized = PRA 0-5%, immunized = PRA 6-99%.
[4]Rejection treatment consisted of methylprednisolone 1000 mg intravenously once daily for three days.
[5]Blood group data of 1 donor-recipient pair is missing, the recipient was BKPyV viremia negative.
[6]HLA mismatched (A, B and DR loci) arranged in groups with no mismatches (completely matched), 1-3 mismatches (haplotype mismatched), and 4 or more mismatches (more than haplotype mismatch).

To investigate the association between BKPyV seroreactivity, BKPyV viremia and BKPyVAN during follow-up, baseline BKPyV VP1 IgG seroresponses were measured in both donors and recipients (Figure S2). In total, 389 (96%) of the donors, and 385 (95%) of the recipients were BKPyV-seropositive (Table 2). In line with the high seroprevalence in both groups, BKPyV-serostatus was not associated with BKPyV viremia and BKPyVAN, nor were specific donor-recipient serostatus combinations (Table 2). However, when the height of donor and recipient BKPyV IgG seroresponses were analyzed, either as a continuous variable or categorized in quartiles (Q1-Q4) (Figure S2), statistically significant associations were observed

between pretransplantation donor seroreactivity and posttransplantation recipient BKPyV viremia ($p < 0.001$ and $p < 0.001$, respectively) (Table 2) and BKPyVAN ($p < 0.001$ and $p = 0.013$, respectively) (Table 2). To illustrate, only 9 (8%) of the viremic recipients had a low seroreactive donor (Q1), whereas 51 (46%) had a high seroreactive donor (Q4) (Table 2). The same statistically significant trend was observed for BKPyVAN, just one (8%) occurred in recipients with an (intermediate) low seroreactive donor (Q1-Q2), while the majority (11 out of 12, 92%) developed

Table 2. Pretransplantation BKPyV-seropositivity and seroreactivity among kidney allograft donors and recipients, related to posttransplantation recipient BKPyV viremia and BKPyVAN.

	Recipients (n = 407)			Viremic recipients (n = 111)		
	No BKPyV viremia (n = 296)	BKPyV viremia (n = 111)	p-value[1]	No BKPyVAN (n = 99)	BKPyVAN (n = 12)	p-value[1]
Donor						
BKPyV seropositive	281 (95%)	108 (97%)	0.420	377 (95%)	12 (100%)	0.670
BKPyV seroreactivity	11511 (7371)	17200 (6605)	<0.001	12883 (7609)	18988 (4199)	<0.001
Seroreactivity quartile groups[2]						
Low (Q1)	93 (31%)	9 (8%)	<0.001	102 (26%)	0 (0%)	0.013
Low intermediate (Q2)	82 (28%)	21 (19%)		102 (26%)	1 (8%)	
High intermediate (Q3)	71 (24%)	30 (27%)		95 (24%)	6 (50%)	
High (Q40	50 (17%)	51 (46%)		96 (24%)	5 (42%)	
Recipient						
BKPyV seropositive	283 (96%)	102 (92%)	0.140	374 (95%)	11 (92%)	1.000
BKPyV seroreactivity	13774 (7834)	12342 (7956)	0.103	13422 (7901)	12119 (7492)	0.573
Seroreactivity quartile groups[2]						
Low (Q1)	68 (23%)	34 (31%)	0.219	98 (25%)	4 (33%)	0.977
Low intermediate (Q2)	76 (26%)	26 (23%)		99 (25%)	3 (25%)	
High intermediate (Q3)	72 (24%)	30 (27%)		99 (25%)	3 (25%)	
High (Q4)	80 (27%)	21 (19%)		99 (25%)	2 (17%)	
Donor/recipient pair						
BKPyV serostatus						
+/+	268 (91%)	100 (90%)	0.107	357 (90%)	11 (92%)	0.707
+/-	13 (4%)	8 (7%)		20 (5%)	1 (8%)	
-/+	15 (5%)	2 (2%)		17 (4%)	0 (0%)	
-/-	0 (0%)	1 (<1%)		1 (<1%)	0 (0%)	

Data are shown as mean (SD) or n (%).

+, BKPyV seropositive; -, BKPyV seronegative; BKPyV, BK polyomavirus; BKPyVAN, BK polyomavirus-associated nephropathy; Q, quartile.

[1]The p-values were calculated using the Chi-Square test, Fisher's exact test or Student's *t*-test. A p-value <0.05 was considered statistically significant.

[2]MFI distributions of the donor and recipient seroreactivity quartile groups can be found in the legend of Figure S2.

in recipients with an (intermediate) high seroreactive donor (Q3-Q4) (Table 2). In contrast, pretransplantation BKPyV seroreactivity of the recipient was not associated with viremia or BKPyVAN (Table 2). To confirm the associations observed for BKPyV seroreactivity, donor BKPyV IgG-levels were reassessed with ELISA by a different lab that generated comparable results (Figures S3A and B).

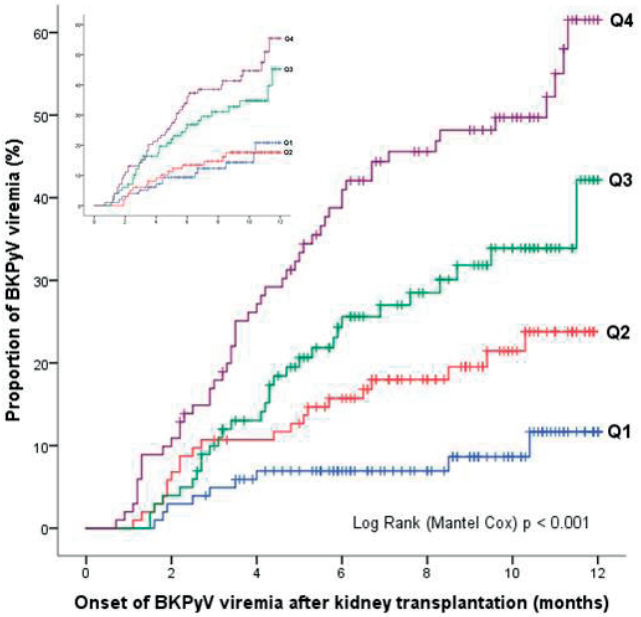
To further substantiate the observed association between pretransplantation donor BKPyV IgG-levels and posttransplantation recipient viremia, Kaplan-Meier curves were generated to compare the onset of BKPyV viremia stratified for baseline BKPyV seroreactivity quartiles of donors and recipients. Again a strong and highly significant correlation was observed between recipient viremia and donor BKPyV seroreactivity ($p < 0.001$) (Figure 2). The Kaplan-Meier curves based on the donor BKPyV seroreactivity results of the conformational ELISA showed the same effect ($p < 0.001$) (Figure 2, inset). For recipient BKPyV seroreactivity, a non-significant reverse trend was found (Figure S4A).

To estimate the risk indicated by baseline BKPyV seroreactivity, the hazard ratio (HR) for recipient viremia was calculated. With every 5000 MFI unit increase of donor seroreactivity, the HR increased with 1.59 (95% confidence interval [CI] 1.38-1.84, $p < 0.001$) (Table 3). In recipients from high BKPyV-seroreactive donors the HR was 6.92 (95% CI 3.41-14.06, $p < 0.001$) (Table 3). In high seroreactive recipients the risk of viremia tended to decrease (HR 0.57, 95% CI 0.33-0.98, $p = 0.041$) (Table 3).

Because opposite trends were observed for donor and recipient baseline BKPyV-seroreactivity, the interplay between these potentially predictive factors of post-transplantation BKPyV viremia was analyzed by calculating the BKPyV viremia risk for donor BKPyV-seroreactivity stratified by recipient seroreactivity. As shown in Figure S4B, a combination of these factors resulted in a substantially increased risk of BKPyV viremia in low BKPyV-seroreactive recipients receiving an allograft from high seroreactive donors (HR 10.07, 95% CI 3.50-28.96, $p < 0.001$) (Table 3).

Finally, Cox regression analyses were performed for the risk to develop BKPyV viremia after transplantation related to the identified serological risk factors and the cohort characteristics presented in Table 1. In the univariate analysis (Table 4), apart from donor BKPyV seroreactivity (HR 1.59, 95% CI 1.38-1.84, $p < 0.001$), only unrelatedness of the living donor (HR 1.49, 95% CI 1.02-2.17, $p = 0.042$) and rejection treatment (HR 1.54, 95% CI 1.02-2.34, $p = 0.040$) were associated with BK-PyV viremia. Recipient BKPyV seroreactivity did not reach statistical significance

Figure 2. Proportion of BKPyV viremia detected in the first year after kidney transplantation according to BKPyV-seroreactivity determined in donors.



Kaplan-Meier (1 - survival function) curves for proportion of BKPyV viremia observed in the recipients according to donor BKPyV seroreactivity quartile groups (shown in Figure S2). The inset shows the Kaplan-Meier (1 - survival function) curves for proportion of BKPyV viremia observed in the recipients according to donor BKPyV seroreactivity quartile groups determined with ELISA (Log Rank [Mantel Cox] $p < 0.001$). MFI distributions of the donor seroreactivity quartile groups are described in the legend of Figure S2. Tick marks represent censored recipients. Groups from the inset are divided by optical density values seroreactivity quartiles. Q1, low: 0 – 0.0150; Q2, low intermediate: 0.151 – 0.6105; Q3, high intermediate: 0.6106 – 1.2225; Q4, high: 1.2226 – 3.1180. Tick marks represent censored recipients. BKPyV, BK polyomavirus; Q, quartile.

(HR 0.90, 95% CI 0.80-1.02, p = 0.088). In line with the stratified analysis above, the multivariate analysis showed a significant protective effect of recipient BKPyV-seroreactivity (HR 0.84, 95% CI 0.75-0.95, p = 0.006) (Table 4). The effect of the unrelated donor (HR 1.35, 95% CI 0.79-2.31, p = 0.268) and rejection treatment (HR 1.53, 95% CI 0.97-2.40, p = 0.066) were lost in the multivariate analysis, whereas donor BKPyV seroreactivity remained a highly significant risk factor for BKPyV viremia (HR 1.61, 95% CI 1.39-1.88, p < 0.001).

Despite the low number of BKPyVAN cases (n = 12), univariate analysis for BKPyVAN (Table 5) showed an association with donor BKPyV IgG-levels (HR 1.95, 95% CI 1.15-3.32, p = 0.013). These associations were also observed in the multivariate analysis (HR 2.89, 95% CI 1.33-6.29, p = 0.007, and HR 23.52, 95% CI 4.57-120.99, p < 0.001, respectively). Recipient seroreactivity showed a reverse, but not sta-

tistically significant protective trend in the univariate and multivariate analysis (HR 0.91, 95% CI 0.64-1.30, p = 0.609, and HR 0.69, 95% CI 0.46-1.04, p = 0.075, respectively). In a subanalysis for BKPyVAN among viremic recipients, in which recipients of donors with high IgG levels are overrepresented, no additional associations were found in either the uni- or multivariate analysis (Table S2).

Table 3. Risk of recipient BKPyV viremia after kidney transplantation according to BKPyV-seroreactivity measured pretransplantation in the donor, the recipients, and in the donor-recipient pair by stratified analysis.

		HR	95%-CI	p-value[1]
Donor				
BKPyV-seroreactivity[2]		1.59	1.38 – 1.84	<0.001
Seroreactivity quartile groups[3]				
Low (Q1)		1.0		<0.001
Low intermediate (Q2)		2.34	1.07 – 5.11	0.033
High intermediate (Q3)		3.82	1.82 – 8.06	<0.001
High (Q4)		6.92	3.41 – 14.06	<0.001
Recipient				
BKPyV-seroreactivity[2]		0.90	0.80 – 1.02	0.088
Seroreactivity quartile groups[3]				
Low (Q1)		1.0		0.221
Low intermediate (Q2)		0.74	0.47 – 1.24	0.257
High intermediate (Q3)		0.85	0.52 – 1.39	0.509
High (Q4)		0.57	0.33 – 0.98	0.041
Donor-recipient pair				
Donor seroreactivity[3]	Recipient seroreactivity[3]			
Low (Q1)	High (Q3-Q4)	1.00		<0.001
	Low (Q1-Q2)	0.90	0.24 – 3.36	0.879
Intermediate low (Q2)	High (Q3-Q4)	1.84	0.55 – 6.12	0.319
	Low (Q1-Q2)	2.52	0.82 – 7.72	0.106
Intermediate high (Q3)	High (Q3-Q4)	2.99	0.97 – 9.16	0.056
	Low (Q1-Q2)	4.31	1.45 – 12.82	0.009
High (Q4)	High (Q3-Q4)	4.89	1.71 – 14.01	0.003
	Low (Q1-Q2)	10.07	3.50 – 28.96	<0.001

BKPyV, BK polyomavirus; CI, confidence interval; HR, hazard ratio; MFI, mean fluorescence intensity; Q, quartile.

[1]The p-values, HRs and 95% CIs were calculated with Cox regression analysis. A p-value <0.05 was considered statistically significant.

[2]Donor and recipient seroreactivity per 5000 increasing MFI.

[3]MFI distributions of the donor and recipient seroreactivity quartile groups can be found in the legend of Figure S2.

Table 4. Uni- and multivariate Cox regression analysis for risk factors of BKPyV viremia development among 407kidney transplantation recipients in the first year after transplantation.

Covariate	Univariate analysis			Multivariate analysis		
	HR	95%-CI	p-value[1]	HR	95%-CI	p-value[1]
Age recipient (years)	1.01	1.00 – 1.03	0.138	1.00	0.99 – 1.02	0.665
Age donor (years)	1.01	0.99 – 1.02	0.498	1.00	0.98 – 1.02	0.858
Gender recipient	1.21	0.82 – 1.80	0.340	1.04	0.68 – 1.60	0.842
Gender donor	0.90	0.62 – 1.33	0.603	0.94	0.61 – 1.44	0.765
Underlying condition[2]						
Inherited	1.00		0.359	1.00		0.476
Glomerular	0.97	0.56 – 1.66	0.903	1.14	0.64 – 2.04	0.657
Vascular	1.45	0.86 – 2.43	0.163	1.62	0.94 – 2.77	0.081
Obstructive	0.78	0.34 – 1.79	0.554	1.04	0.43 – 2.51	0.939
Other	0.94	0.52 – 1.67	0.820	1.18	0.64 – 2.17	0.601
Dialysis pretransplantation	0.85	0.59 – 1.24	0.405	0.89	0.55 – 1.41	0.608
Duration dialysis (months)	0.90	0.77 – 1.04	0.156	0.99	0.98 – 1.01	0.219
Unrelated donor	1.49	1.02 – 2.17	0.042	1.35	0.79 – 2.31	0.268
Retransplantation	1.23	0.66 – 2.30	0.513	1.36	0.68 – 2.68	0.384
PRA immunization pretransplantation	0.72	0.23 – 2.26	0.570	0.72	0.22 – 2.41	0.598
Blood group compatibility	1.43	0.67 – 3.08	0.359	1.22	0.44 – 3.42	0.701
HLA mismatched A, B and DR loci[3]						
0	1.00		0.832	1.00		0.746
1-3	1.08	0.46 – 2.52	0.859	0.82	0.34 – 2.01	0.668
4-6	1.20	0.51 – 2.78	0.678	0.71	0.27 – 1.89	0.495
Basiliximab vs. alemtuzumab	1.04	0.51 – 2.13	0.921	0.99	0.38 – 2.58	0.988
Tacrolimus vs cyclosporin A	0.89	0.58 – 1.37	0.599	0.76	0.48 – 1.23	0.264
Donor BKPyV-seroreactivity[4]	1.59	1.38 – 1.84	<0.001	1.61	1.39 – 1.88	<0.001
Recipient BKPyV-seroreactivity[4]	0.90	0.80 – 1.02	0.088	0.84	0.75 – 0.95	0.006
Rejection treatment[5]	1.54	1.02 – 2.34	0.040	1.53	0.97 – 2.40	0.066

BKPyV, BK polyomavirus; CI, confidence interval; HLA, human leukocyte antigen; HR, hazard ratio; MFI, mean fluorescence intensity; PRA, panel reactive antibody.

[1]The p-values, HRs, and 95% CIs were calculated with uni- and multivariate Cox regression analysis. A p-value <0.05 was considered statistically significant.

[2]Describes the HR of viremia in recipients with each underlying condition group compared to the inherited disease group, the clarification of the categories can be found in the legend of Table 1.

[3]Describes the onset of viremia in recipients with each group of number of HLA mismatches on loci A, B, and DR compared to the group with no HLA mismatches.

[4]Donor and recipient seroreactivity per 5000 increasing MFI.

[5]Rejection treatment consisted of methylprednisolone 1000 mg intravenously once daily for three days.

Table 5. Uni- and multivariate Cox regression analysis for risk of BKPyVAN development among recipients (n = 407) in the first year after kidney transplantation.

Covariate[1]	Univariate analysis			Multivariate analysis		
	HR	95%-CI	p-value[2]	HR	95%-CI	p-value[2]
Age Recipient (years)	1.01	0.97 – 1.05	0.638	0.98	0.92 – 1.03	0.424
Age donor (years	1.03	0.98 – 1.08	0.284	1.06	0.98 – 1.14	0.136
Gender recipient	1.26	0.38 – 4.20	0.702	1.23	0.29 – 4.38	0.864
Gender donor	1.08	0.34 – 3.41	0.893	0.82	0.21 - 3.25	0.779
Underlying condition[3]						
Inherited	1.00		0.838	1.00		0.350
Glomerular	0.68	0.15 – 3.05	0.618	0.49	0.08 – 2.83	0.422
Vascular	0.28	0.03 – 2.46	0.248	0.25	0.02 – 2.54	0.240
Obstructive	0.73	0.08 – 6.56	0.781	3.03	0.23 – 40.27	0.402
Other	0.88	0.20 – 3.94	0.868	2.60	0.42 – 15.91	0.302
Dialysis pretransplantation	0.92	0.29 – 2.90	0.883	0.35	0.07 – 1.75	0.198
Duration dialysis (months)	1.03	0.70 – 1.50	0.894	1.01	0.97 – 1.05	0.657
Unrelated donor	2.90	0.79 – 10.71	0.110	1.58	0.30 – 8.33	0.593
Retransplantation	2.06	0.45 – 9.41	0.350	2.94	0.36 – 24.14	0.316
PRA immunization pretransplantation	2.58	0.33 – 20.00	0.364	3.05	0.22 – 42.12	0.405
Basiliximab vs. alemtuzumab	1.21	0.16 – 9.35	0.857	3.59	0.35 – 37.16	0.283
Donor BKPyV-seroreactivity****	1.95	1.15 – 3.32	0.013	2.89	1.33 – 6.29	0.007
Recipient BKPyV-seroreactivity****	0.91	0.64 – 1.30	0.609	0.69	0.46 – 1.04	0.075
Rejection treatment*****	11.60	3.14 – 42.86	<0.001	23.52	4.57 – 120.99	<0.001

BKPyV, BK polyomavirus, BKPyVAN, BK polyomavirus-associated nephropathy; CI, confidence interval; HR, hazard ratio; PRA, panel reactive antibody.

[1]Covariates blood group compatibility, HLA mismatches, and tacrolimus vs. cyclosporin A, as shown in Table 1, could not be added to this Cox model due to the low number of BKPyVAN cases in our cohort and the distribution of these baseline characteristics among the recipient groups with and without BKPyVAN (Table 1).

[2]The p-values, HRs, 95% CIs were calculated with uni- and multivariate Cox regression analysis. A p-value <0.05 was considered statistically significant.

[3]Describes the HR of BKPyVAN in recipients with each underlying condition group compared to the inherited disease group, the clarification of the categories can be found in the legend of Table 1.

[4]Donor and recipient seroreactivity per 5000 increasing MFI.

[5]Rejection treatment consisted of methylprednisolone 1000 mg intravenously once daily for three days.

Discussion

BKPyV-associated disease is a major problem in the care for kidney transplant recipients for which no antiviral treatment is available (1,2,10,32). As timely reduction of immunosuppression is the only effective treatment so far (11,12), currently all recipients are screened for BKPyV viremia on a regular basis after transplantation (10-12,16). Only a subset of recipients, however, is at risk of de-

veloping BKPyV viremia (15-30%) and subsequently BKPyVAN (1-10%) (10,12-16). Apart from rejection treatment following transplantation, pretransplantation risk factors for BKPyV viremia and BKPyVAN have not been identified thus far and, therefore, no markers are available to predict which recipients are actually at risk. The observed strong positive correlation between donor BKPyV IgG-levels and development of BKPyV viremia and BKPyVAN in recipients could fill this gap.

The kidney allograft plays a key role in the development of BKPyVAN, either because it serves as a transmitting vehicle for BKPyV to the recipient, as suggested by a number of previous reports (19,23), or because of increased renal vulnerability to infection, for example resulting from kidney injury related to transplantation (10,33). Our findings that show strong associations between donor BKPyV seroreactivity, and BKPyV viremia and BKPyVAN provide strong support for the first explanation, indicating that manifest BKPyV infection in recipients originates from the kidney allograft.

The strength of the observed association between donor BKPyV seroreactivity and recipient BKPyV infection, and the height of the calculated hazards are remarkable. As far as we know, previous studies have not compared donor BKPyV seroreactivity with recipient viremia and BKPyVAN. In general, studies that compared recipient BKPyV infection with BKPyV-related virological and immunological characteristics are rare, probably because BKPyV serology was considered not useful in this regard; BKPyV serostatus was shown to be positive in almost all cases, donors as well as recipients. One study compared donor BKPyV IgG levels and recipient BKPyV viruria and noted a correlation, in line with our findings (19). Unfortunately, urine samples could not be analyzed in the present cohort, since they were not routinely archived. Despite this and some other limitations of the study (see below), our findings indicate that BKPyV seroreactivity is the strongest (donor-related) pretransplant factor identified so far, predicting manifest BKPyV infection in kidney allograft recipients.

The predictive value of high donor BKPyV IgG levels for recipient BKPyV infection makes one wonder about the role of humoral BKPyV immunity in BKPyV infection. Obviously it is not donor-derived BKPyV-directed antibodies that confer infection, but the virus itself. Therefore, we assume that the intensity of measured donor BKPyV seroreactivity reflects the amount or virulence of infectious BKPyV present in the persistently infected kidney allograft. Since we are unaware of documented differences in virulence among BKPyV genotypes, it is most likely that BKPyV IgG levels reflect the BKPyV kidney load, as such correlating with

the risk of BKPyV infection in recipients. It should be noted, however, that so far BKPyV genotyping is skewed towards virus isolates obtained from recipients with BKPyV infection, and therefore may not represent the distribution of BKPyV genotypes circulating in the general population including potential donors.

Because BKPyV seroreactivity likely reflects the BKPyV load of infected kidneys, it is important to consider the role that serum IgG antibodies play in the control of persistent BKPyV infection. As previous studies have shown that BKPyV IgG seroresponses increase upon BKPyV DNA detection (21,24,25), and in line with observations suggesting an inverse relationship between recipient BKPyV IgG levels and BKPyV infection (19,21,22), BKPyV-directed antibodies might be directly involved in containment of BKPyV infection. Indeed recent studies by Rhandawa and Buck have provided evidence of efficient BKPyV neutralization by BKPyV-directed serum antibodies (34,35), and proposed the possibility of offering recipients intravenous immunoglobulins (IVIG) in the posttransplantation period. Involvement of BKPyV-specific antibodies in controlling BKPyV infection can also be inferred from the increased risk of BKPyV viremia observed in serologically low responding recipients. Alternatively, especially in the recipients, the measured BKPyV seroresponses may be a marker of another relevant component of the immune system, for example BKPyV-specific T cells that are essential in controlling BKPyV infection after transplantation (21,36). This possibility is underscored by a recent study of Sester and Hirsch that reports a strong correlation in recipients with BKPyV replication between BKPyV IgG levels and the percentage of BKPyV-reactive CD4 T cells (37).

Taken together it is important to realize that, in the context of transplantation and prediction of BKPyVAN, BKPyV seroreactivity might actually reflect both the BKPyV kidney load and BKPyV T cell immunity. In donors, as depicted above, BKPyV seroreactivity likely reflects BKPyV graft load. In recipients, however, BKPyV seroreactivity might primarily be regarded as a reflection of the overall immunity against BKPyV, including T cells. Both high donor BKPyV-specific antibody titers and low (or absent) recipient BKPyV-specific antibody titers are mentioned as risk factors for BKPyVAN in the most recent American Society of Transplantation, Infectious Disease Community of Practice guideline (38). The added value of our study particularly lies in the integrated evaluation of this serological marker among donor-recipient pairs, which provides leads for future algorithms to predict BKPyV-related disease posttransplantation.

Possible limitations of our study include the single-center design, and the fact that not all recipients were sampled on every time point after transplantation. However, we are not aware of geographic variability regarding BKPyV seroreactivity, and have no indication that completion of the sample set would have changed the overall conclusions. The fact that not all recipients with a BKPyV viremia $\geq 10^4$ c/ml were biopsy screened could have caused underrecognition of the number of BKPyVAN cases. Therefore statistics performed to calculate the BKPyVAN risk must be interpreted with caution. Nevertheless, even with this small number, statistically significant results were obtained regarding the association between recipient BKPyVAN and donor BKPyV seroreactivity. Due to the inclusion of living donor-recipient pairs only, it remains uncertain whether these results also apply to deceased donor-recipient pairs. A borderline increased risk of BKPyV viremia was observed in recipients that received a kidney allograft from an unrelated donor compared to recipients from related donors. However, it is not expected that this factor influences the observed associations between donor BKPyV seroreactivity, and BKPyV viremia and BKPyVAN. In general both the incidence and load of viremic episodes observed in the present study population are in line with comparable kidney transplantation cohorts reported in the literature, including cohorts with deceased donors (10,14,16,25).

Despite its possible limitations, this study identified a serological marker that indicates the risk of BKPyV infection after kidney transplantation. The results suggest that a single, pretransplantation BKPyV IgG measurement could be used to assess the risk of BKPyV infection posttransplantation. Since our data show that recipient BKPyV seroreactivity modulates the risk determined by donor BKPyV seroreactivity, it appears most useful to determine BKPyV seroreactivity before transplantation in both the allograft donor and recipient. Subsequent studies are needed to reveal whether a pretransplantation serological BKPyV risk assessment could provide a basis for personalized BKPyV load-monitoring strategies aimed at early identification of BKPyV viremic patients, in order to increase the efficiency of BKPyV screening. Furthermore it might be worthwhile to consider the additive value of donor-recipient BKPyV seroreactivity matching (high seroreactive donor calls for high seroreactive recipient), in order to lower the BKPyV infection risk. Passive immunization of recipients at high risk could also be considered an option, based on the protective effect of high IgG levels in recipients. The relevance of the present findings for other (reactivating) viral infections after solid organ transplantation merits further study.

By studying BKPyV seroresponses a strong correlation was identified between baseline BKPyV IgG levels and posttransplantation BKPyV infection. Use of BKPyV seroreactivity as a practical predictive disease marker could be of great value in the management of BKPyV-associated disease. Moreover, these findings call for further study into approaches aimed at improving humoral BKPyV immunity posttransplantation, such as the administration of (BKPyV-specific) IVIG and BKPyV vaccination.

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Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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Supporting Information

Table S1. Characteristics of BKPyV viremia among recipients that did or did not develop BKPyVAN.

	Viremic recipients (n = 111)		
	No BKPyVAN (n = 99)	BKPyVAN (n = 12)	p-value[1]
First BKPyV-positive sample after transplantation (months)	4.1 (2.2 – 6.0)	2.8 (1.9 – 3.7)	0.100
Peak BKPyV-load after transplantation (months)	4.8 (3.4 – 6.7)	3.3 (2.8 – 5.6)	0.114
Load of first BKPyV-positive sample (c/ml)	1400 (292 – 10000)	19795 (406 – 81225)	0.063
BKPyV load ≥ 10^3 c/ml	70 (71%)	12 (100%)	0.034
BKPyV load ≥ 10^4 c/ml	40 (40%)	12 (100%)	<0.001
BKPyV load ≥ 10^5 c/ml	12 (12%)	5 (42%)	0.019
BKPyV load ≥ 10^6 c/ml	0 (0%)	1 (8%)	0.108
Peak BKPyV-load (c/ml	4244 (682 – 26000)	65650 (29590 – 729250)	<0.001
BKPyV-load AUC during one year after transplantation (c/ml)	8977 (1382 – 46296)	120750 (27293 – 341225)	<0.001
Number of time-points collected	4 (4 – 5)	5 (4 – 5)	0.031
Number of BKPyV-positive time-points	2 (1 – 3)	2 (2 – 3.8)	0.083
Number of BKPyV-positive time-points ≥ 10^3 c/ml	1 (0 – 1)	1 (0.3 – 1.8)	0.446
Number of BKPyV-positive time-points ≥ 10^4 c/ml	0 (0 – 1)	0.5 (0 – 1)	0.132
Number of BKPyV-positive time-points ≥ 10^5 c/ml	0 (0 – 0)	0 (0 – 0.8)	0.041
Number of consecutive BKPyV-positive time-points	1 (1 – 2)	2 (1 – 1.8)	0.684
Number of consecutive BKPyV-positive time-points ≥ 10^3 c/ml	1 (0 – 1)	1 (0.3 – 1.8)	0.413
Number of consecutive BKPyV-positive time-points ≥ 10^4 c/ml	0 (0 – 1)	1 (0 – 1)	0.036
Number of consecutive BKPyV-positive time-points ≥ 10^5 c/ml	0 (0 – 0)	0 (0 – 0.8)	0.041

Data are shown as median number and interquartile range (IQR) or n (%).
AUC, area under the curve; BKPyV, BK polyomavirus; BKPyVAN, BK polyomavirus-associated nephropathy; c/ml, copies/ml; IQR, interquartile range.
[1]The p-values were calculated with Mann-Whitney U test, Chi-Square test or Fisher's Exact test. A p-value <0.05 was considered statistically significant.

Table S2. Uni- and multivariate Cox regression analysis for risk of development of BKPyVAN among BKPyV viremic recipients (n = 111) during the first year after kidney transplantation.

Covariate[1]	Univariate analysis			Multivariate analysis		
	HR	95%-CI	p-value[2]	HR	95%-CI	p-value[2]
Age Recipient (years)	1.00	0.96 – 1.04	0.938	0.98	0.92 – 1.04	0.422
Age donor (years	1.02	0.97 – 1.08	0.393	1.03	0.96 – 1.11	0.350
Gender recipient	1.08	0.32 – 3.58	0.903	1.53	0.33 – 7.16	0.589
Gender donor	1.18	0.38 – 3.73	0.774	0.87	0.20 – 3.72	0.848
Underlying condition[3]						
Inherited	1.00		0.681	1.00		0.584
Glomerular	0.75	0.17 – 3.33	0.700	0.63	0.09 – 4.36	0.639
Vascular	0.20	0.02 – 1.76	0.146	0.24	0.02 – 2.48	0.231
Obstructive	0.98	0.11 – 8.78	0.986	1.48	0.12 – 18.18	0.759
Other	0.94	0.21 – 4.18	0.931	1.84	0.29 – 11.60	0.516
Dialysis pretransplantation	1.03	0.33 – 3.25	0.959	0.62	0.10 – 3.64	0.593
Duration dialysis (months)	1.02	0.98 – 1.06	0.417	1.04	0.97 – 1.10	0.270
Unrelated donor	1.99	0.54 – 7.35	0.303	2.06	0.35 – 11.97	0.422
Retransplantation	1.73	0.38 – 7.88	0.481	2.52	0.35 – 17.94	0.356
PRA immunization pretransplantation	3.35	0.43 – 25.97	0.248	1.55	0.11 – 28.36	0.751
Basiliximab vs. alemtuzumab	1.08	0.14 – 8.40	0.938	1.01	0.10 – 9.97	0.996
Donor BKPyV-seroreactivity[4]	1.27	0.76 – 2.12	0.362	1.64	0.84 – 3.20	0.147
Recipient BKPyV-seroreactivity[4]	0.98	0.68 – 1.40	0.895	0.84	0.52 – 1.36	0.476
Rejection treatment[5]	2.07	0.45 – 9.44	0.349	1.54	0.23 – 10.24	0.653

BKPyV, BK polyomavirus; BKPyVAN, BK polyomavirus-associated nephropathy; CI, confidence interval; HR, hazard ratio; MFI, mean fluorescence intensity; PRA, panel reactive antibody.

[1]Covariates blood group compatibility, HLA mismatches, and tacrolimus vs. cyclosporin A, as shown in Table 1, could not be added to the Cox model due to the low number of BKPyVAN cases in our cohort and the distribution of these baseline characteristics among the recipient groups with and without BKPyVAN (Table 1).

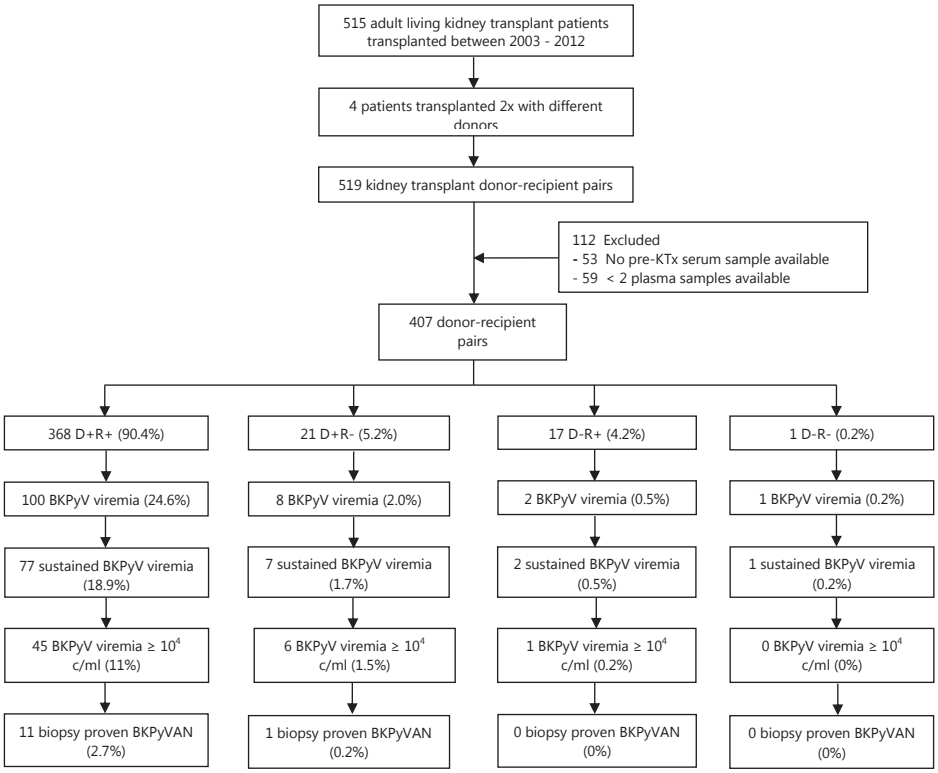
[2] The p-values, HRs, and 95% CIs were calculated with uni- and multivariate Cox regression analysis. A p-value <0.05 was considered statistically significant.

[3]Describes the HR of BKPyVAN in recipients with each underlying condition group compared to the inherited disease group, the clarification of the categories is described in the legend of Table 1.

[4]Donor and recipient seroreactivity per 5000 increasing MFI.

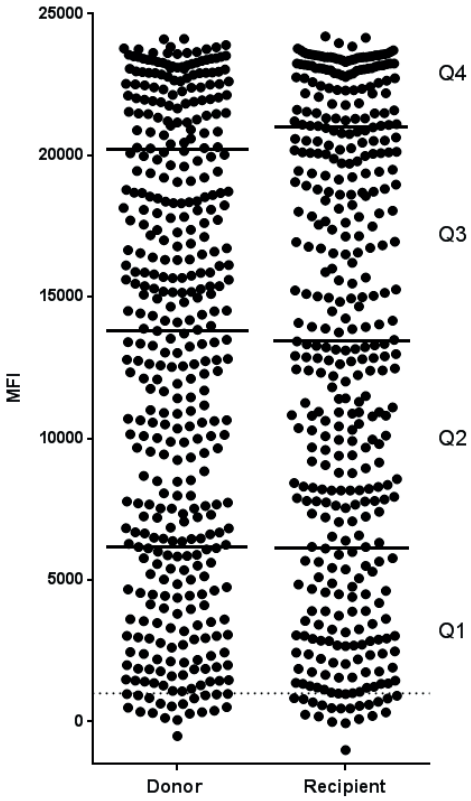
[5]Rejection treatment within 3 months before occurrence of BKPyV viremia was used instead of rejection treatment overall as occurrence of BKPyV viremia always precedes BKPyVAN. Rejection treatment consisted of methylprednisolone 1000 mg intravenously once daily for three days.

Figure S1. Study population, inclusion of kidney transplantation donor–recipient pairs, and development of BKPyV viremia and BKPyVAN divided by donor and recipient pretransplantation BKPyV serostatus.



Inclusion and exclusion criteria and distribution of BKPyV viremia (p = 0.107), sustained BKPyV viremia (p = 0.107), BKPyV viremia of log ≥4(p= 0.155), and BKPyVAN (p = 0.707) in the different BKPyV serostatus pretransplantation donor–recipient pair combinations. The p-values were calculated using the Fisher exact test. A p-value <0.05 was considered statistically significant. +, BKPyV seropositive; -, BKPyV seronegative; BKPyV, BK polyomavirus; BKPyVAN, BK polyomavirus-associated nephropathy; c/mL, copies per milliliter; D, donor; pre-KTx, pre–kidney transplant; R, recipient.

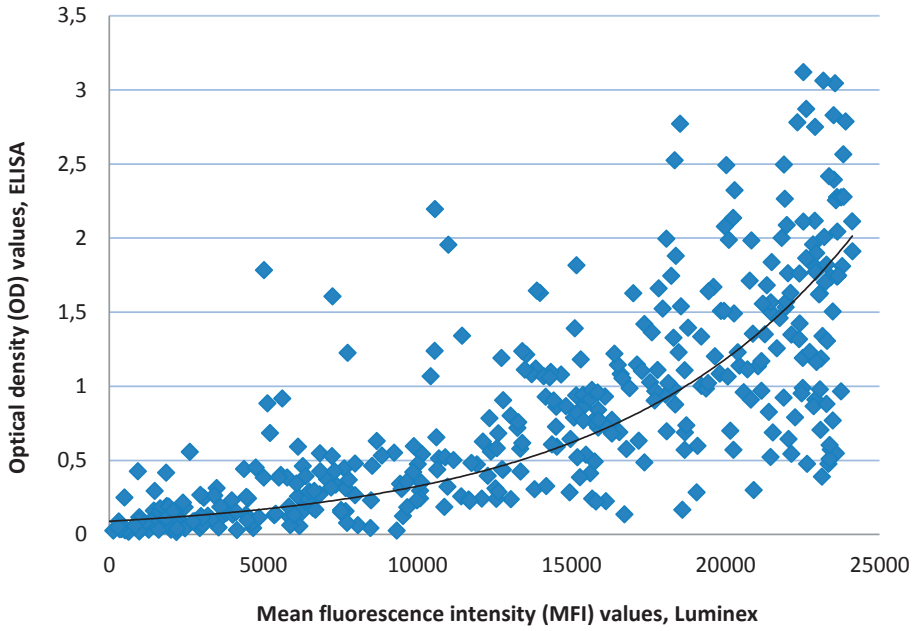
Figure S2. Pretransplantation IgG seroreactivity of 407 kidney transplantation donors and recipients against the BKPyV VP1 antigen.



Pretransplantation IgG seroreactivity of 407 kidney transplantation donors and recipients against the BKPyV VP1 antigen. Each dot represents the pretransplantation BKPyV VP1 IgG seroreactivity of individual donors (left) and recipients (right), tested by a Luminex assay. The measured BKPyV VP1 IgG seroreactivity of donors and recipients is categorized in quartile groups according to the measured mean fluorescence intensity (MFI) values, with quartile 1 (Q1) containing the lowest seroreactive participants and Q4 the highest. The black lines represent the borders between the quartile groups, and the dashed line represents the cutoff value that was used to calculate the percentage of BKPyV seropositivity. MFI ranges for donor seroreactivity quartiles: Q1, low: -1001 to 6169; Q2, low intermediate: 6170–13 842; Q3, high intermediate: 13 843–20 251; Q4, high: 20 252–24 120. MFI ranges for recipient seroreactivity quartiles: Q1, low: -510 to 6178; Q2, low intermediate: 6179–13 490; Q3, high intermediate: 13 491–21 043; Q4, high: 21 043–24 207.

Figure S3. Baseline donor BKPyV seroreactivity comparison between data generated with the Luminex BKPyV GST–VP1 fusion protein assay and the BKPyV VP1 VLP ELISA.

A



B

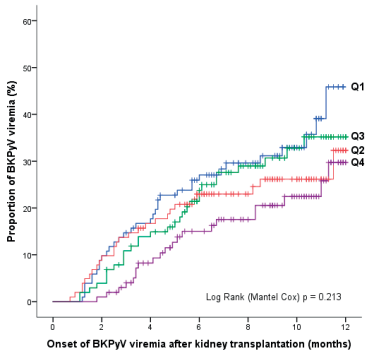
		MFI result (Luminex)
OD result (ELISA)	Correlation coefficient	0.823
	Sig. (2-tailed)	< 0.001
	N	396

A) Overall, 396 of 407 (97.3%) pretransplantation donor sera were analyzed by both the Luminex BKPyV GST-VP1 fusion protein assay and the BKPyV VP1 VLP ELISA. The correlation is shown between the MFI values determined by Luminex and the OD values determined by ELISA.

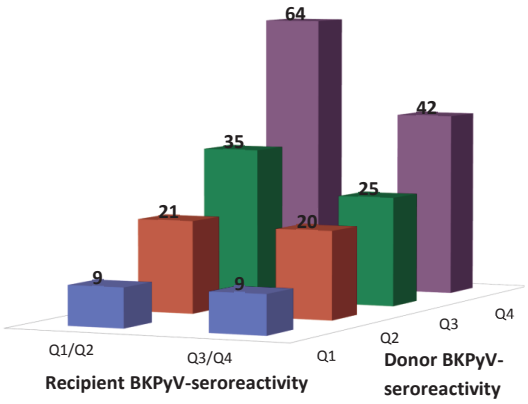
B) The Spearman correlation coefficient was calculated between the MFI and OD values obtained with Luminex and ELISA. A strong positive monotonic correlation was observed between the two variables: $r = 0.823$, $n = 396$, $p < 0.001$. BKPyV, BK polyomavirus; ELISA, enzyme-linked immunosorbent assay; GST, glutathione S-transferase; MFI, mean fluorescence intensity; OD, optical density; VLP, virus like particle; VP1, viral capsid protein 1

Figure S4. Proportion of BKPyV viremia detected in the first year after kidney transplantation according to BKPyV seroreactivity determined in recipients and in donor–recipient pairs.

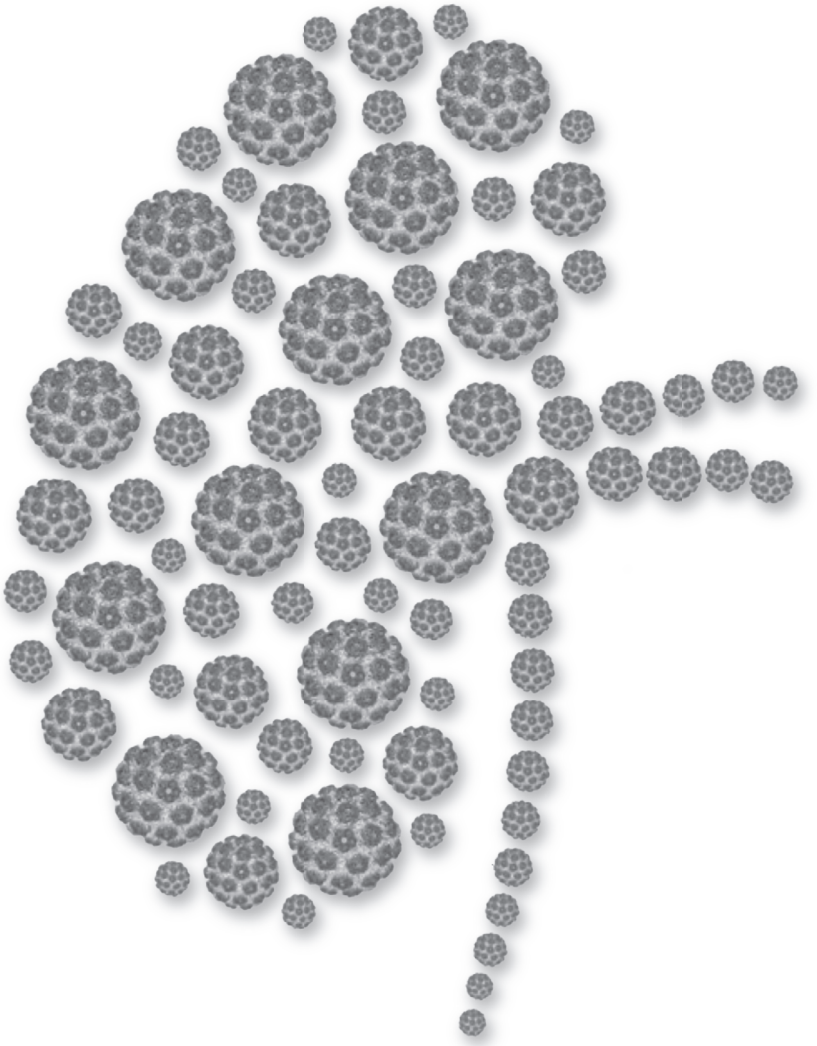
A



B



A) Kaplan–Meier (1 – survival function) curves for proportion of BKPyV viremia observed in the recipients according to recipient BKPyV seroreactivity quartile groups (shown in Figure S2). Mean fluorescence intensity distributions of the recipient seroreactivity quartile groups can be found in the legend of Figure S2. Tick marks represent censored recipients.
B) Incidence of recipient BKPyV viremia during follow-up according to donor BKPyV seroreactivity quartile groups stratified for recipient BKPyV seroreactivity measured before transplantation. The overall percentages of BKPyV viremic recipients are shown within each donor-recipient seroreactivity quartile combination. BKPyV, BK polyomavirus; OD, optical density; Q, quartile.



Chapter 3

Stability of BK polyomavirus seroreactivity and its correlation with preceding viremia

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Abstract

Background

Recently we showed that the level of BK polyomavirus (BKPyV) IgG seroreactivity in kidney donors predicted viremia and BKPyV-associated nephropathy in kidney transplant recipients (KTRs). This observation could be explained by assuming a direct association between BKPyV seroreactivity and the amount of persistent infectious virus in the renal allograft.

Objectives

Since the renal BKPyV reservoir is probably sowed by viremia during primary BKPyV infection, we systematically analysed the dynamics of BKPyV IgG seroreactivity in relation to preceding BKPyV viremia in KTRs and healthy individuals.

Study design

A cohort of 85 KTRs consisting of BKPyV viremic and nonviremic subjects was analysed for BKPyV IgG seroreactivity at five fixed time points until one year after transplantation. A cohort of 87 healthy blood donors (HBDs) was used as controls.

Result

Baseline BKPyV seropositivity was high in both KTRs and HBDs, and the baseline mean BKPyV IgG level comparable. BKPyV IgG levels in nonviremic KTRs and HBDs remained stable during follow-up, while a considerable increase was observed in viremic KTRs ($p = 0.015$). The increase of BKPyV seroreactivity in viremic KTRs was associated with the duration and peak level of BKPyV viremia.

Conclusion

BKPyV IgG seroreactivity was stable over time in immunocompetent subjects, which enables the use of this potential pretransplantation biomarker in kidney donors. The observed dose-dependent relationship of BKPyV IgG seroreactivity with preceding BKPyV replication is in agreement with the assumption that BKPyV seroreactivity reflects past BKPyV activity and correlates with the amount of latent BKPyV residing within a kidney allograft.

Background

BK polyomavirus (BKPyV) is a ubiquitous small, double-stranded DNA virus that infects ~90% of the human population (1,2). Primary infection is asymptomatic and usually occurs in early childhood (3,4). During primary infection BKPyV replicates, disseminates probably via the bloodstream and infects the kidneys. After establishment of host immunity, BKPyV remains latent in the urothelium and renal tubular cells to cause a low-level persistent infection (5,6). From time to time small amounts of viral progeny can be detected in urine of healthy individuals (7-9). Under immunosuppression, however, BKPyV can freely replicate again (reactivate) and cause manifest disease, such as hemorrhagic cystitis in hematopoietic stem cell transplant recipients and BKPyV-associated nephropathy (BKPyVAN) in kidney transplant recipients (KTRs) (3,4,10). The latter condition constitutes a serious problem, since BKPyVAN endangers the allograft function in 5-10% of KTRs and hampers the overall success of kidney transplantation (10-15).

To date, no pretransplantation biomarker exists to reliably predict which KTRs are at risk of developing BKPyVAN, which usually develops within the first year after transplantation (16). Specific antiviral therapy is not available and timely reduction of immunosuppressive therapy is the only effective evidence-based treatment so far (15,17,18). Therefore, frequent blood viral load monitoring of KTRs is recommended to detect BKPyV replication (10,12,15,17). Presence of BKPyV-DNA in blood (viremia), most notably of sustained viremia with loads above 10^4 genome copies/ml (c/ml), is associated with BKPyVAN development (3,4,10).

To understand BKPyV reactivation and optimize and personalize BKPyV monitoring strategies, we recently conducted a large kidney donor-recipient pair study into risk factors of BKPyV viremia and BKPyVAN. We identified a strong association between pretransplantation donor BKPyV IgG seroreactivity and the incidence of BKPyV viremia and BKPyVAN in KTRs posttransplantation (16). The strength of this association surpassed other pretransplantation risk factors including HLA-mismatch and unrelated donor-status. This finding indicated a key role for the donor organ in the development of BKPyV-associated disease, most likely by acting as the transmitting vehicle of infectious BKPyV from donor to recipient, as previously suggested (19,20). Moreover, this finding suggested that the level of BKPyV IgG seroreactivity measured in the donor reflects the amount of latent infectious BKPyV present in the allograft (16).

Objectives

In light of this finding and of the potential use of this parameter as a pretransplantation predictive biomarker of manifest BKPyV infection in KTRs, the present study focused on the relationship between BKPyV IgG seroreactivity and preceding BKPyV replication, as indicated by viremia.

Study design

Study population

Two study populations were defined, one immunosuppressed KTR population and one matched immunocompetent healthy blood donor (HBD) population.

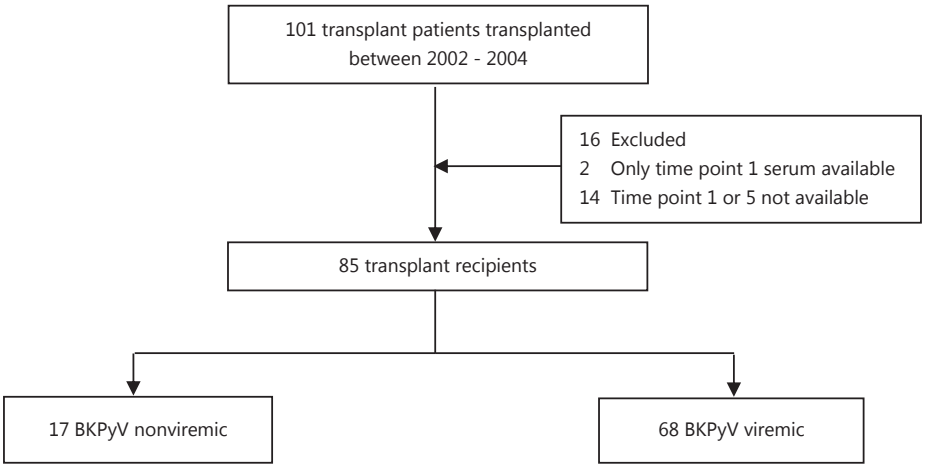
The KTR group was part of a larger prospective European multicenter study designed to investigate the role of human papillomavirus infection in the development of skin cancer in solid-organ transplant patients (21). The study adhered to the Declaration of Helsinki Principles. The medical ethical committee of the Leiden University Medical Center (LUMC) approved the study design and all participants gave written informed consent.

Adult (>18 years of age) KTRs transplanted at the LUMC between 2002 and 2004 were eligible for this cohort as described previously (22). In total, 101 KTRs were identified. Sixteen were excluded because either baseline or follow-up samples were not available for analysis (Figure 1). The remaining 85 KTRs were included in the study.

KTR serum and plasma samples were collected at five regular time-points (T1 - T5), approximately 0, 3, 6, 9 and 12 months after transplantation (Table 1). The median number of time points analysed per KTR was 5 (range 4 – 5). T1 sera were collected with a mean of 11 days (range 2 – 36) after transplantation. For 68 KTRs (80%) all time points were available for BKPyV DNA detection. The remaining 16 KTRs lacked either the T2, T3 or T4 time point.

To obtain a healthy control population, we randomly selected anonymized serum samples from 87 unpaid HBDs, who were matched for age and sex with the KTR study population (Table 1). For each blood donor, two serum samples were studied, collected 1 year apart in 2011 and 2012 (22). For BKPyV DNA analysis only the latter sample was available. BKPyV IgG seroreactivity was determined in both samples.

Figure 1. Kidney transplantation recipient



BKPyV, BK polyomavirus.

Table 1. Baseline characteristics and sampling of kidney transplantation recipients and healthy blood donors.

	Blood donors	Recipients
	Total (n = 87)	Total (n = 85)
Age, mean in years (range)	52 (29 - 68)	47 (21 - 74)
Gender, n (% male)	56 (64%)	58 (68%)
Time points analysed[1]		
T1: baseline	87 (100%)	85 (100%)
T2: 3 months after baseline	NC	84 (99%)
T3: 6 months after baseline	NC	83 (98%)
T4: 9 months after baseline	NC	71 (84%)
T5: 12 months after baseline	87 (100%)	85 (100%)
Number of time points, median (range)	2 (2)	5 (4 - 5)

NC, not collected; T, time point.
[1]In the kidney transplant recipients, baseline refers to around date of transplantation.

BKPyV serology

To detect IgG seroresponses against the major viral capsid protein 1 (VP1) of BKPyV, an antibody-binding assay using Luminex xMAP technology (23) was performed, as previously described (24). Briefly, the assay measures IgG seroreactivity against BKPyV VP1-GST fusion protein bound to glutathione casein-coated fluorescent polystyrene beads (Bio-Rad). Serum samples were tested in a 1:100 dilution and results expressed as median fluorescent intensity (MFI). For background correction, MFI values measured against glutathione S-transferase alone

were subtracted to obtain the BKPyV VP1-specific signals. Quality control was performed on each plate with a serum pool consisting of 4 serum samples that had been analyzed in a 1:4 serial dilution, starting with a dilution of 1:100 up to 1:409,600. The observed interplate variance was small (r 0.963 – 0.999, $p < 0.001$).

The cut-off value was defined based on a group of healthy children 0.5 – 2 years of age and determined as described by van der Meijden et al. (24).

Detection of BKPyV viremia

Total nucleic acids were extracted from 200 µL serum/plasma using the MagNA Pure LC Total Nucleic Acid Isolation Kit–High Performance and MagNA Pure LC Instrument (Roche Diagnostics, Indianapolis, IN, USA). To monitor the quality of DNA extraction and potential PCR inhibition, we added low concentrations of phocine herpesvirus (25) to the lysis buffer. DNA was eluted in a final volume of 100 µL elution buffer, of which 10 µL was used as input for real-time quantitative PCR (qPCR). Using the primers 440BKVs 5'-GAAAAGGAGAGTGTCCAGGG-3' and 441BKVas 5'-GAACTTCTACTCCTCCTTTTATTAGT-3' and a Taqman probe 576BKV-TQ-FAM FAM 5'-CCAAAAAGCCAAAGGAACCC-3'-BHQ1, a 90-bp fragment within the BKPyV VP1 gene was amplified. The BKPyV qPCR and phocine herpesvirus PCR were duplexed for DNA quality and potential PCR inhibition monitoring. Furthermore, the BKPyV qPCR was validated to detect BKPyV genotypes I–IV.

qPCR reactions were performed in a total volume of 50 µL, containing 25 µL Hot-StarTaq Master Mix (QIAGEN, Hilden, Germany), 0.5 µmol/L of each primer, 0.35 µmol/L BKPyV probe, and 3.5 mmol/L $MgCl_2$. Reactions were performed using a CFX96 real-time detection system (Bio-Rad, Hercules, CA, USA) with the following cycle conditions: 15 min at 95°C followed by 45 cycles of amplification (30 s at 95°C; 30 s at 55°C; 30 s at 72°C). For quantification, a standard of a quantified BKPyV-positive urine sample was used. Analytical sensitivity of the BKPyV qPCR was ~10 copies/mL. On each plate, 3 negative controls were included; these controls tested negative in all PCR assays. PCR results with a cycle threshold ≥ 40 were considered negative.

Statistical analyses

Data were analyzed with IBM SPSS Statistics software version 23. Descriptive analyses were used to report cohort characteristics. Differences between KTRs and HBDs or between nonviremic and viremic KTRs were assessed using the

Chi-Square test, Fisher's exact test, Student's *t*-test or Mann-Whitney U test as appropriate. Student's *t*-test was used to analyze differences over a one year period. The course of KTRs BKPyV IgG seroreactivity (based on 5 time points), and of HBDs (based on 2 time points), over a one-year period, was calculated with mixed model analyses. The calculation of Δ BKPyV IgG for the viremic KTRs grouped according to peak viral load was also performed with mixed model analyses. For all performed tests a p-value < 0.05 in a two-sided test was considered statistically significant.

Results

The BKPyV seroprevalence determined at baseline was high in both groups; 95% in the HBDs and 93% in the KTRs (Table 2). The mean baseline BKPyV seroreactivity of both groups was also comparable (Table 2; 12676 and 12472, p = 0.819). After one year of follow-up the mean BKPyV seroreactivity remained stable in the HBDs, while it increased considerably in the KTRs (Table 2; 12162 and 15558, p = 0.007).

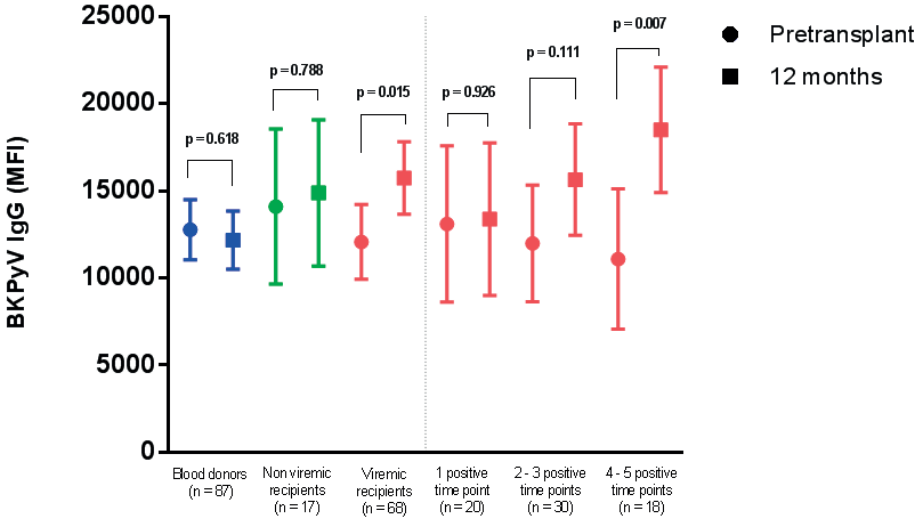
Table 2. BKPyV IgG seroreactivity and BKPyV viremia among kidney transplantation recipients and healthy blood donors.

	Blood donors (n = 87)	Recipients (n = 85)	p-value[1]	Recipients		p-value[1]
				BKPyV nonviremic (n = 17)	BKPyV viremic (n = 68)	
BKPyV seropositivity at baseline[2], n (%)	83 (95%)	79 (93%)	0.533	16 (94%)	63 (93%)	1.000
BKPyV seroreactivity, mean MFI (SD)						
T1: at baseline	12676 (8104)	12472 (8785)	0.819	14090 (8659)	12067 (8833)	0.399
T5: 12 months after baseline	12162 (7849)	15558 (8454)	0.007	14874 (8156)	15728 (8578)	0.712
Δ T1-T5	-605 (4323)	3086 (6971)	<0.001	784 (5481)	3661 (7216)	0.129

BKPyV, BK polyomavirus; MFI, mean fluorescence intensity; T1, time point 1; T5, time point 5. Δ Delta indicates the difference between MFI values measured at T1 and T5.
[1]The p-values were calculated using the Chi-Square test, Fisher's exact test or Student's *t*-test. A p-value <0.05 was considered statistically significant.
[2]In the recipients baseline refers to around date of kidney transplantation.

To investigate the BKPyV seroresponse in relation to BKPyV replication, we determined the presence and degree of viremia by detecting BKPyV DNA in blood of both groups. In total, 68 of 85 KTRs (80%) became viremic during follow-up (Table

Figure 2. Mean BKPyV IgG seroreactivity in healthy blood donors (n = 87) and kidney transplantation recipients (n = 85) during of follow-up.



BKPyV IgG levels determined in blood donors (blue), BKPyV nonviremic recipients (green), and BKPyV viremic recipients (red), at baseline (dots) and after one year of follow-up (squares). For the kidney transplant recipients, baseline indicates time of transplantation. In the right part of the graph, viremic recipients were subdivided in groups according to the number of BKPyV-positive time points. The dots and squares represent the mean BKPyV IgG seroreactivity and the horizontal lines above and below represent the 95% CI.

2) and one HBD. Of the viremic KTRs 23 (34%) had a peak load below 100 c/ml, 30 (44%) between 100 – 999 c/ml, 13 (19%) between 1000 – 9999 c/ml, and 2 (3%) of more than 10000 c/ml. The median peak viral load was 420 c/ml (interquartile range 83 – 1154 c/ml). The single positive HBD had a load of 190 c/ml. The duration of viremia expressed as the number of BKPyV-positive time points ranged from one to five time points with a mean of three. For the HBD case with viremia the duration could not be established, because only T5 samples of HBDs were analysed for BKPyV DNA.

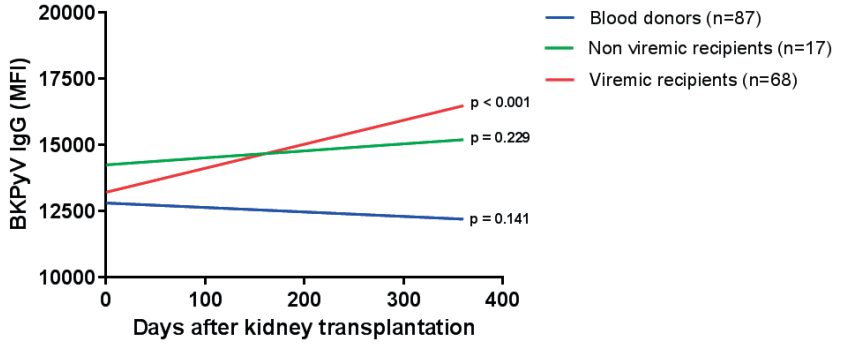
Evaluation among the KTRs showed that the mean BKPyV seroreactivity was lower at time of transplantation and higher after follow-up in viremic compared to nonviremic subjects, but overall these differences were not statistically significant (Table 2; p = 0.399 and p = 0.712, respectively). However, a clear increase in the mean BKPyV seroreactivity between baseline and end of study was only observed in the viremic KTRs (Figure 2; p = 0.015). Stratified analysis for the duration of viremia showed the median BKPyV IgG level in viremic KTRs remained stable in those with one BKPyV DNA positive time point, slightly increased if two or three

time points were positive (p = 0.111), and significantly increased with four or five positive time points (Figure 2; p = 0.007).

To further investigate a potential dose-dependent correlation between BKPyV seroreactivity and BKPyV replication, we evaluated the course of the BKPyV IgG seroresponses measured on all available time points per subject, using mixed

Figure 3. Course of BKPyV IgG seroreactivity in healthy blood donors (n = 87) and kidney transplantation recipients (n = 85) during follow-up evaluated with mixed model analyses.

A



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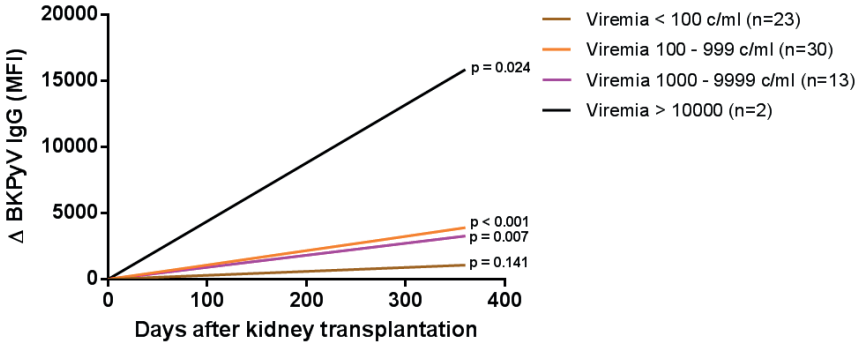

BKPyV, BK polyomavirus; c/ml, copies/milliliter; MFI, mean fluorescence intensity.
A) Mixed model analysis based on the course of the BKPyV IgG seroreactivity, expressed in MFI, per recipient on time point 1-5, in BKPyV nonviremic (green), and BKPyV viremic (red) transplantation recipients during 12 months after transplantation, and in healthy blood donors (blue) based on the course of the BKPyV IgG seroreactivity per blood donor over a 1-year period (time point 1 and 5).
B) The difference (Δ) in BKPyV IgG seroreactivity, expressed in MFI, in BKPyV viremic kidney transplantation recipients during 12 months after transplantation. The BKPyV viremic recipients are divided in groups according to the peak viral load in c/ml.
The p-values were calculated using the Student's *t*-test or with mixed model analyses. A p-value <0.05 was considered statistically significant.

model analyses. This analysis showed BKPyV IgG seroreactivity of both HBDs and nonviremic KTRs remained stable during follow-up, while in the viremic KTRs a substantial increase in BKPyV seroreactivity was observed (Figure 3A; $p < 0.001$).

Stratified analysis for the peak level of viremia during follow-up, showed that viremic KTRs with a peak load below 100 c/ml virtually did not increase in seroreactivity (Figure 3B; $p = 0.141$), while KTRs with a peak load between 100 – 999 c/ml or 1000 – 9999 c/ml did show a significant increase in BKPyV seroreactivity ($p < 0.001$ and $p = 0.007$ respectively). The highest increase of BKPyV seroreactivity was observed in KTRs with a peak load of more than 10000 c/ml ($p = 0.024$).

Discussion

Donor BKPyV IgG seroreactivity potentially represents a timely and practical predictive biomarker of manifest BKPyV infection in KTRs (16). To understand this parameter in more detail, the stability of BKPyV IgG seroresponses over time and its dependence on preceding BKPyV replication was investigated. The latter is important since it could explain why donor BKPyV seroreactivity predicts KTR infection (16), as the level of BKPyV IgG might reflect the amount of infectious BKPyV seeded in a donor kidney during previous viremic episodes of BKPyV replication including primary infection.

Our analysis of BKPyV replication in both immunocompromised and immunocompetent individuals showed a significant dose-dependent correlation between BKPyV IgG seroreactivity and preceding viremia. Increasing seroreactivity was only observed in individuals with a recent episode of viremia, while in individuals without viremia the BKPyV IgG levels remained stable. Previous longitudinal studies that analysed BKPyV seroresponses in either of these groups reported comparable findings (26-30). As far as we know there have been no studies that correlated BKPyV seroreactivity and detection of viremia during primary infection in immunocompetent individuals, for example in children. Despite this gap of knowledge and the relatively small size of our cohorts, based on the observed dose-dependent relationship between BKPyV seroreactivity and viremia, we assume that BKPyV seroreactivity measured in immunocompetent individuals could reflect the level of viremia experienced during primary infection.

In the HBDs a slight decline in mean BKPyV seroreactivity during follow-up was observed, which fits with the earlier observations that BKPyV seroresponses wane

with ageing, and might be explained by immunosenescence or diminished boosting by less virus exposure (1,2). The subtle increase in mean BKPyV IgG observed in the nonviremic KTRs might be explained by the presence of undetected low-level BKPyV viremia that still marginally boosted the BKPyV seroresponse.

To our surprise one of the tested HBDs showed low-level BKPyV viremia. Detection of BKPyV DNA in blood of immunocompetent individuals has been reported before in a healthy kidney donor (31), but a cohort of 400 HBDs tested by Egli et al. was found negative (7). Apparently BKPyV viremia can occur in persistently infected healthy individuals, albeit in a frequency much lower than viruria seen in approximately 7-55% of healthy people (7-9). The clinical relevance of such viremic episodes in healthy individuals is unknown. Since viremia in the HBD was assessed in the T5 samples only, we could not follow-up the BKPyV-IgG seroresponse in this individual. The difference in measured seroreactivity between T1 and T5 was not statistically significant (not shown).

In conclusion, we showed that BKPyV IgG seroreactivity is influenced by preceding BKPyV replication in a dose-dependent manner and is generally stable in immunocompetent individuals. The latter lends support for the tentative use of BKPyV IgG seroreactivity as a possible valuable and reproducible pretransplantation biomarker in donors to predict BKPyV infection and related complications after transplantation in KTRs. The biological relevance of this marker could be its reflection of the amount of latent infectious BKPyV sowed in a donor kidney during previous viremic episodes. Future studies should be directed at establishing this relation by comparing the viral kidney load with seroreactivity, which could further support the development of a useful biomarker that could improve the management and prevention of BKPyV-associated disease in KTRs.

Acknowledgements

The study cohort was part of a larger prospective European multicenter study designed to investigate the role of human papillomavirus infection in the development of skin cancer in solid-organ transplant patients. We want to thank dr. Jan Nico Bouwes Bavinck from the department of Dermatology in the Leiden University Medical Center, Leiden, the Netherlands for assembling the cohort.

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The Dutch kidney Foundation who supported this study had no role in study design;

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Declaration of interests

We declare that we have no conflicts of interest.

Contributors

HFW, EvdM and MCWF initiated and designed the study. EvdM and CSvdB-dB gathered the experimental data. HFW and EvdM analysed the data. EWvZ provided statistical support. HFW, EvdM, HZL, ACMK, JIR, and MCWF interpreted the data. HFW and MCWF drafted the manuscript, including figures and tables. All authors reviewed and approved the final report.

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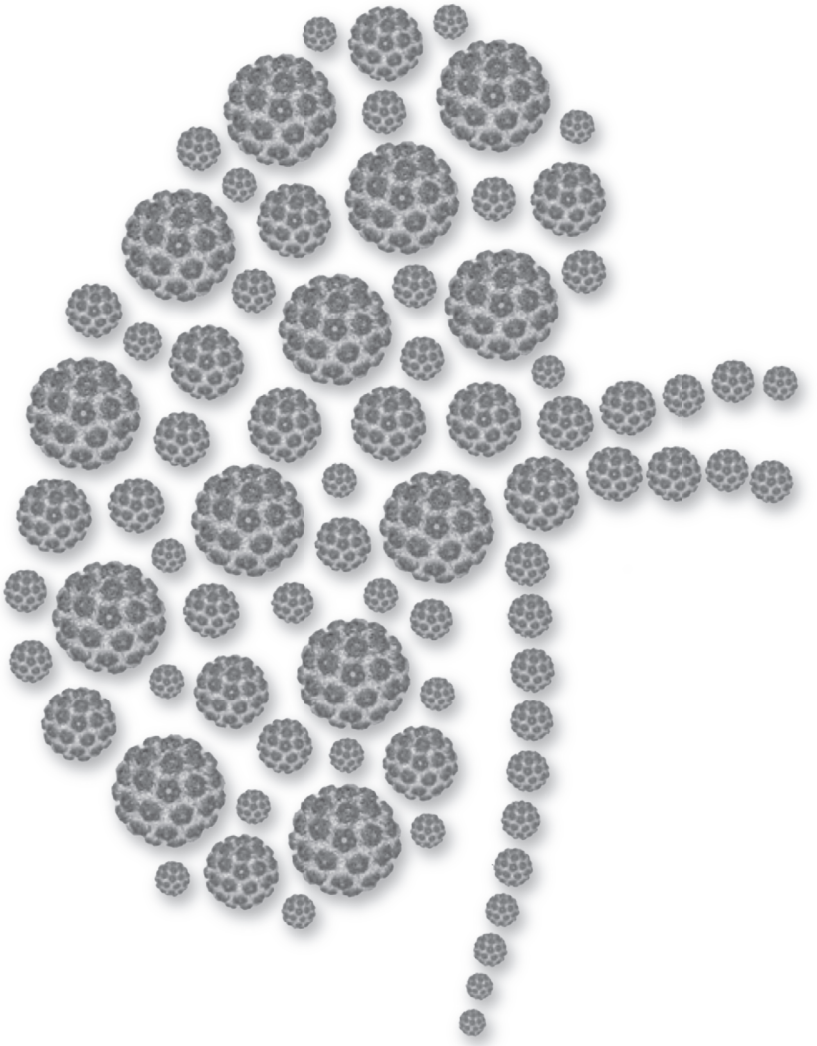

Chapter 4

Reduced risk of BK polyomavirus infection in HLA-B51 positive kidney transplant recipients

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Abstract

Background

Identification of specific HLA alleles and T cell epitopes that influence the course of BK polyomavirus (BKPyV) infection after kidney transplantation (KTx), including development of BKPyV-associated nephropathy (BKPyVAN), can be useful for patient risk stratification and possibly vaccine development.

Methods

In a retrospective cohort of 407 living kidney donor-recipient pairs, donor and recipient HLA class I and II status were correlated with the occurrence of recipient BKPyV viremia and BKPyVAN in the first year after KTx. Relevant HLA alleles were systematically analyzed for candidate peptide epitopes in silico.

Results

While none of the 78 HLA alleles analyzed increased the risk of BKPyV viremia and BKPyVAN, a considerable reduction of BKPyV viremia and BKPyVAN cases was observed in HLA-B51 positive KTx recipients. Multivariate analysis showed that HLA-B51-positivity, found in 36 recipients (9%), reduced the risk of viremia approximately five-fold (HR 0.18, 95% CI: 0.04 – 0.73, p = 0.017).

Four HLA-B51-restricted putative cytotoxic T lymphocyte epitopes were identified, including a previously described HLA-B supermotif-containing peptide (LPLMRKAYL), encoded by two relevant T-antigens (Small T and Large T) and previously shown to be highly immunogenic.

Conclusions

In conclusion, HLA-B51-positive kidney transplant recipients were less susceptible to BKPyV infection, which might be explained by efficient presentation of a particular BKPyV-derived immunogenic peptide.

Introduction

BK polyomavirus-associated nephropathy (BKPyVAN) represents a major burden for kidney transplant recipients (KTRs). After transplantation, BKPyV-DNA is detected in urine (viruria) of at least 50% of KTRs. Progression to viremia (BKPyV-DNA in the circulation) is seen in 20-30% (131, 146-149). A small proportion of viremic KTRs, 1-10% of total, develops BKPyVAN, which has a significant impact on morbidity and graft survival (150-153).

Currently, no BKPyV-specific antiviral drugs are available. Reduction of immunosuppression, with the aim of reconstituting BKPyV immune responses, is the only effective evidence-based treatment (149, 154-157). As sustained viremia and BKPyV-loads above 10^4 genome copies/ml (c/ml) increase the risk of BKPyVAN, KTRs are regularly evaluated for BKPyV viremia to guide timely reduction of immunosuppression, halt BKPyV infection and prevent BKPyVAN (146, 147, 149, 154, 158, 159).

Obviously reducing immunosuppression, resulting in clearance of BKPyV viremia in 80 – 100% of viremic KTRs (149, 151, 155-157), increases the risk of allograft rejection (149, 151, 154, 155, 157). Therefore, the care of KTRs and the overall success of transplantation could improve if current pre-emptive strategies to control BKPyV infection would include BKPyV predictive and preventive strategies (160). With this in mind, we recently put together a cohort of 407 living kidney donor-recipient pairs to identify donor and recipient-related risk factors of BKPyV viremia and BKPyVAN that could be used for pre-KTx risk stratification. In this cohort, we showed that donor BKPyV-directed IgG seroreactivity measured pre-KTx, is a strong determinant of BKPyV infection increasing the risk of KTR viremia up to 10-fold (161). Here, analyzing the same donor-recipient pairs, we investigated the role of individual donor and recipient HLA compositions regarding development of BKPyV infection after KTx.

HLA molecules play essential roles in presenting (viral) antigens to various T cell subsets and at the same time serve as allo-antigens in the context of allogeneic KTx (162). Identification of specific HLA alleles among donors and recipients that influence the risk of BKPyV infection could contribute to personalized BKPyV risk stratification for KTRs. A number of studies have reported certain donor and recipient HLA alleles that seemed to influence development of BKPyV infection after KTx (162-165). For instance (sustained) viremia was found more often in HLA-C7 negative donors and recipients (163), and in HLA-A28 and A68 positive re-

cipients, while recipient HLA-A2 and donor HLA-A9 increased the risk of BKPyVAN (165). Here, we correlated the donor and recipient HLA class I (A, B, C) and class II (DQ and DR) make-up with the development of BKPyV viremia and BKPyVAN in the recipients of our living kidney donor-recipient pair cohort (161). HLA alleles significantly associated with BKPyV infection and BKPyVAN after correction for multiple testing (166), were probed *in silico* for their expected efficiency to present BKPyV-derived peptides, to identify putatively relevant T cell epitopes.

Materials and Methods

Study population and sample collection

The study population includes 407 adult (> 18 years of age) living donor-recipient pairs transplanted at the Leiden University Medical Center (LUMC) between 2003 and 2013, as described previously (161). In brief, donor and recipient sera were collected pre-KTx and recipient plasmas screened for BKPyV-DNA were collected during one year of follow-up at five regular time-points post-KTx. The mean follow-up was 9.1 months and 80%, 95%, 87%, 63% and 36% of the recipient serum samples were available at time point 1, 2, 3, 4 and 5, respectively. The median number of time-points analyzed per recipient was 3.6 and the minimum number was 2 time-points. All samples were originally collected for routine serological and molecular virus-screening and stored at -20˚C. The study protocol was submitted to the Medical Ethical Committee of the LUMC that decided formal approval was not needed, due to the retrospective study design and the use of previously collected and anonymized samples..

BKPyV seroreactivity, detection of BKPyV viremia and assessment of BKPyVAN

Pre-KTx serum samples were analyzed by an in-house Luminex immunoassay detecting IgG-reactivity against the BKPyV-genotype Ib1 major viral capsid protein 1 (VP1), as described (161, 167, 168). For detection of BKPyV-DNA in blood, quantitative BKPyV real-time polymerase chain reaction (PCR) was used to analyze blood plasma. Using the primers 440BKVs 5'-GAAAAGGAGAGTGTCCAGGG-3' and 441BKVas 5'-GAACTTCTACTCCTCCTTTTATTAGT-3' and a Taqman probe 576BKV-TQ-FAM FAM 5'-CCAAAAAGCCAAAGGAACCC-3'-BHQ1, a 90-bp fragment within the BKPyV VP1 gene is amplified. Simultaneous isolation, amplification, and detection of a standard amount of phocid herpesvirus were used for internal control of inhibition.

Routine recipient BKPyV-load screening at 1.5, 3 and 6 months posttransplantation was implemented in May 2007. In case of clinical suspicion of BKPyV infection, BKPyV-loads were also determined later than 6 months posttransplantation. In samples obtained before 2007 and in samples obtained after 2007 that had not been routinely analyzed, BKPyV-loads were determined in retrospect.

A kidney biopsy was performed if indicated in the view of the treating physician, and BKPyVAN was diagnosed was diagnosed based on immunohistological examination of allograft biopsy specimens showing characteristic pathological features, such as intranuclear viral inclusions in tubular epithelial cells, cell enlargement with polymorphic nuclei, interstitial inflammation and tubular atrophy or fibrosis. BKPyVAN diagnosis was confirmed by immunohistochemical staining with a PyV-cross-reacting mouse monoclonal antibody (PAb416, Calbiochem) raised against large T antigen of SV40 polyomavirus (SV40).

HLA genotyping

HLA class I A, B and C typing was performed with a PCR-based reversed sequence specific bead hybridization assay (Lifecodes HLA-SSO Typing Immucor Norcross, Georgia), which involves PCR amplification of targeted regions within the major histocompatibility complex (MHC) class I regions with group specific primers, followed by a process of probing the amplicon with Luminex beads, each coated with sequence specific oligonucleotide probes to identify the presence or absence of specific alleles. The assignment of the HLA allele is then based on the reaction pattern observed, compared to patterns associated with published sequences (Lifecodes HLA-SSO Typing Immucor Norcross, Georgia). HLA class II DR and DQ typing was performed with a reversed approach of the PCR/SSOP technique described previously (169). Briefly, using Biotin-labelled generic primers the polymorphic regions of the HLA genes were amplified by PCR. After amplification, the PCR fragments were hybridized under stringent conditions to HLA specific probes. Signals to discriminate for positive and negative probe hybridization were achieved by adding horseradish peroxidase streptavidin followed by a luminogen (Amersham ECL Kit, GE Healthcare Biosciences Pittsburgh, USA. HLA assignment was done by locally developed HLA allele assignment software.

HLA-B51 epitope prediction

The major structural (VP1, VP2, VP3) and non-structural (agnoprotein, small and large T-antigens) proteins encoded by the BKPyV Dunlop strain (genotype Ia, NCBI Reference Sequence NC_001538.1), were analyzed for containing HLA-B51

presentable, putative CD8+ T cell peptide epitopes using the web-based T cell epitope prediction tool SYFPEITHI (170, 171).

HLA-B51 preferentially binds 9mer peptides, with a stringent requirement for proline in position 2, and enhanced binding by a dominant hydrophobic residue like leucine, isoleucine or valine at position 9 and another hydrophobic residue like leucine or valine at position 3 or 4 (172, 173). As HLA-B51 preferentially binds 9mer peptides, no other peptide lengths were included in the analysis.

HLA-B51 specificity of the predicted T cell epitopes was evaluated by a parallel analysis of the closely related HLA-B52 molecule using the Immune Epitope Database (IEDB) analysis resource Consensus tool (174) which combines predictions from artificial neural network (ANN) aka NetMHC (4.0) (175-177), stabilized matrix method (SMM) (178) and combinatorial peptide libraries (CombLib) (179).

Statistical analyses

Data were analyzed with IBM SPSS Statistics software version 23. Descriptive analyses were used to report cohort characteristics. The association between HLA alleles of donors and recipients with development of viremia in KTRs were analyzed by two-sided Fisher's exact test. Correction of p-values for multiple comparisons was performed according to the Šidák method, which has a bit more power than the Bonferroni correction, to prevent type I errors due to multiple testing for multi-HLA-alleles.(166) The corrected p-values were calculated using the formula 1-(1-p)^N, where p is the obtained p-value and N the number of alleles tested (180-182). P-values were corrected in two ways; by the number of HLA alleles within each locus, and by the total number of tested HLA alleles. The Woolf Haldane test was used to calculate the odds ratios and corresponding 95% confidence intervals (CI) (183, 184). Differences between HLA-B51 negative and HLA-B51 positive recipients and donors were assessed using the Chi-Square test, Fisher's exact test, Student's *t*-test or Mann-Whitney U test as appropriate. To indicate onset of recipient BKPyV viremia in association with the HLA-B51 status of the recipient, Kaplan-Meier curves were generated according to the recipient HLA-B51 status and the Log Rank (Mantel Cox) test was performed. Uni- and multivariate Cox regression were performed to determine if the effect of HLA-B51 on BKPyV viremia was significant if tested alone and together with other covariates. For all performed tests a p-value < 0.05 in a two-sided test was considered statistically significant.

Results

As described previously (161), 111 of 407 KTRs (27%) developed BKPyV viremia within one year after KTx. BKPyVAN was diagnosed in 12 (3%) KTRs (Table 1). An overview of all previously analyzed potential risk factors of BKPyV infection can be found in Tables 4 and 5 in our previous report on this cohort (161).

Table 1. Basic donor and recipient population characteristics sorted for development of BKPyV viremia and BKPyVAN within the first year after kidney transplantation.

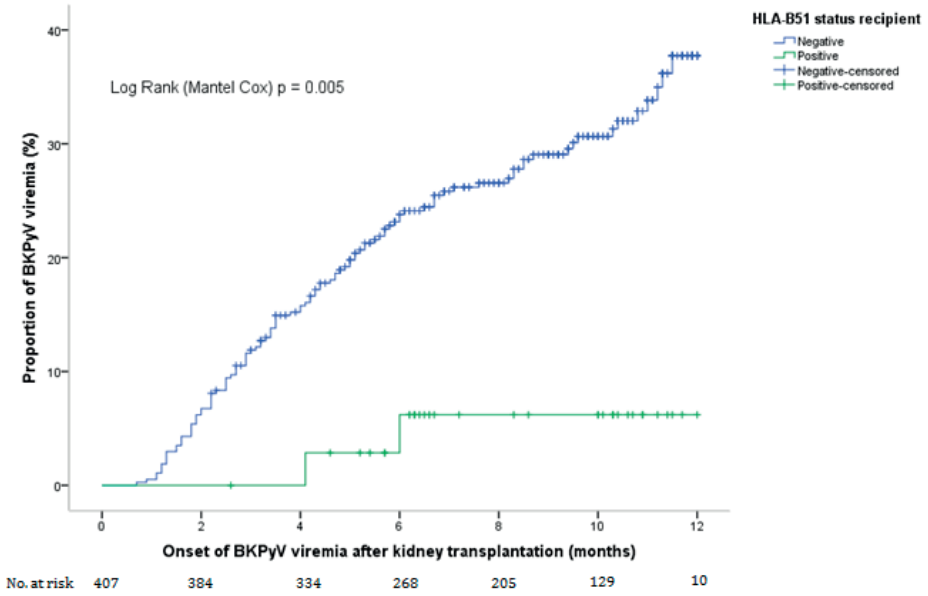
	All recipients (n = 407)			Viremic recipients (n = 111)		
	No BKPyV viremia (n = 296)	BKPyV viremia (n = 111)	p-value[1]	No BKPyVAN (n = 99)	BKPyVAN (n = 12)	p-value[1]
Donor						
Age (years)	53 (11.7)	54 (11.5)	0.354	54 (11.7)	57 (9.6)	0.386
Gender (male)	119 (40%)	42 (38%)	0.664	37 (37%)	5 (42%)	0.763
Recipient						
Age (years)	50 (13.5)	53 (14.2)	0.080	53 (14.1)	53 (16.1)	0.790
Gender (male)	177 (60%)	73 (66%)	0.271	65 (66%)	8 (67%)	1.000

Data are shown as mean (SD) or n (%).
BKPyV, BK polyomavirus; BKPyVAN, BK polyomavirus-associated nephropathy.
[1]The p-values were calculated using the Chi-Square test, Fisher's exact test or Student's *t*-test. A p-value <0.05 was considered statistically significant.

To investigate the association between BKPyV infection in KTRs and the MHC class I and II background of donors and recipients, their HLA typing information including HLA-A, B, C, DQ and DR was retrieved (Tables S1 and S2). The distribution of different HLA alleles across the cohort reflected that of the Dutch general population (182), and did not differ between donors and recipients. Data comparison showed that some donor HLA alleles were less (for example HLA-B27) and some were more frequently (HLA-B56) found among KTRs with BKPyV viremia respectively, but after correction for multiple testing using the Šidák method (166), no specific donor HLA allele was associated with viremia (Table S1). Comparable associations were found for a number of recipient HLA alleles. For example, recipient HLA-C7 and HLA-DR12 were associated with a higher risk of viremia, whereas recipient HLA-A30, B13, B51, C15 and DR13 were associated with a lower risk of viremia (**Table S2**). After correction for multiple testing within the respective HLA loci, recipient HLA-B51 remained significantly associated with a reduced risk of viremia (p = 0.035, Table S2). Correction for multiple testing within the total number of HLA alleles tested resulted in borderline significant associations (Table S1 and S2). The association found for recipient HLA-B51 was studied in more detail.

In total, 11% of donors (n = 44) and 9% of recipients (n = 36) were HLA-B51 positive. Of 111 viremic recipients, only 2 (2%) were HLA-B51 positive compared to 34 (11%) of 296 nonviremic recipients (p = 0.002, Table 2). The HLA-B51 donor status did not affect incidence of viremia (p = 0.720, Table 2). To substantiate the observed association between the recipient HLA-B51 status and BKPyV viremia, a Kaplan-Meier curve was generated to plot the onset of viremia during follow-up stratified for the HLA-B51 status of recipients. A strong and significant correlation was observed between recipient viremia and HLA-B51 status (p = 0.005, Figure 1).

Figure 1. Proportion of BKPyV viremia detected in the first year after kidney transplantation according to HLA-B51 status of the recipient.



Kaplan-Meier (1 - survival function) curves for proportion of BKPyV viremia observed in the 371 HLA-B51 negative and 36 HLA-B51 positive recipients. The numbers at risk below the X-axis indicate the number of recipients still at risk for developing BKPyV viremia. Tick marks represent censored recipients. BKPyV, BK polyomavirus; HLA, human leukocyte antigen.

Comparable to the observed protective effect of HLA-B51 against viremia, BKPyVAN was not diagnosed in any of the HLA-B51 positive recipients, compared to 12 cases of BKPyVAN in the HLA-B51 negative recipients (Table 2). This difference was not statistically significant, possibly related to the low number of BKPyVAN cases in our cohort.

Table 2. Incidence of BKPyV viremia and BKPyVAN in 407 KTRs sorted for HLA-B51 status of themselves and of their donors, and sorted by HLA-B51 matching.

	Recipients (n = 407)			Viremic recipients (n = 111)		
	No BKPyV viremia (n = 296)	BKPyV viremia (n = 111)	p-value[1]	No BKPyVAN (n = 99)	BKPyVAN (n = 12)	p-value[1]
Donor HLA-B51 status						
Negative	265 (90%)	98 (88%)	0.720	86 (87%)	12 (100%)	0.354
Positive	31 (10%)	13 (12%)		13 (13%)	0 (0%)	
Recipient HLA-B51 status						
Negative	262 (89%)	109 (98%)	0.002	97 (98%)	12 (100%)	1.000
Positive	34 (11%)	2 (2%)		2 (2%)	0 (0%)	
Donor/recipient pair HLA-B51 status						
+ / +	14 (5%)	0 (0%)	0.511	0 (0%)	0 (0%)	n.p.
- / +	20 (7%)	2 (2%)		2 (2%)	0 (0%)	
+ / -	17 (6%)	13 (12%)	0.095	13 (13%)	0 (0%)	0.353
- / -	245 (83%)	96 (86%)		84 (85%)	12 (100%)	

Data are shown as n (%).
BKPyV, BK polyomavirus; BKPyVAN, BK polyomavirus associated nephropathy; HLA, human leukocyte antigen; n.p., not possible.
[1]The p-values were calculated using the chi-square test or Fisher exact test. A p-value <0.05 was considered statistically significant.

We also analyzed the effect of donor-recipient HLA-B51 matching on the development of BKPyV viremia and BKPyVAN after KTx. BKPyV viremia nor BKPyVAN occurred in the 14 HLA-B51 +/+ matched pairs (Table 2). In HLA-B51 discrepant (-/+ and +/-, n = 52) donor-recipient pairs, 29% viremia was observed, of which the majority (13 of 15 viremic cases) developed among HLA-B51-negative recipients. A comparable percentage of viremia (28%) was observed among HLA-B51 double negative pairs, which were also the only pairs in which BKPyVAN occurred.

Previous analyses of our cohort revealed a strong association between high donor BKPyV seroreactivity and development of KTR viremia, and a weak inverse association for recipient BKPyV seroreactivity (161). To investigate a possible association (confounding) between BKPyV seroreactivity and HLA-B51 status, the previously determined pre-KTx donor and recipient BKPyV seroresponses were evaluated according to the HLA-B51 status of donors and recipients. No difference in BKPyV seroreactivity among HLA-B51 positive and negative donors and recipients was observed (Figure S1). However, among BKPyV viremic recipients, the mean BKPyV seroreactivity was clearly lower in HLA-B51 positive recipients, which reached statistical significance despite the low number of relevant subjects ($p < 0.001$,

Table 3. Pretransplantation donor and recipient BKPyV seroreactivity of HLA-B51 positive recipients (n = 36) and association with BKPyV viremia and of BKPyV viremic recipients (n = 111) and association with HLA-B51 status.

	BKPyV viremic recipients (n = 111)			HLA-B51 positive recipients (n = 36)		
	HLA-B51 negative (n = 109)	HLA-B51 positive (n = 2)	p-value[1]	No BKPyV viremia (n = 34)	BKPyV viremia (n = 2)	p-value[1]
Donor BKPyV seroreactivity	17140 (6644)	20467 (3044)	0.483	11287 (7593)	20467 (3044)	0.102
Recipient BKPyV seroreactivity	12511 (7928)	3125 (614)	<0.001	14866 (7535)	3125 (614)	<0.001

Data are shown as mean (SD).
BKPyV, BK polyomavirus.
[1]The p-values were calculated using the Student t-test. A p-value <0.05 was considered statistically significant.

Table 3). When BKPyV seroreactivity was evaluated in HLA-B51 positive recipients only, nonviremic HLA-B51 positive recipients (n = 34) were more seroreactive than their viremic equivalents (n = 2, $p < 0.001$, Table 3).

To correct for possible BKPyV viremia confounding between BKPyV seroreactivity and HLA-B51 status, we performed a multivariate analysis and included previously analyzed donor and recipient-related risk factors in the analysis (161), such as unrelatedness between donor and recipient, use of tacrolimus, and rejection treatment consisting of methylprednisolone 1000 mg intravenously once daily for 3 subsequent days. This analysis showed that the risk of viremia in a HLA-B51 positive recipient was approximately 5-fold lower compared to a HLA-B51 negative recipient (HR 0.17; 95% CI: 0.04 – 0.69; p = 0.013; Table 4). For donor HLA-B51 positivity a nonsignificant reverse trend was found (HR1.72; 95% CI: 0.94 – 3.15; p = 0.079; Table 4). As reported previously (161), donor BKPyV seroreactivity was the strongest risk factor for BKPyV viremia identified in our cohort.

Since we observed a significant protective effect of HLA-B51 in KTRs, that might be explained by efficient BKPyV antigen presentation and T cell immunity in HLA-B51 positive subjects, we screened common BKPyV proteins including the T-antigens for containing potentially HLA-B51 presentable peptides using the web-based T cell epitope prediction tool SYFPEITHI (170). This analysis resulted in the identification of four 9mer peptides that fulfilled the HLA-B51 primary and secondary anchor specificities explained in the Material and Methods and therefore might represent putative CTL-epitopes (Table 5). Three of them are found within the major capsid protein VP1 and one, LPLMRKAYL, within the N-terminal part of both the Small and the Large T-antigen expressed by alternative splicing from the same exon (152, 153). As a HLA-B51- pecificity check, a parallel analysis

Table 4. Uni- and multivariate Cox regression analysis for risk factors of BKPyV viremia development among 407 kidney transplant recipients in the first year after transplantation.

Covariate	Univariate analysis			Multivariate analysis		
	HR	95%-CI	p-value[1]	HR	95%-CI	p-value[1]
Age donor (years)	1.01	0.99 – 1.02	0.498	1.01	0.99 – 1.03	0.608
Age recipient (years)	1.01	1.00 – 1.03	0.138	1.00	0.99 – 1.02	0.774
Gender donor	0.90	0.62 – 1.33	0.603	1.07	0.69 – 1.64	0.777
Gender recipient	1.21	0.82 – 1.80	0.340	1.05	0.69 – 1.61	0.811
Underlying condition[2]						
Inherited	1.00		0.359	1.00		0.410
Glomerular	0.97	0.56 – 1.66	0.903	1.05	0.59 – 1.87	0.874
Vascular	1.45	0.86 – 2.43	0.163	1.61	0.94 – 2.77	0.083
Obstructive	0.78	0.34 – 1.79	0.554	1.00	0.41 – 2.42	0.997
Other	0.94	0.52 – 1.67	0.820	1.17	0.63 – 2.18	0.627
Dialysis pretransplantation	0.85	0.59 – 1.24	0.405	0.96	0.60 – 1.53	0.861
Duration dialysis (months)	0.90	0.77 – 1.04	0.156	0.99	0.97 – 1.00	0.176
Unrelated donor	1.49	1.02 – 2.17	0.042	1.18	0.76 – 1.85	0.459
Retransplantation	1.23	0.66 – 2.30	0.513	1.26	0.63 – 2.52	0.510
PRA immunization pretransplantation	0.72	0.23 – 2.26	0.570	0.77	0.23 – 2.58	0.669
Blood group compatibility	1.43	0.67 – 3.08	0.359	1.28	0.48 – 3.42	0.622
Donor HLA-B51 positivity	1.17	0.66 – 2.08	0.599	1.72	0.94 – 3.15	0.079
Recipient HLA-B51 positivity	0.17	0.04 – 0.69	0.013	0.18	0.04 – 0.73	0.017
Basiliximab vs. alemtuzumab	1.04	0.51 – 2.13	0.921	1.06	0.43 – 2.60	0.902
Tacrolimus vs cyclosporin A	0.89	0.58 – 1.37	0.599	0.79	0.49 – 1.26	0.317
Donor BKPyV seroreactivity[3]	1.59	1.38 – 1.84	< 0.001	1.61	1.38 – 1.88	< 0.001
Recipient BKPyV seroreactivity[3]	0.90	0.80 – 1.02	0.088	0.85	0.75 – 0.95	0.006
Rejection treatment[4]	1.54	1.02 – 2.34	0.040	1.51	0.96 – 2.37	0.073

BKPyV, BK polyomavirus; CI, confidence interval; HLA, human leukocyte antigen; HR, hazard ratio; PRA, panel reactive antibodies.

[1]The p-values, HRs, and 95% CIs were calculated with uni- and multivariate Cox regression analysis. A p-value <0.05 was considered statistically significant.

[2]Describes the HR of viremia in recipients with each underlying condition group compared to the inherited disease group; Inherited diseases include autosomal dominant polycystic kidney disease, medullary cystic disease, cystic kidney disease not otherwise specified, arteriovenous malformation due to Klippel-Trénaunay-Weber syndrome, familiar erythrocyturia, Alport syndrome, familiar focal segmental glomerulosclerosis by NPHS2-mutation, familiar haemolytic uremic syndrome, and kidney dys- and agenesis; Glomerular diseases include membranous nephropathy, IgA nephropathy, systemic lupus erythematosus, proliferative glomerulonephritis, membranoproliferative glomerulonephritis, focal segmental glomerulosclerosis, pauci-immune crescentic glomerulonephritis, Morbus Wegener, ANCA-associated vasculitis, anti-glomerular basement membrane nephritis, global glomerulosclerosis, and immunotactoid glomerulonephritis; Vascular diseases include diabetes mellitus type I and II, hypertension, nephrosclerosis, haemolytic uremic syndrome, arteria renalis stenosis, and thrombotic microangiopathy; Obstructive diseases include reflux nephropathy, urethral valves, nephrolithiasis, obstructive uropathy, and prostate hypertrophy; Other include chronic pyelonephritis, acute tubular necrosis, tubulointerstitial nephritis, lithium nephropathy, urate and analgesic nephropathy, iatrogenic, and unknown underlying condition.

[3]Donor and recipient seroreactivity per 5000 increasing mean fluorescence intensity.

[4]Rejection treatment consisted of methylprednisolone 1000 mg intravenously once daily for 3 days.

Table 5. Potential HLA-B51 presented nonamer epitopes encoded by the major BKPyV proteins, predicted with the web based tool SYFPHEITHI by use of the whole BKPyV genome (Dunlop strain).

BKPyV protein[1]	Amino acid position	Amino acid sequence BKPyV Dunlop strain	SYFPHEITHI score[2]
VP1	20	E P V Q V P K L L	20
VP1	158	E P L E M Q G V L	24
VP1	252	G P L C K A D S L	20
Large T[3]	27	L P L M R K A Y L	20
Small T[3]	27	L P L M R K A Y L	20

BKPyV, BK polyomavirus; HLA, human leukocyte antigen.
[1]The following viral proteins were analysed: small T-antigen, large T-antigen, VP1, VP2 and VP3.
[2]The SYFPHEITHI score ranges from 0 to 25, the higher the score the higher the probability that the peptide is being processed and presented to T cells.
[3]The first exon of the Large T and Small T antigen where the identified peptide LPLMRKAYL is located is shared.

for the closely related HLA-B52 molecule was performed for the predicted BKPyV T cell epitopes using the IEDB analysis resource Consensus tool (174). This analysis showed that HLA-B51 had a much higher predicted affinity for all of the predicted epitopes than the closely related HLA-B52 (Table S3).

Discussion

We systematically analysed the effect of specific HLA alleles on the occurrence of BKPyV viremia and BKPyVAN among donors and recipients post-KTx. HLA molecules are expressed on all nucleated cells and play an essential role in activation of the immune response and control of infection, for example in case of BKPyV (185). They induce adaptive immunity by presenting pathogen-derived peptides to T cells and innate immunity by activation of natural killer (NK) cells via ligation to killer-cell immunoglobulin-like receptors (KIRs). The recognition of HLA-peptide complexes by a peptide-specific T cell receptor leads to activation of specific CD8+ cytotoxic T cells through HLA class I-peptide interaction or CD4+ T helper cells through HLA class II. Activation of virus-specific CD8+ cytotoxic T cells results in specific killing of infected cells, whereas activation of virus-specific CD4+ T cells supports the generation of effector T cells that provide help to CD8+ T cells and augment generation of B cells for production of virus-specific antibodies.

For some viruses, such as hepatitis C virus and the human immunodeficiency virus (HIV), it has been shown that certain HLA alleles are significantly associated with viral clearance or slow progression of disease (186, 187). Often it is unknown why these HLA alleles provide a host advantage during viral infection, but sometimes it can be explained by the preferential presentation of epitopes from highly conserved viral proteins by these alleles (187). In this study, we observed a reduced risk of BKPyV viremia and BKPyVAN in HLA-B51 positive KTRs, also when corrected for multiple testing within the respective HLA loci. When corrected for the total number of HLA alleles, statistical significance was lost, indicating a false-positive finding due to a type I error cannot be excluded. However, in the exploratory context of our research, we argue that such a stringent multiple testing correction is overly conservative and could preclude any interesting discoveries. Moreover, we note that the association we found is biologically plausible as the particular HLA-B allele has been previously reported by independent laboratories in conjunction with protection against other viruses (see below). We believe that the combination of statistical and biological evidence makes our finding interesting and merits further research, despite the risk of a type I error.

HLA-B51 is a prevalent HLA allele in Europe, North America and the Far and Middle East (151, 188, 189), and relevant in the course of other infectious diseases. For example, HLA-B51 positive KTRs have lower risk of developing CMV viremia (190). The protective effect of HLA-B51 was also seen with reduced progression of HIV and development of adequate antibody responses to the measles vaccine (191-193). On the other hand, a higher risk of post-transplant lymphoproliferative disorder due to Epstein-Barr virus was reported, as well as increased CMV interstitial pneumonia after bone marrow transplantation, CMV retinitis and encephalitis in HIV patients (194-197). Finally, HLA-B51 is clearly associated with the non-infectious disorder Behçet's disease (188). The etiology and pathogenesis of this systemic vasculitis are unclear but the general accepted hypothesis includes an intense inflammatory reaction elicited by an infectious agent in HLA-B51 positive subjects (188, 198-201).

The basis behind the clinical associations with HLA-B51 is not known. Since BKPyV peptide presentation by HLA-B51 and CD8+ cytotoxic T cell recognition may play a crucial role (151, 202, 203) in the association found in our study, we looked for BKPyV-derived peptides that meet the primary and secondary anchor specificities defined for HLA-B51. Interestingly, a previous study by Li et al. showed that the BKPyV T antigen-derived 9mer LPLMRKAYL, one of the HLA-B51 candidate epitopes that we identified, is recognized by T cells from healthy donors in the context of

HLA-B7 and HLA-B8, and induces interferon-γ production by CD8+ T cells (203). Another study used a bioinformatics approach to characterize potential BKPyV-specific CD8+ T cell epitopes for 14 common HLAs in Europe and North America (151), and identified the same 9mer peptide as a promising BKPyV epitope, which was confirmed by inducing interferon-γ production by CD8+ T cells in HLA-B7 and HLA-B8 BKPyV-seropositive individuals.

Both studies cited above indicate that LPLMRKAYL is a HLA-B7 and HLA-B8 restricted BKPyV epitope (151, 203). As our study indicates that this particular 9mer could also be a HLA-B51-restricted epitope, this peptide might comply with the BKPyV 'supermotif' and bind to several HLA-B molecules (173, 204, 205). In line with our observation, Leboeuf et al. showed that LPLMRKAYL, named 9m127 in their study, bound to HLA-B7, B8 and B51, and to HLA-A2 (202). Moreover, LPLMRKAYL-specific T cell responses measured by interferon-γ production increased significantly in KTRs that cleared BKPyV viremia, and LPLMRKAYL showed the highest frequency of BKPyV-specific T cell responses in expanded T cells from 97 KTRs. Another 9mer peptide that we identified (EPLEMQGVL) was shown to bind to HLA-B51 as well (202).

In general, identification of BKPyV 'supermotif' epitopes that can bind to a family of HLA molecules broadly represented in the worldwide population could be worthwhile for developing BKPyV-specific T cell response monitoring strategies, adoptive T cell transfer for prophylaxis and therapy, and for the design of BKPyV peptide vaccines. Why HLA-B alleles such as HLA-B7 and B8, that should be able to present LPLMRKAYL, were not found associated with BKPyV infection in our study is unclear. Possibly this is related to subtle differences in preferential peptide (amino acid) binding, or perhaps to higher interferon-ɣ production by T-cells elicited by HLA-B51 than by other HLA alleles (202).

Another explanation for the association between HLA-B51 and BKPyV infection could be the interaction between certain KIRs and HLA-B51. KIRs expressed on NK cells can transmit inhibitory or activating signals upon engagement with specific HLA class I ligands. The balance between these signals is considered to initiate or suppress NK cell activation (185, 206). For CMV infection for example, it was shown that the number of activating KIR genes inversely correlates with CMV infection after KTx (207, 208). Although one study did not find a KIR-linked protective effect for BKPyV infection after KTx, two other studies reported effects of activating KIRs on BKPyV infection (206, 207, 209). Unfortunately, KIR typing was not performed in our cohort.

It is generally assumed that HLA mismatching of the donor-recipient pair reduces the efficacy of eliminating virus infected donor cells, and increases the risk of alloimmune responses and therefore the need for immunosuppression (210). In line with this assumption, BKPyV viremia did not occur in HLA-B51 matched (+/+) donor-recipient pairs compared to 29% of viremia in HLA-B51 mismatched pairs (-/+ and +/-). It should be mentioned that we did not observe a statistically significant association between overall HLA-matching and BKPyV viremia, as shown and discussed in our previous article dealing with this cohort population (161).

In conclusion, the role of specific HLA alleles and HLA allele matching in development of BKPyV infection is still poorly understood. By analyzing a large cohort of living kidney donor-recipient pairs, we demonstrated a negative (protective) association between HLA-B51 positivity in KTRs and development of BKPyV infectious complications. This might be potentially useful for BKPyV risk stratification, for example to customize viral load screening (reduced frequency for HLA-B51 positive KTRs) and prevent unnecessary tapering of immunosuppression in HLA-B51 positive viremic KTRs. Furthermore, identification and further study of (potential) BKPyV-derived T cell epitopes can be useful to prevent or treat BKPyV infection and associated diseases in the future. The T-antigen derived 9mer LPLMRKAYL seems a promising candidate in this regard, and further investigations into the role of this peptide in developing immunity against BKPyV seem warranted. Considering the possible risk for a type I error, validation of the association between HLA-B51 positivity of KTRs and a reduced risk of BKPyV infection, preferably in larger, independent cohorts is needed.

Authorship

The author's specific contributions are as follows: HFW and MCWF initiated the study. HFW, ACMK, JWdF, JIR, FHJC and MCWF designed the study. HFW, CSdB, GWH and JIR collected the samples and gathered the data. CSdB performed the serological tests and the PCR assays. HFW analysed the data. EWvZ and GWH provided statistical support. HFW, ACMK, JWdF, JIR, FHJC, and MCWF interpreted the data. HFW and MCWF drafted the manuscript, and designed the figures and tables. All authors reviewed and approved the final report.

Disclosure

The authors declare no conflicts of interest

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Supporting Information

Table S1. Distribution of HLA genotypes among donors of 407 BKPyV nonviremic and viremic KTRs

HLA type[1]	Donors (n=407)	No BKPyV viremia (n=296)	BKPyV viremia (n=111)	OR[2]	95% CI[2]	p-value[3] crude	p-value[4] HLA-locus adjusted	p-value[5] HLA total adjusted
A*01	138 (34%)	93 (31%)	45 (41%)	1.49	0.95 – 2.34	0.100	0.849	1.000
A*02	199 (49%)	149 (50%)	50 (45%)	0.81	0.52 – 1.25	0.374	1.000	1.000
A*03	113 (28%)	78 (26%)	35 (32%)	1.29	0.80 – 2.08	0.321	0.999	1.000
A*11	50 (12%)	37 (13%)	13 (12%)	0.95	0.49 – 1.84	1.00	1.000	1.000
A*23	13 (3%)	11 (45)	2 (2%)	0.57	0.14 – 2.27	0.528	1.000	1.000
A*24	58 (14%)	40 (14%)	18 (16%)	1.25	0.69 – 2.28	0.525	1.000	1.000
A*25	6 (1%)	3 (1%)	3 (3%)	2.71	0.61 – 12.10	0.352	1.000	1.000
A*26	16 (4%)	13 (4%)	3 (3%)	0.68	0.21 – 2.24	0.573	1.000	1.000
A*29	22 (5%)	17 (6%)	5 (5%)	0.83	0.31 – 2.21	0.807	1.000	1.000
A*30	20 (5%)	12 (4%)	8 (7%)	1.87	0.76 – 4.60	0.202	0.983	1.000
A*31	25 (6%)	19 (6%)	6 (5%)	0.88	0.35 – 2.19	0.820	1.000	1.000
A*32	25 (6%)	21 (7%)	4 (4%)	0.54	0.19 – 1.52	0.249	0.994	1.000
A*33	11 (3%)	10 (3%)	1 (<1%)	0.37	0.07 – 2.08	0.302	0.998	1.000
A*34	3 (1%)	2 (<1%)	1 (<1%)	1.60	0.21 – 12.25	1.000	1.000	1.000
A*36	1 (<1%)	0 (0%)	1 (<1%)	8.05	0.33 – 199.09	0.273	0.997	1.000
A*66	1 (<1%)	1 (<1%)	0 (0%)	0.83	0.04 – 21.85	1.000	1.000	1.000
A*68	32 (8%)	27 (9%)	5 (5%)	0.51	0.20 – 1.30	0.150	0.946	1.000
A*80	1 (<1%)	1 (<1%)	0 (0%)	0.88	0. 04 – 21.85	1.000	1.000	1.000
B*07	101 (25%)	69 (23%)	32 (29%)	1.34	0.82 – 2.18	0.250	1.000	1.000
B*08	105 (26%)	71 (24%)	34 (31%)	1.40	0.87 – 2.27	0.203	0.999	1.000
B*13	12 (3%)	7 (2%)	5 (5%)	1.99	0.65 – 6.13	0.322	1.000	1.000
B*14	13 (3%)	7 (2%)	6 (5%)	2.38	0.81 – 6.96	0.126	0.980	1.000
B*15	74 (18%)	51 (17%)	23 (21%)	1.27	0.73 – 2.18	0.471	1.000	1.000
B*18	40 (10%)	33 (11%)	7 (6%)	0.57	0.25 – 1.29	0.190	0.998	1.000
B*27	21 (5%)	19 (6%)	2 (2%)	0.33	0.09 – 1.24	0.077	0.902	0.998
B*35	70 (17%)	53 (18%)	17 (15%)	0.84	0.47 – 1.52	0.658	1.000	1.000
B*37	12 (3%)	8 (3%)	4 (4%)	1.42	0.44 – 4.55	0.742	1.000	1.000
B*38	14 (3%)	10 (3%)	4 (4%)	1.14	0.37 – 3.52	1.000	1.000	1.000
B*39	9 (2%)	7 (2%)	2 (2%)	0.88	0.21 – 3.75	1.000	1.000	1.000
B*40	58 (14%)	47 (16%)	11 (10%)	0.60	0.30 – 1.19	0.152	0.992	0.992
B*41	3 (1%)	2 (<1%)	1 (<1%)	1.60	0.21 – 12.25	1.000	1.000	1.000
B*42	1 (<1%)	0 (0%)	1 (<1%)	8.05	0.33 – 199.09	0.273	1.000	1.000
B*44	82 (20%)	62 (21%)	20 (18%)	0.84	0.48 – 1.46	0.580	1.000	1.000
B*45	13 (3%)	9 (3%)	4 (4%)	1.27	0.40 – 3.97	0.757	1.000	1.000
B*46	2 (<1%)	2 (<1%)	0 (0%)	0.53	0.03 – 11.09	1.000	1.000	1.000
B*47	5 (1%)	4 (1%)	1 (<1%)	0.88	0.14 – 5.67	1.000	1.000	1.000

Table S1. (continued)

HLA type[1]	Donors (n=407)	No BKPyV viremia (n=296)	BKPyV viremia (n=111)	OR[2]	95% CI[2]	p-value[3] crude	p-value[4] HLA-locus adjusted	p-value[5] HLA total adjusted
B*48	2 (<1%)	2 (<1%)	0 (0%)	0.53	0.03 – 11.09	1.000	1.000	1.000
B*49	8 (2%)	7 (2%)	1 (<1%)	0.52	0.09 – 3.07	0.689	1.000	1.000
B*50	13 (3%)	9 (3%)	4 (4%)	1.27	0.40 – 3.97	0.757	1.000	1.000
B*51	44 (11%)	31 (11%)	13 (12%)	1.16	0.59 – 2.28	0.722	1.000	1.000
B*52	8 (2%)	8 (3%)	0 (0%)	0.15	0.01 – 2.66	0.114	0.970	1.000
B*53	5 (1%)	2 (1%)	3 (3%)	3.80	0.74 – 19.55	0.128	0.981	1.000
B*55	24 (6%)	19 (6%)	5 (5%)	0.74	0.28 – 1.94	0.637	1.000	1.000
B*56	3 (1%)	0 (0%)	3 (3%)	19.13	0.98 – 373.38	0.020	0.442	0.791
B*57	28 (7%)	17 (6%)	11 (10%)	1.83	0.84 – 3.98	0.185	0.997	1.000
B*58	9 (2%)	8 (3%)	1 (<1%)	0.46	0.08 – 2.65	0.454	1.000	1.000
B*73	1 (<1%)	1 (<1%)	0 (0%)	0.88	0.04 – 21.85	1.000	1.000	1.000
C*01	24 (6%)	18 (6%)	6 (6%)	0.95	0.38 – 2.39	1.000	1.000	1.000
C*02	22 (5%)	17 (6%)	5 (5%)	0.85	0.32 – 2.26	0.807	1.000	1.000
C*03	130 (32%)	100 (34%)	30 (28%)	0.75	0.47 – 1.22	0.278	0.986	1.000
C*04	81 (20%)	62 (21%)	19 (18%)	0.81	0.46 – 1.43	0.485	1.000	1.000
C*05	58 (14%)	46 (16%)	12 (11%)	0.69	0.36 – 1.35	0.336	0.995	1.000
C*06	76 (19%)	49 (17%)	26 (24%)	1.60	0.94 – 2.73	0.111	0.783	1.000
C*07	219 (54%)	154 (53%)	65 (61%)	1.38	0.88 – 2.17	0.173	0.916	1.000
C*08	18 (4%)	11 (4%)	7 (7%)	1.83	0.71 – 4.71	0.276	0.985	1.000
C*12	37 (9%)	25 (9%)	12 (11%)	1.37	0.67 – 2.81	0.438	0.999	1.000
C*14	12 (3%)	9 (3%)	3 (3%)	1.00	0.29 – 3.47	1.000	1.000	1.000
C*15	24 (6%)	17 (6%)	7 (7%)	1.18	0.49 – 2.85	0.813	1.000	1.000
C*16	23 (6%)	17 (6%)	6 (6%)	1.01	0.40 – 2.55	1.000	1.000	1.000
C*17	6 (1%)	4 (1%)	2 (2%)	1.52	0.32 – 7.25	0.661	1.000	1.000
DQB1*02	157 (39%)	112 (38%)	45 (41%)	1.12	0.72 – 1.75	0.648	0.995	1.000
DQB1*03	230 (57%)	168 (57%)	62 (56%)	0.96	0.62 – 1.49	0.911	1.000	1.000
DQB1*04	28 (7%)	19 (6%)	9 (8%)	1.32	0.59 – 2.96	0.518	0.974	1.000
DQB1*05	118 (29%)	89 (30%)	29 (26%)	0.83	0.51 – 1.35	0.464	0.96	1.000
DQB1*06	181 (44%)	136 (46%)	45 (41%)	0.81	0.52 – 1.25	0.371	0.901	1.000
DRB1*01	75 (18%)	57 (19%)	18 (16%)	0.82	0.46 – 1.47	0.566	1.000	1.000
DRB1*03	116 (29%)	79 (27%)	37 (33%)	1.38	0.86 – 2.20	0.218	0.959	1.000
DRB1*04	117 (29%)	84 (28%)	33 (30%)	1.07	0.67 – 1.73	0.806	1.000	1.000
DRB1*07	76 (19%)	58 (20%)	18 (16%)	0.81	0.45 – 1.43	0.478	1.000	1.000
DRB1*08	28 (7%)	18 (6%)	10 (9%)	1.56	0.71 – 3.43	0.378	0.998	1.000
DRB1*09	12 (3%)	10 (3%)	2 (2%)	0.62	0.15 – 2.52	0.525	1.000	1.000
DRB1*10	18 (4%)	12 (4%)	6 (5%)	1.40	0.53 – 3.71	0.590	1.000	1.000

Table S1. (continued)

HLA type[1]	Donors (n=407)	No BKPyV viremia (n=296)	BKPyV viremia (n=111)	OR[2]	95% CI[2]	p-value[3] crude	p-value[4] HLA-locus adjusted	p-value[5] HLA total adjusted
DRB1*11	71 (17%)	50 (17%)	21 (19%)	1.16	0.66 – 2.03	0.661	1.000	1.000
DRB1*12	19 (5%)	15 (5%)	4 (4%)	0.76	0.26 – 2.22	0.792	1.000	1.000
DRB1*13	106 (26%)	83 (28%)	23 (21%)	0.68	0.40 – 1.14	0.163	0.901	1.000
DRB1*14	26 (6%)	21 (7%)	5 (5%)	0.66	0.25 – 1.73	0.495	1.000	1.000
DRB1*15	100 (25%)	74 (25%)	26 (23%)	0.93	0.56 – 1.54	0.797	1.000	1.000
DRB1*16	8 (2%)	7 (2%)	1 (<1%)	0.52	0.09 – 3.07	0.689	1.000	1.000

Data are shown as n (%).

BKPyV, BK polyomavirus; CI, confidence interval; HLA, human leukocyte antigen; OR, odds ratio.

[1]From all donors the complete information of HLA A, B, DQ and DR were available, HLA C was missing in 8 donor cases.

[2]Odds ratios and corresponding 95% CI were calculated with the Woolf Haldane test.

[3]The p-values were calculated using the two-sided Fisher's exact test.

[4]The p-values were corrected for multiple testing according to the Šidàk method (Šidàk 1967). The formula of the Šidàk correction is 1-(1-p)^N, were N is the number of antigens (comparisons) per locus.

[5]The p-values were corrected for multiple testing according to the Šidàk method (Šidàk 1967). The formula of the Šidàk correction is 1-(1-p)^N, were N is the number of HLA alleles tested, which is 78 for donors.

A p-value <0.05 was considered statistically significant.

Table S2. Distribution of HLA genotypes among 407 BKPyV nonviremic and viremic KTRs

HLA type[1]	Recipients (n = 407)	No BKPyV viremia (n = 296)	BKPyV viremia (n = 111)	OR[2]	95% CI[2]	p-value[3] crude	p-value[4] HLA locus adjusted	p-value[5] HLA total adjusted
A*01	115 (28%)	80 (27%)	35 (32%)	1.24	0.77 – 1.99	0.389	1.000	1.000
A*02	213 (52%)	151 (51%)	62 (56%)	1.20	0.78 – 1.86	0.436	1.000	1.000
A*03	111 (27%)	83 (28%)	28 (25%)	0.87	0.53 – 1.43	0.618	1.000	1.000
A*11	48 (12%)	31(11%)	17(15%)	1.56	0.83 – 2.92	0.226	0.984	1.000
A*23	16 (4%)	12 (4%)	4 (4%)	0.95	0.32 – 2.85	1.000	1.000	1.000
A*24	57 (14%)	45 (15%)	12 (11%)	0.69	0.36 – 1.35	0.336	0.999	1.000
A*25	10 (2%)	8 (3%)	2 (2%)	0.77	0.19 – 3.22	0.734	1.000	1.000
A*26	23 (6%)	15 (5%)	8 (7%)	1.49	0.63 – 3.54	0.470	1.000	1.000
A*29	12 (3%)	10 (3%)	2 (2%)	0.62	0.15 – 2.51	0.525	1.000	1.000
A*30	15 (4%)	15 (5%)	0 (0%)	0.08	0.01 – 1.37	0.014	0.208	0.660
A*31	27 (7%)	18 (6%)	9 (8%)	1.39	0.62 – 3.14	0.504	1.00	1.000
A*32	22 (5%)	16 (5%)	6 (5%)	1.04	0.41 – 2.66	1.000	1.000	1.000
A*33	15 (4%)	12 (4%)	3 (3%)	0.73	0.22 – 2.44	0.768	1.000	1.000
A*34	5 (1%)	4 (1%)	1 (<1%)	0.88	0.14 – 5.65	1.000	1.000	1.000
A*66	2 (<1%)	1 (<1%)	1 (<1%)	2.67	0.27 – 25.89	0.473	1.000	1.000
A*68	39 (10%)	31 (11%)	8 (7%)	0.69	0.31 – 1.52	0.352	0.999	1.000
B*07	89 (21%)	63 (21%)	26 (23%)	1.14	0.68 – 1.91	0.687	1.000	1.000
B*08	93 (23%)	66 (22%)	27 (24%)	1.13	0.68 – 1.88	0.692	1.000	1.000
B*13	13 (3%)	13 (4%)	0 (0%)	0.09	0.01 – 1.60	0.024	0.477	0.831
B*14	17 (4%)	15 (5%)	2 (2%)	0.42	0.11 – 1.61	0.173	0.994	1.000
B*15	79 (19%)	54 (18%)	25 (23%)	1.31	0.77 – 2.23	0.328	1.000	1.000
B*18	33 (8%)	22 (7%)	11 (10%)	1.40	0.66 – 2.95	0.419	1.000	1.000
B*27	27 (7%)	20 (7%)	7 (6%)	0.97	0.41 – 2.30	1.000	1.000	1.000
B*35	70 (17%)	53 (18%)	17 (15%)	0.84	0.47 – 1.52	0.658	1.000	1.000
B*37	15 (4%)	9 (3%)	6 (5%)	1.87	0.67 – 5.18	0.252	1.000	1.000
B*38	18 (4%)	13 (4%)	5 (5%)	1.09	0.39 – 3.00	1.000	1.000	1.000
B*39	20 (5%)	17 (6%)	3 (3%)	0.52	0.16 – 1.66	0.303	1.000	1.000
B*40	64 (16%)	46 (16%)	18 (16%)	1.07	0.59 – 1.92	0.879	1.000	1.000
B*41	8 (2%)	5 (2%)	3 (3%)	1.71	0.44 – 6.65	0.455	1.000	1.000
B*42	3 (1%)	2 (<1%)	1 (<1%)	1.60	0.21 – 12.25	1.000	1.000	1.000
B*44	92 (23%)	64 (22%)	28 (25%)	1.23	0.74 – 2.04	0.429	1.000	1.000
B*45	5 (1%)	3 (1%)	2 (2%)	1.92	0.37 – 9.85	0.617	1.000	1.000
B*47	3 (1%)	2 (<1%)	1 (<1%)	1.60	0.21 – 12.25	1.000	1.000	1.000
B*49	9 (2%)	7 (2%)	2 (2%)	0.88	0.21 – 3.75	1.000	1.000	1.000
B*50	13 (3%)	9 (3%)	4 (4%)	1.27	0.40 – 3.97	0.757	1.000	1.000
B*51	36 (9%)	34 (12%)	2 (2%)	0.17	0.05 – 0.64	0.001	0.035	0.093

Table S2. (continued)

HLA type[1]	Recipients (n = 407)	No BKPyV viremia (n = 296)	BKPyV viremia (n = 111)	OR[2]	95% CI[2]	p-value[3] crude	p-value[4] HLA locus adjusted	p-value[5] HLA total adjusted
B*52	10 (2%)	8 (3%)	2 (2%)	0.78	0.19 – 3.23	0.735	1.000	1.000
B*53	3 (1%)	2 (<1%)	1 (<1%)	1.60	0.21 – 12.25	1.000	1.000	1.000
B*55	21 (5%)	14 (5%)	7 (6%)	1.40	0.56 – 3.47	0.615	1.000	1.000
B*56	5 (1%)	2 (<1%)	3 (3%)	3.80	0.74 – 19.55	0.128	0.975	1.000
B*57	31 (8%)	21 (7%)	10 (9%)	1.33	0.61 – 2.87	0.532	1.000	1.000
B*58	5 (1%)	5 (2%)	0 (0%)	0.24	0.01 – 4.33	0.329	1.000	1.000
B*73	1 (<1%)	1 (<1%)	0 (0%)	0.88	0.04 – 21.85	1.000	1.000	1.000
C*01	24 (6%)	20 (7%)	4 (4%)	0.56	0.20 – 1.59	0.344	0.996	1.000
C*02	41 (10%)	27 (9%)	14 (13%)	1.45	0.73 – 2.85	0.356	0.997	1.000
C*03	123 (30%)	85 (29%)	38 (35%)	1.28	0.81 – 2.04	0.332	0.995	1.000
C*04	70 (17%)	54 (19%)	16 (15%)	0.76	0.42 – 1.39	0.380	0.998	1.000
C*05	48 (12%)	34 (12%)	14 (13%)	1.12	0.58 – 2.16	0.863	1.000	1.000
C*06	70 (17%)	50 (17%)	20 (18%)	1.08	0.61 – 1.91	0.883	1.000	1.000
C*07	222 (55%)	153 (53%)	69 (63%)	1.51	0.97 – 2.37	0.073	0.624	0.996
C*08	21 (5%)	16 (6%)	5 (5%)	0.87	0.32 – 2.34	0.806	1.000	1.000
C*12	51 (13%)	41 (14%)	10 (9%)	0.63	0.31 – 1.29	0.239	0.971	1.000
C*14	9 (2%)	8 (3%)	1 (<1%)	0.46	0.08 – 2.63	0.454	1.000	1.000
C*15	27 (7%)	25 (9%)	2 (2%)	0.24	0.07 – 0.90	0.013	0.162	0.633
C*16	13 (3%)	10 (3%)	3 (3%)	0.87	0.26 – 2.99	1.000	1.000	1.000
C*17	12 (3%)	8 (3%)	4 (4%)	1.41	0.44 – 4.51	0.743	1.000	1.000
DQB1*02	150 (37%)	110 (37%)	40 (36%)	0.96	0.61 – 1.50	0.908	1.000	1.000
DQB1*03	223 (47%)	156 (53%)	67 (60%)	1.36	0.88 – 2.12	0.181	0.631	1.000
DQB1*04	27 (7%)	21 (7%)	6 (5%)	0.79	0.32 – 1.95	0.658	0.995	1.000
DQB1*05	138 (34%)	104 (35%)	34 (31%)	0.82	0.51 – 1.31	0.413	0.930	1.000
DQB1*06	176 (43%)	134 (45%)	42 (38%)	0.74	0.47 – 1.15	0.216	0.705	1.000
DRB1*01	89 (21%)	71 (24%)	18 (16%)	0.62	0.36 – 1.10	0.106	0.768	1.000
DRB1*03	112 (28%)	79 (27%)	33 (30%)	1.17	0.72 – 1.89	0.536	1.000	1.000
DRB1*04	123 (30%)	88 (30%)	35 (32%)	1.09	0.68 – 1.75	0.718	1.000	1.000
DRB1*07	65 (16%)	50 (17%)	15 (14%)	0.78	0.42 – 1.45	0.451	1.000	1.000
DRB1*08	25 (6%)	20 (7%)	5 (5%)	0.70	0.27 – 1.83	0.492	1.000	1.000
DRB1*09	10 (2%)	6 (2%)	4 (4%)	1.87	0.55 – 6.35	0.471	1.000	1.000
DRB1*10	15 (4%)	10 (3%)	5 (5%)	1.41	0.49 – 4.05	0.565	1.000	1.000
DRB1*11	69 (17%)	50 (17%)	19 (17%)	1.03	0.58 – 1.83	1.000	1.000	1.000
DRB1*12	15 (4%)	7 (2%)	8 (7%)	3.17	1.16 – 8.68	0.034	0.364	0.924

Table S2. (continued)

HLA type[1]	Recipients (n = 407)	No BKPyV viremia (n = 296)	BKPyV viremia (n = 111)	OR[2]	95% CI[2]	p-value[3] crude	p-value[4] HLA locus adjusted	p-value[5] HLA total adjusted
DRB1*13	104 (26%)	85 (29%)	19 (17%)	0.52	0.30 – 0.90	0.021	0.243	0.795
DRB1*14	18 (4%)	13 (4%)	5 (5%)	1.09	0.39 – 3.00	1.000	1.000	1.000
DRB1*15	98 (24%)	68 (23%)	30 (27%)	1.25	0.76 – 2.05	0.435	0.999	1.000
DRB1*16	14 (3%)	10 (3%)	4 (4%)	1.14	0.37 – 3.52	1.000	1.000	1.000

Data are shown as n (%).
BKPyV, BK polyomavirus; CI, confidence interval; HLA, human leukocyte antigen; OR, odds ratio.
[1]From all recipients the complete information of HLA B, DQ and DR were available, whereas HLA A and C were missing in 1 and 6 recipient cases.
[2]Odds ratios and corresponding 95% CI were calculated with the Woolf Haldane test.
[3]The p-values were calculated using the two-sided Fisher's exact test. A p-value <0.05 was considered statistically significant.
[4]The p-values were corrected for multiple testing according to the Šidàk method (Šidàk 1967). The formula of the Šidàk correction is 1-(1-p)^N, were N is the number of antigens (comparisons per locus).
[5]The p-values were corrected for multiple testing according to the Šidàk method (Šidàk 1967). The formula of the Šidàk correction is 1-(1-p)^N, were N is the number of HLA alleles tested, which is 74 for recipients.
A p-value <0.05 was considered statistically significant.

Table S3. Potential HLA-B51 presented nonamer epitopes encoded by the major BKPyV proteins, predicted with the IEDB analysis resource Consensus tool by use of the whole BKPyV genome (Dunlop strain)

HLA allele	BKPyV protein[1]	Amino acid position	Amino acid sequence BKPyV Dunlop strain	IEDB analysis Percentile rank[2]
B51	VP1	20	E P V Q V P K L L	3.1
B52	VP1	20	E P V Q V P K L L	42
B51	VP1	158	E P L E M Q G V L	1.5
B52	VP1	158	E P L E M Q G V L	36
B51	VP1	252	G P L C K A D S L	4.3
B52	VP1	252	G P L C K A D S L	30
B51	Large T[3]	27	L P L M R K A Y L	0.9
B52	Large T[3]	27	L P L M R K A Y L	18
B51	Small T[3]	27	L P L M R K A Y L	0.9
B52	Small T[3]	27	L P L M R K A Y L	18

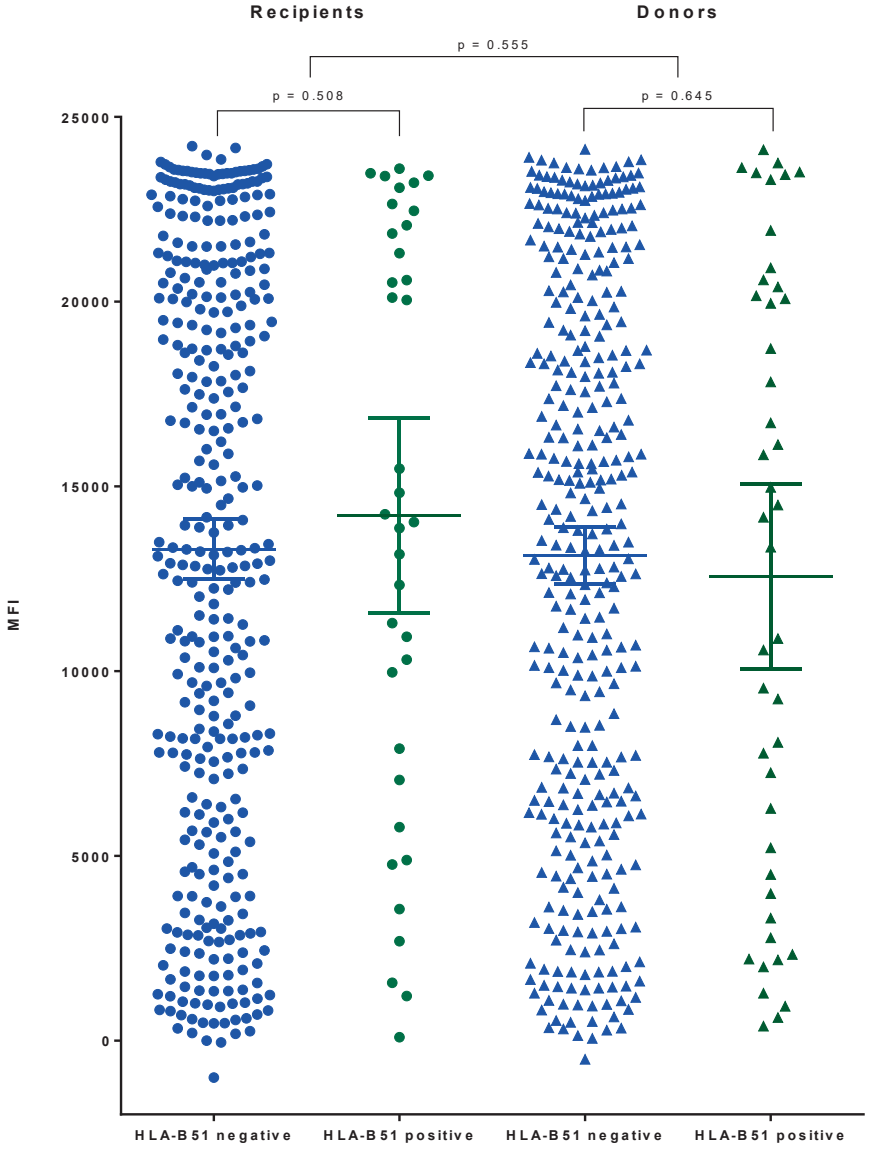
BKPyV, BK polyomavirus; HLA, human leukocyte antigen.

[1]The following viral proteins were analysed: small T-antigen, large T-antigen, VP1, VP2 and VP3.

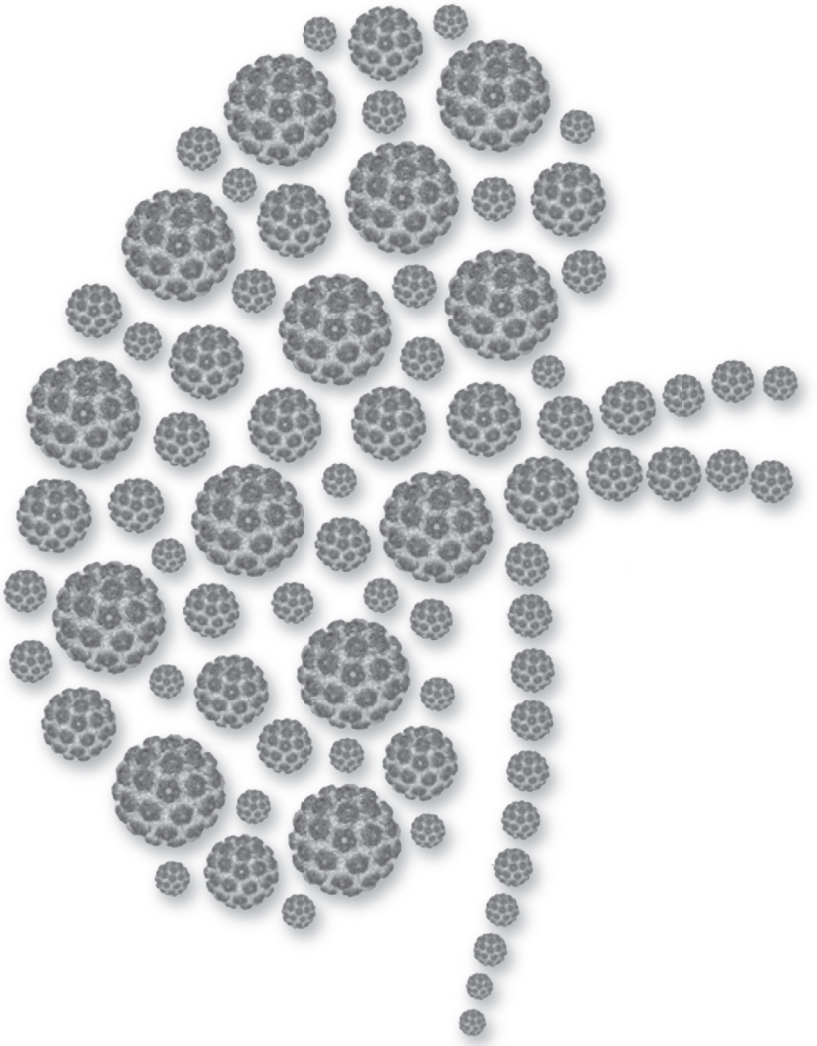
[2]The IEDB analysis percentile rank ranges from 0 to 100, the lower the score the higher the probability that the peptide is being processed and presented to T cells.

[3]The first exon of the Large T and Small T antigen where the identified peptide LPLMRKAYL is located is shared.

Figure S1. Pretransplantation IgG seroreactivity against BKPyV among 407 kidney transplant recipients and donors sorted for HLA-B51 status.



Pretransplantation IgG seroreactivity of 407 kidney transplant recipients and donors against the BKPyV VP1 antigen. Each dot represents the pretransplantation BKPyV VP1 IgG seroreactivity of individual recipients (left) and each triangle represents the pretransplantation BKPyV VP1 IgG seroreactivity of individual donors (right), tested by Luminex. The BKPyV VP1 IgG seroreactivities of recipients and donors are divided in two columns based on their HLA-B51 status; HLA-B51- negative (blue) and HLA-B51-positive (green). The horizontal lines represent the mean and 95% CI. P-values were calculated using the Student's *t*-test. A p-value < 0.05 was considered statistically significant. BKPyV, BK polyomavirus; CI, confidence interval; HLA, human leukocyte antigen; VP1, viral capsid protein 1.



Chapter 5

Development and evaluation of a BK polyomavirus serotyping assay using Luminex technology

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Abstract

Background

The BK polyomavirus (BKPyV) is subdivided into four genotypes. The consequences of each genotype and of donor-recipient genotype (mis)match for BKPyV-associated nephropathy (BKPyVAN) in kidney transplant recipients (KTRs) are unknown.

Objectives

To develop and evaluate a genotype-specific IgG antibody-based BKPyV serotyping assay, in order to classify kidney transplant donors and recipients accordingly.

Study design

VP1 antigens of six BKPyV variants (Ib1, Ib2, Ic, II, III and IV) were expressed as recombinant glutathione-s-transferase-fusion proteins and coupled to fluorescent Luminex beads. Sera from 87 healthy blood donors and 39 KTRs were used to analyze seroreactivity and serospecificity against the different BKPyV genotypes. Six sera with marked BKPyV serotype profiles were analyzed further for genotype-specific BKPyV pseudovirus neutralizing capacity.

Results

Seroreactivity was observed against all genotypes, with seropositivity rates above 77% comparable for KTRs and blood donors. Strong cross-reactivity ($r > 0.8$) was observed among genotype I subtypes, and among genotypes II, III and IV. Seroresponses against genotypes I and IV seemed genuine, while those against II and III could be out(cross)competed. GMT (Luminex) and IC_{50} (neutralization assay) values showed good agreement in determining the genotype with the strongest seroresponse within an individual.

Conclusions

Despite some degree of cross-reactivity, this serotyping assay seems a useful tool to identify the main infecting BKPyV genotype within a given individual. This information, which cannot be obtained otherwise from nonviremic/nonviruric individuals,could provide valuable information regarding the prevalent BKPyV genotype in kidney donors and recipients and warrants further study.

Background

BK polyomavirus-associated nephropathy (BKPyVAN) is one of the major causes of graft dysfunction and loss in kidney transplant recipients (KTRs). BKPyV DNA is detected in urine and blood in 50-70% and 20-30% of KTRs after transplantation, respectively (1-6). BKPyVAN generally develops in 1-10% of KTRs, usually in those with sustained viremia and viral DNA-loads above 10^4 genome copies/ml (3, 6-8). Unfortunately, the burden of BKPyVAN continues to increase, as the population of KTRs is still growing (9-12).

Despite the clinical need, BKPyV-specific antiviral drugs are not available, and reduction of immunosuppression is the only effective evidence-based treatment (6, 13-15). Therefore, current guidelines recommend regular screening of KTRs to detect BKPyV viremia and guide timely reduction of immunosuppression (3, 4, 6, 14), which improves BKPyV immunity, but at the same time increases the risk of acute rejection (6, 14, 15). This makes management of BKPyV infection challenging for transplantation physicians and calls for reliable pretransplantation predictive markers that identify KTRs at risk. Such markers could, for example, guide physicians toward more frequent monitoring of BKPyV viremia or use of a lower viremia threshold for at-risk patients.

The overall seroprevalence for BKPyV exceeds 90% (16, 17), and it is believed that nearly all adults are persistently infected with at least one BKPyV genotype (18, 19). Recently, we provided compelling evidence that the level of BKPyV-directed IgG seroreactivity measured before kidney transplantation (KTx), especially in donors, predicts the risk of BKPyV infection, in KTRs after transplantation (20). In line with previous studies that showed associations between donor and recipient seroreactivity and recipient BKPyV infection risk (21-23), our results showed that strongly BKPyV-seroreactive kidney donors conferred an approximately 10-fold increased risk of viremia to their recipients. In the prior study, BKPyV genotype Ib1 VP1 antigen was used to analyze seroresponses. To learn more about the specificity of BKPyV-directed seroresponses and to investigate the impact of BKPyV genotype disparity between donors and recipients (9), we set out to complement our BKPyV-immunoassay with the most common circulating BKPyV subtypes. The availability of a high-throughput BKPyV serotyping assay could overcome the limitation of BKPyV genotyping, requiring a certain amount of viral DNA, which is usually not detectable in healthy donors without viruria and viremia.

BKPyV is classified into four genotypes based on single nucleotide polymorphisms (SNPs) (24, 25). Genotype I is the most prevalent and widespread BKPyV genotype worldwide, genotype IV accounts for most of the remaining subjects, while genotypes II and III are rarely detected in all geographic regions (26, 27). Phylogenetic sequence analysis has been used to classify BKPyV strains, resulting in further subdivision of genotypes I and IV into subtypes Ia, Ib1, Ib2, Ic, and IVa1, IVa2, IVb1, IVb2, IVc1, and IVc2, respectively (26-29). Ia is most prevalent in Africa, Ib1 in Southeast Asia, Ib2 in Europe, America and West Asia, and Ic in Northeast Asia (26, 27, 30, 31). All subtypes of genotypes IV except IVc2, are prevalent in East Asian populations, with subtype IVc2 occurring mainly in Europe, America and Northeast Asia (32).

It is generally believed that each BKPyV genotype represents a distinct serotype, which fits with the majority of SNPs being located in the VP1 capsid protein (9, 33, 34). Genotype-specific vaccination studies in mice have confirmed this (34), and indicated antibody-mediated genotype-specific BKPyV neutralization. Furthermore, it was shown that subtypes Ib1 and Ib2 can behave as distinct serotypes in some individuals, while Ib2 and Ic seem to represent a single serotype, as are all subtypes of genotype IV (33, 34).

Objectives

The aim of this study was to develop and evaluate a serological BKPyV IgG genotyping assay with the help of separate cohorts of healthy blood donors and KTRs, that detects BKPyV genotype-specific IgG antibody responses. This could be useful to detect pretransplantation BKPyV genotype-specific IgG antibody responses in kidney transplant donors and recipients. Availability of such a serotyping system could shed light on previously reported, sometimes conflicting results regarding associations between specific BKPyV genotypes and pathogenic replication (28, 35-39). Furthermore, BKPyV serotyping could reveal whether the current BKPyV genotype distribution pattern deduced from viremic KTRs reflects that of asymptomatic immunocompetent people. Finally, if BKPyV serotyping can reliably differentiate between genotypes, this method could be used to analyze and predict the clinical impact of BKPyV genotype mismatch between donor and recipient.

Study design

Study population

For evaluation of the BKPyV multiplex serotyping immunoassay, anonymized serum samples from a cohort of 87 adult Dutch HBDs (40, 41), and a cohort of 39 adult Dutch KTRs (42, 43) were tested. Basic demographic details, such as age, sex and year of collection can be found in the references. The study adhered to the Declaration of Helsinki Principles and all participants gave informed consent.

BKPyV VP1 bead-based immunoassay

To detect IgG seroresponses against the BKPyV major viral capsid protein (VP1), an antibody-binding assay using Luminex xMAP technology (44) was previously developed, equipped with the VP1 antigen of BKPyV subtype Ib1 as described (16, 20). The assay showed a good intra (r 0.963 – 0.999, $p < 0.001$) and intertest variability (r 0.891, $p < 0.001$) (20). To detect seroresponses against other BKPyV genotypes, synthetic VP1 gene fragments (gBlocks, IDT, San Jose, CA, USA) of BKPyV Ib2, Ic, II, III and IVb1, were cloned into pGEX-5x-3 vectors (GE Healthcare Life Sciences, Chicago, IL, USA) and expressed as GST-VP1.tag fusion proteins in BL21 Rosetta bacteria. The synthetic gene fragments used in this study were reported previously (45); BKV-Ib2 (PittVR2; DQ989796), BKV-Ic (RYU-2; AB211377), BKV-II (GBR-12; AB263920), BKV-III (KOM-3; AB211386), and BKV-IVb1 (THK-8; AB211390). The different GST-VP1 fusion proteins were individually coupled to differently coloured Luminex bead sets. BKPyV Ia was not included in the antigen set, because BKPyV Ia and Ib1 differ by only three synonymous SNPs in VP1, and are identical in their amino acids (46). Only one genotype IV VP1 antigen was included in the analyses, BKPyV IVb1, since all BKPyV genotype IV subtypes are thought to belong to one serotype (33, 34).

Serostatus (positive or negative) was identified and interpreted according to the calculated cut-off values, Ib1 763 MFI, Ib2 515 MFI, Ic 475 MFI, II 446 MFI, III 366 MFI and IV 298 MFI, as described in the supplemental information.

A high agreement was observed between test plates for all 6 BKPyV variants as described in supplementary information together with further information regarding the previously described BKPyV VP1 bead-based immunoassay.

Serum competition analysis

To study the cross-reactivity between the different BKPyV serotypes, VP1 antigen competition experiments were performed, where a fixed amount of unbound

competitor VP1 antigen is added to a serum dilution series, in addition to the bead-bound targeted VP1 antigen, as described previously (16, 42). Selected serum samples were diluted from 1:100 up to 1:409.600 and incubated with regular blocking buffer containing either GST or GST-VP1 fusion proteins (~2 mg/ml).

BKPyV Neutralization assay

Of the 39 KTR samples, six were independently analyzed for serological confirmation with a BKPyV genotype-specific pseudovirion based neutralization assay (PVNA) (dilution 1:100 to 1:39.062.500), as described (45). The neutralization titer was defined as the half maximal inhibitory concentration (IC_{50}) and was calculated using Prism Software (Graphpad) by fitting a variable-slope sigmoidal dose-response curve for each serum dilution series. The IC_{50} values of the PVNA were compared with the geometric mean titers (GMT) determined on seroreactivities of the six serum samples measured in the BKPyV serotyping multiplex immunoassay.

Seroreactivities against BKPyV serotype specific VP1 antigens were measured in healthy blood donors (HBDs, n = 87, panel A) and in kidney transplant recipients (KTRs, n = 39, panel B). Results are depicted as mean fluorescence intensity (MFI), and are shown in box plots. The bottom and top of the boxes represent the first and third quartiles, the band inside the boxes represents the median, and the end of the whiskers represents the minimum and maximum seroreactivities. The differences between the seroreactivities against BKPyV genotype-specific VP1 antigens were statistically significant: BKPyV Ib1 HBDs mean 10976 MFI, standard deviation 7586 MFI and KTRs mean 19163 MFI, standard deviation 7019 MFI, $p < 0.001$; BKPyV Ib2 HBDs mean 7631 MFI, standard deviation 6419 MFI and KTRs mean 14996 MFI, standard deviation 8468 MFI, $p < 0.001$; BKPyV Ic HBDs mean 8201 MFI, standard deviation 6615 MFI and KTRs mean 15850 MFI, standard deviation 8016 MFI, $p < 0.001$; BKPyV II HBDs mean 4428 MFI, standard deviation 4768 MFI and KTRs mean 10867 MFI, standard deviation 7307 MFI, $p < 0.001$; BKPyV III HBDs mean 2543 MFI, standard deviation 3028 MFI and KTRs mean 8859 MFI, standard deviation 7649 MFI, $p < 0.001$; BKPyV IV: HBDs mean 2961 MFI, standard deviation 3209 MFI and KTRs mean 10370 MFI, standard deviation 7365 MFI, $p < 0.001$.

Statistical analysis

Data were analyzed with IBM SPSS Statistics software version 21. Differences between HBDs and KTRs were assessed using the chi-square test, Fisher exact test or Student t-test, as appropriate. Pearson correlation coefficients were calculated to determine intertest reliability. Correlation between assessed BKPyV serotypes was further examined by calculating Spearman rank correlation coefficients.

Results

BKPyV genotype-directed seroreactivity

VP1 antigens of the common BKPyV subtypes, Ib1, Ib2, Ic, II, III and IVb1 were analysed for seroreactivity BKPyV Ia was not included in the antigen set, because BKPyV Ia and Ib1 VP1 are identical (46). Only one genotype IV antigen was included in the analyses, BKPyV IVb1, since all BKPyV genotype IV subtypes are thought to belong to one serotype (33, 34).

Each selected VP1 was analyzed for antigenicity using serum samples from 87 immunocompetent (blood donors) and 39 immunocompromised (immunosuppressed KTRs) individuals. Figure 1 shows boxplots of the measured MFI values obtained for each BKPyV genotype at 1:100 dilution. The seroreactivity measured against any BKPyV genotype was significantly stronger in KTRs compared to HBDs ($p < 0.001$), indicated by higher median MFI values, probably as the result of boosted seroresponses by replicating virus under immunosuppression, as we have shown previously (41). The highest median seroreactivities in HBDs and KTRs, were observed for BKPyV subtypes belonging to genotype I. The seropositivity rate was high for all BKPyV serotypes, ranging from 91-100% for genotype I, 86-90% for genotype II, 77-87% for genotype III and 80-95% for genotype IV, and comparable between HBDs and KTRs (Table 1). On average, both HBDs and KTRs were seropositive against at least five of the six BKPyV subtypes and three of the four genotypes (Table 1).

Figure 1. Seroreactivity against BKPyV genotype-specific VP1 antigens measured by the Luminex multiplex immunoassay in healthy blood donors (A) and kidney transplant recipients (B)

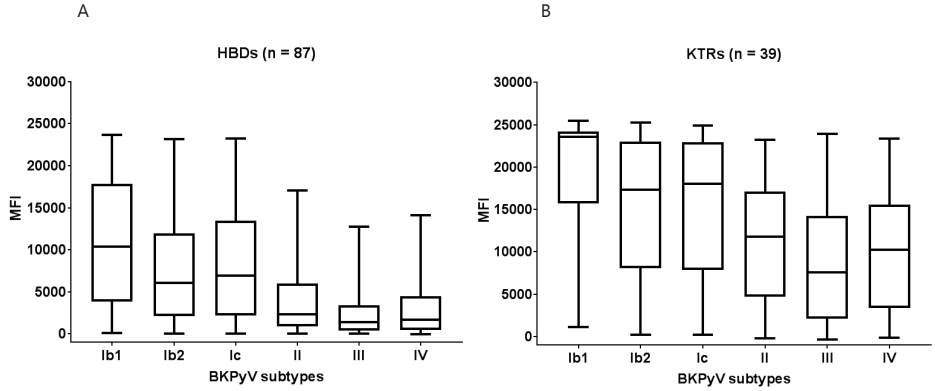


Table 1. Seropositivity against BKPyV geno(sub)types measured in sera from 87 healthy blood donors and 39 kidney transplant recipients

BKPyV genotype	HBDs n (%)	KTRs n (%)
I, subtype b1	81 (93%)	39 (100%)
I, subtype b2	79 (91%)	37 (95%)
I, subtype c	81 (93%)	38 (97%)
II	75 (86%)	35 (90%)
III	67 (77%)	34 (87%)
IV	70 (80%)	37 (95%)
Mean number of seropositive geno(sub)types per individual	5.24 (1.50)	5.64 (1.01)
Mean number of seropositive genotypes per individual	3.41 (1.08)	3.72 (0.79)

BKPyV, BK polyomavirus; HBDs, healthy blood donors; KTRs, kidney transplant recipients.

Correlation between seroresponses against individual BKPyV variants

To learn about crossreactivity between the different BKPyV genotypes and subtypes analyzed in our assay, a correlation matrix of the serotype-specific seroresponses was generated for the HBDs and KTRs (Figure 2A). Spearman rank correlation coefficients were calculated for each BKPyV serotype combination (Figure 2B). Strong correlations were observed between BKPyV subtypes belonging to genotype I, and between genotypes II, III and IV. Between the seroresponses against genotype I and genotypes II–IV, moderately strong correlations were generally observed. The observed cross-reactivity pattern matched with the VP1 amino acid sequence similarity between the genotypes, with strong correlations among BKPyV genotypes with ≥95% similarity (data not shown).

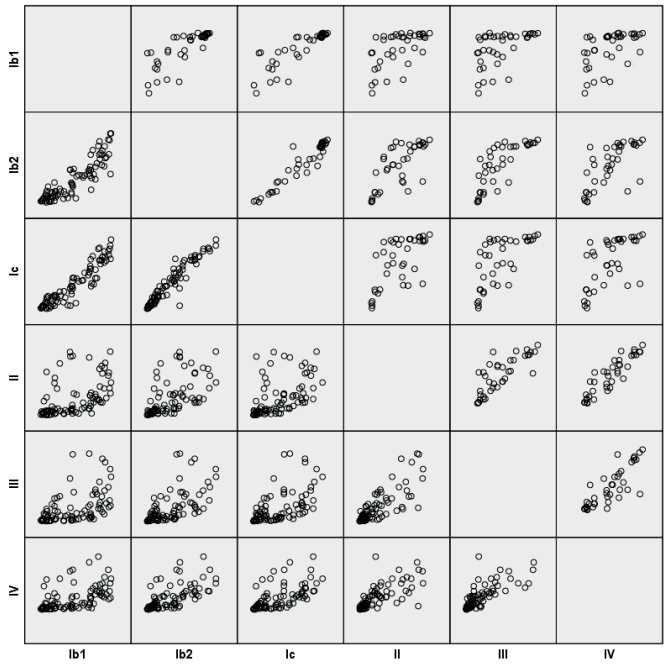
Cross-reactivity of seroresponses against BKPyV

To explore the BKPyV crossreactivity in more detail, six serum samples with a high seroreactivity (> 15.000 MFI) to at least one of the genotypes were selected, diluted and tested against each VP1 antigen to calculate their GMT for each genotype (Table 2A). Furthermore, soluble, competing heterologous VP1-antigens were added to the serum titration series, while assaying seroreactivity against bead-bound VP1 antigen of the relevant BKPyV variants. Figure 3 shows a selection of these analyses for each analyzed genotype, while a comprehensive overview of the VP1-antigen inhibition experiments can be found in supplementary Figure S2.

Although each serum sample proved different in these analyses, seroresponses against BKPyV genotype Ib1 were only efficiently blocked by the homologous Ib1 VP1 antigen. Pre-incubation with other genotype I subtype VP1 antigens caused only a slight reduction in seroreactivity, comparable to the inhibition caused

Figure 2. Cross-reactivity matrices (A) and Spearman rank correlation coefficients (B) between the VP1 antigens from different BKPyV genotypes and subtypes in serum samples of HBDs and KTRs

A



B

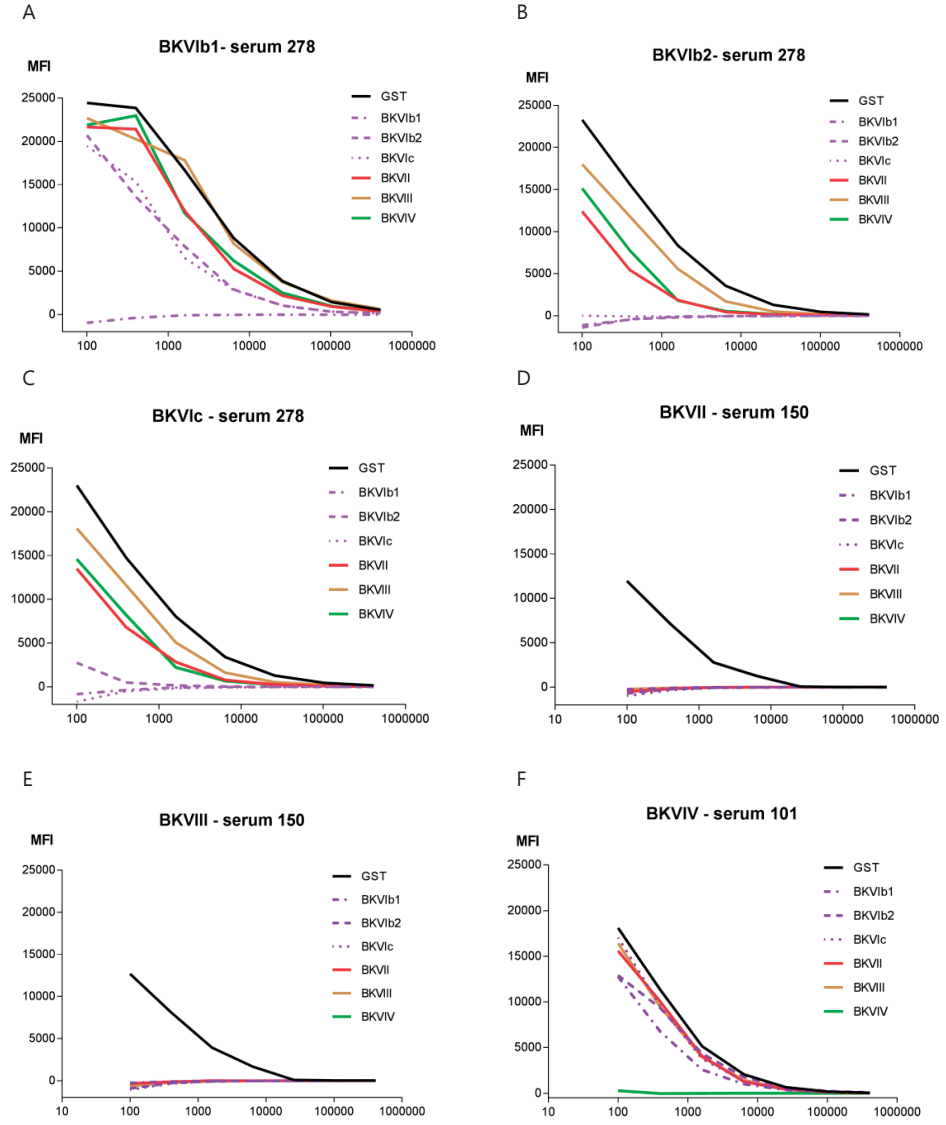
		Spearman correlation serotiters of KTRs (n = 39)					
		Ib1	**Ib2**	**Ic**	**II**	**III**	**IV**
Spearman correlation serotiters of HBDs (n = 87)	**Ib1**		0.850	0.911	0.618	0.621	0.615
	Ib2	0.893		0.940	0.742	0.854	0.766
	Ic	0.926	0.953		0.682	0.746	0.700
	II	0.630	0.740	0.678		0.819	0.912
	III	0.601	0.734	0.680	0.823		0.826
	IV	0.612	0.745	0.711	0.831	0.858	

In panel A correlation graphs are shown as scatter plots for the healthy blood donor population (lower left part, n = 87) and for the kidney transplant recipients (upper right part, n = 39), with each circle representing one serum sample. In panel B, the numbers in the lower left part of the Table show the Spearman correlation coefficients calculated between seroresponses measured against VP1 of the BKPyV genotypes and subtypes tested in the healthy blood donor population and the upper right part of the kidney transplant recipients. The color of the cells represents the degree of correlation between the different BKPyV variants; red = high correlation ($r \geq 0.8$), yellow = moderate correlation ($r = 0.6 - 0.8$).

by the more distant genotypes II, III and IV (Figure 3A). Seroresponses against genotype Ib2 and Ic, in most of the cases could be inhibited by any genotype I VP1 antigen, but not by the other genotypes (Figure 3B-C). Seroresponses against

BKPyV VP1 of genotypes II and III were inhibited by all of the heterologous VP1 antigens (Figure 3D-E), whereas responses against genotype IV VP1 were not inhibited by the heterologous VP1 antigens (Figure 3F). Altogether, these data

Figure 3. Cross-reactivity analysis of BKPyV serotype seroresponses by VP1-specific competition



Titrated serum samples were pre-incubated with crude bacterial extract containing GST only (in black), or containing GST-VP1 of the autologous BKPyV geno(sub)type (target subtype shown on top of each graph) or non-target heterologous BKPyV geno(sub)types. Results are depicted as median fluorescence intensity (MFI) and are shown for the seroresponses to Ib1 (A), Ib2 (B), Ic (C), II (D), III (E) and IV (F) measured in each serum sample.

indicate that the immunoassay detects seroreactivity against BKPyV genotype I subtypes, especially Ib1, and genotype IV with little chance of cross-reactivity,

Table 2. Comparison between results obtained with the BKPyV Luminex multiplex serotyping immunoassay and the pseudovirion based neutralization assay for six selected sera

A

Test method	BKPyV serotypes						
	Serum sample	Ib1	Ib2	Ic	II	III	IV
Luminex assay	101	17717	8433	8376	20434	5985	**22321**
(MFI values)	150	**24875**	24009	24443	20532	22753	22109
	256	**24853**	23334	24084	19259	20531	19804
	258	12994	4464	7780	14526	7726	**17326**
	278	**25243**	24143	22973	20414	21081	20017
	312	**17318**	170	1876	-243	-385	1087
Luminex assay	101	187	58	55	624	32	**1403**
(GMT values)	150	**2233**	1265	1848	302	434	511
	256	1225	929	**1265**	459	568	526
	258	490	125	186	487	144	**887**
	278	**5874**	2667	2602	1834	1602	1722
	312	**476**	2	8	1	1	2
Neutralization assay	101	4825	195	333	20939	497	**36516**
(PVNA IC_{50} values)	150	**73673**	27286	21557	24346	20477	10942
	256	**20672**	2925	5856	8921	5580	2894
	258	5452	260	683	**20407**	4810	14587
	278	**26768**	925	1051	5513	896	311
	312	**4974**	0	0	237	0	O

B

	Luminex assay (MFI value)	**Luminex assay** (GMT value)	**Neutralization assay** (IC_{50} value)
Main genotype serum samples			
101	IV	IV	IV
150	I	I	I
256	I	I	I
258	IV	IV	II
278	I	I	I
312	I	I	I

Panel A shows the MFI values of six serum samples with a 1:100 dilution from kidney transplantation recipients, the geometric mean values of the serial dilutions (1:100 up to 1:409.600) of these six serum samples, and the IC_{50} values of the serial dilutions (1:100 up to 1:39.062.500) of six selected serum samples. The highest values per serum sample are depicted in bold. Panel B shows for each serum sample the BKPyV genotype that reached the highest MFI, GMT and IC_{50} value determined in the Luminex and the neutralization assay, respectively.

while seroresponses against genotypes II and III often seemed to lack specificity as they were completely inhibited by the heterologous VP1 antigens.

Virus neutralization by BKPyV genotype VP1-specific sera identified in the bead-based assay

To further evaluate the performance of the BKPyV serotyping immunoassay with regard to specificity and capacity to detect seroresponses with neutralizing activity, the selected serum samples were also tested in a previously described BKPyV PVNA (33, 34). The PVNA IC_{50} values obtained for each BKPyV genotype and each serum are shown in Table 2A, in comparison with the Luminex-obtained MFI and GMT values mentioned above.

For comparison, in Table 2B we show for each selected serum the BKPyV genotype that generated the highest MFI, GMT and IC_{50} value in each test method. Genotype ranking based on MFI and GMT Luminex values showed a 100% concordance. Comparing MFI/GMT ranking values with the PVNA IC_{50} value ranking, revealed one discrepant result (serum 258), since Luminex indicated the highest MFI/GMT values for genotype IV, while the PVNA indicated genotype II. In both cases, however, the obtained MFI, GMT, and IC50 values were rather close to each other, and the second-best response for the relevant serum was directed against the reciprocal genotype, being IV with PVNA, and II with Luminex (Table 2).

Discussion

Little is known about the distribution of BKPyV genotypes among kidney donors and recipients and their association with the risk, course and severity of BKPyV infection and complications after KTx. Studies assessing this association in KTRs have reported conflicting results. These studies were mainly focused on isolates obtained from recipients with manifest BKPyV infection (viremia or viruria) and thus may not represent the distribution of BKPyV genotypes circulating in the general population, including kidney donors (28, 35-39).

Current BKPyV genotyping mainly relies on sequencing of BKPyV DNA in clinical samples. In healthy subjects however, BKPyV DNA is seldom detectable in sufficient amounts to allow sequence analysis (24, 25, 46-48). This makes BKPyV genotyping of donor-recipient pairs before KTx almost impossible. Serum neutralization assays to detect infecting BKPyV genotypes have been described using pseudovirion systems (9, 33, 34), but these are not suitable for routine use, as they are laborious

in terms of production of the infectious pseudovirus and in the conduct of the neutralization assays. The BKPyV serotyping Luminex immunoassay could potentially fill this gap, as it creates the possibility of a fast and efficient high-throughput assay detecting multiple BKPyV genotype-specific VP1 antigens at the same time in only a small amount of sample, eventually saving time and costs. A previous comparison between BKPyV GST-VP1 antigen presented on a bead and BKPyV VP1 VLP antigen showed good agreement between the two (20, 42).

Based on our results with this new serotyping assay, the prevalence of BKPyV genotype I infections was high (> 90%) in both blood donors and KTRs, which is in accordance with literature (16, 17). The measured seroprevalence of serotypes II and III ranged between 77-90% in both groups. These percentages are higher than generally reported in the literature (9, 26, 27, 45). However, as most serotype II and III seroresponses were completely inhibited by heterologous VP1 antigens, we believe the high serotype II and III seroprevalence could be a reflection of the cross-reactivity with heterologous serotypes and should be interpreted with extreme caution. Whether this caution applies as well to BKPyV genotype II and III seroprevalences obtained in other studies we do not know. Since genotype IV-directed seroresponses could not be inhibited by other VP1 antigens, we consider the prevalence of infections with this genotype to be genuinely high.

The cross-reactivity analysis showed high correlations between the seroreactivities against subtypes belonging to genotype I, and between genotypes II, III and IV, indicating that cross-reactivity with these two groups is likely. The antigen competition experiments, on the other hand, showed that the immunoassay detects seroreactivity against BKPyV genotypes I and IV with little chance of cross-reactivity, as the responses to the VP1 of these genotypes were not inhibited by the heterologous VP1 antigens. As genotypes I and IV are the most prevalent and widespread BKPyV genotypes worldwide and BKPyV genotypes II and III are only rarely detected in all geographic regions (26, 27), BKPyV I and IV serotyping based on this method could be of potential interest to explore the risk of BKPyV (genotype-specific) infection in KTRs. Ideally, further study in that direction should include a direct comparison between serotyping and genotyping results, and therefore can only be performed in viruric or viremic subjects.

Comparison between the MFI and GMT values of the Luminex immunoassay with the IC50 values of the neutralization assay showed good agreement in determination of the main genotype. A previous study also showed a good correlation between BKPyV-VLP and BKPyV VP1 antibody responses (49). The only possible

disagreement was a type IV in the Luminex compared to type II with the neutralization assay, which, if interpreted as a discrepancy although MFI and IC50 values for both genotypes were rather similar, can be explained by cross-reactivity between these serotypes in the neutralization assay (34). Based on this limited comparison, we assume that serum samples that show high seroreactivity in the Luminex assay have BKPyV neutralizing activity, as shown in the PVNA.

To conclude, we described the development and evaluation of a BKPyV genotype-specific VP1 directed IgG immunoassay. The results indicate that this immunoassay is a potentially useful tool for the detection of BKPyV infection with the most prevalent genotypes I and IV, in individuals without detectable viral DNA available. Whether the assay can detect and discriminate genotype II and III-specific seroresponses remains unclear and should be further evaluated with sera from individuals with molecularly proven genotype-specific BKPyV infections.

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Declaration of interests

We declare that we have no conflicts of interest

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Role of the funding source

The Dutch Kidney Foundation who supported this study had no role in study design; collection, analysis, and interpretation of data; writing of the report; or in the decision to submit the paper for publication.

Contributors

HFW and MCWF initiated and designed the study. ACMK provided the infrastructure. CSdB, EvdM, and DVP performed the experiments and gathered the experimental data. HFW analyzed the data. HFW, CBB, and MCWF interpreted the data. HFW and MCWF drafted the manuscript, including figures and tables. All authors reviewed and approved the final report.

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Supporting Information

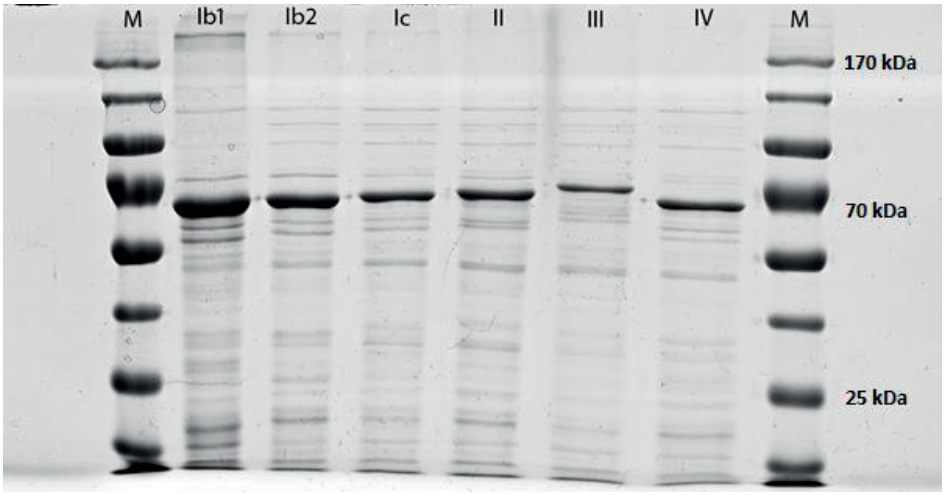
BKPyV VP1 bead-based immunoassay

Expression and coupling to the corresponding Luminex bead set of each GST-VP1 fusion protein was performed and checked (Figure S1), as previously described for other human polyomaviruses (16, 39). To detect seroreactivity against the different BKPyV genotypes, serum samples were incubated for 1h in blocking buffer (1 mg/ml casein, 0,5% polyvinylalcohol, 0,8% polyvinylpyrrolidone, 2,5% Super ChemiBlock (Chemicon International, Billerica, MA, USA) and 2 mg/ml GST bacterial lysate in PBS) to suppress potential non-specific binding to the beads or to GST (41, 47). Of each serum sample, a 10-fold diluted series was tested (1:100 to 1:100.000). In the meantime, the GST-BKPyV VP1 fusion proteins for the different serotypes were coupled to glutathione-casein linked polystyrene beads and the serum samples were subsequently incubated with the mixture of GST-BKPyV VP1 beads (one hour in the dark at room temperature). For detection of a VP1-directed human IgG response, biotinylated goat-α-human IgG (H+L) (1:1000 Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) followed by streptavidine-R-phycoerythrin (SAPE) (1:1000 Invitrogen, Waltham, MA, USA), were used, incubated for 30 minutes each in the dark at room temperature.

A serially diluted (1:100 up to 1:409.600) mixture of 4 serum samples with known seroreactivity against BKPyV was included in each run (n = 34), to control for interplate test variance. High agreement was observed between test plates for all serotypes (Ib1 r = 0.942 – 1.000, Ib2 r = 0.915 – 1.000, Ic r = 0.873 – 1.000, II r = 0.965 – 1.000, III r = 0.950 – 1.000, and IV r = 0.979 – 1.000, $p < 0.001$).

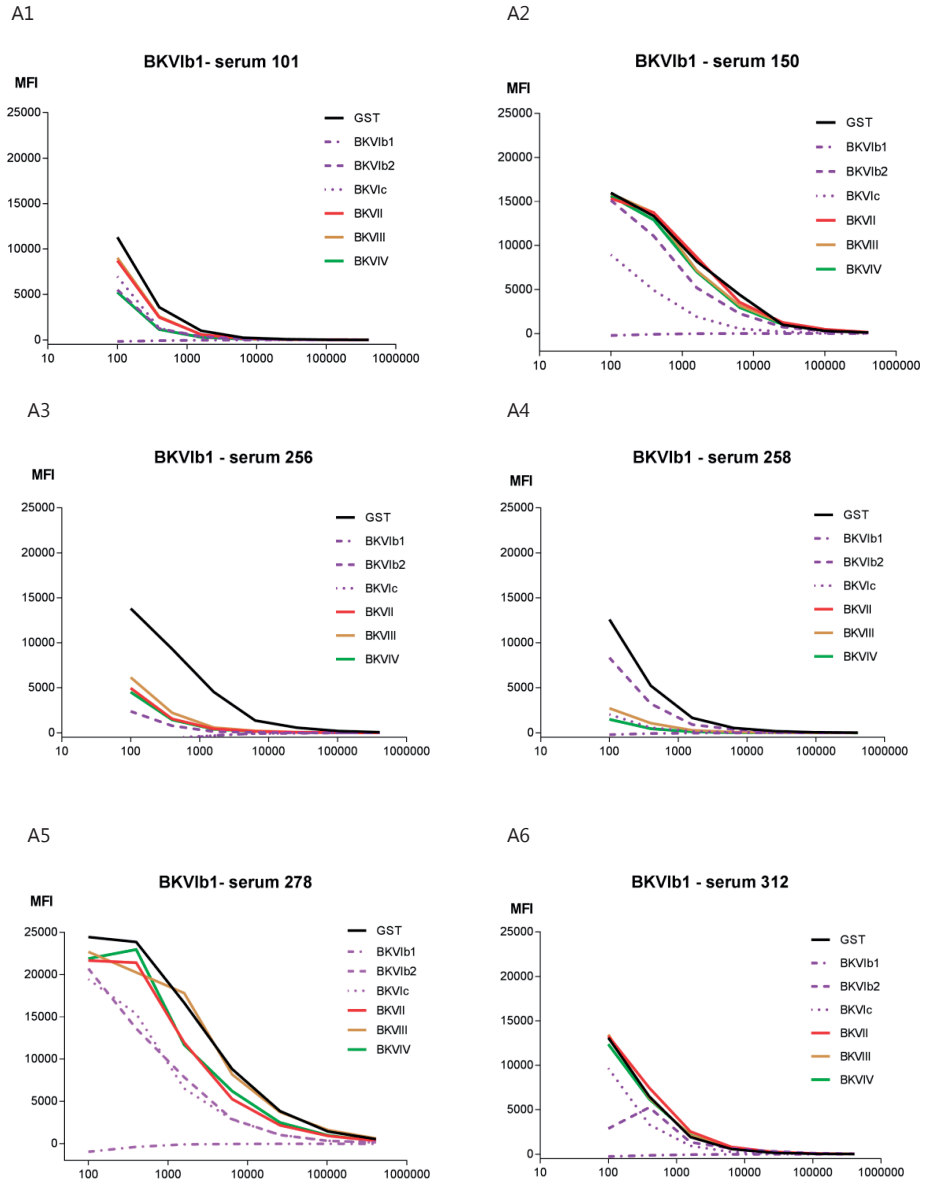
Seropositivity cut-off values were calculated with the help of a group of immunocompetent children aged 7–24 months (n = 63), as described (16). The following mean fluorescence intensity (MFI) cut-off values were obtained: Ib1 763, Ib2 515, Ic 475, II 446, III 366, and IV 298 MFI.

Figure S1. Expression of BKPyV geno(sub)type GST-VP1 proteins



Coomassie blot showing glutathione-purified GST-VP1 bacterial lysates of BKPyV serotypes Ib1, Ib2, Ic, II, III, and IV. The molecular mass of BKPyV GST-VP1 fusion proteins = 45 + 25 = 71 kilodalton (kDa). Molecular mass in kDa of the pageruler prestained protein ladder (Thermofisher Scientific, Waltham, MA, USA) is indicated on the right.

Figure S2. Cross-reactivity analysis of BKPyV serotype seroresponses by VP1-specific competition



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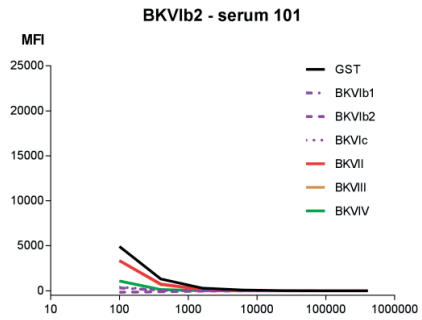

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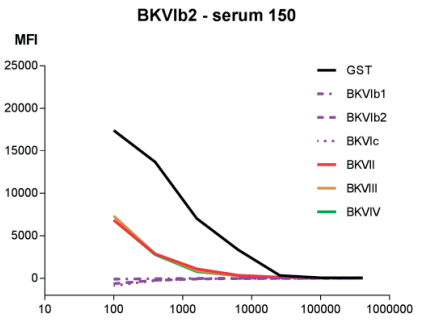

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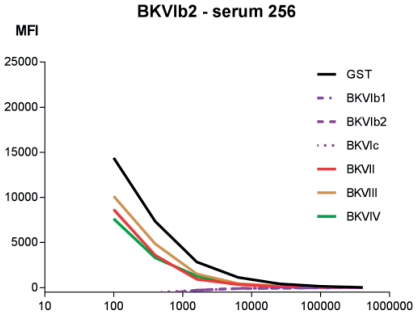

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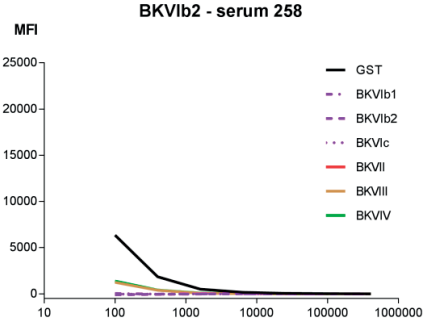

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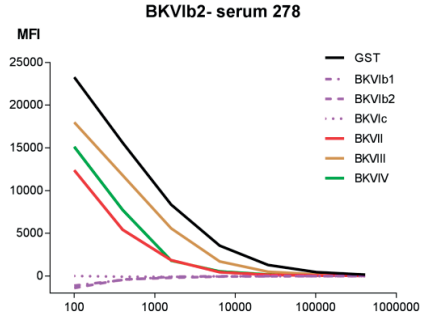

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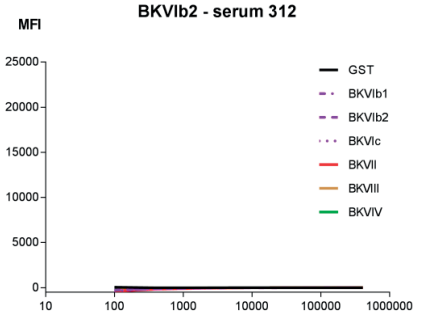

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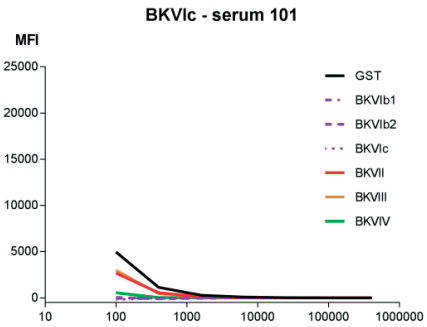

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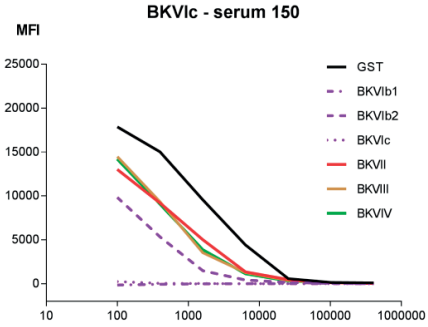

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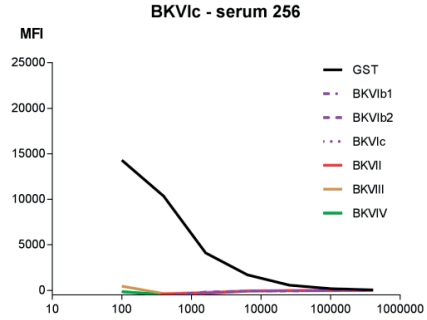

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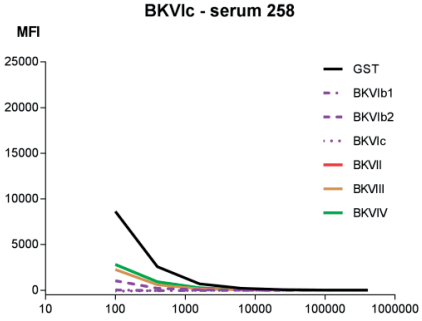

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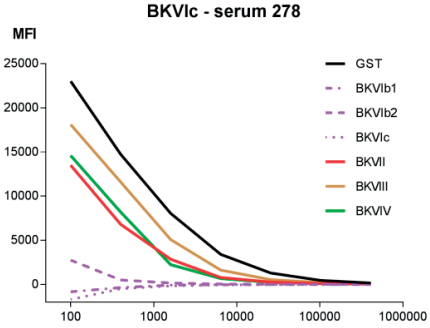

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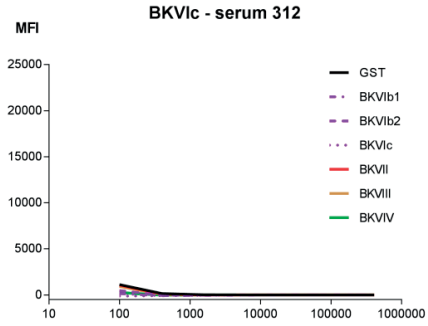

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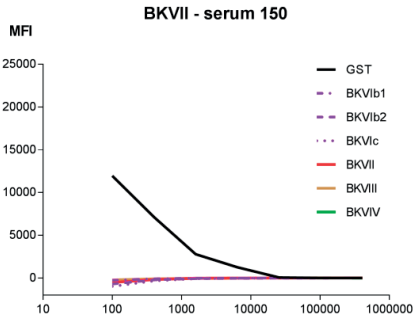
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MFI
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20000
15000
10000
5000
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10
100
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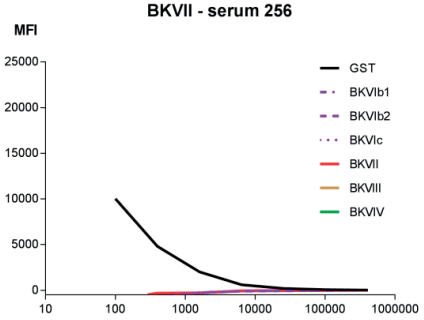
1000000
GST
BKVIb1
BKVIb2
BKVIc
BKVII
BKVIII
BKVIV

D2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10
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GST
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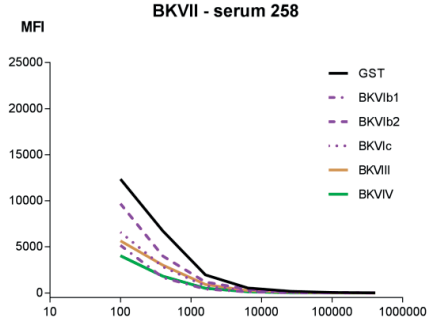
BKVIb2
BKVIc
BKVII
BKVIII
BKVIV

D3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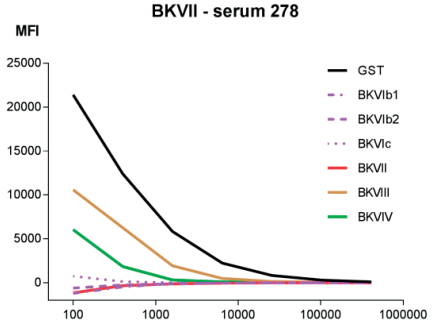
BKVIb2
BKVIc
BKVIII
BKVIV

D4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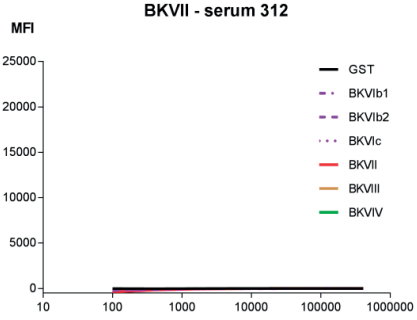
BKVIII
BKVIV

D5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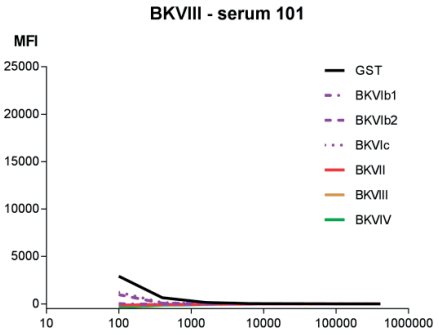
GST
BKVIb1
BKVIb2
BKVIc
BKVII
BKVIII
BKVIV

E1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20000
15000
10000
5000
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10
100
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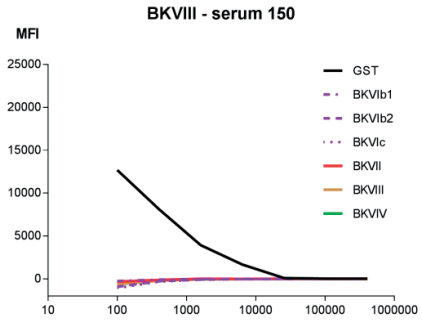
10000
100000
1000000
GST
BKVIb1
BKVIb2
BKVIc
BKVII
BKVIII
BKVIV

E2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15000
10000
5000
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10
100
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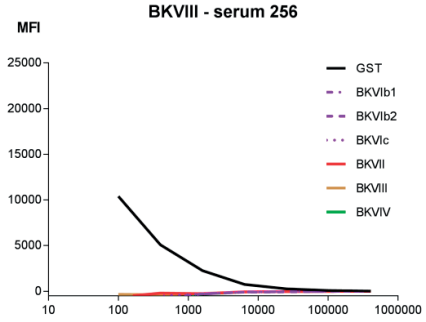
BKVIb2
BKVIc
BKVII
BKVIII
BKVIV

E3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15000
10000
5000
0
10
100
1000
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100000
1000000
GST
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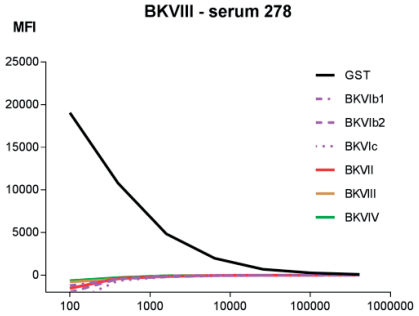
BKVIb2
BKVIc
BKVII
BKVIII
BKVIV

E4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15000
10000
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GST
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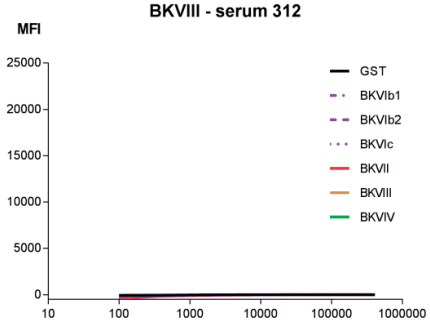
BKVIb2
BKVIc
BKVII
BKVIII
BKVIV

E5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15000
10000
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100
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GST
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BKVIb2
BKVIc
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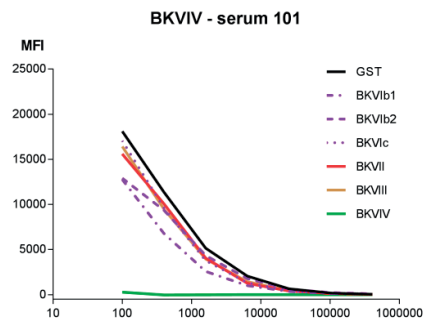
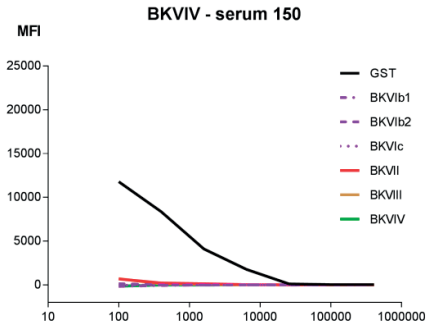
BKVIII
BKVIV

F1



F2



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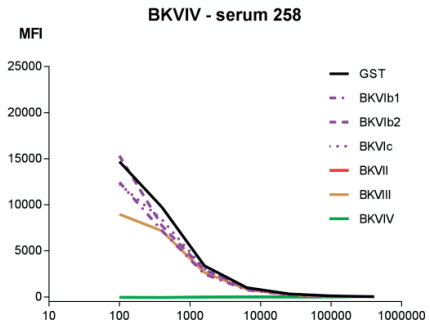

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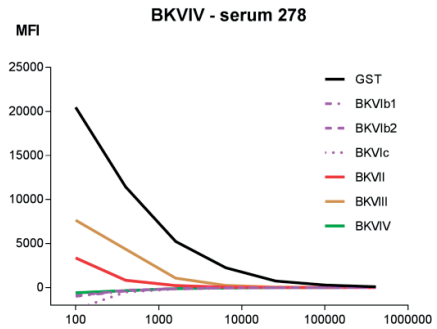

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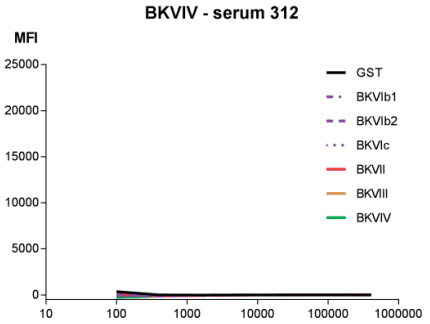
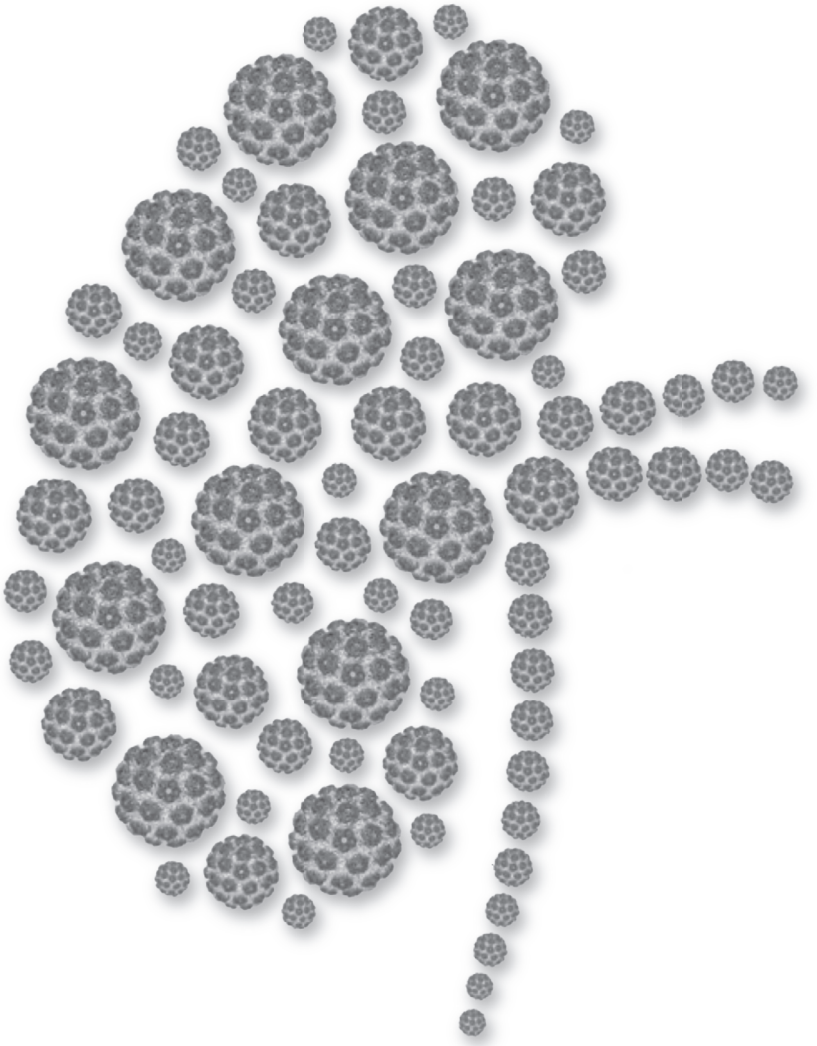


Titrated serum samples were pre-incubated with crude bacterial extract containing GST only (in black), or containing GST-VP1 of the autologous BKPyV subtype (target subtype shown on top of each graph) or non-target heterologous BKPyV subtypes. Results are depicted as median fluorescence intensity (MFI) and shown for the seroresponses to Ib1 (A1-6), Ib2 (B1-6), Ic (C1-6), II (D1-5), III (E1-5) and IV (F1-5) measured in each serum sample. The graphs displaying results for serum 258 lack inhibition data for BKPyV genotype II, which antigen unfortunately was not added to the analysis.



Chapter 6

Source and relevance of the BK polyomavirus genotype for infection after kidney transplantation

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Abstract

Background

BK polyomavirus-associated nephropathy (BKPyVAN) is a major threat for kidney transplant recipients. The role of specific BKPyV genotypes/serotypes in development of BKPyVAN is poorly understood. Pretransplantation serotyping of kidney donors and recipients and posttransplantation genotyping of viremic recipients, could reveal the clinical relevance of specific BKPyV variants.

Methods

A retrospective cohort of 386 living kidney donor-recipient pairs was serotyped before transplantation against BKPyV genotype I-IV specific VP1 antigen using a novel BKPyV serotyping assay. Replicating BKPyV isolates in viremic recipients after transplantation were genotyped by real-time PCR and confirmed by sequencing. BKPyV serotype and genotype data were used to determine the source of infection and analyse the risk of viremia and BKPyVAN.

Results

Donor and recipient BKPyV genotype and serotype distribution was dominated by genotype I (>80%), especially Ib, over II, III and IV. Donor serotype was significantly correlated with the replicating genotype in viremic recipients ($p < 0.001$). Individual donor and recipient serotype, serotype (mis)matching and the recipient replicating BKPyV genotype were not associated with development of viremia or BKPyVAN after transplantation.

Conclusions

BKPyV donor and recipient serotyping and genotyping indicates donor-origin of replicating BKPyV in viremic kidney transplantation recipients, but provides no evidence for BKPyV genotype-specific virulence.

Introduction

BK polyomavirus (BKPyV) causes asymptomatic infection early in life (1, 2), reaching a seroprevalence of ~90% in adults (3, 4). Thereafter, BKPyV latently persists in the urothelium and renal tubular cells (5, 6). In immunocompromised patients, BKPyV infections can cause manifest disease, such as BK polyomavirus-associated nephropathy (BKPyVAN) in kidney transplant recipients (KTRs) (1, 2). BKPyVAN represents a major problem for KTRs (7-9), causing graft dysfunction and graft loss in 1-10% of recipients (10-13). Currently, reduction of immunosuppressive therapy is the only effective evidence-based treatment with the disadvantage of increasing the risk of allograft rejection (13, 14).

BKPyV is classified into four genotypes, I-IV (15-18), and several subtypes, Ia, Ib1, Ib2, Ic, IVa1, IVa2, IVb1, IVb2, IVc1, and IVc2 (19-22). The various genotypes and respective subtypes show a different geographical distribution (19, 21-24). Genotype I is the most prevalent and widespread worldwide (~80%), followed by genotype IV (~15%) mainly found in Europe and East Asia. Genotypes II and III are rare in all geographic regions (~5%) (19, 21-27). Reported prevalence percentages are generally based on BKPyV isolates from viruric and viremic (immunocompromised) individuals, and therefore may not represent the BKPyV genotype distribution in the general (immunocompetent) population. Coinfection of a dominant genotype with other BKPyV genotypes/subtypes (quasispecies) is common (24, 28, 29).

Little is known about the association between specific BKPyV genotypes and the risk, course and severity of BKPyV-associated infection after kidney transplantation (KTx). It has been shown that genotype I replicates more efficient than genotype IV in human renal epithelial cells *in vitro* (30), possibly suggesting more efficient infection *in vivo*. Some studies reported associations between BKPyVAN and especially genotypes I and IV (16, 31-34). However, these studies were all performed in regions where I and IV are the most prevalent genotypes, thereby introducing a potential bias (16, 31-35). A recent report investigating BKPyV genotype-specific neutralizing antibody profiles of KTRs, showed that the absence of antibodies specifically neutralizing the replicating genotype rather than the genotype itself increased the risk of BKPyV viremia (36).

Altogether, these studies provide conflicting evidence for BKPyV genotype-specific associations with BKPyV-associated disease. To solve a number of these issues, we recently developed a BKPyV serotyping assay based on Luminex technology (37). This assay enables simultaneous detection of seroresponses against the major

viral capsid protein 1 (VP1) of BKPyV genotypes I, II, III and IV, and its main subtypes. With the help of this assay, by calling the genotype that elicits the strongest seroresponse the serotype, each seropositive individual can be BKPyV-serotyped. Based on validation of this approach by mutual comparison of measured seroreactivity against individual BKPyV genotypes, we found this assay to reliably serotype infections with the common BKPyV genotypes I and IV, while infections with genotypes II and III were hard to detect and distinguish serologically (37).

Here, with the help of this new method, we serotyped a large retrospective cohort of KTRs and their donors prior to transplantation (37). These data were mutually compared and compared with the replicating BKPyV genotype identified in recipients that developed viremia and BKPyVAN after transplantation. In this way we could determine the origin (donor or recipient) of the replicating BKPyV strain, the presence of genotype-specific associations with development of viremia and BKPyVAN, and the relevance of donor-recipient pair BKPyV genotype (mis)matching for developing viremia and BKPyVAN.

Materials and Methods

Study population and sample collection

The study cohort was extensively described previously (38), and initially included 407 living donor-recipient pairs transplanted at the Leiden University Medical Center (LUMC) between 2003 and 2013. For the current study, twenty-one pairs were excluded, because not enough serum was available from either donor or recipient for determination of BKPyV genotype IgG-levels. The remaining 386 donor-recipient pairs were included in the study (Figure S1). Donor and recipient sera were collected at a median of 125 and 6 days pretransplantation, respectively, and recipient blood plasmas were collected posttransplantation at five regular time-points, during one year of follow-up with a mean follow-up of 9.1 months. The study protocol was submitted to the Medical Ethical Committee of the LUMC that decided formal approval was not needed, due to the retrospective study design and the use of previously collected anonymized samples.

BKPyV serotyping

Serum samples were analyzed by a lab-developed Luminex immunoassay detecting IgG-reactivity against VP1 of BKPyV Ia/Ib1, Ib2, Ic, II, III and IVb1, as described recently (37). As BKPyV variants Ia and Ib1 have a 100% VP1 amino acid sequence similarity, they represent one serotype (37, 39). Since BKPyV genotype IV subtypes

belong to one serotype (39), IVb1 included in this analysis accounts for all IV subtypes.

BKPyV serotype-specific immunoassay cut-off values were based on immunocompetent children aged 7–24 months (n=36), as described (4, 37). The following serotype-specific mean fluorescence intensity (MFI) cut-off values were obtained: Ib1 478, Ib2 1013, Ic 1451, II 792, III 758, and IV 356 MFI. The geometric mean titers (GMT) of all BKPyV serotypes were determined for donors and recipients, as described (37), by testing serum dilution series of 1:100; 1:1000; 1:10.000 and 1:100.000.

Detection of BKPyV viremia and assessment of BKPyVAN

Viremia was detected by quantitative BKPyV PCR analysis of blood plasma, as described previously (38, 40). All BKPyVAN cases included in our analysis were biopsy-confirmed. A kidney biopsy was performed if indicated in the view of the treating physician, and BKPyVAN was diagnosed based on the criteria described (38).

BKPyV genotyping

Total nucleic acid extracted from recipient BKPyV DNA-containing plasma samples was analyzed to determine the infecting genotype with the help of a BKPyV genotype-specific real-time PCR assay and VP1 sequencing.

The BKPyV genotype-specific real-time PCR assay was performed according to a published protocol (28). In brief, the BKPyV genotype-specific real time PCR assay consists of four BKPyV genotype-specific real-time PCRs targeted to the most conserved region of the VP1 gene for each genotype. Primers and probes were designed in a region of the VP1 gene with low variability between the subtypes of a genotype, but with high variability between the genotypes.

For VP1 sequencing, serum samples with a BKPyV load ≥10.000 copies/ml were selected. Primers (sense primer 5'-CCTCAATGGATGTTGCCTTT-3', antisense primer 5'-ACCACCCCCAAAATAACACA-3') were chosen just outside the VP1 gene (BKPyV Dunlop strain, Genbank:V01108) with the help of Primer3 (http://primer3.sourceforge.net/). The BKPyV genotype was determined by Sanger sequencing of the generated PCR products, using the selected and four additional PCR primers (sense primer 5'-CTAACCTGTGGAAATCTACT-3', antisense primer 5'-TACWGTYACAGCCTCCCACA-3', sense primer 5'-CAGCTACCACAGTGTTGCT-3', antisense primer 5'-CCCCACACCCTGTTCATC-3').

Statistical analyses

Data were analyzed with IBM SPSS Statistics software version 21. Differences between viremic and non-viremic recipients and viremic recipients with or without BKPyVAN were assessed using the Chi-Square test or Fisher's exact test. The GMT and MFI values of the Luminex immunoassay were compared and assessed by Cohen's kappa agreement analysis. For all performed tests a p-value < 0.05 in a two-sided test was considered statistically significant.

Results

BKPyV serotyping of donors and recipients

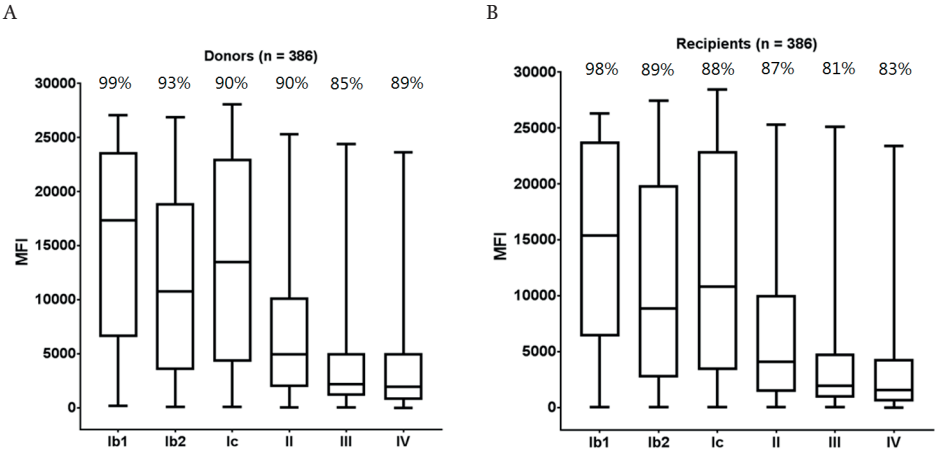
To serotype all donors and recipients, seroreactivity against six common BKPyV genotypes/subtypes (Ia/Ib1, Ib2, Ic, II, III, and IVb1) was determined in the serum samples collected pretransplantation. Both the MFI-value measured at 1:100 serum dilution and the calculated GMT based on a 10-fold serum dilution series (1:100 - 1:100.000) were recorded. Comparable to what we reported before (37), among both donors and recipients strong agreement was observed between the BKPyV genotype with the highest seroreactivity expressed as MFI value or expressed as GMT (Kappa > 0.8; Tables S1 A-B). In the remainder of this article, we will use the MFI values obtained with the 1:100 serum dilution for further analyses.

Overall, seroresponses were observed against all of the analysed genotypes, and measured MFI values did not differ between donors and recipients (Figure 1). The seropositivity rate of all BKPyV variants in donors and recipients was high (>80%). The highest mean seroreactivity was measured for BKPyV genotype I subtypes, followed by genotypes II, III and IV, respectively. Ranking of seroresponses according to the BKPyV genotype VP1 antigen that obtained the highest MFI value within a subject indicated that most donors and recipients were seroresponsive to BKPyV belonging to genotype I, primarily Ib1, followed by II, IV and III, respectively (Table 1), suggesting that most subjects were primarily infected with genotype I.

BKPyV genotyping of viremic recipients

In total, 103 of the 386 recipients (27%) developed viremia during one-year of follow-up after KTx. To identify the replicating BKPyV genotype, DNA isolated from each recipient with a viral load exceeding 10^3 genome c/ml (n = 92) was analysed by genotype-specific real-time PCR. This analysis revealed 76 replicat-

Figure 1. Seroreactivity against BKPyV genotype-specific VP1 antigens in kidney transplantation donors and recipients



Seroreactivity against BKPyV genotype-specific VP1 antigens was measured in serum samples collected before transplantation from kidney transplantation donors (panel A) and recipients (panel B). Results are depicted as the mean fluorescence intensity (MFI) obtained at a 1:100 serum dilution. The bottom and top of each box represent the first and third quartiles. The band inside each box represents the median, and the end of the whiskers include the minimum and maximum recorded seroreactivities. The percentage shown above each box represents the seroprevalence of each BKPyV genotype/subtype.

Table 1. BKPyV serotype distribution among 386 kidney transplant donors and recipients

	BKPyV serotype					
	I			**II**	**III**	**IV**
	Ib1	Ib2	Ic			
Donors	331 (86%)			45 (12%)	3 (1%)	7 (2%)
	223 (58%)	19 (5%)	89 (23%)			
Recipients	331 (86%)			38 (10%)	11 (3%)	6 (2%)
	223 (58%)	22 (6%)	86 (22%)			

Data are shown as n (%). BKPyV, BK polyomavirus.

ing infections with BKPyV genotype I (87%), six with genotype IV (7%), five with genotype II (6%) and none with genotype III (Table 2). In five recipients genotyping failed, probably because the DNA load was close to the detection limit. VP1 sequencing and subsequent genotyping, which required a higher concentration of input DNA of at least 10^5 genome c/ml, succeeded in 45 of the 92 recipients. The obtained sequences showed complete agreement with the genotype-specific PCR results (Table S2).

Table 2. Association between donor and recipient BKPyV serotype determined pretransplantation and the BKPyV genotype replicating after transplantation

BKPyV serotype		Genotype of replicating BKPyV strain in 87 viremic recipients				
		I (n = 76)	II (n = 5)	III (n = 0)	IV (n = 6)	p-values*
Donor	I (n = 79)	75 (99%)	1 (20%)	0 (0%)	3 (50%)	< 0.001
	II (n = 6)	1 (1%)	4 (80%)	0 (0%)	1 (17%)	
	III (n = 0)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
	IV (n= 2)	0 (0%)	0 (0%)	0 (0%)	2 (33%)	
Recipient	I (n = 77)	69 (91%)	4 (80%)	0 (0%)	4 (67%)	0.082
	II (n = 8)	6 (8%)	1 (20%)	0 (0%)	1 (17%)	
	III (n = 1)	1 (1%)	0 (0%)	0 (0%)	0 (0%)	
	IV (n= 1)	0 (0%)	0 (0%)	0 (0%)	1 (17%)	

Data are shown as n (%). *P-values were calculated using the Fisher's exact test. P-values < 0.05 were considered statistically significant. BKPyV, BK polyomavirus.

Correlation between replicating BKPyV genotype and donor serotype

The BKPyV serotype distribution among donors and recipients was comparable to the distribution of replicating genotypes among viremic recipients, with a predominance of sero/genotype I in all groups (Table 2). We compared the BKPyV genotyping results obtained from viremic recipients after KTx with the donor and recipient BKPyV serotyping results obtained before KTx, to assess the source of the replicating virus in the recipient. A strong association was observed between the recipient replicating genotype and the donor serotype ($p < 0.001$) (Table 2), suggesting similarity between the donor BKPyV and the virus replicating in the recipient.

Lack of association between viremia and BKPyVAN development and BKPyV serotype

Next we looked for associations between the donor and recipient BKPyV serotype and development of viremia and BKPyVAN after KTx. In this regard, no significant differences were observed between viremic and non-viremic recipients, and between viremic recipients with and without BKPyVAN (Table 3). Moreover, donor-recipient pair BKPyV serotype (mis)matching showed no difference in incidence of viremia and BKPyVAN (Tables 3 and S3).

Table 3. Association of donor and recipient BKPyV serotype with development of viremia and BKPyVAN in the recipients during follow-up

	Recipients (n = 386)			Viremic recipients (n = 103)		
	No BKPyV viremia (n = 283)	BKPyV viremia (n =103)	p-values*	No BKPyVAN (n = 92)	BKPyVAN (n = 11)	p-values*
Donor BKPyV serotype						
I	238 (84%)	93 (90%)	0.419	82 (89%)	11 (100%)	0.676
II	37 (13%)	8 (8%)		8 (9%)	0 (0%)	
III	3 (1%)	0 (0%)		0 (0%)	0 (0%)	
IV	5 (2%)	2 (2%)		2 (2%)	0 (0%)	
Recipient BKPyV serotype						
I	243 (86%)	88 (85%)	0.877	78 (85%)	10 (91%)	1.000
II	27 (10%)	11 (11%)		10 (11%)	1 (9%)	
III	9 (3%)	2 (2%)		2 (2%)	0 (0%)	
IV	4 (1%)	2 (2%)		2 (2%)	0 (0%)	
Donor-recipient pair						
BKPyV serotype matching						
Matched	205 (72%)	79 (77%)	0.401	71 (77%)	8 (73%)	0.715
Mismatched	78 (28%)	24 (23%)		21 (23%)	3 (27%)	

Data are shown as n (%). *P-values were calculated using the Chi-Square or Fisher's exact test. P-values < 0.05 were considered statistically significant. BKPyV, BK polyomavirus.

Discussion

By serotyping and genotyping of a retrospective cohort of KTx donor-recipient pairs, we aimed to determine the source (donor or recipient) of the replicating BKPyV strain, evaluate BKPyV genotype-specific associations with BKPyV infection after KTx, and determine the role of donor-recipient BKPyV genotype matching in development of viremia and BKPyVAN.

The observed seropositivity rate of all analysed BKPyV variants in both donors and recipients was high (>80%). The rates were higher than expected for BKPyV genotypes II, III, and IV, which could mean that genotypes II, III, and IV circulate more often in the general population than expected based on BKPyV-viremic KTx patient screening only (36, 41), and that mixed infection with different BKPyV variants is common. Three previous studies also reported the occurrence and detection of mixed BKPyV infections in healthy and immunocompromised patients (24, 28, 29). Although we believe that BKPyV genotyping generally underestimates the prevalence of different BKPyV genotypes among study populations, we think

the seropositivity rates of genotypes II and III are generally overrated, because of a substantial amount of cross-reactivity with especially genotype IV (37).

To determine the main infecting BKPyV genotype by serotyping, we ranked the genotype-specific seroresponses according to the BKPyV genotype VP1 antigen that obtained the highest MFI and GMT values. We recently showed good agreement between these measures and the presence of neutralizing antibodies against the relevant BKPyV genotype (37). Our serotyping results suggest that most subjects, donors as well as recipients, are primarily infected with BKPyV genotypes belonging to serotype I (86%), especially Ib1 (58%), while some individuals seem primarily infected with II (10-12%), IV (2%) and III (1-3%), respectively. This serotype distribution is somewhat different from what has been reported elsewhere in Europe with genotype Ib2 as most prevalent subtype (~75%), and genotype IV accounting for most of the remaining subjects (15%), respectively (22, 26). Geographic differences in genotype distribution may account for these differences, however, it should be kept in mind, that both serotyping and genotyping have their limitations and their data may be difficult to compare.

Overall, the distribution of BKPyV genotypes among viremic KTRs in our cohort was comparable with the serotype distribution obtained from donors and recipients pretransplantation, likely representing the distribution of the BKPyV genotypes present in the general population (19, 22, 26). The observed agreement between the genotype and serotype distributions suggests that serotyping represents a useful surrogate method for genotyping, especially in (immunocompetent) populations that do not shed (sufficient amounts of) BKPyV for genotyping.

Since BKPyV infection in recipients is thought to originate from the kidney allograft (38, 42-45), we analyzed if the serotype of donors and recipients, determined before transplantation, was correlated with the BKPyV genotype found in viremic KTRs. The replicating BKPyV genotype in viremic KTRs was significantly correlated with the serotype of the donor and not of the recipient, indicating that BKPyV infection after KTx is indeed donor-derived.

For some viruses, for example hepatitis C virus, it is known that the different genotypes influence the course, treatment response and outcome of disease (46). For BKPyV we found no indication that the genotype is relevant to any of the analyzed aspects of BKPyV infection. Furthermore, we observed no differences in virological and clinical outcome between BKPyV genotype-matched and mismatched donor-recipient pairs. Specific associations between BKPyVAN develop-

ment and genotype I and IV infection, as described in other studies (16, 31-34), were not confirmed by this study.

To conclude, donor and recipient serotyping shows that BKPyV genotype I infections dominate the picture and that replicating BKPyV strains in KTRs are donor-derived. Furthermore, no direct effect of specific BKPyV genotypes or genotype (mis)matching was shown for development of viremia or BKPyVAN in our study.

Acknowledgements

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Disclosure

The authors of this manuscript have no conflicts of interest to disclose.

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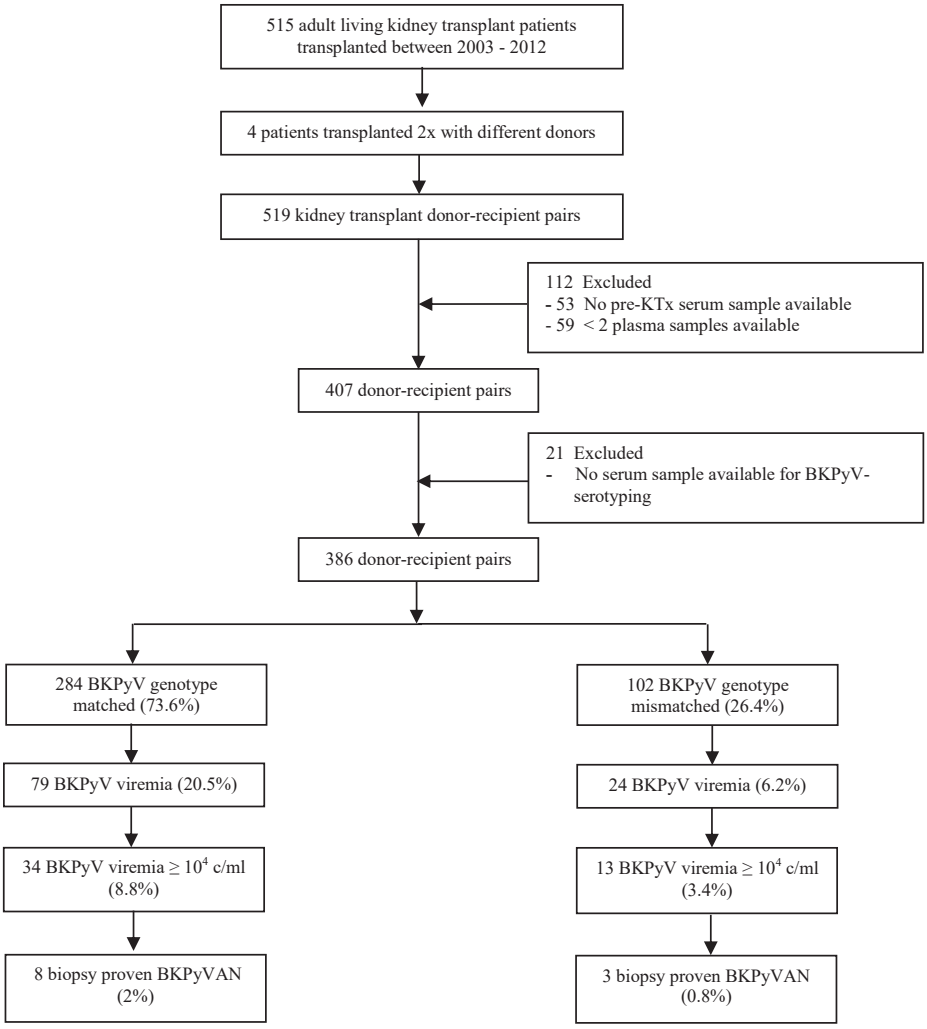
Supporting Information

Figure S1. Study population, inclusion of kidney transplantation donor-recipient pairs, and development of BKPyV viremia and BKPyVAN divided by pretransplantation BKPyV donor-recipient pair genotype matching



Inclusion and exclusion criteria and distribution of BKPyV viremia ($p = 0.401$), BKPyV viremia ≥ log 4 ($p = 0.338$), and BKPyVAN ($p = 0.715$) in the pretransplantation donor-recipient pair genotype matched and mismatched group. P-values were calculated using the Chi-Square or Fisher's exact test. P-values < 0.05 were considered statistically significant. BKPyV, BK polyomavirus; BKPyVAN, BK polyomavirus-associated nephropathy; c/ml, copies/ml; pre-KTx, pre-kidney transplantation.

Table S1. Agreement within an individual between the BKPyV genotype that induced the highest seroresponses expressed as MFI values obtained at 1:100 serum dilution and the genotype that induced the highest geometric mean titer in donors and recipients

A

Donor	**BKPyV genotype with highest 1:100 MFI value (n = 386)**			
	I (n = 331)	II (n = 45)	III (n = 3)	IV (n = 7)
BKPyV genotype with highest geometric mean titer				
I	323 (97%)	3 (7%)	0 (0%)	0 (0%)
II	5 (2%)	40 (89%)	0 (0%)	0 (0%)
III	3 (1%)	0 (0%)	3 (100%)	0 (0%)
IV	1 (<1%)	2 (4%)	0 (0%)	7 (100%)
Observed agreement	97%			
Expected agreement	74%			
Kappa coefficient	0.871			
95% CI	0.80 – 0.94			
P value	< 0.001			
Classification	Strong			

B

Recipient	**BKPyV genotype with highest 1:100 MFI value (n = 386)**			
	I (n = 331)	II (n = 38)	III (n = 11)	IV (n = 6)
BKPyV genotype with highest geometric mean titer				
I	319 (96%)	1 (3%)	1 (9%)	0 (0%)
II	9 (3%)	33 (87%)	3 (27%)	0 (0%)
III	3 (1%)	0 (0%)	7 (64%)	0 (0%)
IV	0 (0%)	4 (10%)	0 (0%)	6 (100%)
Observed agreement	95%			
Expected agreement	73%			
Kappa coefficient	0.802			
95% CI	0.72 – 0.88			
P value	< 0.001			
Classification	Strong			

Panel A shows the observed agreement and Cohen's kappa analysis between highest BKPyV genotype titers of 1:100 MFI values and geometric mean titers in donors and panel B in recipients.

Table S2. Association between BKPyV genotyping by real-time PCR and sequencing

	Recipient replicative BKPyV genotype Real-time PCR (n = 45)				
	I (n = 38)	II (n = 2)	III (n = 0)	IV (n = 5)	p-values*
Recipient replicative BKPyV genotype Sequencing (n = 45)					
I	38 (100%)	0 (0%)	0 (0%)	0 (0%)	< 0.001
II	0 (0%)	2 (100%)	0 (0%)	0 (0%)	
III	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
IV	0 (0%)	0 (0%)	0 (0%)	5 (100%)	

Data are shown as n (%). *The p-value was calculated using the Fisher's exact test. A p-value < 0.05 was considered statistically significant. BKPyV, BK polyomavirus.

Table S3. Lack of association between BKPyVAN and replicating BKPyV genotype of the recipient after transplantation determined by sequencing (A) and real-time PCR (B)

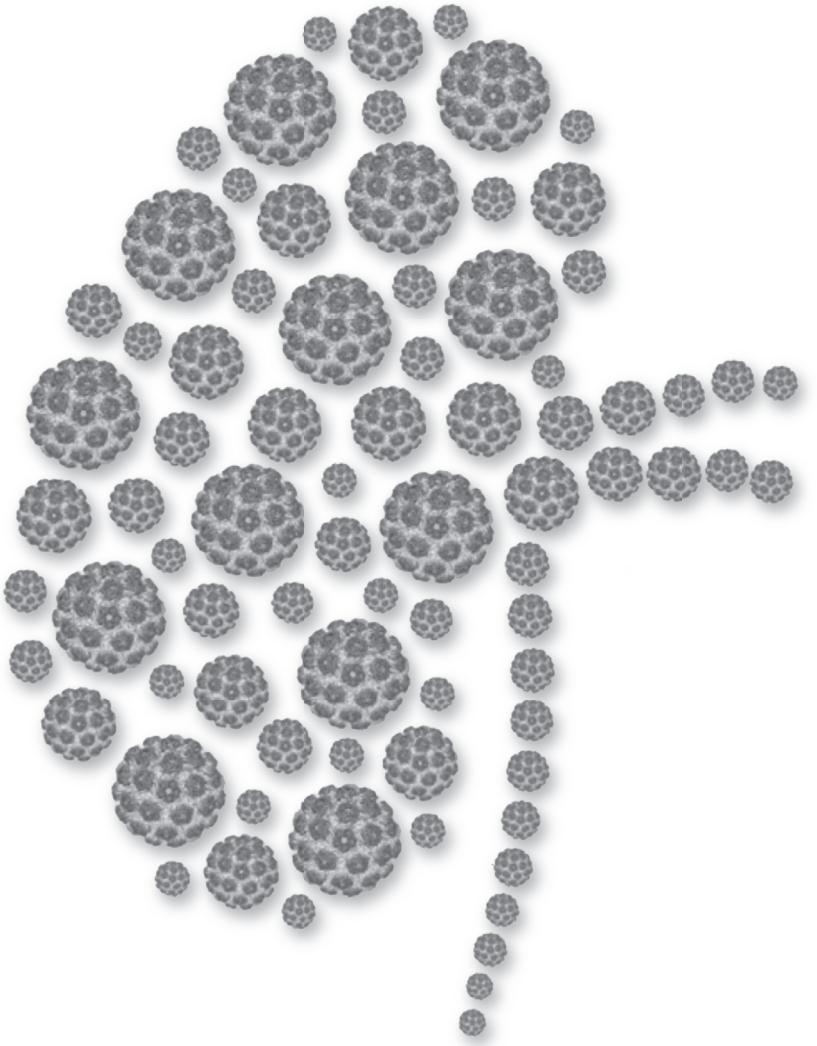
A

	Viremic recipients (n = 46)		
	No BKPyVAN (n = 36)	BKPyVAN (n = 10)	p-values*
Recipient replicative BKPyV genotype			
Sequencing			
I	30 (83%)	9 (90%)	1.000
II	2 (6%)	0 (0%)	
III	0 (0%)	0 (0%)	
IV	4 (11%)	1 (10%)	

B

	Viremic recipients (n = 87)		
	No BKPyVAN (n = 76)	BKPyVAN (n = 11)	p-values*
Recipient replicative BKPyV genotype			
Real-time PCR			
I	66 (87%)	10 (91%)	1.000
II	5 (7%)	0 (0%)	
III	0 (0%)	0 (0%)	
IV	5 (7%)	1 (9%)	

Data are shown as n (%). *The p-value was calculated using the Fisher's exact test. A p-value < 0.05 was considered statistically significant. BKPyV, BK polyomavirus.



Chapter 7

General Discussion

BKPyV infection after KTx; current challenges

BKPyV infection remains a major threat for KTRs and is regarded as the most challenging opportunistic infection after KTx (1, 2). BKPyVAN represents one of the major causes of graft dysfunction and loss in KTRs (3-11), and despite the clinical need, BKPyV-specific antiviral drugs are not available.

Although the process of KTx has been revolutionized during the last decades (12, 13), the ever expanding waiting list and the worldwide shortage of kidney organ donors (14, 15), call for further damage prevention and outcome improvement of the available kidney grafts. One way to reach this goal, is to better understand, prevent and manage BKPyV infections occurring after KTx.

BKPyV infection after KTx can only be prevented if the source of infection, either donor or recipient-derived, is identified. Furthermore, identification of risk factors that predict KTRs at risk for developing BKPyV-related complications, will be essential to optimize the current BKPyV screening strategy employed after KTx (6, 16). This strategy is aimed at early identification of viremic KTRs, because sustained viremia and BKPyV-loads above 10^4 genome c/ml are associated with development of BKPyVAN (6, 8, 11). In this way, early identification of viremic KTRs ensures that timely reduction of immunosuppression, which is the only effective evidence-based treatment thus far, is possible to prevent or halt progression of BKPyVAN.

But why then is optimization of the BKPyV screening strategy necessary? At this moment in most centers, BKPyV screening includes all KTRs, while only 20-30% of them develop viremia and only 1-10% BKPyVAN (6, 7, 17-20). Therefore, the current screening strategy including all KTRs seems overtly abundant. Furthermore, the current pre-emptive BKPyV screening strategy, which does not prevent viremia but identifies KTRs that already show replication, does not fully eliminate the risk of BKPyVAN and also increases the risk of donor-specific antibodies, rejection, graft loss and death. The Kidney Disease Improving Global Outcomes (KDIGO) guideline therefore already noted in 2010, that studies to determine the most cost-effective strategies for BKPyV screening are needed (16).

Optimization of the current strategy could be accomplished by identification of risk factors that correlate with the risk of developing BKPyV viremia or BKPyVAN. The most valuable risk factors will be the ones that can be used as biomarkers that timely predict BKPyV infection, for instance already before transplantation. This would allow treating physicians to take the identified risk for BKPyV infection

into account, while deciding on (immunosuppressive) treatment of the respective recipients in the posttransplantation period.

Identification of pretransplantation predictive markers of BKPyV infection after KTx

To address the above-mentioned issues we assembled a large retrospective cohort of 407 living kidney transplantation donor-recipient pairs, called the PAIR cohort. With the help of the PAIR cohort we identified a predictive pretransplantation serological biomarker of BKPyV viremia and BKPyVAN in KTRs, described in Chapters 2 and 3. At the same time, we provided compelling evidence for the kidney allograft as source of BKPyV infection after KTx in Chapter 2 and 6. In Chapter 4, we described the protective effect of a recipient HLA-B51 positive status against development of BKPyV viremia after KTx, and identified four potential cytotoxic T cell epitopes, including a previously described highly immunogenic peptide (LPLMRKAYL) that induces interferon-γ production by $CD8^+$ T cells. Furthermore, in Chapter 5 we described the development of a BKPyV serotyping method that, despite its shortcomings, reliably detects infections of the two most prevalent BKPyV genotypes worldwide, namely BKPyV genotype I and IV. In Chapter 6, with the help of this method, we showed the absence of BKPyV genotype specific associations with BKPyV viremia and BKPyVAN in KTRs.

Implications and future directions

What do the results of this thesis contribute to the current knowledge about BKPyV infection after KTx, what are the implications, and which questions still remain? Both high donor BKPyV-specific antibody titers and low (or absent) recipient BKPyV-specific antibody titers were already mentioned as risk factors for BKPyVAN in the American Society of Transplantation Infectious Disease Community of Practice guideline of 2013 (21). However, due to the high BKPyV seroprevalence in the general population and the shortage of organ donors (22, 23), (high) BKPyV seroreactive donors cannot be excluded from kidney donation.

The added value of our findings in Chapter 2, particularly lie in the integrated evaluation of the BKPyV seroreactivity among donor-recipient pairs, providing leads for future algorithms to predict BKPyV-related disease after transplantation. The use of donor-recipient pair BKPyV seroreactivity as a practical, single determination, easy to perform, low-cost and early predictive disease marker could be of great value in the prevention and management of BKPyV-associated disease. The identification and use of this potential predictive disease marker raises several questions related to a number of areas:

I. Clinical management-related questions

a. can the current BKPyV screening strategy after KTx be optimized/personalized by the use of pretransplantation BKPyV serological testing of (potential) donors and recipients, and is BKPyV seroreactivity matching, low seroreactive recipient calls for low seroreactive donor and vice versa, clinically feasible and will it lead to a better outcome of the allograft?
b. can the use of a pretransplantation BKPyV seroreactivity risk assessment guide treating physicians in deciding on (immunosuppressive) treatment of KTRs in the posttransplantation period?
c. is measurement of the seroreactivity against genotype I alone, sufficient for the prediction of BKPyV infection after KTx, or does the seroreactivity against the three other genotypes contribute to a better prediction model?

II. Scientific questions

a. is BKPyV seroreactivity a reflection of the BKPyV viral kidney load?
b. how important is humoral immunity to control BKPyV infection and should we explore approaches aimed at improving humoral BKPyV immunity post-transplantation, such as the administration of (BKPyV-specific) IVIG and BKPyV vaccination, to improve the outcome of available kidney grafts?

Ia

BKPyV seroreactivity matching and optimization of the current BKPyV screening strategy

Subsequent studies are needed to reveal whether a pretransplantation serological BKPyV risk assessment could provide a basis for personalized BKPyV load-monitoring strategies aimed at early identification of BKPyV viremic patients and improving the efficiency of the current BKPyV screening strategy. A well designed, preferably randomized controlled, prospective (multi-center) trial is needed to evaluate the potential benefits of BKPyV seroreactivity matching and the use of a BKPyV serological risk assessment (Figure 1). Such a trial will hopefully refine the interpretation of the serological values, and reduce or discard the need for frequent blood sampling and BKPyV load monitoring in a certain percentage of KTRs with very little risk of BKPyV infection. For example by determination of bottom threshold values below which recipients get reduced or do not need frequent BKPyV load monitoring. On the other hand, upper threshold values for donors could be established, above which recipients need more frequent monitoring of BKPyV viremia. An example of such a serological stratification strategy is cur-

rently recommended in multiple sclerosis patients being treated with or initiating natalizumab (24, 25). In these patients the risk of developing progressive multifocal leukoencephalopathy and the frequency of MRI monitoring is determined by measuring the anti-JCV antibody index (24, 25). Therefore, the included recipients will need to be categorised according to their predicted risk of BKPyV infection by the pretransplantation donor-recipient pair seroreactivity, for example in low donor-high recipient (low risk), low donor-low recipient (intermediate low risk), high donor-high recipient (intermediate high risk), and high donor-low recipient (high risk) groups. On the basis of this categorisation and the resultant estimate of BKPyV infection, the frequency of BKPyV load monitoring can then be adjusted (Figure 1C). Mathematical modeling with the data generated from this prospective trial could hopefully lead to such refinement. The trial can possibly also lead to the use of a lower viremia threshold for reduction of immunosuppression in the subset of recipients at high risk of BKPyV infection after KTx.

Ib

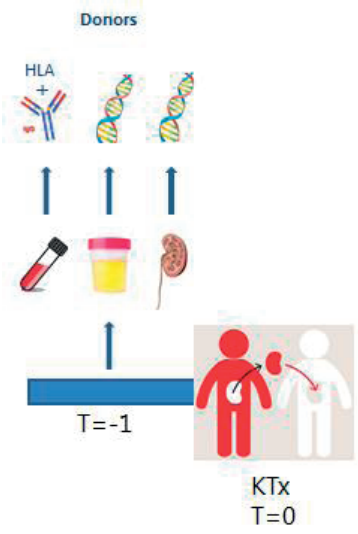
BKPyV seroreactivity risk assessment and potential adjustment of immunosuppression

To prevent rejection of the allograft, KTRs receive both induction and maintenance immunosuppression. The induction treatment with mono- or polyclonal antibodies that deplete host lymphocytes (thymoglobulin/ATG and alemtuzumab) or anti-CD25 monoclonal antibodies (basiliximab or dacluzimab), is very immunosuppressive and administered early after KTx when the risk for acute rejection of the allograft is the highest. The maintenance treatment, with calcineurin inhibitors (CNI) (cyclosporine and tacrolimus), mTOR inhibitors (everolimus and sirolimus), antiproliferative agents (mycophenolate mofetil (MMF) and azathioprine), and corticosteroids, is needed throughout life to prevent chronic rejection and improve long-term allograft survival (3). The maintenance regimen for most KTRs consists of three agents, typically a CNI, an antiproliferative agent, and a corticosteroid. In some cases, the CNI is exchanged by an mTOR inhibitor to decrease the risk of allograft failure or CNI induced nephrotoxicity (26).

With the development of more potent immunosuppressive agents like the CNI, the reduction of allograft rejection was inversely correlated with an increased incidence of BKPyV infection (3, 27). Induction treatment with a lymphocyte depleting antibody has been shown to be associated with a higher rate and a longer duration of BKPyV viremia and a higher incidence of BKPyVAN than induc-

Figure 1. Design of future prospective study investigating BKPyV infection after KTx

A



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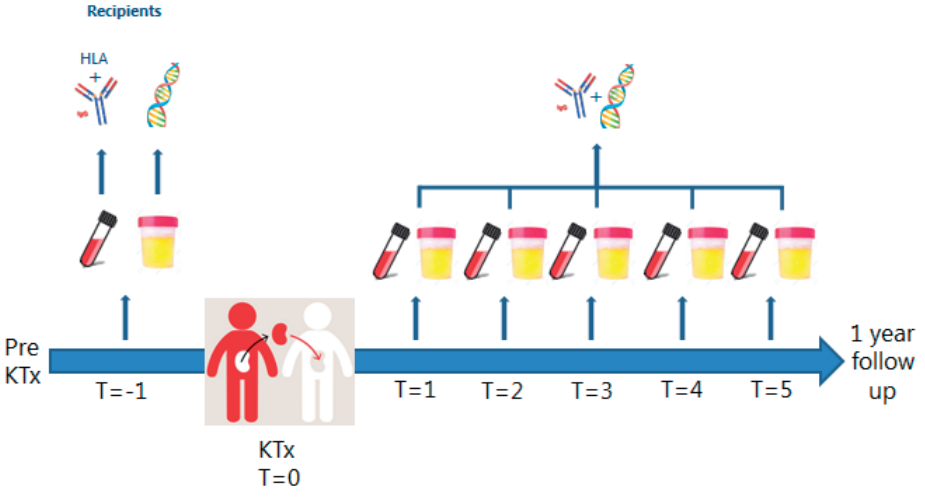

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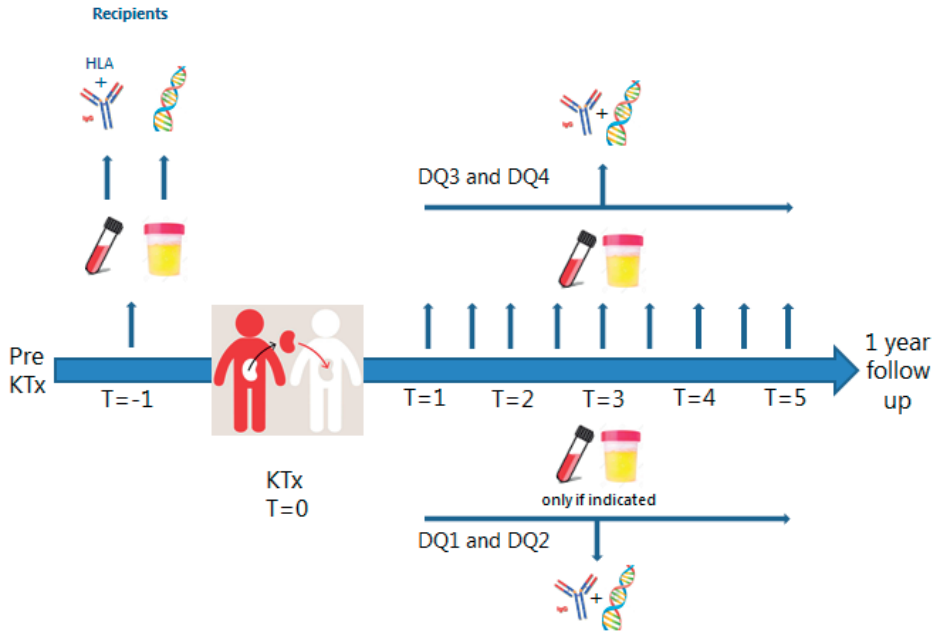

Outline of an optional future prospective study design. A) Donor sampling: Pretransplantation serum samples will be tested for HLA class I and class II status and with the Luminex BKPyV serotyping immunoassay for BKPyV serotype specific IgG levels. Pretransplantation urine samples, protocolled kidney biopsies taken at implantation and superfluous ureters removed at implantation will be tested for BKPyV DNA with a BKPyV genotype specific real-time PCR. B) Recipient sampling: Pretransplantation serum samples will be tested for HLA class I and class II status and with the Luminex BKPyV serotyping immunoassay for BKPyV serotype specific IgG levels. Pretransplantation urine samples will be tested for BKPyV DNA with a BKPyV genotype specific real-time PCR. Posttransplantation serum and urine samples will be taken at five different time-points after transplantation: 1.5, 3, 6, 9 and 12 months after transplantation, or more frequent if indicated by the treating physician. Posttransplantation serum samples will be tested with the Luminex BKPyV serotyping immunoassay for BKPyV serotype specific IgG levels and for BKPyV DNA with a BKPyV genotype specific real-time PCR. Posttransplantation urine samples will be tested for BKPyV DNA with a BKPyV genotype specific real-time PCR. C) Future option for recipient sampling according to pretransplantation donor or donor-recipient pair BKPyV seroreactivity if the prediction rules for seroreactivity levels are better defined. Pretransplantation serum samples will be tested for HLA class I and class II status and with the Luminex BKPyV serotyping immunoassay for BKPyV serotype specific IgG levels. Pretransplantation urine samples will be tested for BKPyV DNA with a BKPyV genotype specific real-time PCR. Recipients with a donor with a low pretransplantation BKPyV seroreactivity (DQ1 and DQ2) will be sampled only if indicated by the treating physician and recipients with a donor with high pretransplantation BKPyV seroreactivity (DQ3 and DQ4 are tested more frequently at regular time-points.

tion with anti-CD25 monoclonal antibodies (17, 28-31). When considering the maintenance immunosuppressive regimen, tacrolimus has been associated with a higher risk of BKPyV infection than cyclosporine (3, 7, 17, 21, 32-34). Tacrolimus with MMF and corticosteroids seems to confer the highest risk of BKPyV viremia (17, 35, 36), and a greater exposure to corticosteroids has also been associated with viremia (7, 21, 37). In comparison to the CNI, the mTOR inhibitors are considered less potent immunosuppressive agents and also seem to confer a lower risk of BKPyV viremia (28, 38, 39).

Thus, specific immunosuppressive agents or combinations thereof used as induction or maintenance treatment influence the risk of BKPyV infection after KTx. A pretransplantation BKPyV donor-recipient pair seroreactivity risk assessment could potentially help transplantation nephrologists in deciding which immunosuppressive agents compose the best immunosuppressive regimen for a particular KTR after KTx. For instance, when the risk for BKPyV infection is low or absent, a regimen of tacrolimus with MMF and corticosteroids can probably be chosen to optimally reduce the risk of rejection. However, when the risk of BKPyV infection is high it might be better to go for cyclosporine or an mTOR inhibitor instead of tacrolimus, and maybe lower the corticosteroid exposure. Further studies are needed to investigate if a BKPyV seroreactivity risk assessment can contribute to personalized treatment or the adjustment of the net degree of immunosuppression given to specific KTRs in the future.

Ic

Measuring BKPyV seroreactivity only against genotype I or against multiple genotypes

While BKPyV seroreactivity is common and detectable against all main genotypes, the most prevalent and intense seroresponses are directed against BKPyV genotype I. In Chapter 6, we found no direct effect of a specific BKPyV genotype and of genotype donor-recipient (mis)matching on viremia and on BKPyVAN. This questions the relevance of BKPyV serotyping in the pretransplantation work-up and could indicate that measurement of the pretransplantation donor-recipient seroreactivity based on BKPyV genotype I, subtype Ib1 as described in Chapter 2, is sufficient to predict the risk of BKPyV infection after transplantation. Solis et al. also showed no significant difference in the prevalence of the different BKPyV genotypes among KTRs with viruria, viremia, and BKPyVAN (40). However, they did show that a pretransplantation neutralization antibody titer <4 $\log_{10}$ of

the recipient against the donor strain was significantly associated with BKPyV replication after KTx and that donor-recipient genotype mismatching was associated with transition from viruria to viremia (40). This would imply that BKPyV genotype-specific neutralizing antibody titers may be a meaningful predictive marker allowing patient stratification by BKPyV disease risk before and after transplantation (40). Unfortunately, we did not have any viruria data available to evaluate the transition from viruria to viremia.

To evaluate if the seroresponse to the BKPyV genotypes other than genotype I, are relevant for the prediction of BKPyV infection after KTx, the pretransplantation donor-recipient pair seroreactivity against the respective BKPyV genotypes should be investigated. In Chapter 2 we analyzed the risk of BKPyV viremia and BKPyVAN by dividing the BKPyV Ib1 seroreactivity of donors and recipients in seroquartiles and calculated the hazard ratio of BKPyV infection according to the donor seroreactivity quartiles stratified by the recipient seroreactivity quartiles. To evaluate if the seroresponse to BKPyV genotypes other than genotype I, are relevant for the prediction of BKPyV infection after KTx, the pretransplantation donor-recipient pair seroreactivity against the respective BKPyV genotypes should be evaluated in the same way, especially in donor-recipient pairs of which the recipient shows posttransplantation replication of a BKPyV genotype other than genotype I.

Regarding the Luminex immunoassay developed in Chapter 5, the BKPyV genotype I and IV seroresponses seem genuine, but it remains unclear if the assay can detect and discriminate genotype II and III-specific seroresponses. Therefore, studies evaluating the detection of these serotypes are necessary. The fact that mixed BKPyV infections in healthy and immunocompromised patients occur frequently (41-43), but that their relevance is still unclear also demands further study. In conclusion, the added value of donor-recipient pair BKPyV sero/genotyping and the height of the respective seroresponses of donor-recipient pairs against the different BKPyV genotypes in the context of KTx and management of KTRs needs to be further evaluated (Figure 1).

IIa

BKPyV seroreactivity as reflection of the BKPyV viral kidney load

The observation in Chapter 2 that the pretransplantation donor-recipient pair seroreactivity against BKPyV, especially the BKPyV seroreactivity of the donor, is correlated with development of BKPyV viremia and BKPyVAN after KTx, is in line

with the reported correlation between donor BKPyV IgG levels and the onset, duration and peak level of recipient BKPyV viruria (44). Obviously, it is the virus itself that is causing the infection after KTx, not the BKPyV-directed antibodies. This indicates that the donor BKPyV seroreactivity reflects the amount of latently persistent BKPyV present in the kidney allograft.

In Chapter 3, we showed that the increase of BKPyV seroreactivity is associated with the duration and peak level of preceding BKPyV viremia. The proportional relation between donor BKPyV seroreactivity and recipient viremia supports the postulation that donor BKPyV seroreactivity reflects the infectious load of the kidney allograft. Unfortunately, we could not confirm this hypothesis by detecting BKPyV DNA in protocolled kidney allograft biopsies at implantation. Even if we had these biopsies available, the detection of BKPyV DNA would be hampered by the random (multi)focal nature of BKPyV infection, in which sporadically (latently) infected areas likely coexist with large areas of unaffected kidney parenchyma (45).

Based on the observed dose-dependent relationship between BKPyV seroreactivity and preceding viremia in viremic KTRs, and the stability of BKPyV seroreactivity over time in immunocompetent subjects and nonviremic KTRs (Chapter 3), we assume that BKPyV seroreactivity measured in immunocompetent individuals, which also represents potential donors, reflects the level of viremia experienced during primary infection and possibly during re-exposures. Future studies should establish this relation by comparing the allograft load of an individual with its BKPyV seroreactivity. In this context, it is important to notice that BKPyV latently persists in the urothelium and renal tubular cells (46, 47), and autopsies from immunocompetent patients have shown that BKPyV DNA can also be detected in ureter and bladder samples (46). Therefore, to overcome the issues related to BKPyV DNA detection in protocolled kidney allograft biopsies at implantation, it would be worthwhile to investigate other urinary tract samples as well. Residual kidney allograft ureters that are superfluous after implantation could represent a good option (Figure 1A). Pretransplantation urinary sampling could also prove valuable in this regard (Figure 1), as it has been shown that small amounts of viral progeny can be temporarily detected in urine of 7-55% of healthy individuals, depending on the sampling frequency (48-50). The amount of BKPyV DNA in donor ureters or urine of donors and recipients could be compared to their BKPyV eroreactivity, and correlated with the posttransplantation replicative BKPyV DNA in viremic KTRs. The effect of pretransplantation urinary shedding of BKPyV in the recipient has been shown to increase the risk of posttransplantation viruria

but not of viremia and BKPyVAN (51). Pretransplantation BKPyV urinary shedding of the donor however, was associated with development of BKPyV viremia and BKPyVAN in KTRs after transplantation (52, 53)

IIb

Improving the outcome of KTx by improving the humoral immunity

The additive effect of a low BKPyV seroreactive recipient in combination with a high seroreactive donor to the risk of BKPyV infection after KTx, suggests that BKPyV-directed antibodies are directly involved in containment of BKPyV infection. This is supported by studies that show efficient BKPyV neutralization by BKPyV-directed serum antibodies (54, 55). Neutralization by BKPyV-directed antibodies, suggests that IVIG administration in the posttransplantation period could prove beneficial in viremic KTRs, especially in those that do not respond to adjustment of immunosuppression. This is supported by the demonstration that IVIG contains neutralizing antibodies against BKPyV (55, 56), and by several studies reporting the safe and successful clearance of BKPyV viremia after IVIG administration (33, 57, 58).

Besides reflecting humoral immunity, BKPyV seroreactivity could also be a reflection of the BKPyV-specific T cell immunity (59), which is generally considered essential for controlling BKPyV infection after KTx (60, 61). Therefore, in the context of KTx, BKPyV seroreactivity might actually reflect the BKPyV viral kidney load in donors and the overall BKPyV-specific immunity in KTRs. The observation in Chapter 4, that HLA-B51 positive recipients have a lower risk of developing BKPyV infection after KTx, and the identification of four putative BKPyV-specific cytotoxic T lymphocyte epitopes makes one wonder about possible interventions strategies like BKPyV vaccination and transfer of primed BKPyV-reactive T-cells to boost or restore the BKPyV-specific immunity in KTRs.

Validation of the association between recipient HLA-B51 positivity and a reduced risk of BKPyV infection is needed (Figure 1), as it might be potentially useful for BKPyV risk stratification, customization of viral load screening, and adjustment in tapering of immunosuppression. Furthermore, it has been shown that BKPyV-specific T cell immunity is essential for controlling BKPyV infection after KTx (59-61). The identification of immunogenic BKPyV-specific peptides like the 9mer LPLMRKAYL derived from the LT and ST antigens, calls for analyses to comprehensively map T cell epitopes within all five antigenic proteins of BKPyV. It is also

important to expand the HLA restriction to all common HLA alleles worldwide, like Cioni et al. did by specifically mapping 39 BKPyV-specific T cell epitopes for 14 major HLA class I alleles prevalent in Europe and North America (62). The identification of BKPyV-derived T cell epitopes could be useful for developing BKPyV-specific T cell response monitoring strategies, adoptive T cell transfer for prophylaxis and therapy, and for the design of BKPyV peptide vaccines.

Conclusion

To conclude, by identifying pretransplantation predictive markers and enabling a better donor-recipient match, this thesis provides a number of important leads to reduce the frequency and severity of BKPyV-induced infection and prevent graft loss due to BKPyVAN in KTRs. Moreover, we provided compelling evidence that BKPyV infection after KTx is derived from the kidney allograft, and that high BKPyV seroreactive donors confer the highest risk for both viremia and BKPyVAN. The risk caused by donor BKPyV seroreactivity is modulated by the BKPyV seroreactivity of the recipient, and the use of a pretransplantation BKPyV-seroreactivity risk assessment among donor-recipient pairs could identify KTRs at increased risk of BKPyV infection among those at low or absent risk.

Furthermore, we identified a protective association between recipient HLA-B51 positivity and a reduced risk of BKPyV infection and suggest that potential BKPyV-derived T cell epitopes might be useful for developing BKPyV-specific T cell response monitoring strategies, adoptive T cell transfer for prophylaxis and therapy, and for the design of BKPyV peptide vaccines.

Finally, BKPyV serotyping provides an alternative method to genotyping for the two most common BKPyV genotypes (I and IV), bypassing the need for viral DNA required for genotyping.

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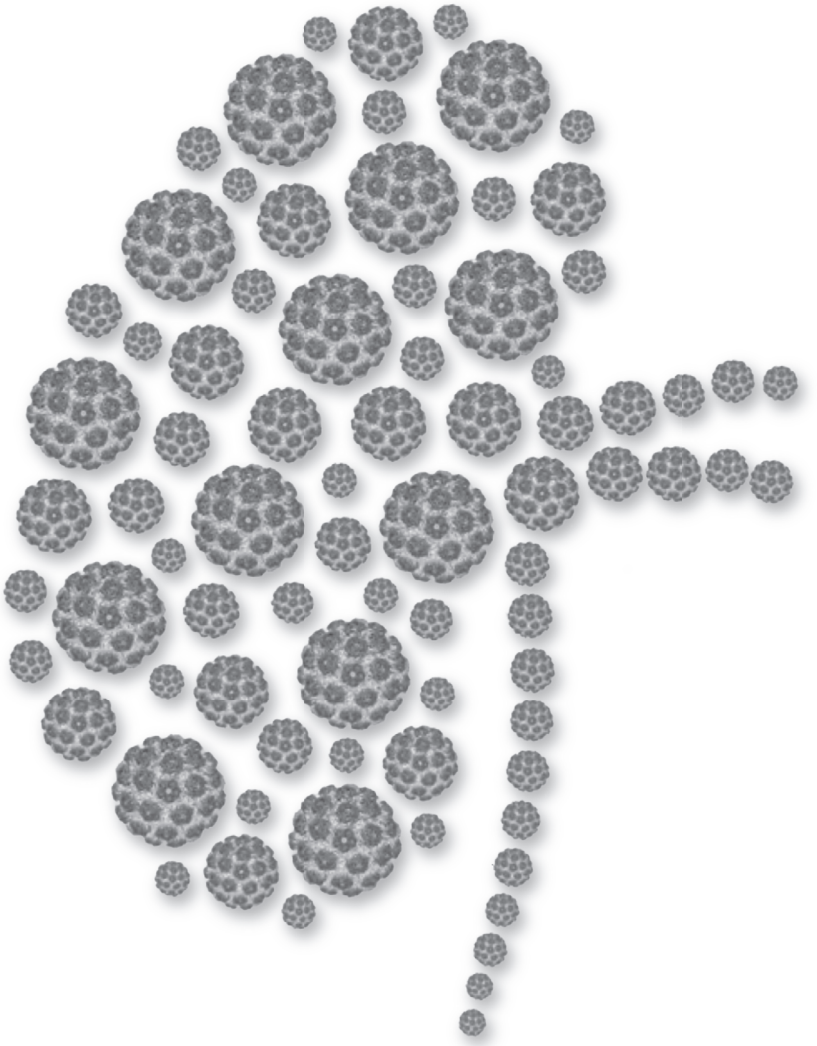


Nederlandse samenvatting
Dutch summary

Nederlandse samenvatting

Ruim 1.7 miljoen mensen van de Nederlandse bevolking, ~10%, heeft last van chronische nierschade. Oorzaken voor chronische nierschade zijn o.a. hoge bloeddruk, diabetes, nierbekkenontsteking, ongezonde leefstijl (roken, overgewicht, hoge zoutinname) en erfelijke aandoeningen zoals cystenieren. Door het verhoogde risico op nierfalen en hart- en vaatziekten hebben mensen met chronische nierschade een levensverwachting die gemiddeld 7-12 jaar lager ligt dan die van mensen met een normale nierfunctie.

Veel mensen met nierfalen komen in aanmerking voor een nierfunctievervangende behandeling. Een dergelijke behandeling vind over het algemeen plaats als de nier nog 15% van de normale nierfunctie overheeft. De twee opties voor nierfunctievervangende therapie zijn nierdialyse en niertransplantatie (NTx). Bij dialyse wordt de nierfunctie overgenomen door een kunstnier. Dialyse heeft vaak heftige bijwerkingen tot gevolg, zoals schommelingen in de bloedwaarden en het vochtgehalte van het lichaam, maar ook infecties. Dit zorgt o.a. voor chronische vermoeidheid en een ziektegevoel. Omdat dialysepatiënten vaak meerdere malen per week voor een langere aaneengesloten periode aan de kunstnier dienen te worden aangesloten zijn ze ook veel minder mobiel. De meeste patiënten ervaren dialyse dan ook als een enorme belasting en beperking van hun bewegingsvrijheid. Vanwege deze redenen heeft een NTx de voorkeur, waarbij een donornier, die afkomstig kan zijn van een levende of overleden donor, de nierfunctie overneemt

Om de donornier, lichaamsvreemd materiaal, niet af te stoten is langdurige afweer-onderdrukking noodzakelijk, m.n. als er geen goede match was tussen donor en ontvanger. Een onderdrukte afweer leidt echter tot een verhoogd risico op infecties. Ziekteverwekkers profiteren van deze situatie, waaronder ook virussen die bij mensen met een normale afweer geen ziekte kunnen veroorzaken. Een van deze virussen, het BK polyomavirus (BKPyV), kan na ongestoorde vermenigvuldiging bij een onderdrukte afweer, ernstige schade/infectie van de donornier veroorzaken. Dit ziektebeeld noemt men ook wel BK polyomavirus geassocieerde nefropathie (BKPyVAN).

Bij ongeveer de helft van de NTx patiënten gaat BKPyV zich vermenigvuldigen gedurende het eerste jaar na transplantatie. BKPyV DNA kan worden aangetoond in de urine (virurie) en bij ongeveer een kwart ook in de bloedcirculatie (viremie). Van deze viremische patienten zal een gedeelte (1-10%) BKPyVAN ontwikkelen, uiteindelijk leidend tot nierfalen en verlies van de donornier. Er zijn geen BKPyV-

specifieke antivirale middelen beschikbaar en BKPyV infectie is momenteel alleen te behandelen door de afweerremmende medicijnen te verminderen en de afweer te herstellen. Het nadeel van het verminderen van de afweerremmende medicijnen is echter dat de kans op afstoting (rejectie) van de donornier toeneemt. De behandeling van BKPyV stelt de niertransplantatie artsen derhalve voor een lastig dilemma, waarbij het risico op rejectie dient te worden afgewogen tegenover de complicaties van BKPyV infectie.

Aangezien er nog geen duidelijke risicofactoren voor het oplopen van een BKPyV infectie na NTx bekend zijn, hebben wij ons gebogen over de vraag welke NTx-patiënten de meeste kans hebben op het ontwikkelen van BKPyV infectie na transplantatie en of dit op basis van bepaalde risicofactoren al voor transplantatie te voorspellen is. Het uiteindelijke doel is om risicofactoren en voorspellende biomarkers te identificeren die in de toekomst te gebruiken zijn om BKPyV geassocieerde complicaties na NTx te voorspellen en te voorkomen, ten einde het huidige BKPyV test (screening) beleid na NTx meer af te stemmen op het risicoprofiel van de individuele NTx patiënt. Zoals genoemd worden momenteel alle NTx-ontvangers na transplantatie op gezette tijden getest op de aanwezigheid van BKPyV DNA in het bloed, terwijl maar 20-30% van de ontvangers BKPyV viremie ontwikkeld en 1-10% BKPyVAN. Het huidige BKPyV testbeleid na NTx zou dus vele malen efficiënter kunnen als we vooraf kunnen voorspellen welke ontvangers wel *at risk* zijn en welke ontvangers helemaal geen risico lopen op BKPyV infectie na NTx.

Pretransplantatie donor-ontvanger paar BKPyV seroreactiviteit

In hoofdstuk 2 hebben we alle NTx uitgevoerd in het LUMC tussen 2003 en 2012 in kaart gebracht waarbij er een nier van een levende donor werd getransplanteerd. In totaal werden 407 donor-ontvanger paren onderzocht op aanwezigheid van BKPyV-antistoffen in bloed afgenomen voorafgaand aan de NTx en op het optreden van BKPyV viremie en nefropathie bij de ontvangers gedurende het eerste jaar na transplantatie. Deze data werd samen met vele andere klinische en laboratoriumgegevens geanalyseerd, en leverde aan aantal belangrijke bevindingen op. De meest relevante was de associatie tussen aanwezigheid van BKPyV-antistoffen bij de donor en in mindere mate de afwezigheid daarvan bij de ontvangers, welke een tot 10x verhoogd risico gaf op BKPyV viremie en BKPyVAN. Deze associatie was sterker dan die van alle anderen factoren die in deze studie werden geëvalueerd, waaronder de vorm van immuunsuppressie en de mate van HLA-matching tussen donor en ontvanger..

Stabiliteit BKPyV seroreactiviteit en relatie met voorafgaande viremie

In hoofdstuk 3 lieten we zien dat toename van de BKPyV seroreactiviteit geassocieerd is met actieve BKPyV infectie, en afhankelijk is van de viremie duur en de piek-hoeveelheid virus in het bloed tijdens een viremie. Het niveau van BKPyV seroreactiviteit onder NTx-ontvangers zonder BKPyV viremie en onder gezonde volwassen bloed donoren bleek stabiel.

Verlaagd risico op BKPyV infectie bij HLA-B51 positieve NTx-ontvangers

In hoofdstuk 4 werd de relatie tussen de HLA klasse I en klasse II status van de nier donor en ontvanger en BKPyV viremie na NTx onderzocht. Er werd een aanzienlijke reductie van BKPyV viremie en BKPyVAN gevonden in HLA-B51 positieve NTx-ontvangers. Multivariate analyse liet een vijfvoudige reductie van BKPyV viremie zien. Het effect van HLA-B51 bleef ook behouden bij correctie voor meervoudige vergelijkingen binnen de respectievelijke HLA loci. In silico analyse naar mogelijke HLA-B51 restrictieve T-cel epitopen leverde vier potentiele cytotoxische T-cel epitopen op, waaronder LPLMRKAYL welke reeds eerder beschreven was als hoog immunogeen peptide dat CD8+ T-cellen aanzet tot interferon-γ productie.

BKPyV serotypering en relevantie van BKPyV genotypen en genotype mis(matching)

BK polyomavirus kan worden verdeeld in vier verschillende genotypen, I-IV, en een aantal subtypen. De betekenis van deze specifieke genotypen of van genotype (mis)matching tussen de donor en de ontvanger voor het risico op BKPyV infectie na NTx is niet bekend. Voor BKPyV genotypering is een bepaalde hoeveelheid BKPyV DNA nodig, die normaalgesproken niet detecteerbaar is bij latent BKPyV-geïnfecteerde gezonde mensen.

Aangezien eerdere studies hebben laten zien dat elk BKPyV genotype een serotype vertegenwoordigt, zou BKPyV genotypering o.b.v. serotypering de noodzaak van detecteerbaar BKPyV DNA omzeilen. Dit was voor ons de reden om de in hoofdstuk 2 gebruikte serologische Luminex BKPyV IgG test gebaseerd op het BKPyV VP1 antigeen afkomstig van genotype Ib1, uit te breiden met de overige meest voorkomende BKPyV varianten; Ib2, Ic, II, III en IV. De ontwikkeling en validatie van deze BKPyV serotypering methode is beschreven in hoofdstuk 5. De resultaten laten zien dat deze test gebruikt kan worden voor het detecteren van de IgG seroresponsen tegen BKPyV genotype I en genotype IV, die weinig kruisreactiviteit tegen de andere genotypen lieten zien. Dit is van belang aangezien genotype I en IV wereldwijd de twee meest voorkomende genotypen zijn en samen 95% van de BKPyV infecties voor hun rekening nemen. De seroresponsen

tegen genotypen II en III blijken gevoelig voor kruisreactiviteit en dienen derhalve voorzichtig geïnterpreteerd te worden.

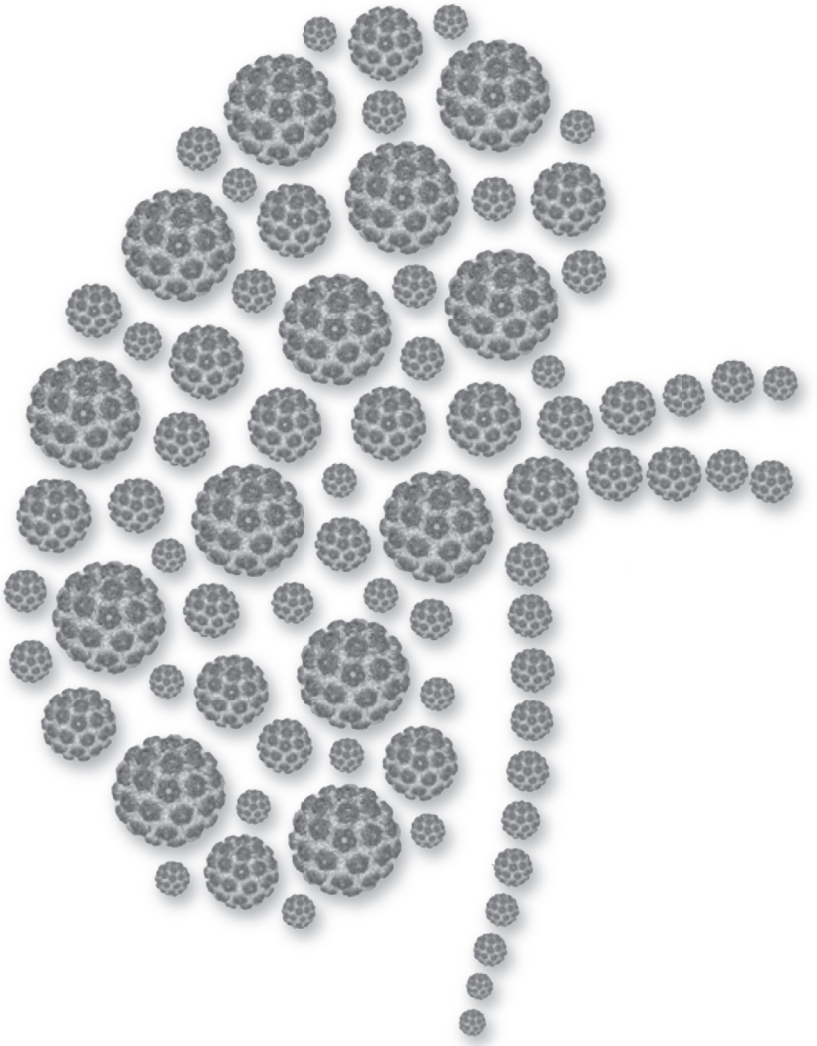
BKPyV serotypering werd ontwikkeld met als doel de seroresponsen tegen de verschillende genotypen te kunnen bepalen in donoren en ontvangers voorafgaand aan NTx, zodat het effect van de specifieke BKPyV genotypen bij donors en ontvangers op BKPyV infectie na NTx bepaald kon worden. In hoofdstuk 6 werden de pretransplantatie BKPyV serotypering resultaten van de donoren en ontvangers vergeleken met die het replicerende BKPyV genotype in de ontvangers na NTx, gebruikmakend van hetzelfde studie cohort als in hoofdstuk 2. Deze analyse toonde aan dat de meeste donoren en ontvangers geïnfecteerd waren met serotype I. Specifieke pretransplantatie donor of ontvanger serotypen lieten geen associatie zien met het ontstaan van BKPyV viremie of BKPyVAN. Donor-ontvanger paar BKPyV genotype (mis)matching liet ook geen associatie zien met BKPyV infectie na NTx. Het pretransplantatie BKPyV serotype van de donor bleek significant gerelateerd te zijn aan het replicerende BKPyV genotype van de ontvangers na transplantatie.

Conclusie

Concluderend blijkt dat BKPyV infectie na NTx afkomstig is van de donornier en dat de pretransplantatie donor-ontvanger paar seroreactiviteit veruit de beste voorspeller is voor het risico op BKPyV infectie na transplantatie. Dit betekent dat er potentieel een simpele, eenmalige en goedkope biomarker voorhanden is, die voorafgaand aan transplantatie het risico op BKPyV infectie en complicaties kan voorspellen. Toekomstige prospectieve studies dienen de voorspellende waarde van deze biomarker te bevestigen en verder te verfijnen. Daarnaast dient onderzocht te worden of de bepaling van de pretransplantatie donor-ontvanger paar seroreactiviteit een plaats dient te krijgen in de pretransplantatie work-up procedure van NTx patienten, of donor-ontvanger paar BKPyV seroreactiviteit matching zinvol is en leidt tot minder BKPyV infecties na NTx, of de bepaling van de pretransplantatie donor-ontvanger paar BKPyV seroreactiviteit kan leiden tot een gepersonaliseerde BKPyV screening beleid na NTx en of aanpassing van het immunosuppressief regime o.b.v. deze biomarker mogelijk is.

Andere bijdragende factoren die aanvullend onderzoek vereisen t.a.v. hun mogelijke rol bij het verlagen van het risico op BKPyV infectie na NTx, zijn (1) de HLA-B51 status van de NTx-ontvanger, (2) de relevantie van BKPyV specifieke T-cel

epitopen voor de monitoring van de BKPyV specifieke T-cel respons, adoptieve T-cel profylaxe en/of therapie en voor het samenstellen van een BKPyV vaccin, en als laatste (3) de beoordeling van het belang van de donor-ontvanger paar seroreactiviteit tegen de verschillende BKPyV genotypen.



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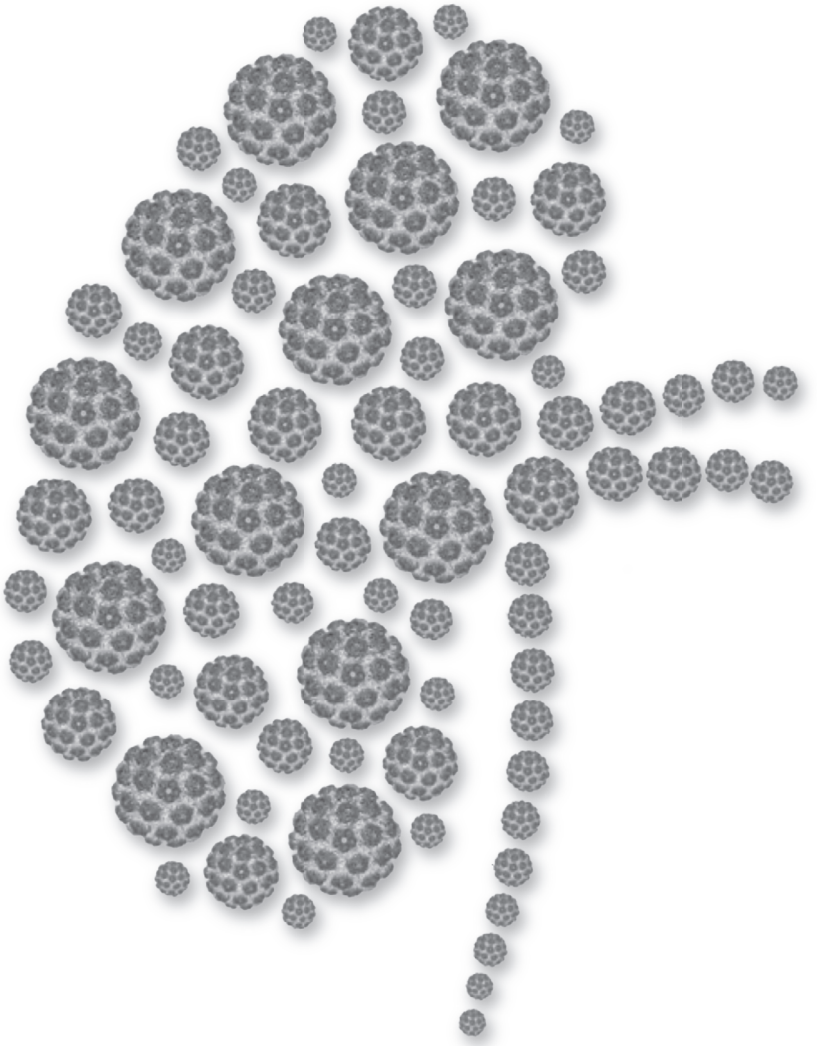
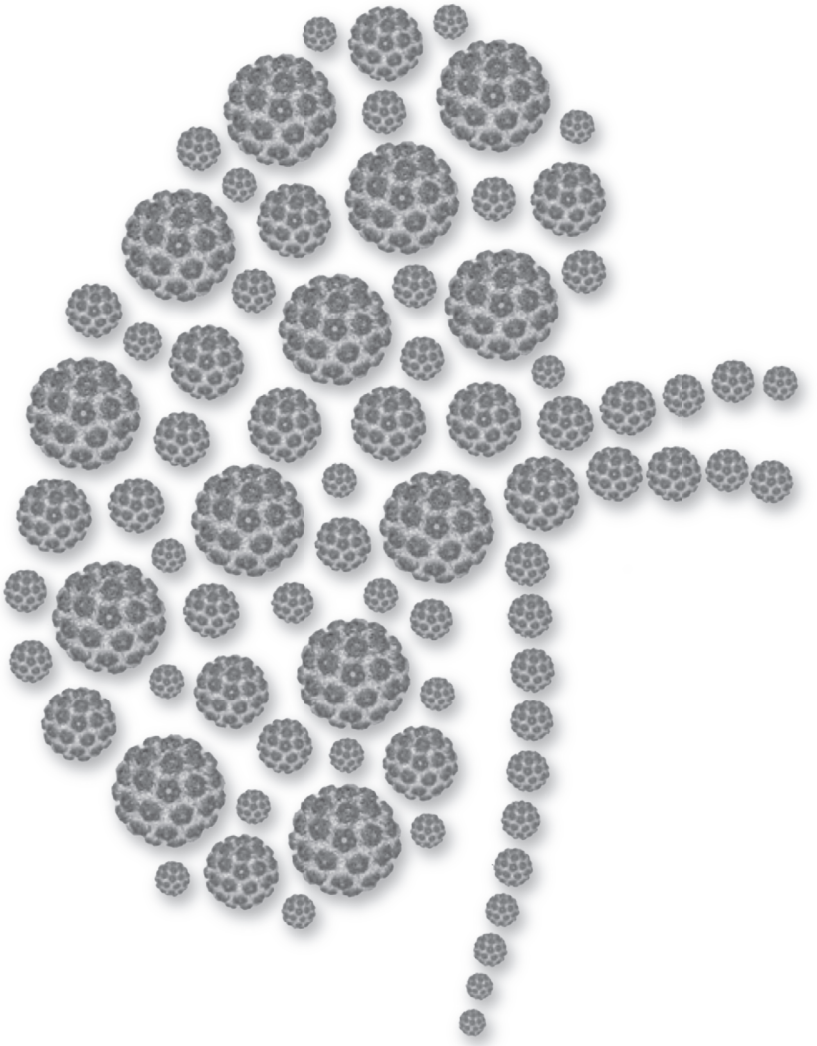


Curriculum Vitae

Curriculum vitae

Herman Wunderink werd op 22 maart 1984 geboren te Rotterdam. Hij behaalde zijn VWO-diploma met de profielen 'Natuur en Techniek' en 'Natuur en Gezondheid' in 2002 aan het Titus Brandsma College te Dordrecht. Vervolgens begon hij met de studie Geneeskunde aan de Rijksuniversiteit Groningen alwaar de propedeuse werd behaald in 2003 en het artsexamen in 2008. Naast zijn opleiding Geneeskunde nam hij deel aan Junior Scientific Masterclass van de Rijksuniversiteit Groningen en was hij als student-onderzoeker betrokken bij onderzoek naar de chaperonne functie van Heat Shock proteïne 27 onder begeleiding van prof. dr. H.H. Kampinga op de afdeling Straling en Stress Celbiologie van het Universitair Medisch Centrum Groningen.

Na het artsexamen werkte hij een half jaar als ANIOS beschouwende vakken in het Martini ziekenhuis te Groningen, waarna hij in 2009 begon met de opleiding tot arts-microbioloog bij opleider prof. dr. A.C.M. Kroes op de afdeling Medische Microbiologie van het Leids Universitair Medisch Centrum (LUMC). Tijdens de opleiding begon hij met zijn promotieonderzoek onder begeleiding van dr. M.C.W. Feltkamp en prof. dr. A.C.M. Kroes. In oktober 2014 werd hij geregistreerd als arts-microbioloog, waarna hij als zodanig tot en met oktober 2017 werkzaam was op de afdeling Medische Microbiologie van het LUMC. Sinds september 2017 is hij werkzaam als arts-microbioloog met de aandachtsgebieden bacteriologie en infectiepreventie bij de afdeling Medische Microbiologie in het Universitair Medisch Centrum Utrecht. In april 2018 kreeg hij op het jaarlijkse Bootcongres van de Nederlandse Transplantatie Vereniging de Novartis Transplantation Award uitgereikt voor het als beste beoordeelde gepubliceerde artikel in 2017 op het gebied van de klinische transplantatiegeneeskunde.



Dankwoord

Dankwoord

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Inmiddels ben ik werkzaam in het Universitair Medisch Centrum Utrecht (UMCU), alwaar ik al mijn collega's wil bedanken voor de fijne start en de tijd die me naast het werk is gegund om mijn promotieonderzoek succesvol af te ronden.

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