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The impact of pre-graft serology on the risk of BKPyV infection reactivation post-renal transplantation

Fatima El Zahraa Dakroub

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Thèse de Doctorat

*Mention Biologie santé
Spécialité Virologie*

Présentée à l'Ecole Doctorale en Sciences Technologie et Santé (ED 585)
de l'Université de Picardie Jules Verne
et
à l'Ecole Doctorale des Sciences et de la Technologie
de l'Université Libanaise

Par

Fatima El Zahraa DAKROUB

Pour obtenir le grade de Docteur de l'Université de Picardie Jules Verne

The Impact of Pre-Graft Serology on the Risk of BKPyV Infection Reactivation Post-Renal Transplantation

Soutenue le 23 Octobre 2020 après avis des rapporteurs, devant le jury d'examen:

M^{me} Constance DELAUGERRE, PU-PH,

M^{me} Mireille LAFORGE, CR-CNRS,

M. Antoine TOUZÉ, PU,

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Abstract

BK polyomavirus associated nephropathy (BKPyVAN), is a troublesome disease induced by BK polyomavirus (BKPyV) reactivation in immunocompromised renal graft recipients. BKPyVAN can progress to graft dysfunction and has no current treatment, making immunosuppression reduction the only management choice. Thus, predictive BKPyV infection reactivation markers are needed for high-risk patient identification. We conducted a retrospective study to assess the correlation between the BKPyV pre-transplant serostatus and post-transplant BKPyV infection incidence. Sera from 329 recipients and 222 matched donors were tested for anti-BKPyV antibodies against four BKPyV serotypes by a VLPs- based IgG ELISA, and BKPyV DNA load was monitored for at least 1-year post transplantation. 80 (24%) recipients were viruric and 59 (18%) recipients were viremic post transplantation. An elevated BKPyV viremia risk was observed for recipients who had a mean antibody titer for all serotypes ≤ 400 before transplantation (odds ratio [OR], 5.58; 95% confidence interval [CI], 2.60-11.79; $P < 0.0001$). In addition, kidney recipients from donors with a mean BKPyV antibody titer ≤ 400 had a lower BKPyV viremia risk (OR, 0.47; CI, 0.21-0.95; $P = 0.055$). Both donor and recipient mean BKPyV antibody titer may serve as a predictive tool to manage clinical BKPyV infection by identification of patients at high reactivation risk. In addition, a high donor's pre-transplant BKPyV antibody titer may predict the severity of the BKPyV infection in the recipient after transplantation.

Keywords: BKPyV; BKPyV associated nephropathy; BKPyV reactivation; BKPyV serostatus; BKPyV seroprevalence; serological technique; BKPyV virus serology; kidney transplantation

Résumé

La néphropathie associée au virus BK (BKPyVAN) est une pathologie observée chez les receveurs d'une greffe rénale suite à une réactivation du virus BK (BKPyV). BKPyVAN peut évoluer vers un dysfonctionnement de la greffe et n'a actuellement aucun traitement, rendant la réduction de l'immunosuppression comme seul choix thérapeutique. Cependant, cette réduction s'avère inadaptée ou non applicable conduisant à une augmentation du risque de rejet aigu. Ainsi, des marqueurs prédictifs en pré-greffe de réactivation de l'infection à BKPyV sont nécessaires pour l'identification des patients à haut risque. Nous avons mené une étude rétrospective pour évaluer la corrélation entre le statut sérologique du BKPyV en pré-transplantation et l'incidence de l'infection par BKPyV en post-transplantation. Des sérums de 329 receveurs et 222 donneurs appariés ont été testés pour les anticorps anti-BKPyV contre quatre sérotypes de BKPyV par un test IgG ELISA à base de VLPs, la charge virale du BKPyV a été surveillée pendant au moins 1 an après la transplantation par PCR. 80 (24%) receveurs étaient viruriques et 59 (18%) receveurs étaient virémiques en post-transplantation. Un risque élevé de virémie à BKPyV a été observé pour les receveurs qui avaient un titre moyen d'anticorps pour tous les sérotypes ≤ 400 avant la transplantation (Odds ratio [OR], 5.58; intervalle de confiance à 95% [IC], 2.60-11.79 ; $P < 0.0001$). De plus, les receveurs de reins à partir de donneurs avec un titre moyen d'anticorps anti- BKPyV ≤ 400 avaient un risque de virémie à BKPyV plus faible (OR, 0.47; CI, 0.21-0.95 ; $P = 0.055$). Le titre moyen d'anticorps anti-BKPyV du donneur et du receveur peut servir d'outil prédictif pour gérer l'infection clinique avec BKPyV, en identifiant les patients à haut risque de réactivation du virus.

Mots-clés : virus BK; néphropathie associée au BKPyV; réactivation du BKPyV; statut sérologique du BKPyV; séroprévalence du BKPyV; technique sérologique; sérologie du virus BK; transplantation rénale

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ABBREVIATIONS

A

aa, Amino acids; ATG, Anti-thymocyte globulin.

B

BCR, B cell receptor; BKTGR, BK virus typing and grouping region; BKPyVAN, BKPyV associated nephropathy; BKPyV, BK polyomavirus; BMI, body mass index.

C

cAMP, Cyclic adenosine monophosphate; CI, Confidence interval; c/ml, Copies per milliliter; CNI, Calcineurin inhibitor; CRE, cAMP responsive-element; CTLs, Cytotoxic T-lymphocytes.

D

D-/R+, Negative donor positive recipient; D-/R-, Negative donor negative recipient; D+/R+, Positive donor positive recipient; D+/R-, Positive donor negative recipient; DBD, DNA-binding domain; DC, Dendritic cells; DNA, Deoxyribonucleic acid.

E

EBV, Epstein Barr virus; ECBS, Expert Committee on Biological Standardization; ELISA, Enzyme linked immunosorbent assay; EM, Electron microscope; ER, Endoplasmic reticulum; ERAD, ER-associated protein degradation; ERE, Oestrogen responsive- element.

F

FBS, Fetal bovine serum.

G

GFP, Green fluorescent protein; GRE/PRE, Glucocorticoid/progesterone responsive-element; GST, Glutathione S-transferase.

H

HIA, Hemagglutination inhibition assays; HLA, Human leukocyte antigen; HPyVs, Human polyomaviruses; HPyV6, Human Polyomavirus-6; HPyV7, Human Polyomavirus-7; HPyV8, Human Polyomavirus-8; HPyV9, Human Polyomavirus-9; HPyV10, Human Polyomavirus-10; HRP, Horse radish peroxidase; Hsc70, Heat shock cognate 70; HSCT, Hematopoietic stem cell transplantation.

I

ICTV, International Committee on Taxonomy of Viruses; IgG, Immunoglobulin G; IL-12, Interleukin-12; IVIg, Intravenous immune globulin.

J

JCPyV, JC polyomavirus.

K

KIPyV, Karolinska Institute Polyomavirus; KTRs, Kidney transplant recipients.

L

LB, Lenox Broth.

M

mAb, Monoclonal antibody; MD, Molecular dynamics; mDC, Myeloid dendritic cells; MCPyV, Merkel Cell Carcinoma-Associated Polyomavirus; miRNA, MicroRNA; MMF, Mycophenolate mofetil; mRNA, Messenger RNAs; mTORi, Inhibitors of the mammalian target of rapamycin; MVBKV, BKPyV peptide-based multi-epitope vaccine; MWPyV, Malawi Polyomavirus.

N

NCCR, Non-coding control region; NF-KB, Nuclear factor KB; NIA, Neutralization inhibition assay; NIH, American National Cancer Institute; NLS, Nuclear localization signal.

O

OD, Optical density; OR, Odds ratio; ORI, Origin of replication.

P

P53, DNA protein 53; PBDC, Peripheral blood dendritic cells; PBS, Phosphate buffered saline; PCa, Prostate cancer; PCR, Polymerase chain reaction; pDC, Plasmacytoid DC; PKA, Protein Kinase A; PKC, Protein Kinase C; PKD, Protein Kinase D; Pol α -primase, DNA polymerase α -primase; pRb, Retinoblastoma protein; PsV, Pseudovirion.

R

rATG, Rabbit antithymocyte globulin; RNA, Ribonucleic acid; RPA, Replication Protein A.

S

SD, Standard deviation; Ser, Serine; SV40, Simian virus 40; SNAP, α -soluble N-ethylmaleimide-sensitive fusion attachment protein; SNFG, Symbol nomenclature for Glycans; STLPyV, St Louis polyomavirus 19.

T

Tag, Large tumor antigen; tAg, Small tumor antigen; Th1, T helper-1; Th2, T helper-2; Thr, Threonine; TLR, Toll-like Receptor; TRE, Phorbol ester responsive-element; trunc Tag, Truncated tumor antigen; TSPyV, Trichodysplasia Spinulosa-Associated Polyomavirus.

U

ULBP3, UL16 binding protein 3; US, United States.

V

VLPs, Virus like particles; VP1, Viral capsid protein 1; VST, Virus-specific T-cells.

W

WHO, World Health Organization; WUPyV, Washington University Polyomavirus.

X

xMAP[®], Multi-Analyte Profiling[®]; X^2 test, *Chi-Square* test.

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I. INTRODUCTION

The best treatment for patients with end-stage renal disease is kidney transplantation. However, technically successful transplantations can be complicated by renal dysfunction episodes in the following months [1]. There are many reasons for this renal dysfunction: Failure to control opportunistic infections, the antiviral and immunosuppressant drugs' nephrotoxicity, and both acute and chronic immune-mediated graft rejection. The guidelines on the kidney transplant recipients' treatment, suggest that the immune mediated graft rejection can be mitigated by intensive immunosuppressant treatment in the immediate post-transplantation period [2]. The immunosuppression required for the graft function maintenance increases the risk of viral infections in kidney recipients [3]. A typical condition in immunosuppressed individuals is the BK polyomavirus (BKPyV) reactivation [4]. Even though we still lack specific anti-BKPyV treatments, there are no methods for reliably predicting the onset of BKPyV-associated infectious complications. However, it has been postulated that the kidney allograft is the infection source. Consequently, the donor's BKPyV seroreactivity may reflect the subsequent BKPyV load in the recipient. Conversely, the recipient's seroreactivity reflects his/her immune status against BKPyV. Hence, BKPyV serostatus represents a valuable tool for predicting the BKPyV-associated disease occurrence after transplantation [5]. Here, we review and compare the different assay techniques used to assess BKPyV seroreactivity. We also consider the clinical BKPyV infection management as a function of the patient's BKPyV serostatus. Lastly, we discuss the obstacles in the routine BKPyV serostatus assessment in a clinical setting.

A. The BK polyomavirus (BKPyV)

1. History and classification

Polyomaviruses, members of the *polyomaviridae* family, are small double stranded deoxyribonucleic acid (DNA) viruses. They are characterized by an icosahedral capsid surrounding the circular DNA genome and lacking a lipoprotein envelope [6]. Before the establishment of the designation *Polyomaviridae* which includes Polyomavirus as the sole genus, these viruses were formerly in designed in 1999 to the *Papovaviridae* family which included both papillomaviruses and polyomaviruses [7]. In 2019 however, the International

Committee on Taxonomy of Viruses (ICTV) created the *Papovaviricetes* class which comprises the two families *Papillomaviridae* and *Polyomaviridae*. The founding member after which the *Polyomaviridae* family was named was discovered in mice and described as an agent which induces “many tumors” hence the name “polyoma” [8]. This was followed by the discovery of simian virus 40 (SV40); a primate polyomavirus from the rhesus monkey [9]. In 1971, the first two human polyomaviruses were discovered: The BK polyomavirus (BKPyV) and JC polyomavirus (JCPyV). Each virus was named after the patient’s initials from which it was isolated [10,11]. BKPyV was isolated from the urine of a 39-year-old male Sudanese renal allograft patient suffering from ureteric obstruction, and advanced renal failure. Electron microscopy was utilized to observe BKPyV particles in the cells lining the ureter, and the patient’s serum demonstrated high and rising BKPyV antibody titers. It was suggested then that the infection was induced by latent viral reactivation either in the transplanted kidney or in the patient [10]. Then, nine novel human polyomaviruses (HPyVs) were discovered between 2007 and 2012 due to recent technologies such as rolling circle amplification and digital transcriptome subtraction. They are listed here in order of their discovery: Karolinska Institute Polyomavirus (KIPyV), Washington University Polyomavirus (WUPyV), Merkel Cell Carcinoma-Associated Polyomavirus (MCPyV), Human Polyomavirus-6 (HPyV6), Human Polyomavirus-7 (HPyV7), Trichodysplasia Spinulosa-Associated Polyomavirus (TSPyV; HPyV8), Human Polyomavirus-9 (HPyV9), Malawi Polyomavirus (MWPyV; HPyV10) [6] and St Louis polyomavirus 19 (STLPyV) [12]. The evolution of the polyomaviruses’ nomenclature has continued with the discovery of these viruses. The Polyomaviridae Study Group of the ICTV classified the polyomaviruses into three genera. The Orthopolyomavirus and Wukipolyomavirus contain all the mammalian species and Avipolyomavirus includes the bird polyomaviruses [7]. The distances of relationships between the human polyomaviruses are revealed in the simplified phylogenetic tree in Figure 1. The groupings are perhaps not surprising, since related species often display similarities in the tissue or sample types from which they were isolated. For instance, HPyV6 and HPyV7, WUPyV and KIPyV and MWPyV and STLPyV are observed in pairs in their own distinct branches. This reflects the fact that they were isolated from skin, nasopharyngeal and stool sources respectively. The distinct tissue tropism of each pair might be reflected by this close protein homology amongst those pairs [13].

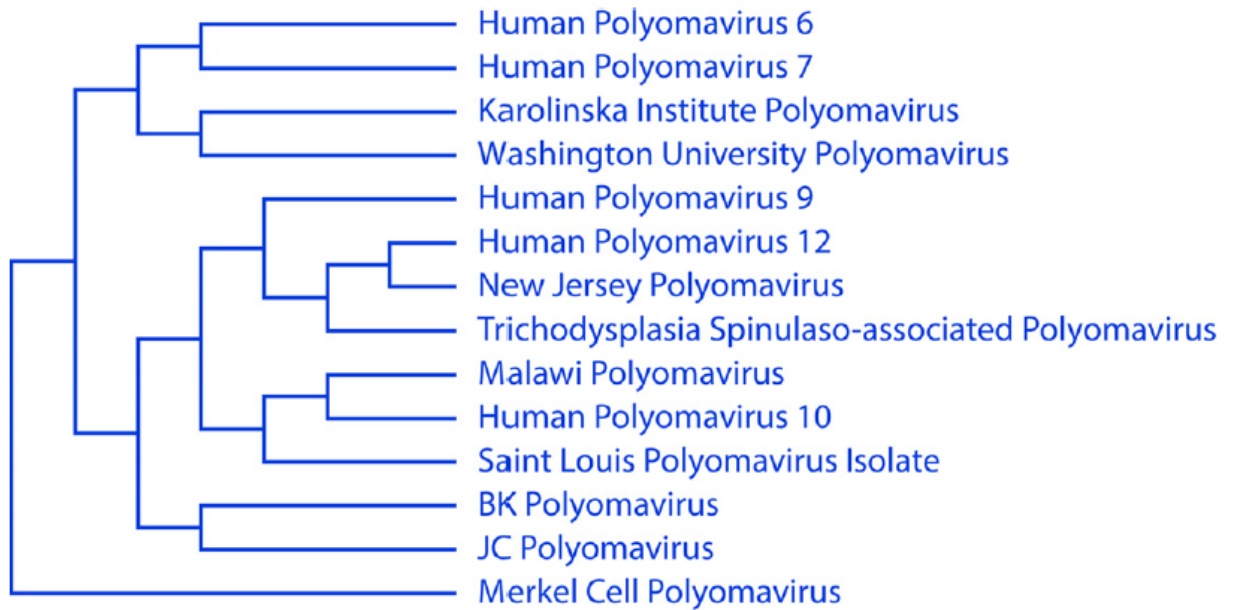


Figure 1: Phylogenetic tree showing the distances of relationships between the human polyomaviruses discovered to date.

Ambalathingal et al., *Clinical Microbiology Reviews*, 2017 [4]

2. BKPyV seroprevalence and epidemiology

Four major subtypes of BKPyV (I to IV) have been described. Subtype I was the most prevalent worldwide and subtype IV in Europe and East Asia. Subtypes II and III were rarely observed [14]. These subtypes were further divided into subgroups, and it has been shown that genotypes I, II, III, and IV behave as fully distinct serotypes [15] (Table1). There exists a correlation between human populations and BKPyV lineages, and it was suggested that the polyomavirus evolution mode was a host-linked evolution. Unlike subtype I; which is prevalent in all human populations, there exists an uneven subtype IV geographic distribution. A switch in BKV host during evolution could explain this pattern. It was thus postulated that BKV subtype IV may have been transmitted to ancestral Asians from a primate and then spread to the Eurasian Continent mainly. Another theory suggested that African ancestors were the first humans to be infected with subtype-IV, and then spread it to the world through migrations out-of-Africa [16].

Table 1: Serotype classification of the different BKPyV subtypes and subgroups.

Sub type/Subgroup	Serotype
Ia Ib1	✓
Ib2 Ic	✓
II	✓
III	✓
IV a1 IV a2 IV b1 IV b2 IV c1 IV c2	✓

BKPyV subtype IV proportions in Africa and parts of Asia and Europe might have subsequently decreased. This could be explained by a possible alteration in the hosts' susceptibility to the virus. A link was also observed between the BKPyV subgroups and human races. Each human population showed a close affinity to one of the subtype-I subgroups as follows: Ib1 to Southeast Asians, Ib2 to Europeans, Ia to Africans and Ic to Northeast Asians. Further BKPyV and human populations relationship understanding requires more BKPyV information, including its transmission mode [16].

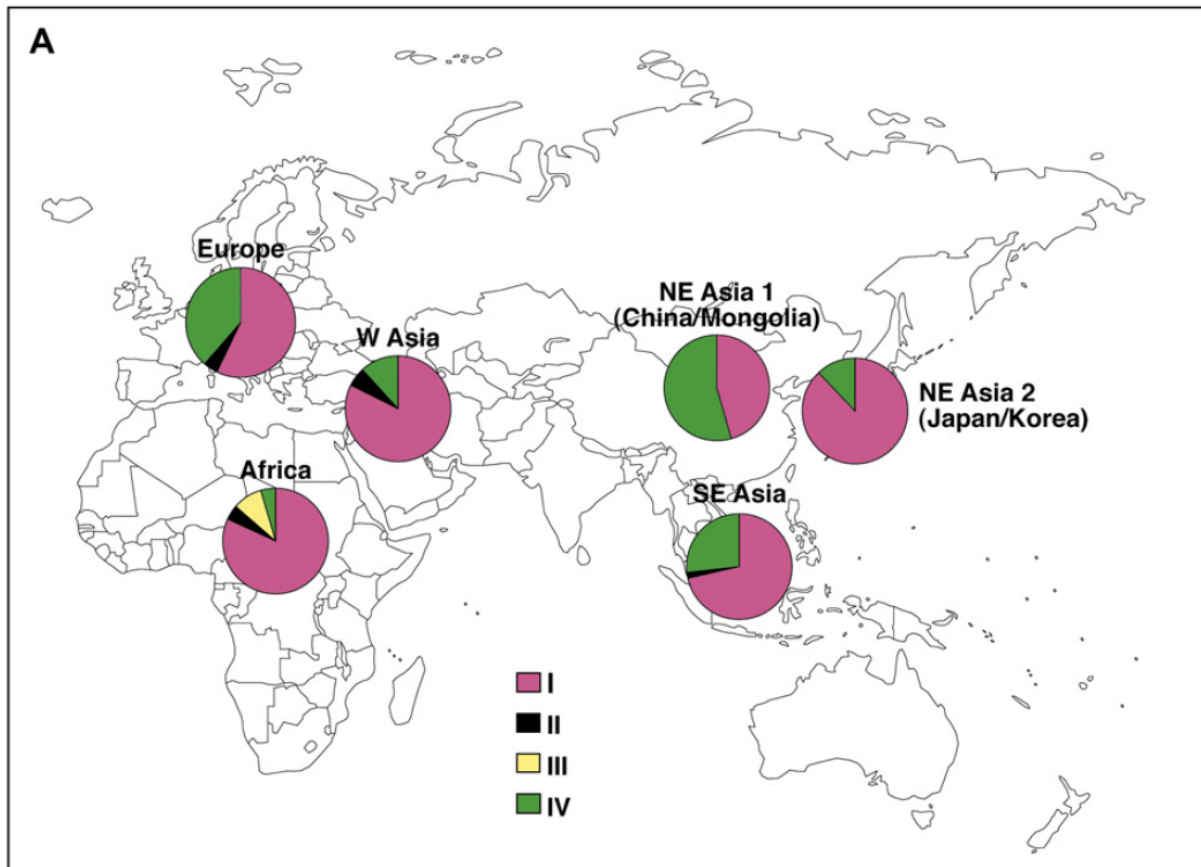


Figure 2: BKPyV subtypes distribution in major geographic areas.

The frequencies of BKPyV subtypes (A) are represented by different colors in the pie charts.

Zheng et al., *Microbes and Infection*, 2006 [16]

Studies demonstrated that by the age of 10 years, 70% of children were infected with BKPyV indicating the BKPyV primary infection occurrence during early childhood. The common childhood BKPyV infection predominantly occurs without major clinical symptoms. Although the transmission route remains unclear, several pathways have been proposed [17] (Table 2).

Table 2: Proposed BKPyV transmission routes.

Ambalathingal et al., *Clinical Microbiology Reviews*, 2017 [4]

Route of transmission	Source of BKV identification
Respiratory	Upper respiratory tract Tonsils Waldeyer's ring
Fecal-oral	BKV DNA in sewage
Blood transfusion	Leukocytes
Transplacental	Fetus

2.1. Population-based BKPyV epidemiological studies

A study exploring BKPyV infection in the United States (US) population of Maryland was conducted in 1973. It noted a BKPyV seroprevalence increase from 50% to 100% between the three years and 10-11 years age groups respectively. The authors concluded that the BKPyV infection was highly wide-spread during early childhood [18]. In their cohort, Portolani et al. assessed 453 sera samples from healthy Italian people of various ages for BKPyV antibodies. They found that antibodies to BKV were already prevalent by the age of two years. BKV seropositivity reached a maximum rate of 82.9% in the 16-25 years age group. Although still high, the authors noted a slight seroprevalence decrease after the age of 45 years [19]. A study in 2003 demonstrated that the overall BKPyV seroprevalence rate among the population of England and Wales was 81%. A highly significant association was observed between age and antibody titer with a BKPyV titer linear decrease rate of 8.7% per 10 years. BKPyV seroprevalence was greater than that of JCPyV and reached 91% at five–nine years of age. That of JCPyV, however, only reached 50% by age 60–69 years [20]. Another study from 2003 demonstrated a rapid increase in BKV seropositivity with age when examining sera, from Swedish children aged 1-13 years, for anti-BKV antibodies. It demonstrated a rapid increase in BKPyV seropositivity with age. BKPyV seroprevalence reached 98% in the 7-9 years age group, followed by a minor decline. The authors concluded that the overtime polyomavirus seropositivity stability represented a valid cumulative virus exposure marker [21]. A study of 400 healthy immunocompetent blood donors at the time of regular blood donation was performed in Switzerland. It reported 82% BKPyV overall IgG seroprevalence in the mentioned cohort, significantly higher than that of JCPyV (58% seroprevalence). Urinary shedding of BKPyV, however, was observed to be only 7%. The authors concluded the lack of gross alterations in BKPV infection epidemiology between the years 1994 and 2009 [22]. The BKPyV phylogenetic distribution pattern was investigated amongst the German population. In all study subjects, the BKPyV subtype I was found predominant with 90.9% of the BKPyV strains classified as I and only 6.1% classified as IV. Subgroup Ic represented the majority of the German sequences [23]. Antonsonn et al. investigated both the BKPyV seroprevalence and the antibody stability over time in the Australian population. They have revealed that over 4.5 years, the BKPyV and JCPyV seroprevalence were 97% and 63 %, respectively. BKPyV seroprevalence showed elevated stability over an 11 years period, with 96% of people

remaining seropositive and 2% staying seronegative [24]. A BKPyV seroprevalence study of the general Czech Republic Population demonstrated that 69% exhibited anti-BKPyV serum antibodies. In addition, it seemed that the seroprevalence rate was associated with age: anti-BKPyV antibodies occurrence was highest among the 10-19 and 20-29 age groups [25]. If seroprevalence was assessed against all BKPyV serotypes, it would show that all population members have been infected by at least one BKPyV serotype. Hence, it is possible to say that across human populations, BKPyV infection prevalence is virtually 100%.

3. Viral characteristics

3.1. The BKPyV particle

The Polyomaviridae family member BKPyV is a non-enveloped virus with a 45 nm diameter and a ~5 kb double-stranded DNA genome. The viral capsid's outer surface is composed of 72 VP1 protein pentamers arranged in a T = 7 d icosahedral structure stabilized by calcium cations and disulfide bonds. The viral proteins VP2 and VP3 reside at the capsid's inner part. VP1, the major capsid protein, can form flexible interactions with the neighboring pentamers thanks to its protruding C- arms [26]. A hairpin structure is formed by a single copy of VP1 or VP2 C-terminus, and inserted in the VP1 pentamer cavity [27]. DNA binding is mediated by the VP1 N-terminal domain, which lies inside the virion. A copy of VP2 or VP3 interacts with a VP1 pentamer through hydrophobic interactions. On the icosahedral vertices of the capsid, twelve pentamers are located, whilst the other sixty pentamers are coordinated with six adjacent pentamers [26]. In an intact BKPyV particle, H-bonding interactions occur 60 times providing a key source of BKPyV virion stability. Another critical factor for capsid stability is the disulfide bond formation, and it is very likely that the BKPyV virion exhibits decreased structure rigidity under reducing conditions [28]. Actually, an essential virion un-coating step is the disulfide bond reduction/isomerization in the infected host cell's endoplasmic reticulum. Eight to twelve hours post-infection, the BKPyV capsid's extensive inter-pentameric and intra-pentameric disulfide bond network starts to disintegrate. The polyomavirus disassembly process is believed to start by these conformational changes or VP1 shedding [29]. The BKPyV structural protein components architecture is similar to that of previous structures from other hosts. However, a direct interaction between the BKPyV capsid and its packaged genome has

been observed; unlike previously studied polyomavirus structures. This is consistent with the biochemical description of the VP1 DNA-binding properties [26].

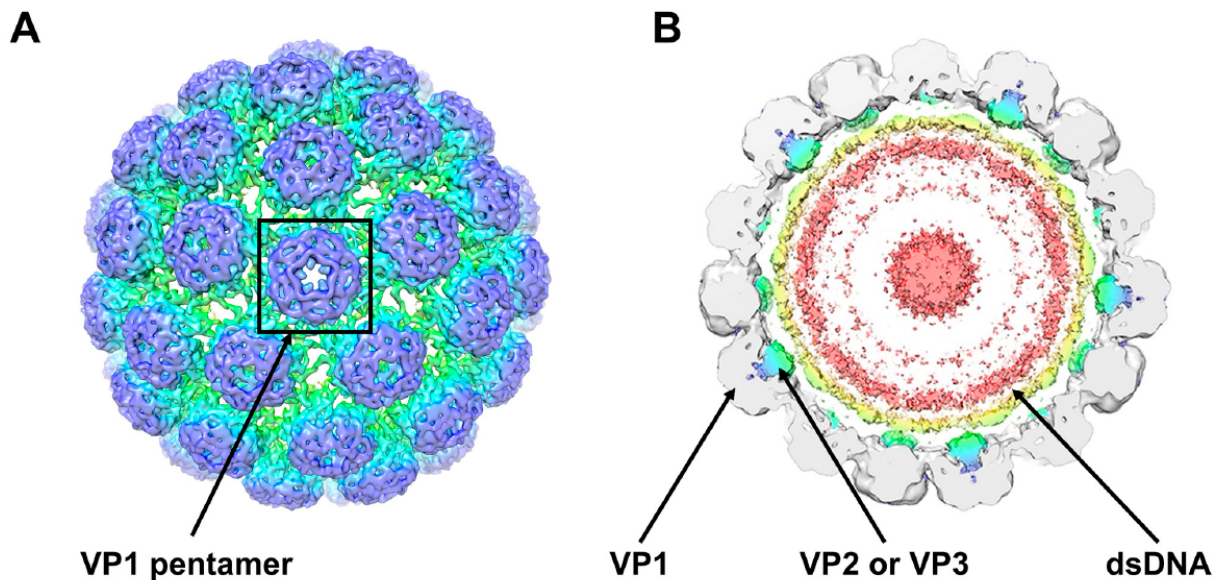


Figure 3: Cryo-electron microscopy structure of BK polyomavirus (BKPyV) viral particles.

(A) External view of the BKPyV virion. A viral protein VP1 pentamer is highlighted.

(B) View of a 40-Å-thick slab through the unsharpened/unmasked virion map. 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 double stranded DNA (dsDNA) yellow/pink.

Hurdiss et al., *Cell Press*, 2016 [26]

A key determinant of viral infection efficiency is the host cell recognition by the virus. The route that the virus will take to the nucleus is determined by the initial interaction at the plasma membrane level. This infection's stage integrity is critical for proper conformational changes and subsequent disassembly of the capsid. Gangliosides GD1b and GT1b are the major host cell receptors for the BKPyV. The virions bind to the carbohydrate moiety—a disialic acid motif in particular—rather than the identical ceramide lipid moiety present among all gangliosides. This was demonstrated by the inability of BKPyV to bind to gangliosides other than GD1b and GT1b. In addition, proteinase K and neuraminidase treatment showed independence of the BKPyV-membrane interaction from proteins [30].

The interaction of GT1b with BKPyV is summarized in (Fig 4). “Left” and “right” arms were described to be specific components of GT1b (Fig 4 E). GT1b uses the right arm- located disialic acid motif to interact with VP1 on the BKPyV capsid. The right arm is well resolved in (Fig 4 B).

Functional and mutagenic studies demonstrate that the left arm, which was shown to be a dynamic structure, enhances BKPv infectivity. Low-resolution electron microscope (EM) density suggests a likely GT1b left arm multiple and weak interactions with the VP1 surface (Fig 4 C) [28].

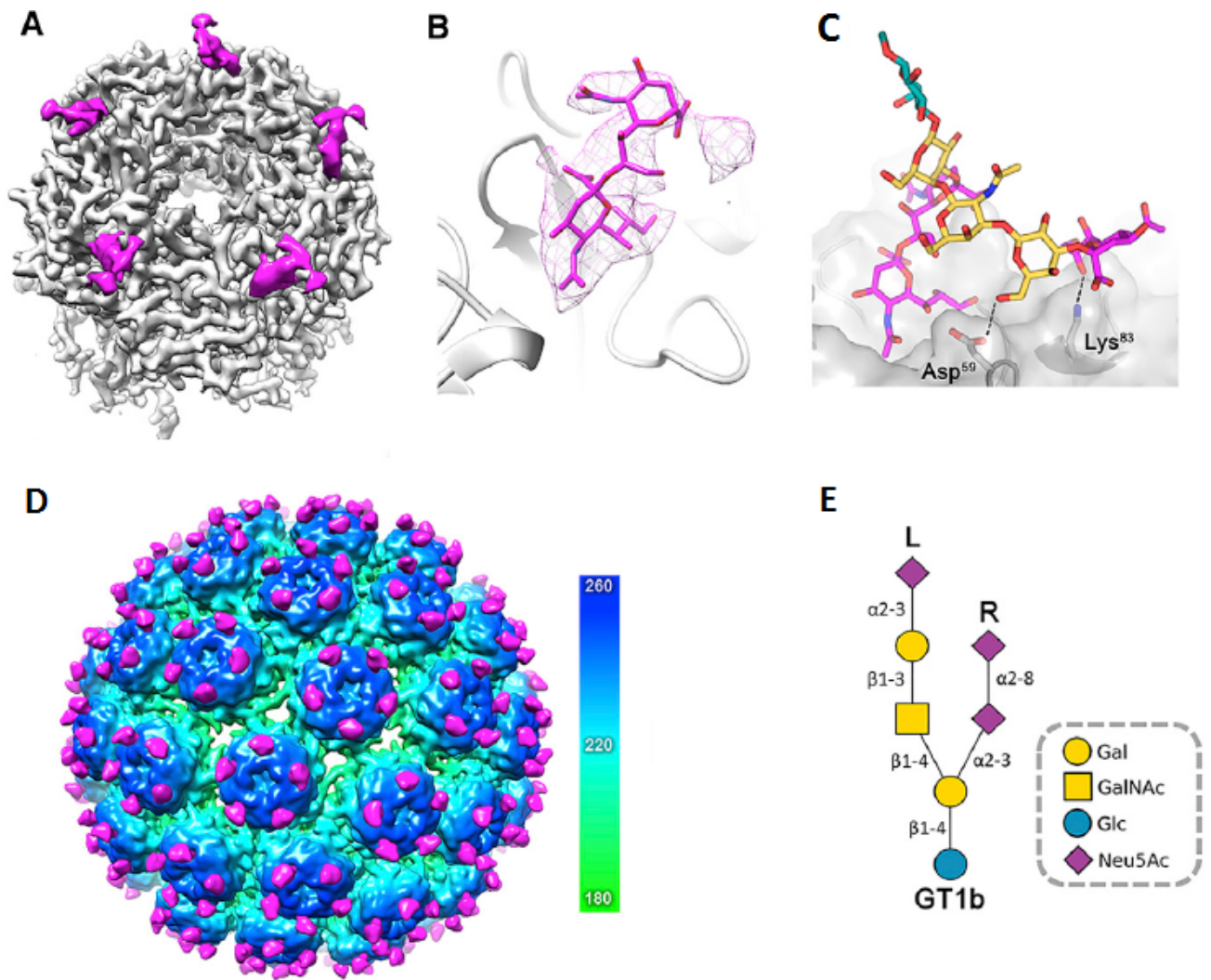


Figure 4: BKPv interaction with gangliosides GT1b.

- (A) Iso-surface representation of a single pentamer of the BKPv-GT1b complex within the capsid.
- (B) Enlarged view of the GT1b density containing the corresponding atomic model for the disialic acid motif of the right arm.
- (C) A snapshot of the molecular dynamics (MD) simulated structure of GT1b showing possible interactions of the left arm with Asp 59 and Lys 83.
- (D) Isosurface representation of the BKPv-GT1b map viewed down the icosahedral 2-fold axis and colored according to the radial coloring scheme shown.
- (E) Symbol nomenclature for Glycans (SNFG)-representation of GT1b oligosaccharide showing the left and right arms of the molecule.

Adapted from Hurdiss et al., Cell Press, 2018 [28]

3.2. The BKPyV genome

The BKPyV capsid encapsulates a circular double stranded DNA genome of around five kb. This DNA molecule exhibits bidirectional replication from a unique origin. Two highly conserved regions, separated by a 400bp non-coding control region (NCCR), encode early and late BKPyV proteins. Shortly after infection, the large tumor antigen (TA_g), the small tumor antigen (tAg) and the truncated tumor antigen (truncTA_g) are expressed by mRNA alternative splicing. After genomic replication initiation, late genes' expression produces BKPyV structural proteins including VP1, VP2, VP3 and Agno proteins. Two late ribonucleic acid (RNA) classes; 16S and 19S, are produced by alternative splicing from a common pre-messenger RNA (mRNA). The 16S RNA is translated to produce Agno and VP1, while the 19S RNA's translation produces VP2 and VP3 [14].

Five sequence blocks constitute the NCCR starting from the early side: O (142 bp), P (68 bp), Q (39 bp), R (63 bp) and S (63 bp) (Fig 5). The O block includes the origin of replication and a TATA box. Blocks P, Q, R and S contain regulatory regions for early and late gene expression, as well as TATA-like elements [31–33]. Among the approximately 30-transcription factor binding sites, SP1 plays a principal role. The number of Sp1 binding sites and affinity affect the early and late viral gene region expression bidirectional balance. Evidence was also identified for the contribution of other transcription factors like Ets-1, NF-KB or NF1 [32,34]. Transcription and replication regulation is also conferred by glucocorticoid/progesterone-, oestrogen-, Cyclic adenosine monophosphate (cAMP)- and phorbol ester- responsive-elements [35].

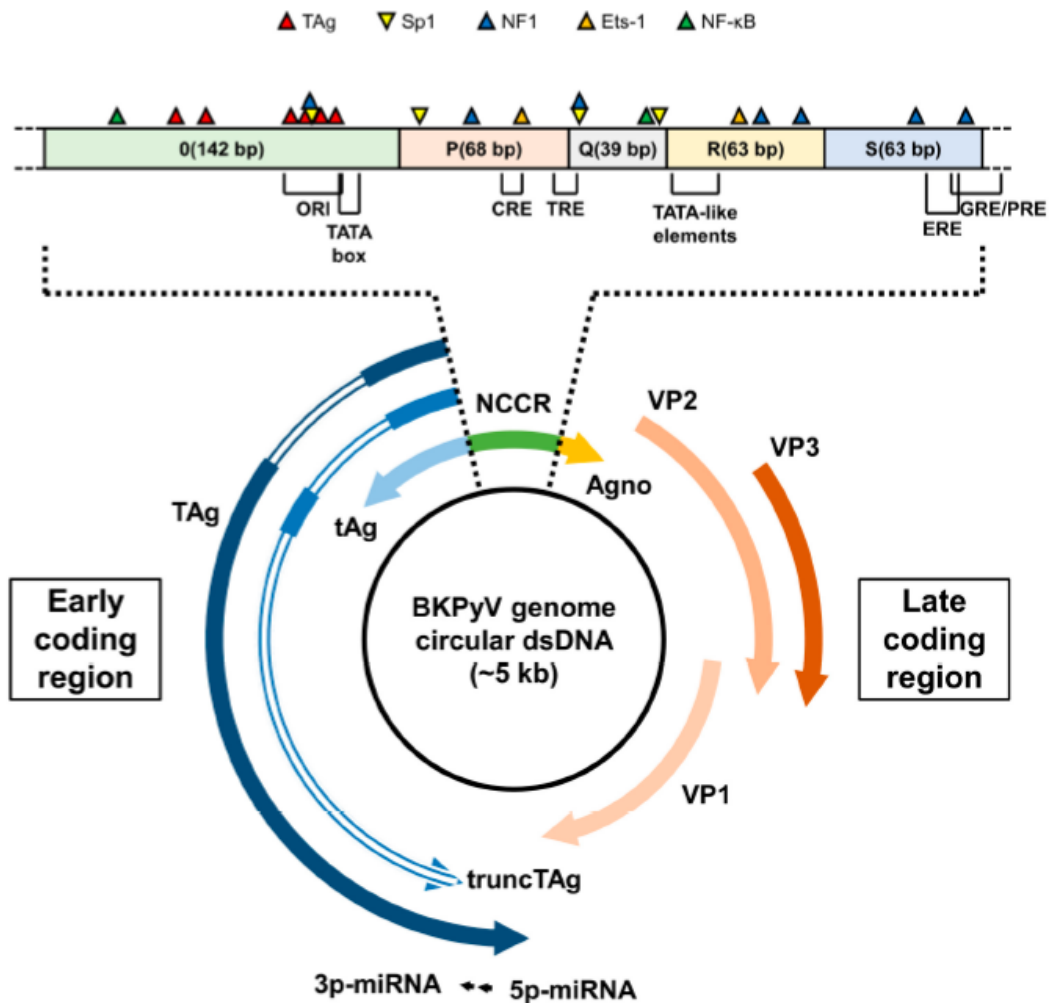


Figure 5: Genome structure of the BKPyV.

Early and late coding regions transcription proceeds in a bidirectional way from the origin of replication (ORI) located within the noncoding control region (NCCR). The early coding region encodes large tumour antigen (Tag), small tumour antigen (tAg) and truncated TAG (truncTAG). Double lines represent introns in the early coding region. The late coding region encodes the structural proteins VP1, VP2 and VP3 as well as the Agno protein. The BKPyV genome also encodes two miRNAs –5p-miRNA and 3p-miRNA– perfectly complementary to the Tag encoding mRNAs. (Top) Schematic representation of the BKPyV archetype non-coding control region (NCCR). It is divided into five sequence blocks (O, P, Q, R and S). It includes the origin of replication (ORI), TATA box and TATA-like elements. The positions of different sites important for Tag binding and the transcription factors Sp1, NF1, Ets-1 and nuclear factor KB (NF-KB), as well as cAMP-, phorbol ester-, glucocorticoid/progesterone- and oestrogen responsive-elements (CRE, TRE, GRE/PRE and ERE, respectively) are also mentioned. CRE: cAMP responsive-element; TRE: phorbol ester responsive-element; GRE/PRE: glucocorticoid/progesterone responsive-element; ERE: oestrogen responsive-element.

Adapted from Helle et al, viruses, 2017 [14]

Considerable NCCR variation is observed between distinct BKPyV isolates, unlike most viral strains, which exhibit strong sequence conservation. Usually associated with disease, the kidney and other tissues frequently reveal rearranged NCCR forms [31]. The P and S blocks are retained in most NCCR variants. This observed selection to preserve the P and S blocks indicates the importance of these two sequence blocks. Anywhere within the P, Q, R or S regions, deletions are found to occur but they typically include all or part of the R region. Most naturally occurring NCCRs rearrangements involve the P region triplication or duplication including portions of the O and Q sequences [36]. Rearranged NCCRs revealed a strong early gene expression and relatively weak late gene expression compared to archetypal NCCRs. This observation occurred irrespectively of deletion or insertion architectures. In addition, increased viral replication and cytopathology are observed in BKPyV with rearranged NCCR which emerges in vivo in renal transplant patients [37]. The polyomaviruses ability to adapt to new cellular environments is likely reflected by the NCCR heterogeneity. With disease progression, the BKPyV NCCR may progressively become more rearranged [14].

3.3. The BKPyV proteins

VP1, VP2, and VP3: A key component of BKPyV life cycle's cell entry and virion assembly steps are the BKPyV structural proteins VP1, VP2, and VP3. The VP1 polypeptide's different strands are connected by five loops known as BC, DE, EF, GH and HI which have been demonstrated to be important for capsid assembly. VP1 is made of 362 amino acids (42 kDa) and forms pentamers that assemble into the BKPyV viral capsid [38]. An inter- and intra-pentameric disulphide bonds extensive network stabilizes the VP1 capsid [39]. Five β -barrel-shaped VP1 monomers form a ring that constitutes one capsid pentamer [26]. Binding between neighboring pentamers is mediated by the C-terminal subdomains of VP1. Although C-terminally truncated VP1 did form capsomers, these couldn't assemble into normal virus-like particles [38]. The VP1 N-terminal region is located on the virion's inside, and is involved in DNA-binding mediation [26]. Each VP1 pentamer binds a copy of either VP2 or VP3 internal proteins. VP2 and VP3 are inserted into the capsid cavity in a hairpin-like manner through hydrophobic interactions. Site directed mutagenesis was used to identify key VP1 residues for BKPyV attachment to susceptible cells. BKPyV propagation was strongly dependant on VP1 Serine (Ser) -80 phosphorylation [40]. A shallow groove formed by the VP1 BC and HI loops has been predicted as the location of the BKPyV receptor-binding site location [39]. The

difference between the distinct BKPyV serotypes lies in epitopes found in the VP1 BC loop. The different serotypes of BKPyV are identified by a VP1 subregion of only 100 bp (1977 through 2076) named BKPyV virus typing and grouping region (BKTGR) [41].

VP2 (351 amino acids; 38 kDa) and VP3 (232 amino acids; 27 kDa) are translated from the same late mRNA transcript. They share the same C-terminal amino acid sequence which contains a nuclear localization signal, a DNA-binding region and a VP1-binding region [42]. A unique N-terminal amino acid set that contains a putative Gly-2 myristoylation was identified in VP2 [43]. Although essential for viral infectivity, VP2 and VP3 proteins were unnecessary for viral assembly or stability. VP2 and VP3 start codon mutations alone or together appeared to induce a 99% infection reduction compared to the wild type BKPyV [42]. This demonstrated the VP2 and VP3 essential role in creating infectious virions. It is worth mentioning that BKPyV propagation was suggested to be dependent on VP2 Ser-254 phosphorylation [40].

Large T-antigen (TAg), small T-antigen (tAg) and truncated T-antigen (trunc TAg): The large T-antigen composed of 695 amino acids (aa) and 80 kDa is translated by the first intron removal, leading to first exon splicing with the next one. The alternate retention of this first intron facilitates the small T-antigen tAg (172 aa, 20 kDa) translation by reaching a stop codon within the first intron (Fig 6). This means that TAg and tAg share the first 82 aa [14]. Evidence of a truncated T-antigen (trunc TAg) of 136 aa (17 kDa) encoding viral mRNA was provided by Abed et al in a 2009 study [44]. The TAg encoding mRNA excision of a second intron results in the trunc TAg expression (Fig 6). Consequently, truncTAg and TAg's first 133 aa are identical. It is the additional splice that leads to a different reading frame translation [14].

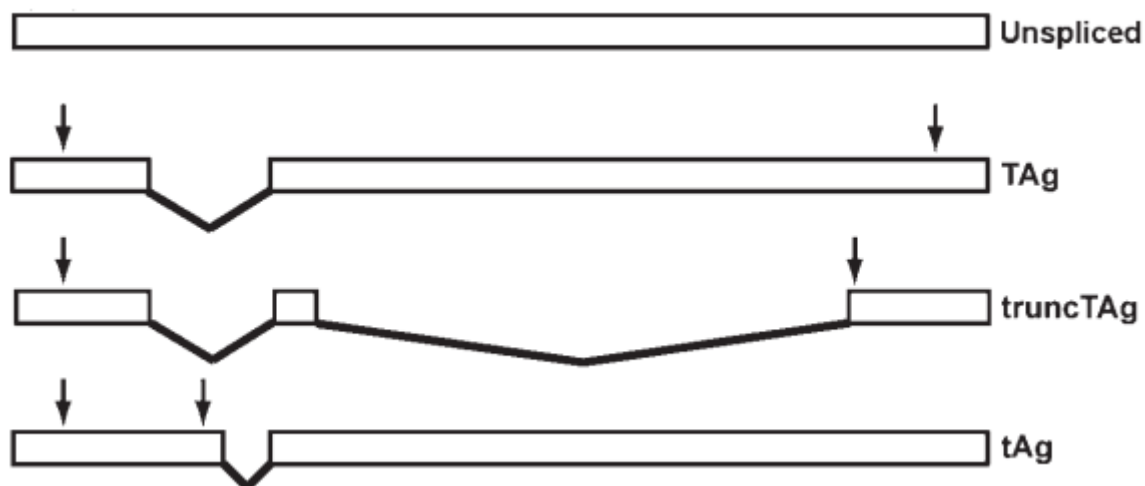


Figure 6: Diagram of the four early region mRNAs produced by BKPyV.

Boxes indicate exons and lines indicate introns. Vertical arrows indicate the positions of the start and stop codons for each transcript.

Adapted from Abend et al., Journal of General Virology, 2009 [44]

A nuclear localization signal (NLS) localizes the trunc Tag and Tag primarily in the nucleus [14]. Both proteins revealed a J domain in their N-terminus, with extensive homology to the Dnal chaperone proteins family. Heat shock cognate 70 (Hsc70) interacted specifically with this domain to assure efficient viral replication [45,46]. The TAg and trunc TAg sequence contain a conserved 105-LXCXE-109 motif that binds to retinoblastoma protein (pRb) and its family members: p107 and p130. This interaction promoted viral replication by driving cell cycle entry/progression through E2F transcription factor family members displacement [47]. The TAg encompasses a Zinc-binding domain, a DNA-binding domain (DBD) and an ATPase domain. These domains conferred the Tag DNA helicase activity, essential for viral genome replication initiation. The origin of replication located in the NCCR includes a GAGGC sequence present in four copies, to which the Tag DBD binds. Moreover, the DBD interaction with Replication Protein A (RPA) was also required for viral replication. The Zinc-binding domain allowed the TAg hexamers formation, which represented the helicase's active form. To prevent cell cycle arrest and apoptosis, the helicase domain's external surface showed an interaction with the tumour suppressor protein 53 (p53) [45,47,48]. The tAg is located in both the nucleus and cytoplasm. It contains a unique region with two zinc-fingers that promote cell cycle progression by inactivating protein phosphatase 2A [45].

Agno protein: It has been shown in vivo and in vitro that BKPyV infected cells express the Agno protein (8 kDa) abundantly. It is a basic and relatively small protein, composed of 66 amino acids. During the BKPyV infection cycle's last phase, this protein is localized mainly in the cytoplasm; most intensely in the peri-nuclear area. But a minor fraction of Agno protein can also be detected in the nucleus [49]. Putative phosphorylation sites of BKPyV Agno are: Ser-7, Ser-11, Ser-64, and Threonine (Thr)-21. BKPyV propagation as well as Agno protein stabilization was controlled by Ser-11 phosphorylation. Protein Kinase C (PKC) mediated Ser-11 phosphorylation in cell culture. Studies showed that Protein Kinase A (PKA) and Protein Kinase (PKD) also phosphorylated Ser-11, while Ser-7 and Thr-21 could be phosphorylated by PKC and PKD. BKPyV with an Agno gene start codon mutation that halted Agno production retained the ability to infect Vero cells. However, these BKPyV mutants displayed a reduced infection capacity compared to the wild type virions. In this manner, the Agno protein role in the BKPyV life cycle is important but not crucial [50]. Some BKPyV strains with an Agno coding sequence 5' end deletion in the NCCR region didn't release an infectious progeny to cell culture supernatants. Such BKPyV strains only produced non-infectious VLPs in the nucleus. Agno trans-complementation to these strains resulted in the rescue of infectious virions synthesis and release in cell culture supernatants. This demonstrated an Agno role in infectious virions assembly, maturation, and/or release [51]. An interaction between Agno and a human proteins subset has been identified. Johanessen et al. identified an interaction between the cellular α -soluble N-ethylmaleimide-sensitive fusion attachment protein (-SNAP) and the viral Agno protein. Vesicles disassembly during secretion is mediated by SNAP. Agno seemed to exert a negative influence on exocytosis, but the functional consequence of this modulation is still unknown. It was proposed that antigen presentation could be impeded by this process. In addition, it might promote immune evasion by reducing cytokines and/or interferons secretion by infected cells [50]. Ultimately, it was observed that BKPyV particles lacking the Agno protein exhibited an increase in VP1 protein expression [50].

3.4. BKPvV encoded μ iRNAs

Similar to SV40 and JCV, BKPvV was found to encode two distinct and mature microRNA (miRNA) molecules: 5p-miRNA and 3p-miRNA. Both originate from a common pre-miRNA hairpin encoded by the BKPvV genome. Each of these two miRNA molecules exhibits perfect complementarity to Tag BKPvV messenger RNAs (mRNAs) [52]. Both BKPvV miRNAs were implicated in regulating the early gene expression by cleavage of early viral transcripts. This might be implicated in reducing the infected cells' susceptibility to being destroyed by cytotoxic T-cells (similar to SV40) [53]. A recent 2019 study examined the small RNA expression changes in tubular epithelial cells infected with BKPvV. It was shown that over the course of 12 days, BKPvV-miR-B1-5p and BKPvV-miR-B1-3p increased by 1000 folds in these cells with more expression of 3p than 5p miRNA species. Still, this increase didn't prevent host cell lysis. It was observed that host miR-10b and miR-30a were both downregulated by BKPvV infection [54]. Lagatie et al. summarized the polyomavirus encoded miRNAs auto-regulatory role (Fig 7). It occupies a crucial role in viral replication by targeting the TAG. The host immune response is also regulated by the BKPvV viral miRNA by targeting certain host factors. A stress-induced ligand called UL16 binding protein 3 (ULBP3) —important for infected cells recognition by the immune system— is targeted by miRNA. In addition, host miRNAs can also influence TAG expression and consequently viral replication. They might also directly hinder viral protein expression by binding to the viral transcripts 3'UTR. Next to this virus specific role, the viral infection-induced immune response was also affected by miRNAs [55].

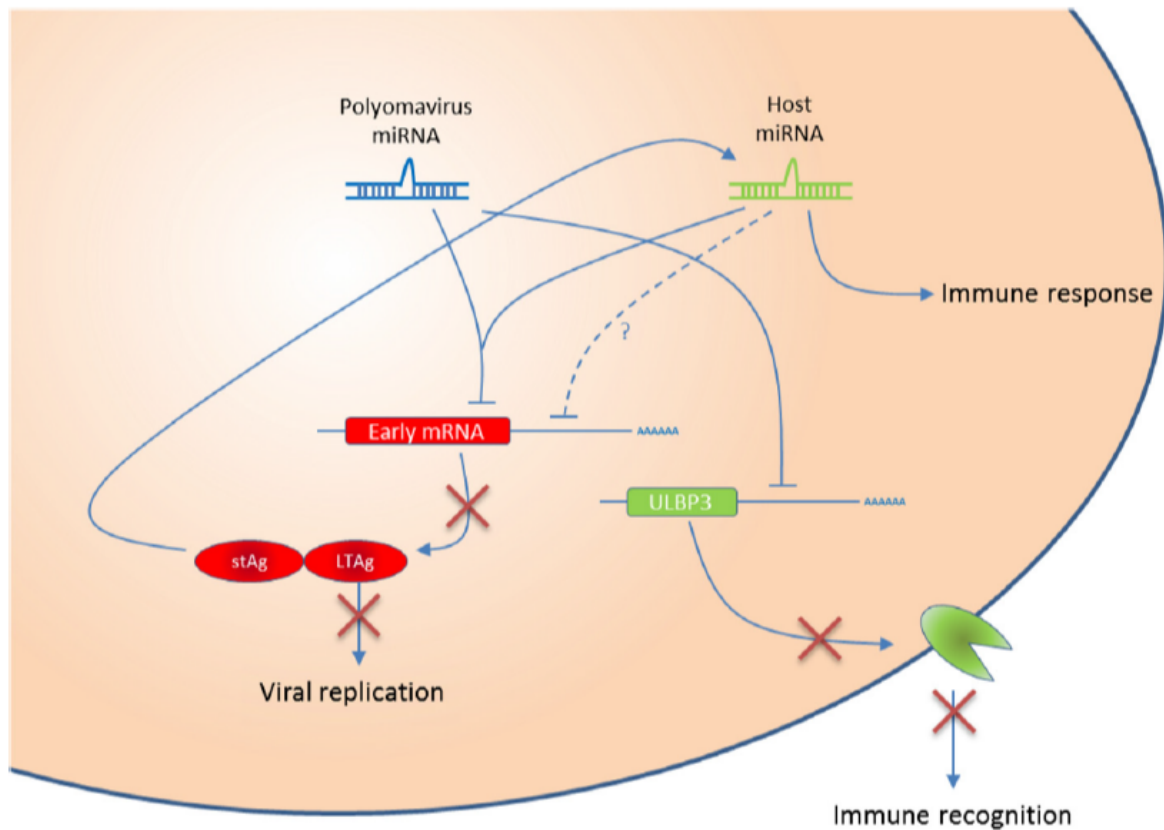


Figure 7: Host and viral miRNA functions related to Polyomavirus infection overview.

Lagatie et al, *Virology Journal*, 2013 [55]

Overlapping BKPyV NCCR elements control the BKPyV miRNA expression levels. Archetype BKPyV represents the BKPyV form that is transmissible and persistent in a host. Its replication is largely controlled by miRNA. A study by Broekema et al. showed that high early promoter activity led to the expression of high messenger RNA (mRNA) levels in BKPyV variants with a rearranged NCCR (Fig 8 A). However, the miRNA expression level in these variants was very weak. mRNA produced in BKPyV variants was translated into TAg, which bonded to the origin of replication and drove DNA replication. In Archetype virus, miRNA was robustly expressed and targeted early mRNA leading to its degradation (Fig 8 B). Early mRNA was weakly expressed from the early promoter, and DNA replication was thus inhibited in archetype virus in RPTE cells [56].

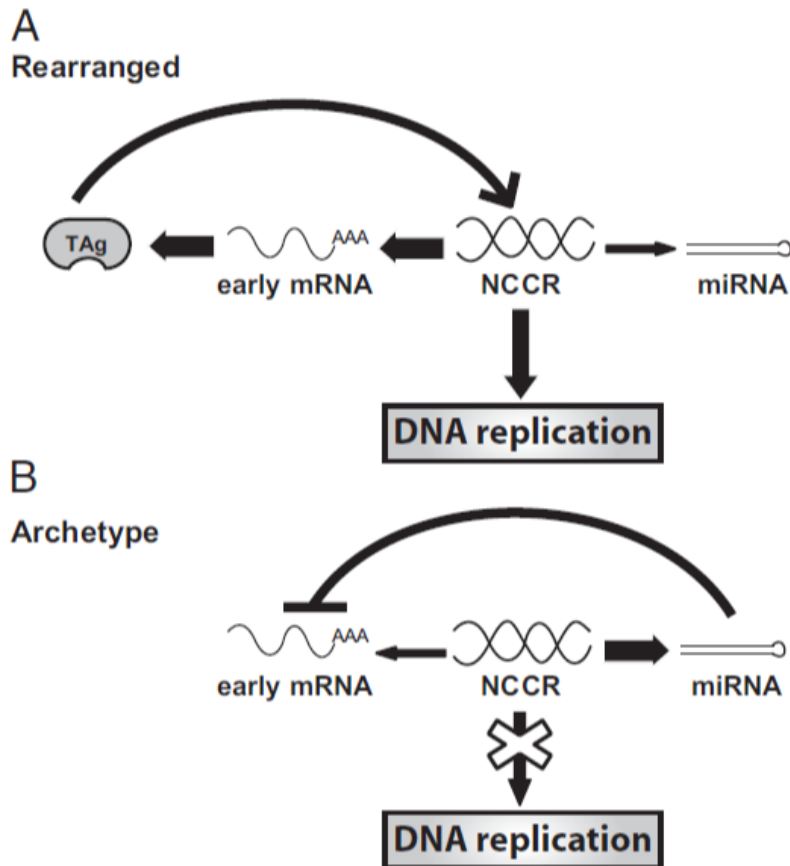


Figure 8: Model of miRNA control of archetype BKPyV replication.

Broekema et al, PNAS, 2013 [56]

4. The BKPyV life cycle

4.1. BKPyV entry

Attachment: For a DNA virus, the viral genome must be transported to the nucleus to facilitate its replication. BKPyV VP1 attachment to cell receptors represents the BKPyV infection's initial step. Pastrana et al. suggested that distinct BKPyV subtypes/serotypes sustain different tropism, because each binds to a different spectrum of cell surface receptors [15]. A cell membrane's lipid raft portion is rich in a type of glycosphingolipids called gangliosides. The latter consists of a carbohydrate and a ceramide moiety with one or more sialic acid residues. Polysialylated gangliosides exhibit a crucial role in the initial BKPyV and target cell interaction. In addition, they facilitate human type O red blood cells hemagglutination by BKPyV [57]. The minimal BKPyV binding epitope was found to be the conserved (2, 8)-disialic acid motif on the b-series gangliosides right arm. Additional contacts are mediated by the variable ganglioside's

left arm. Consequently, BKPyV can interact with several b-series gangliosides types ex: GD1b, GD2, GD3 and GT1b. In contrary, the monosialylated a-series gangliosides can't interact with BKPyV virions [58].

Internalization: BKPyV is internalized into the target cell following initial attachment. Eash et al. showed that BKPyV entry into Vero cells was dependent on caveola-mediated endocytosis rather than clathrin-coated-pit assembly. In addition, they demonstrated that BKPyV particles reached a neutralizing antibody-resistant compartment 2 h post-infection [59]. Using RPTE cells, Zhao et al. contradicted previous findings by showing BKPyV used a caveolin and clathrin-independent pathway to enter host cells [60].

Endoplasmic reticulum trafficking: BKPyV virions traffic to smooth tubular structures contiguous with the rough endoplasmic reticulum (ER). This observation was made according to BKPyV-infected cells' transmission electron microscopy [61]. In both Vero and RPTE cells, BKPyV intracellular trafficking relied on an intact microtubule network but not on an intact actin cytoskeleton. In addition, BKPyV trafficking was dynein-independent in both cell types [62]. A pH-dependent step was involved during the first two hours of BKPyV entry. Thus, endosomes acidification and maturation were required for BKPyV infection [29]. BKPyV virions likely pass too rapidly through the Golgi apparatus to be detected or utilize a non-Golgi apparatus involving pathway. Introducing a Golgi apparatus morphology disruptor didn't inhibit BKPyV infection, supporting the Golgi apparatus bypass idea [63]. After endosomal sorting, BKPyV reaches the ER at ~10 h post-entry [14].

Release from the ER and nuclear entry: Reductases, chaperones, and disulphide isomerases contribute to BKPyV benefits from ER trafficking. These proteins facilitate capsid uncoating process and mediate viral ER-to-cytosol translocation. The proteasome along with the ER-associated protein degradation (ERAD) pathway have been implicated in BKPyV transport from the ER to the cytosol [64]. In the cytosol, the VP2 and VP3 NLS is exposed and utilized to transport the BKPyV virions to the nucleus through the nuclear pore complex. This process is mediated by the importin α/β import pathway [65].

4.2. BKPyV replication

Early viral genes transcription occurs right after nuclear entry. Then, early viral proteins translation takes place in the cytoplasm. TAg next enters the nucleus using the NLS. TAg binds P53 and pRb to promote cell cycle progression and prevent apoptosis. This allows the viral genome to exploit the host DNA synthetic machinery for its own viral replication [14]. Tag represents the only multifunctional viral protein required by BKPyV for replication. The virus doesn't encode DNA polymerase in its genome. In addition, the host cell supplies all other replication factors needed by BKPyV. TAg initiates viral DNA replication by binding to GAGGC motifs in the replication origin, after which it acquires its helicase activity. Next, TAg locally unwinds the double stranded DNA, in an ATP-dependent and bidirectional manner. Then, short RNA primers are synthesized by DNA polymerase α -primase (Pol α -primase). These are elongated by the enzyme complex's DNA polymerase function [66].

4.3. BKPyV assembly and release

VP1, VP2 and VP3 represent the components of the BKPyV viral capsid. After cytoplasmic translation, they are transported to the nucleus for virion assembly. High nuclear calcium concentration may allow the viral capsomers assembly around newly synthesized genomes [38]. Progeny virions take two days after infection to start appearing in the infected cell's nucleus [67]. An infected cell is estimated to produce a mean of 6,000 BKPyV virions as determined by renal biopsies with polyomavirus-associated nephropathy [68]. Consequently, nuclear inclusion structures in infected cells can be revealed by electron microscopy due to dense crystal-like arrays of BKPyV [61]. The general assumption is that non-enveloped viruses are released by passive means like host cell lysis. In addition, a BKPyV lytic replication cycle in RPTE cells has been demonstrated [67]. However, BKPyV-infected cells rarely exhibit strong cytopathic effects. Moreover, it has been reported that a BKPyV infection of RPTE cells resulted in a non-lytic egress [69]. Recently, a study by Handala et al. demonstrated the BKPyV virions' release into Extracellular vesicles, enabling them to infect cells using an alternative entry pathway [70]. The BKPyV life cycle is illustrated in Figure 9.

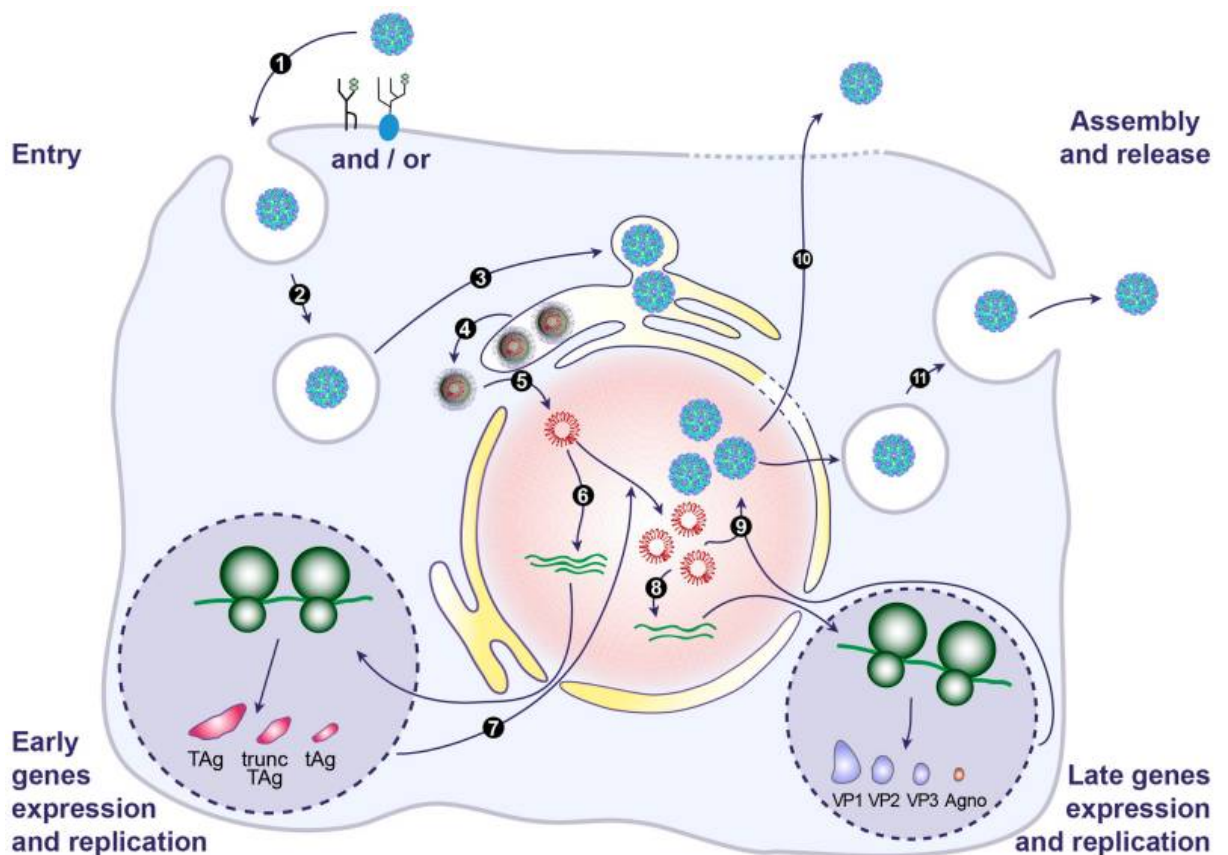


Figure 9: Model of the BKPyV life cycle.

BKPyV virions bind to the host cell receptors (1). Virions are internalized (2) and the virus traffics to the endoplasmic reticulum (ER) (3). Aided by ER-associated protein degradation (ERAD) machinery, partially uncoated viruses are released into the cytosol (4). The viral genome is transported into the nucleus via the nuclear pore complex (5). Early genes expression (6) is followed by early proteins nuclear translocation to initiate viral DNA replication (7). Late genes are expressed (8). VP1, VP2 and VP3 are translocated into the nucleus where they form capsids to package the newly synthesized viral DNA (9). Progeny virions are mainly released from infected cells after cell lysis (10). However, a small fraction of progeny virions may also be released through a non-lytic egress (11).

Adapted from Helle et al., *viruses*, 2017 [14]

5. BKPyV infection

After a primary BKPyV infection (which usually occurs during childhood), the virus becomes latent in the kidneys and the urinary tract. It can be reactivated in an immunosuppression context, leading in many cases to the virus particles excretion in the urine. It has been reported however, that occasional BKPyV excretion in the urine was detected in healthy adults and children as well [71]. A leading risk factor for manifesting polyomavirus renal graft infection after transplantation is high dose immunosuppressive therapy [72]. BKPyV can also induce other diseases in immunocompromised patients (e.g., hemorrhagic cystitis in bone marrow

transplant recipients and in cyclophosphamide treated cancer patients). The guidelines for these conditions recommend regular BKPyV replication monitoring and immunosuppressant dose adjustment for patients with significant viral loads [2].

5.1. BKPyV primary infection

Usually, the BKPyV Primary infection occurs in childhood and is generally asymptomatic. When symptoms are noted, the most common ones are non-specific upper respiratory tract infection and fever [73]. BKPyV infection sites include the kidneys, lungs, eyes, liver and brain indicating a notable viral tropism. In addition, BKPyV may target genitals and bone cells. In rare cases, BKPyV primary infection of the kidneys can induce critical manifestations such as hemorrhagic and non-hemorrhagic cystitis, nephritis and ureteric stenosis. Acute upper respiratory tract infection and pneumonitis are pulmonary diseases that can be triggered by a BKPyV lung infection [74]. Moreover, Primary and reactivated CNS disease can be triggered by BKPyV. Following the primary infection, BKPyV resides latently in many sites most commonly in renal tissue [75].

5.2. BKPyV associated nephropathy (BKPyVAN)

Post-transplantation immunosuppression may lead to BKPyV replication reactivation, which in turn may result in BK polyomavirus-associated nephropathy (BKPyVAN). This disease is a major renal allograft dysfunction cause (with a 1–10% incidence in kidney transplant patients) and can sometimes progress to interstitial nephritis with ureteric stricture and stenosis [76]. Several candidate biomarkers for BKPyV replication have been identified, such as decoy cells detection in the urine and BKPyV DNA load in urine and plasma [77]. Although BKPyVAN can appear as early as 1 month after transplantation, some cases are undetected until more than 80 months after the procedure. The viral reactivation is asymptomatic, and the infection is often only revealed by kidney failure. Despite a significant increase in clinical awareness and a better understanding of BKPyV infections, BKPyVAN still poses a fundamental problem for kidney transplant patients [78]. BKPyV in the renal allograft can cause BKPyVAN after reactivation due to immunosuppression. However, it is also possible that a patient becomes infected with a serotype, which he/she has never been exposed to before transplantation. This novel de novo post-transplantation infection can also lead to nephropathy (Fig 10).

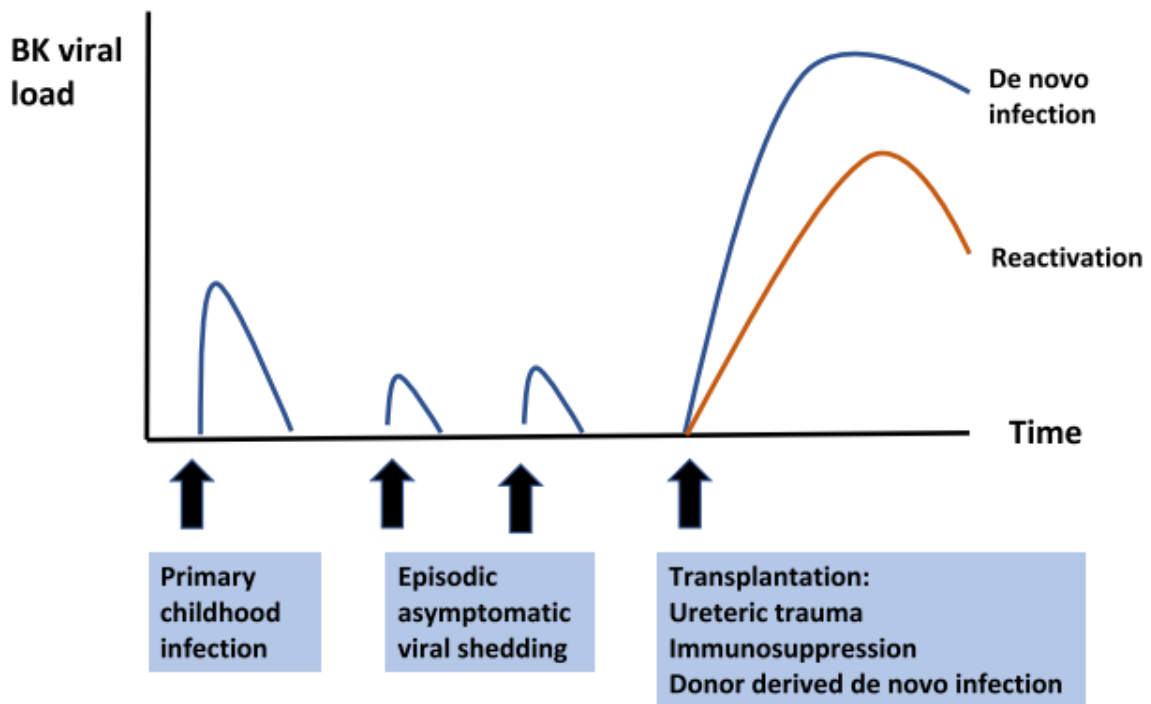


Figure 10: Natural progression of BK polyomavirus (BKPyV) infection.

Chong et al, Wiley, 2019 [79]

The exclusively available treatment strategy for BKPyVAN seeks to reduce virus replication while avoiding graft rejection; this corresponds to a timely level reduction of immunosuppression and (in some cases) antiviral therapy initiation [76]. Although a partial immune function restoration controls BKPyV replication, it increases the risk of the allograft immune rejection. There is a genuine need for controlled studies to find safe and effective treatment for BKPyVAN, especially for those in whom immunosuppression reduction is impossible [79].

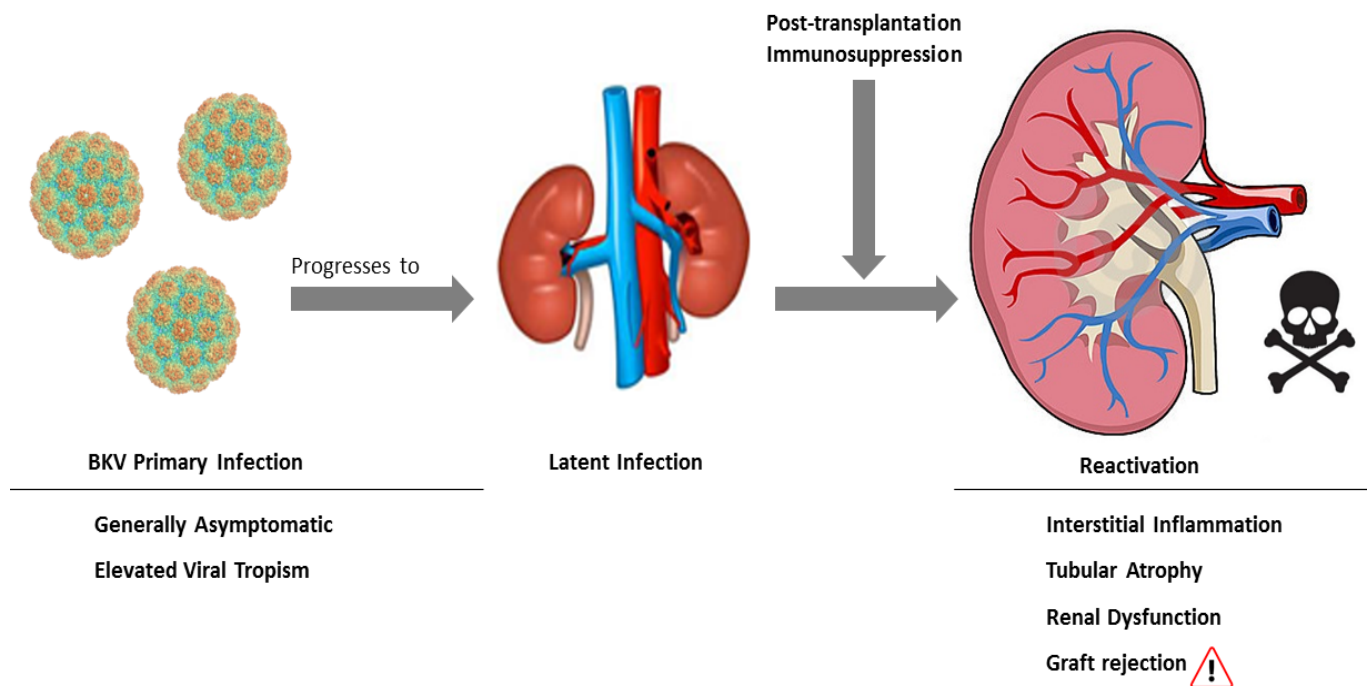


Figure 11: The different phases of the BKPyV infection.

5.3. BKPyV and cancer

Malignant transformation of BHK21 clone 13 cells [80] and hamster kidney cells [81] were induced by BKPyV. Several mechanisms by which BKPyV may induce transformation are presented in Table 3. In addition, the notion of BKPyV as a potential cofactor in human prostate cancers has been supported. Biological agents such as oncogenic viruses that can interfere with the cell cycle may induce Gene alterations. BKPyV, alongside chemical and physical agents, might be implicated in the prostate cancer (PCa) putative genomic evolution [82]. Sufficient BKPyV in vivo and in vitro carcinogenicity evidence prompted the WHO BKPyV classification as “possibly carcinogenic to human” [83]. BKPyV DNA sequences were found by Fiori and Di Mayorca in three of four human tumor cell lines and five of 12 human tumors by using DNA–DNA reassociation kinetics. Full-length BKPyV genomes, in addition to rearranged and defective BKPyV DNA molecules were found in these tumors [84]. Free episomal BKPyV DNA was detected at a generally low copy number in four out of nine (44%) human pancreatic islets tumors and in 19 out of 74 (26%) human brain tumors [85].

Table 3: Proposed mechanisms by which BKPyV may induce neoplasiaReploeg et al., *Clinical Infectious Diseases*, 2001 [74]

BK virus substrate	Interaction	Host cell substrate	Mechanism of neoplasia
BK T Ag	Binding	p53	Blockade of apoptosis
BK T Ag	Unknown	pRb, p107, p130	Blockade of apoptosis
BK T Ag	Unknown	E2F	Increased cell growth or division
BK T Ag	Unknown	Host cell DNA	Chromosomal aberrations
BK T Ag	Unknown	Estrogen receptors	Estrogen-mediated cell growth or division
BK DNA	Binding	HIV Tat1 protein	Induce expression of BK virus genes
BK DNA promoter	Rearrangement	BK DNA promoter	Increased BK viral replication

6. BKPyV immune controls

The chronic viral infections pathogenesis has a key element: The balance disruption between the host immune control and viral replication. Transplant patients are particularly pre-disposed to BKPyV reactivation by many host immune factors like insufficient BKPyV humoral immunity and ineffective T-lymphocytes immune surveillance. Although indispensable for graft tolerance, immunosuppression places transplant recipients at a high risk for viral disease reactivation. It has been suggested that the greatest BKPyV reactivation risk's factor is the immunosuppression overall intensity rather than its specific type [86]. In fact, one of the most common viral complications in kidney transplant recipients is the BKPyV infection. The BKPyV has been increasingly acknowledged since its discovery as an important human pathogen in the immunocompromised population [87].

6.1. Innate immune control

Despite the vital role of innate immunity in anti-viral defense, only limited data exist on its involvement in the BKPyV infection. A type of antigen presenting cells that can regulate and induce an immune response is dendritic cells (DC). These cells are responsible for antigen presentation and T-cell activation, and at least two distinct lineages have been shown to exist: myeloid precursors-derived myeloid DC (mDC) which promote T helper-1 (Th1)-polarized immune response and synthesize high interleukin-12 (IL-12) levels, and Plasmacytoid DC (pDC). The latter lack myeloid cell markers, exhibit a plasma cell-like morphology, produce high interferon- α levels, and drive T helper-2 (Th2) responses [86]. Investigating DC role in

persistent and acute polyomavirus infection in mice models revealed that both macrophages and DC are permissive for mouse polyomavirus infection. However, the efficiency at presenting the immune-dominant viral epitope to CD8+ T cells was markedly higher in dendritic cells. In addition, only infected DC, were able to prime anti-polyomavirus CD8+ T cells in vivo [88].

A study by Womer et al. demonstrates that due to immunosuppressive therapy and/or allo-transplantation itself, both DC subsets are profoundly reduced after renal transplantation. In addition, the same study reported lower levels of peripheral blood dendritic cells in patients with BKPyVAN compared to renal recipients with stable graft function. The pDC/mDC ratio was also significantly lower in renal recipients, reflecting an important reduction in pDC levels. The total DC levels and pDC/mDC ratio post-transplant decrease varied from patient to patient, but was observed in all the recipients. However, transplant recipients receiving an anti-thymocyte globulin (ATG) and corticosteroids combination displayed significantly lower mDC levels compared to other recipients. ATG is a polyclonal antibody against human thymocytes, which also has clinical effects on non-T lymphocytes cells such as hematopoietic cells. It was suggested that post-transplant complications such as infection and rejection risks may be predicted by DC levels monitoring in the peripheral blood. It's because these levels can mirror the individual immunosuppression extent in each patient [89]. In another study, Womer et al. validated the findings above in a larger patient population. They also showed that renal graft recipients who developed BK viremia post-transplantation had a peripheral blood dendritic cells (PBDC) deficiency before the transplantation procedure. Hence, pretransplantation PBDC levels can represent a viral reactivation risk factor. Since viremia was absent before transplantation, the circulating DC deficiency can't be attributed to a direct virus depressive effect, but rather linked to the host-specific immune factor. The authors suggested the need for investigating whether in vivo cytokines or growth factors administration before transplantation is efficient in endogenous DC levels boosting [86].

6.2. Humoral immune control

Both humoral and cellular immune responses are involved in the BKPyV infection control. In a cohort of renal recipients with BKPyVAN, Hariharan et al. observed significantly higher BKPyV-specific antibody titers in subjects after BKPyVAN resolution as compared to titers at BKPyVAN diagnosis time. The authors suggest that BKPyV-specific antibody titers are associated with viral clearance [90], but the increase in IgG levels can also be linked to viral replication. It was demonstrated that the BKPyV-specific antibody response course follows the BKPyV replication level and duration in KTRs who experience high viruria or viremia levels [91].

Accessory molecules and the clonally unique B cell receptor (BCR) produce antigenic stimulation. B cells respond to it by forming an immunoglobulin (Ig)-secreting plasma cells and high-affinity memory B cells network. The memory B cells recurring activation and the long-lived plasma cells persistent production of serum antibodies keeps the BKPyV infection under control in healthy subjects. Natural or therapeutically-induced immunodeficiency circumstances lead to antibody production suppression and infection control lack, which may result in severe pathological consequences [92]. Chen et al. illustrated the complex humoral and cellular immune responses interplay in renal graft recipients with BKPyVAN. The humoral immune response may not be induced in the case of a viremic phase absence. In addition, the renal function recovery may be achieved by BKPyV clearance from the kidney orchestrated by T-cells. Conversely, abundant viruria followed by a viremic phase can result from the inability of an absent or inadequate cellular immune response to combat the BKPyV infection. In this case, a humoral immune response can be induced by the virus' presence in the blood. Subsequently, high BKPyV antibody titers may be able to resolve the viremic phase. However, they may not succeed to clear the virus from its intracellular replication site in the kidney. This may lead to a sustained viruria state. Recovery of renal function may be prevented by BKPyV replication persistence associated with kidney inflammation [93]. Lindner et al. demonstrated a potent neutralizing antibody response produced by a BKPyV infection. In fact, cross-reactive antibodies were harbored by the clonally diverse anti-BKPyV repertoire. The latter was characterized by a high monoclonal antibodies' frequency and a clonal complexity regarding both immunoglobulin sequences and isotypes. Viral binding to its cellular receptor may be disrupted by anti-BKPyV antibodies, which may also be implicated in blocking viral uncoating processes post-entry. The authors called for the identification of antibody candidates that can

be integrated in a potent curative strategy for polyomaviruses in immunosuppressed individuals, such as organ transplant recipients (Fig 12) [94].

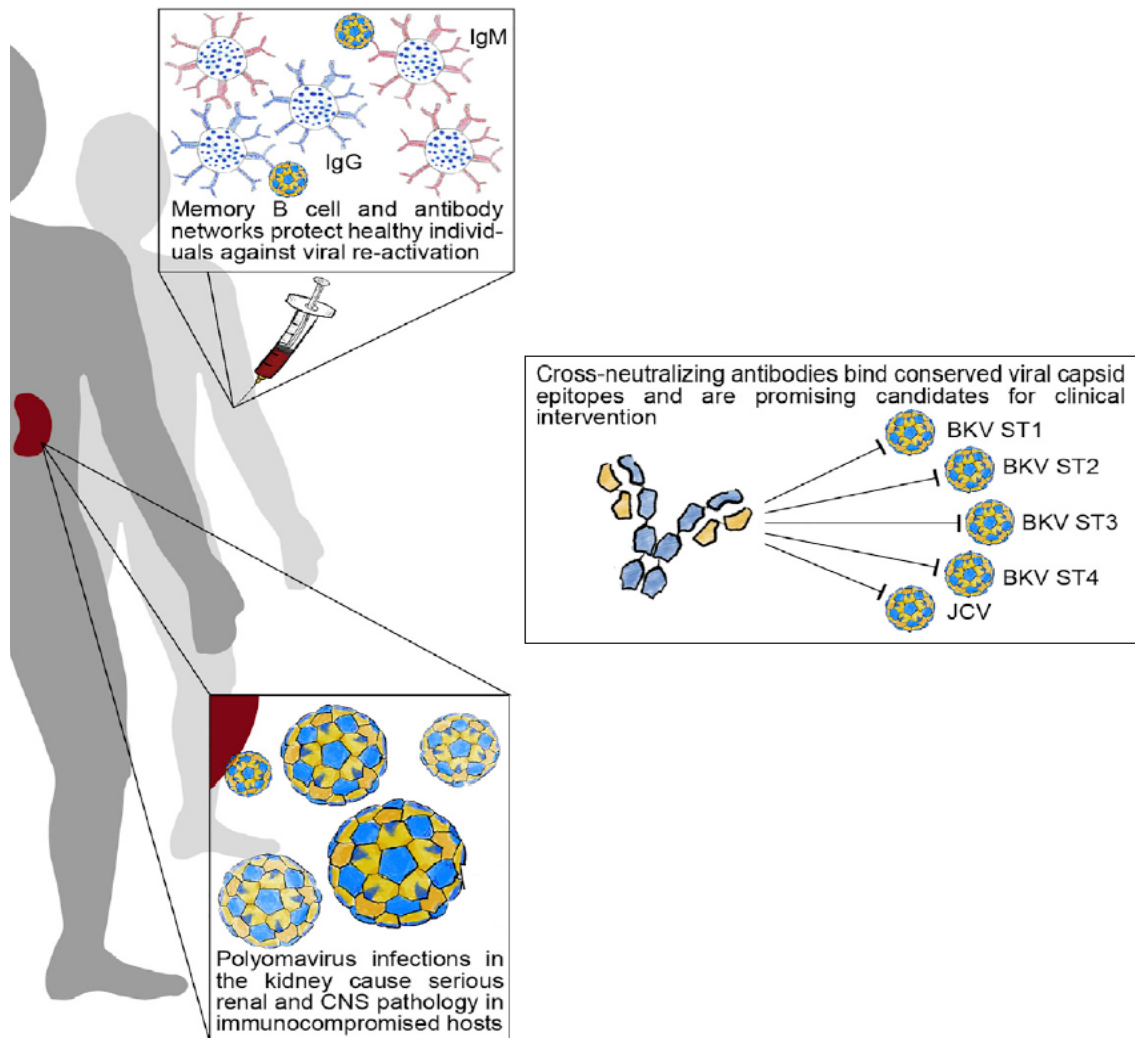


Figure 12: Cross-neutralizing antibodies as part of a potential infection inhibition strategy in renal recipients.

Adapted from Lindner et al., *Immunity*, 2019 [94]

6.3. Cellular immunity

BKPyV specific-T cells regulate the BKPyV replication control and latency maintenance. Weak T-cell responses are associated with recurrent and ongoing BKPyV viremia, whilst viral replication cessation is correlated with the BKPyV-specific cellular immunity reconstitution. In fact, it has been suspected that the BKPyVAN pathogenesis common denominator was the balance between BKPyV-specific cellular immune functions and BKPyV replication. Along the patient, graft and virus different interaction points, this balance can be perturbed and lead to BKPyV reactivation [95]. There exists a time-dependent direct correlation between the BKPyV-specific T-cell immunity reconstitution rate and the viral load decline rate. A T-cell response can be elicited not only by VP1 and large T-antigen (LT) proteins, but also by other BKPyV proteins such as VP2, VP3 and small T-antigen (st). Positive T-cell responses to VP3 were detected in a noteworthy proportion of patients lacking VP1 or LT proteins detectable T-cell responses [87]. It was demonstrated that renal graft recipients with past or active BKPyV replication had CD4+ IFN- γ -producing T cells that were predominantly VP1-specific. The CD8+ population was predominantly specific to the large T-antigen protein. The severity of the previous BKPyV infection was correlated with the magnitude of memory multifunctional CD4+ T cells. The function of these cells was speculated to not wholly be directed at providing helper functions, but also at exerting direct virus replication control [96].

Flow cytometry and ELISpot assays were both used to study the healthy BKPyV seropositive individuals' T-cell response. They revealed that 33 % of individuals had a CD8+ T- cell response, whilst 91% generated a CD4+ T-cell response. Thus polyfunctional CD4+ T-cells dominate the anti-BKPyV T-cell response, predominantly expressing polyfunctional cytokines and displaying a high proliferative activity [97]. In renal transplantation patients however, it has been suggested that BKPyV-specific T-cells occupy a dual role in the BKPyV infection control. Immunopathogenic phenomena can be induced by BKPyV-specific T lymphocytes. Peripheral blood CD4+ and CD8+ T cells and kidney biopsies derived T-cell lines of BKPyVAN patients were analyzed. They revealed that BKPyVAN patients with high viral loads exhibited a robust BKPyV-specific T-cell response. The clinical course of BKPyV infection can be determined by the BKPyV T-cell immunity extent and intra-graft inflammation. When viral clearance is achieved by BKPyV-specific cytotoxic T cells at an early stage, intra-graft inflammation remains

low grade, and BKPyVAN progression doesn't occur. However, in case viral clearance is unachieved by the cytotoxic T-cell response, intra-graft inflammation caused by BKPyV replication persists. Rather than controlling viral replication, activated T- cells migrating to the inflammatory zone, where they attack BKPyV or donor MHC antigens presenting graft cells. Intra-graft inflammation is further increased by the induced tissue damage, thus accelerating BKPyV proliferation and attracting even more cytotoxic T cells. These events can ultimately lead to BKPyVAN [87].

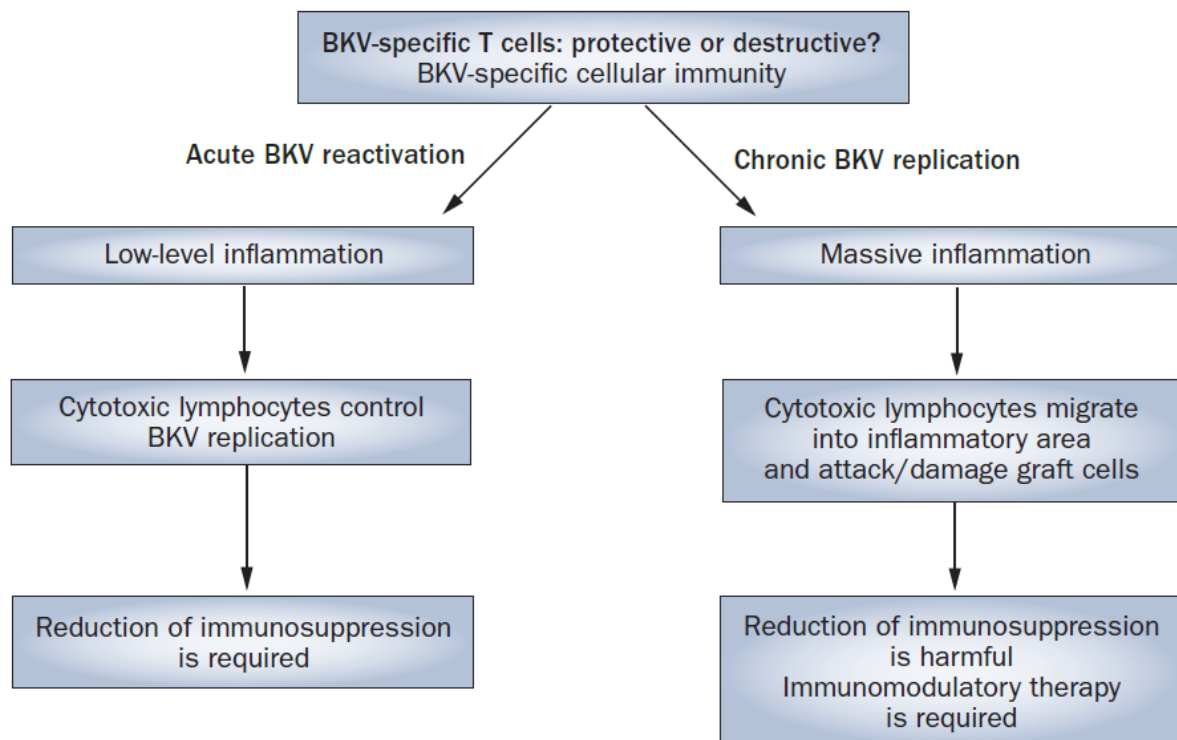


Figure 13: The dual role of BKPyV-specific cytotoxic T cells in BKPyV infection.

Babel et al, nature reviews, 2011 [87]

7. BKPyV diagnostic tools

7.1. Direct BKPyV diagnosis

Higher BKPyVAN risk is associated with sustained BKPyV viremia in renal patients. Viral load quantification by polymerase chain reaction (PCR) represents the standard BKPyV reactivation-monitoring tool in clinics. BKPyV DNA can be measured in the plasma, urine or cerebrospinal fluid (for CNS infection) samples from transplantation patients [98]. An international standard for BKPyV PCR-based assays studies has been published by the World Health Organization (WHO) Expert Committee on Biological Standardization (ECBS) [99]. Hirsch et al. tested urine for decoy cells presence at routine visits from patients. Whenever decoy cells were detected, the authors moved to BKPyV DNA PCR testing [77].

A highly specific and sensitive non-invasive method for BKPyV mRNA level detection in the urine was described. Using 6.5×10^5 BKPyV VP1 mRNAs/ng RNA as a cutoff limit in urinary cells, this method revealed 93.9% specificity. Although promising, this mRNA-based method for BKPyV replication detection still requires further validation [100].

Singh et al described a polyomavirus Haufen test recently as a non-invasive BKPyVAN biomarker. BKPyV aggregates in the urine are described by the term Haufen. Electron microscopy is implemented to detect cast-like three-dimensional BKPyV aggregates. A considerable positive predictive value (more than 90%) for BKPyVAN is obtained by this method. However, high cost and limited availability of electron microscopy decrease this assays feasibility as a routine clinical practice [101]. Direct diagnostic tests for BKPyV infection are summarized in Table 4.

Table 4: Diagnostic testing and prognostic values for BKPyV infection and disease.

Ambalathingal et al., *Clinical Microbiology Reviews*, 2017 [4]

Diagnostic method	Sensitivity for detection of BKV infection (%)	Specificity for detection of BKV infection (%)	Positive predictive value (PPV) for diagnosis of BKVAN (%)	Negative predictive value (NPV) for diagnosis of BKVAN (%)	Comment(s)
Urine cytology for decoy cells	>80	70–84	20–35	>95	Useful for determining BKV reactivation but low PPV for BKVAN
Urine PCR	>98	78	30–40	>95	Effective at detecting possible BKVAN with BKV loads of $>1 \times 10^7$ copies/ml
Serum PCR	90–100	83–96	50–80	>95	Highly specific and sensitive for detecting BKV reactivation; PPV for BKVAN increases with a higher BKV load; a cutoff of 1×10^4 copies/ml has been suggested as a threshold for biopsy specimens to exclude BKVAN
Haufen detection (electron microscopy)	100	>95	>95	100	Higher reported PPV than that for any other method, but the method is expensive
Histopathology			>98	100	Kidney biopsy is the gold standard for determining disease progression Stage/class A: infection/cytopathic changes, <25%; interstitial inflammation/tubular atrophy/fibrosis, <10% Stage/class B: infection/cytopathic changes, 11–50%; interstitial inflammation/tubular atrophy/fibrosis, <50% Stage/class C: infection/cytopathic changes, >50%; interstitial inflammation/tubular atrophy/fibrosis, >50

7.2. Indirect BKPyV diagnosis

Serology diagnostic tests are unimplicated yet in clinics because of the BKPyV ubiquitous nature (practically all population members are seropositive). However, it has been suggested that the donor and recipient pre-transplant serostatus represent predictive markers for BKPyV infection [5,102]. Comprehensive description of the BKPyV serology topic is included later in this manuscript.

8. The BKPyV treatment approaches

8.1. Immunosuppression modulation

A combination treatment with a corticosteroid, an anti-proliferative agent (mycophenolate or azathioprine) and a calcineurin inhibitor (tacrolimus or cyclosporine), usually constitutes the immunosuppression regimen for renal transplant patients. A sequential immunosuppression regimen is adopted by many medical centers where at the transplantation time; an induction agent is administered to prevent acute rejection. Induction treatment is followed by a triple maintenance immunosuppression regimen. To promote the allograft survival, maintenance immunosuppression is required indefinitely post-transplantation. Induction treatment is achieved by a lymphocytes-depleting polyclonal or monoclonal antibody: anti-thymocyte globulin or interleukin-2 receptor antibody. These agents' combinations proved to be successful in maintaining very low rejection rates at many transplant centers [103].

When BKPyV viral load exceeds 10,000 copies/ml, viremia is considered significant. The main treatment would be early immunosuppression reduction guided by serial viremia PCR monitoring. Schaub et al. reduced immunosuppression in patients with sustained BKPyV-viremia (i.e. $\geq 1,000$ copies/ml) by setting tacrolimus trough levels one step lower. Tacrolimus trough levels were further reduced by one step if BKPyV viremia wasn't reduced. Then, a 50% Mycophenolate mofetil (MMF) dose reduction was introduced in case of viremia persistence [104]. Treating BKPyV-associated disease by immunosuppression can have a long-term consequence. One study reported an increased chronic rejection incidence by demonstrating an excess in de novo donor-specific anti-human leukocyte antigen (HLA) antibody development in viremic recipients [105].

8.2. Anti-viral therapy

Unfortunately, anti-BKPyV drug treatment benefits have only been shown in small trials or limited by toxicity. Several anti-viral agents were employed to date for BKPyV anti-viral therapy:

DNA synthesis is inhibited by the immunomodulatory drug Leflunomide. Activity against DNA viruses—including BKPyV—through virion assembly disruption has been demonstrated by in vitro studies [106]. However, a large side effects number has limited Leflunomide use.

Originally developed for use in cytomegalovirus (CMV) infection, Cidofovir is an intravenous nucleotide analogue that acts through viral DNA synthesis inhibition. However, the BKPyV viral DNA polymerase lack makes the anti-BKPyV mechanism unclear. BKPyV treatments meta-analysis didn't reveal clear Cidofovir benefits. In addition, this drug's nephrotoxicity side effect limits its use in renal recipients [79].

Promising anti-viral action against DNA viruses was demonstrated by the oral lipid ester Brincidofovir. Higher intracellular release potency and reduced renal toxicity are conferred by the drug's formula compared to Cidofovir [107].

Several fluoroquinolones have been trialed in BKPyV infection prophylaxis and treatment. They act by inhibiting DNA helicase and impeding viral replication [79]. Fluoroquinolone prophylaxis to prevent BKPyVAN has been shown to be ineffective according to a systematic review [108].

High BKPyV neutralizing IgG titers are found in commercially available IVIg preparations making it an attractive BKPyV treatment option. However, IVIg clinical benefits for BKPyVAN treatment possess insufficient current evidence [79]. When administered together with leflunomide, ciprofloxacin and intravenous Cidofovir, IVIg produced faster viral clearance [109]. Still more studies are required to assess the IVIg effect in this context.

The Inhibitors of the mammalian target of rapamycin (mTORi) drugs sirolimus and everolimus have both been used in clinical practice. Superior outcomes in renal recipients with BKPyV were reported in small case series. Unfortunately, larger studies didn't reproduce these findings.

The successful use of BKPyV-specific cytotoxic T-lymphocytes (CTLs) was documented in a hematopoietic stem cell transplantation (HSCT) patient with hemorrhagic cystitis. Virus-specific T-cells (VST) from allogeneic donors have proven promising and safe in treating HSCT

complicated by viral disease [79]. The current clinical trials on BKPyV treatment agents are summarized in Table 5.

Table 5: The current clinical trials exploring treatments for BKPyV infection or BKPyV associated disease.

These trials were retrieved from the website www.clinicaltrials.gov.

Start Date	Intervention/Treatment	Status	Targeted condition	Location	Phase
March 2011	Drug: Cidofovir	Active, not recruiting	BKPyV-induced hemorrhagic cystitis	USA	Phase 2
July 2015	Allogeneic BKPyV-specific cytotoxic T-lymphocytes	Recruiting	Patients with malignancies with BKPyV and/or JCPyV	USA	Phase 2
September 2015	Switching tacrolimus to equivalent dose of cyclosporine in BKPyV viremic patients	Recruiting	Renal patients with BKPyV viremia or BKPyVAN	USA	N/A
September 2015	Viral specific CTL infusion	Recruiting	Viral infections in immunocompromised patients	USA	Phase 2
May 2016	Intravenous immune globulin (IVIg; Privigen®)	Recruiting	Kidney transplant recipients With BKPyV viremia	Israel and USA	Phase 1
January 2018	Everolimus	Recruiting	Kidney transplant recipients with BKPyV viremia	France	Phase 4
October 2019	BKPyV-virus specific CTLs	Recruiting	post- transplantation or - chemotherapy BKPyV viremic patients	USA	Early Phase 1
April 2020	BKPyV cytotoxic T cells (CTLs)	Not recruiting yet	Refractory BKPyV infection post allogeneic hematopoietic stem cell transplantation	USA	Phase 1/ Phase 2
April 2020	Drug: MAU868	Not recruiting yet	Renal recipients with active BKPyV	N/A	Phase 2
August 2020	Biological: Vivalym-M	Not recruiting yet	Patients with BKPyV viruria and hemorrhagic cystitis	N/A	Phase 3
September 2020	HLA-matched virus specific T-cells (VSTs)	Not recruiting yet	Viral infections after an allogeneic hematopoietic stem cell transplant (HSCT).	N/A	Phase 1

8.3. Putative vaccines

There exist several promising BKPyV vaccine candidates. VaxiGen developed a vaccine targeting both CMV and BKPyV. The vaccine was developed by three DNA plasmids incorporation and is currently in a phase 1 clinical trial under evaluation [79]. Development of a pre-clinical BKPyV vaccine by researchers at the American National Cancer Institute (NIH) was initiated. This vaccine by Buck and Pastrana is multivalent against all four known BKPyV serotypes, and aims to prevent BKPyV associated disease development. Moreover, Keshewani et al designed a BKPyV peptide-based multi-epitope vaccine (MVBKV) that remains to be validated experimentally. A synthetic Toll-like Receptor (TLR) 4 peptide ligand (RS09) was added to the final vaccine construct to improve MVBKV's immunogenic properties [110].

B. BKPyV serology

1. Current clinical approaches for assessing BKPyV serology

The immunosuppression required for graft tolerance in kidney transplant patients can trigger latent BK polyomavirus (BKPyV) reactivation, and the infection can progress to nephropathy and graft rejection. It has been suggested that pre-transplantation BKPyV serostatus in donors and recipients is a predictive marker for post-transplantation BKPyV replication. Two risk factors for early post-transplantation BKPyV replication have been identified: A low BKPyV antibody titer in the recipient, and a high titer in the donor (Fig 14) [111]. It has therefore been hypothesized that a single BKPyV serostatus assessment before transplantation can predict the post-transplantation BKPyV replication risk [5]. Despite these findings, a standardized, commercially available, regulatory-agency-approved assay for anti-BKPyV antibodies is not available [112]. In addition, research laboratories have used many different assay techniques to determine BKPyV Serostatus, which complicates their result's data analysis. Even studies based on the same technique differed in their standard controls choice, the antigenic structure type used for detection, and the cut-off for seropositivity [113]. More sensitive, standardized immunoassays would facilitate the donor/recipient immune status assessment and thus enable the clinician to more closely monitor patients with a high predicted viral

replication risk [114]. Around the world, thousands of patients are on organ transplant waiting lists, and transplantation is becoming a major financial burden in the developed world [115]. Consequently, it is essential to improve BKPyV serologic assays and donor–recipient BKPyV seroreactivity matching with a view to increasing the kidney graft survival rate. To achieve this objective, the most cost-effective strategies for BKPyV screening in different patient populations must be determined—as noted in the Kidney Disease: Improving Global Outcomes guidelines [2].

Most serological assays detect antibodies against the immunodominant BKPyV capsid protein VP1; including enzyme-linked immunosorbent assays (ELISAs), neutralization assays, multiplex immunoassays, and hemagglutination inhibition assays. Most serologic assays detect the immunodominant BKPyV capsid protein VP1 (the virus’s major surface protein) [116]. Cost-effective strategies for BKPyV screening have been sought in various patient populations [117]. It is known that systemic BKPyV infections induce strong, stable, prolonged antibody responses against viral structural proteins. Thus, past BKPyV infections can be detected with high sensitivity by measuring the anti-VP1 antibodies accumulation. In contrast, antibodies against the large T-antigen (TAg) are infrequent and have low titers—making them unsuitable infection markers in most cases [118]. The low antibody response against TAg might be due to poor immune accessibility and/or poor recognition; the latter is thought to be due to the similarity between the TAg functional domains and that of cellular proteins [119].

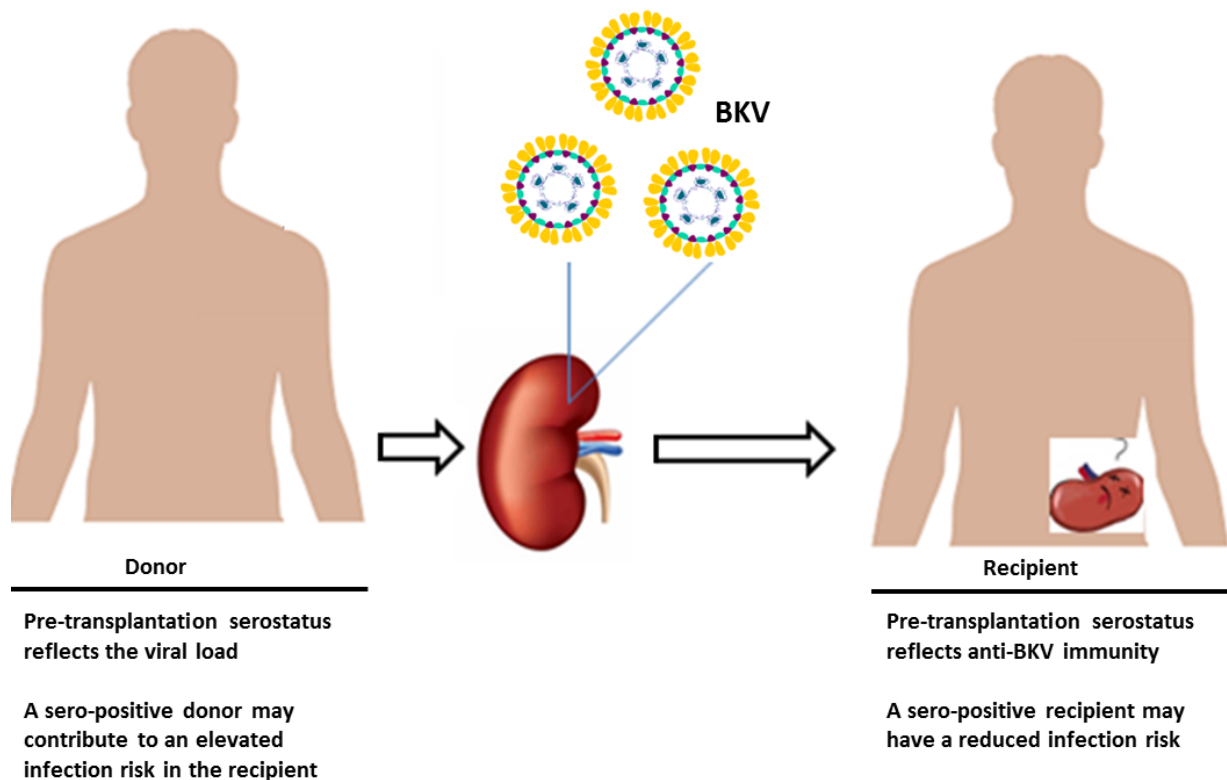


Figure 14: Model of the donor and recipient serostatus as BKPyV infection risk markers.

2. VP1 antigens used in BKPyV serologic assays

Although all serologic assays reviewed here detect anti-BKPyV VP1 antibodies, they differ regarding the target antigens. Furthermore, several different VP1 antigen types can be detected. Below, we briefly describe the VP1 antigens that have been incorporated into the serologic assays developed by research laboratories. A summary of the different BKPyV immunoassay antigens is illustrated in Figure 15.

2.1. Virus-like particles

Virus-like particles (VLPs) are most commonly generated from VP1 structural proteins, but VLPs with both VP1 and VP2/VP3 proteins have been synthesized. Although VLPs resemble native virions assembled into capsids (comprising 72 capsomers with a T = 7 symmetry), they do not contain viral genetic material. They can be used for diagnostic antigens for detecting serum specific antibodies against BKPyV VP1. The VLPs' structure, transduction efficiency, and tropism are similar to those of native virions, except for the fact that VLPs do not undergo

post-translational modification [21]. BK polyomavirus VLPs can encapsidate DNA fragments derived from the cells in which they were produced; consequently, the VLPs in each production batch contain VLPs with differing densities, depending on the incorporated DNA amount and size [27]. The VLPs quantity and quality can be affected by many factors, including the used production system type and the purification method. Virus-like particles can be produced in insect cells giving them the advantage of being free of mammalian pathogens; however, the yields are rather low, with a high cost and risk of contamination with enveloped baculovirus particles and host DNA [38]. Yeast production systems have the advantage of producing safe, DNA-free VLPs, which makes them perfect to produce VLP vaccines. In fact, a study found that recombinant VLPs synthesized in yeast and used in an ELISA for human polyomaviruses have many advantages in ease of production, protein yield, and cost terms [21]. The 293TT mammalian cell line is most commonly used for VP1 VLPs synthesis because it allows authentic assembly and folding of recombinant proteins. Still, the production costs in the mammalian system are high, yields are low, and the cells are vulnerable to infection with mammalian pathogens [38]. One must also consider the VLPs' purity and integrity prior to the use in immunoassays. In fact, VLPs can be coupled to biotin for use in ELISAs. VLPs can be treated with the EZ-Link Sulfo-NHS-LC biotinylation kit and then bound to streptavidin plates, after which a sample diluent is added. Kardas et al. reported that standard polyomavirus VP1 VLPs and biotinylated VLPs did not differ significantly with regard to assay variability at the population level [120]. The VLP profile may vary even when the same production, purification, and quantification methods are applied. It is important to assess each batch's quality by ensuring that the VLPs' hemagglutination activity and immunogenicity make them suitable for serologic assays [27]. After production, SDS-PAGE can be used to confirm that the VLP batch has a major protein band at 40 kDa, and thus can be qualified for use in ELISAs [121]. It is known that native VLPs and denatured VLPs have different antigenic epitopes; denatured VLPs react less efficiently with BKPyV-positive human serum. BK polyomavirus VLPs are stable at relatively high pH values, which enables them to be used in conventional ELISAs [27]. These VLPs are therefore the best tools for detecting BKPyV seroreactivity and have also been extremely valuable in BKPyV epidemiological studies.

2.2. Pseudovirions and native virion

The term “pseudovirion” (PsV) is used to describe synthetic viruses produced by the plasmid transfection of genes encoding capsid proteins and artificial genetic material used as a reporter. Although PsVs are similar to native virions in many ways (e.g., their behavior within cells), these synthetic viruses cannot replicate and do not propagate infection in cell cultures or *in vivo*. Hence, PsVs have become common tools for studying cellular entry and neutralization, and might be valuable in the future as vaccine vehicles or gene transfer tools [122]. Pastrana et al. generated pseudovirions by co-transfecting BKPyV capsid protein expression plasmids coding for VP1, VP2, and VP3 with a reporter plasmid encoding luciferase into 293TT cells. The cells were suspended and lysed 48 h post-transfection. The lysate was incubated overnight to allow capsid maturation, and then clarified. Ultracentrifugation using an iodixanol gradient was then used to purify the pseudovirions from the clarified supernatant [123]. Pseudovirions are mainly used in serum neutralization assays, where they contain a luciferase or green fluorescent protein reporter plasmid [124]. Apart from PsVs and VLPs, native virus particles can also be used as antigens in immunoassays. Native BKPyV particles are usually grown in HEK, Vero, or 293TT cells, harvested, purified, and quantified prior to their use in serologic assays [125]. It is also noteworthy that only the subtype Ia BKPyV (Dunlop or Gardner strain) can be propagated easily in culture, which means that the use of whole-virion antigens is not practical when the measurement of antibodies against different BKPyV serotype strains is required.

2.3. Soluble VP1 proteins

Both recombinant and synthetic soluble VP1 proteins have been used as antigens in ELISAs. In a computer-assisted analysis of the late viral region, Pirtrobon et al. produced two specific, synthetic BKPyV VP1 peptides with a stable secondary structure. The synthetic peptides were incorporated in ELISAs that could detect anti-BKPyV antibodies in the absence of cross-reactivity with other small DNA tumor viruses [126]. The use of uniform, well-defined synthetic peptides with a high epitope density advantageously limits inter- and intra-assay variability and increases sensitivity. However, cross-reactivity can still be a problem, since synthetic peptides may not be able to bind specifically enough to the target antibodies; the

short peptides may have a different conformation when compared with full-length VP1 molecules assembled into capsomers during VLP synthesis [127]. Transfecting E.coli with pGEX VP1 plasmids produced VP1 pentamers; the resulting VP1 protein is fused to an N-terminal glutathione S-transferase (GST). After affinity purification on glutathione resin, the fusion proteins can be bound to 96-well polysorp plates (using a casein-glutathione conjugate) and used in a capture ELISA [128]. Alternatively, the VP1-GST fusion proteins can be directly affinity-purified on polystyrene beads for use in a multiplex immunoassay [118].

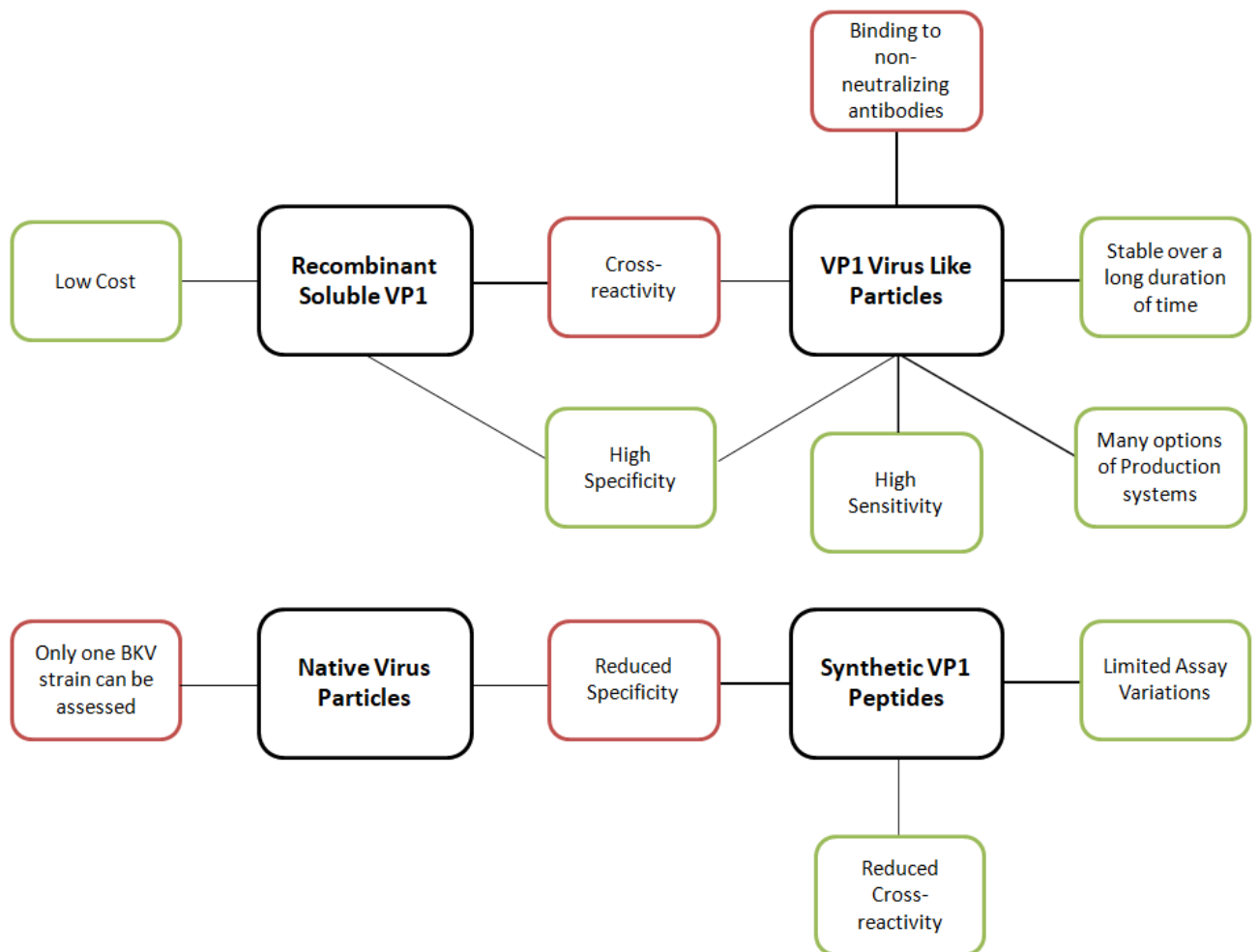


Figure 15: A comparison between the different advantages and disadvantages of BKPyV VP1 antigens used in serology assays.

3. Assay techniques for BKPyV seroreactivity

Four different techniques can be used to evaluate seroreactivity to BK polyomavirus. The techniques' respective advantages and disadvantages are summarized in Table 6. Each technique's methodology is illustrated in Figure 16.

Table 6: Advantages and disadvantages of assay techniques for BK polyomavirus (BKPyV) seroreactivity.

Technique	Advantages	Disadvantages	Time Requirement
Enzyme Immunoassay	Small quantities of sample required Versatile and customizable Inexpensive once set up	Can only measure one analyte at a time Cross-reactivity Relatively expensive initial investment Time consuming Elevated risk of error when testing a large number of samples	2 days (if wells are coated with antigen overnight)
Hemagglutination Inhibition Assay	Highly specific	Technically demanding Cannot distinguish between antibody classes Requires either intact virions or VLPs	1 day
Multiplex assay	Simultaneous detection of multiple antigens High speed and dynamic range Customizable Reduced workflow	Expensive especially if a small number of antigens is analyzed Specialized equipment and analysis software are not available in most clinical settings Lack of normalization	Around 2 days (if beads are prepared in advance)
Neutralization Inhibition Assay	Highly Specific Measures neutralizing antibodies	Can only be used with PsV or viruses that can be grown Technically demanding Very time consuming	Around 5 days

3.1. Enzyme immunoassays

Enzyme-linked immunosorption is a rapid, high-throughput, sensitive, and highly reproducible method for antibody detection. Furthermore, colorimetric, chemiluminescent, or fluorescence ELISAs typically have a broad dynamic range [125]. The ELISA plates can be coated with any of the above-mentioned BKPyV antigens' types. Kean et al. studied several human polyomaviruses and found that a VP1 pentamer-based ELISA performed better than the more common VLP-based ELISA. The casein-glutathione conjugate used to capture the GST-VP1 capsomers on Polysorp 96-well plates fully exposed the bound capsomers to the serum sample and facilitated all the VP1-reactive antibodies measurement [128]. However,

Bodaghi et al. reported that ELISAs with VP1 VLPs as coating antigens are more specific and sensitive than those with VP1 monomers or pentamers. Furthermore, the researchers' denaturation experiments experimentally confirmed the antigen's three-dimensional structure importance [119]. In the absence of standardized ELISAs for BKPyV, research and clinical laboratories have developed their own in-house ELISAs using various antigens, protocols, and standards. This complicated the comparison of BKPyV ELISA serology results between one lab and another, especially in the absence of guidelines on quantitative cut-offs. As mentioned above, the BKPyV VP1 antigen can be used in different forms. Even labs that use the same type of antigen (VLPs, for example) can differ regarding the antigen production and purification methods and the final concentration used to coat wells. Another variable is the reference material used to optimize the assay, which may differ from one lab to another. For example, the negative control is a blank well in some studies [120,121] and a bovine serum albumin-coated well in others [129,130]. Bodaghi et al. used an SF9 extract as a negative control [119], while Abend et al. used human anti-chicken lysozyme IgG [117].

Similarly, the normalization well composition may vary, and some labs even skip this step. Inter-plate normalization usually involves diluting an internal reference serum close to 1 optical density. Hence, the absence of a standardized, commercially available antibody prevents labs from using the same identical normalization step. In addition to technical variables, the cut-off or positivity can be set differently in each laboratory. A clear BKPyV seropositive sample definition is currently lacking, and each laboratory uses its own in-house method to determine the cut-off. In summary, inter-ELISA variability is caused by differences in the reference material (normalization antibodies and negative controls), the VP1 antigen's type and concentration, the experimental protocol, the cut-off, and the seropositivity definition. A growing body of evidence suggests that pre-transplantation testing for BKPyV exposure can help to predict the occurrence of BKPyV-associated diseases after transplantation. Despite that, there are currently no consensus guidelines on an ELISA technique that healthcare institutions could use to determine the BKPyV serostatus of kidney or bone marrow transplant recipients. It will be difficult (but not impossible) to implement a technique that can be universally applied for pre-transplantation BKPyV serology assessments in a clinical setting. In the light of research performed over the last decade (i.e., strong evidence of a relationship between pre-transplantation BKPyV serology and post-

transplantation BKPyVAN), it is now more important than ever to develop a standard ELISA for pre-transplantation BKPyV serostatus.

3.2. Multiplex immunoassays

ELISA and other conventional serologic assays measure the presence of serum antibodies against a single antigen per well. In contrast, multiplex technologies enable the production of arrays of sensors—each of which provides its own unique detection signal. Multiple antigens can be measured simply by placing the sample in contact with the array [131]. Protein–protein interactions have been explored in multiplexed planar and suspension arrays, both of which requiring pre-purified proteins [132]. In a multiplex suspension array, a template (e.g., a micro well) is filled with different sensing elements in solution [131]. One of the best suspension array examples that efficiently detects antiviral antibodies in serum is the LUMINEX Multi-Analyte Profiling® (xMAP®) technology, in which indicator molecules are covalently attached to 5.6- μm polystyrene bead sensor elements. The beads have an internal color code that is obtained by filling them with different proportions of two or three spectrally distinct fluorochromes—resulting in an array of at least 500 separate bead sets [132]. Thus, the difference in the internal classification dye quantity in each microsphere results in a unique emission profiles generation, even though these same-sized beads have similar emission requirements [133]. LUMINEX indirect serologic assays have been extensively validated for the detection of antibodies against several polyomaviruses types [134–136] including BKPyV [5,24,137]. The BKPyV VP1 protein was expressed in *Escherichia coli* as a fusion protein with GST, and then affinity-purified using LUMINEX beads coupled to glutathione-casein. The modified beads could to be used directly for the detection of anti-BKPyV antibodies [132]. When LUMINEX beads are used in serologic assays, non-specific background binding is a major drawback; human sera may contain antibodies that bind directly to the beads. Serum panels vary in the proportion of these sera, which frequently exceeds 5%. Using SeroMap beads (rather than xMAP® beads) to minimize binding to heterophilic serum antibodies only partially solves the problem, so the sera pretreatment with background inhibitors was recently suggested [138].

Furthermore, seroepidemiologic studies require many samples to be tested for several analytes in a rapid, sensitive, specific manner. This kind of analysis is facilitated by multiplex

assay formats. Hence, if one seeks to detect anti-BKPyV antibodies against several viral serotypes, multiplex technology will be a time saver. This technique allows the simultaneous analysis of each serum sample against all the BKPyV serotypes at once. Furthermore, multiplex technology minimizes the experimental variability associated with conventional serology methods because multiple data points are obtained from a single measurement. The technique's requirement for a very low sample volume also maximizes data collection. In contrast, multiplex technology may offer fewer advantages in a clinical setting; the costly, specialized equipment and analytical software are unlikely to be available in all hospital laboratories. Compared with epidemiological studies, the number of subjects to be assessed at a given time point in a hospital or a transplant center is much lower. This means that the cost of performing these assays will be higher than for conventional serologic tests (e.g., ELISAs). Furthermore, it is harder to define a clear cut-off in multiplex assays, since the result for each sample is usually expressed as mean fluorescence intensity. Lastly, it is noteworthy that multiplex assays use soluble VP1 proteins (rather than VLPs); this may constitute a slight drawback because many studies have suggested that the conformational structure of VP1 inside VLPs offers more specificity and sensitivity than that of VP1 monomers or capsomers.

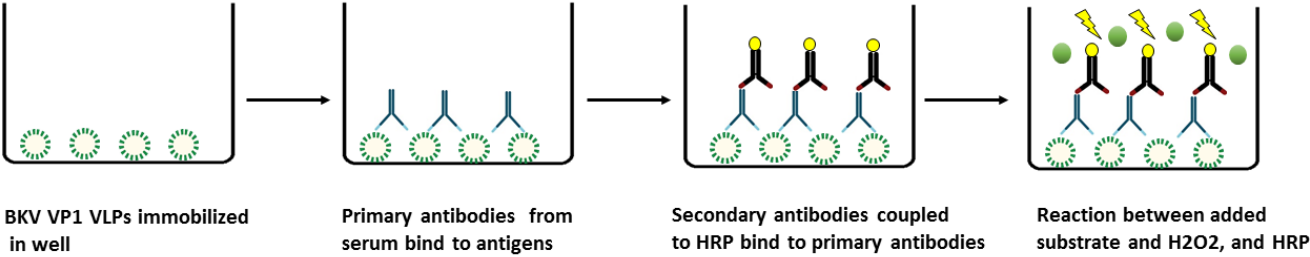
3.3. Neutralization inhibition assays

A neutralization inhibition assay for BKPyV serology has been reported in the literature. In general, serum samples are serially diluted, pre-incubated with PsV or native virions, added to seeded cells, and then incubated for a period of at least 48 or 72 h. The cell lysate is then analyzed: The greater the neutralizing antibodies titer in the serum is, the lower is the PsV-transduced or virion-infected cells' number and thus the weaker is the signal [124]. Solis et al. synthesized three different PsV types and then measured the antibody titers against BKPyV in the sera of 156 kidneys transplant recipients at six different time points. The researchers demonstrated that this technique could quantify antibody titers in many samples [139]. This technique's greatest drawback is probably the need for cell culture—making it time-consuming, technically demanding and therefore unsuitable for clinical measurements. Furthermore, there is no standard method for a reliable neutralization inhibition assay so far, and as in the ELISA case, the seropositivity definition differs from one lab to another. Other variables include the BKPyV antigen type and the cell type used in the assay. For instance, RPTEC [117] and 293TT cells [7, 32] have both been used to determine BKPyV serostatus.

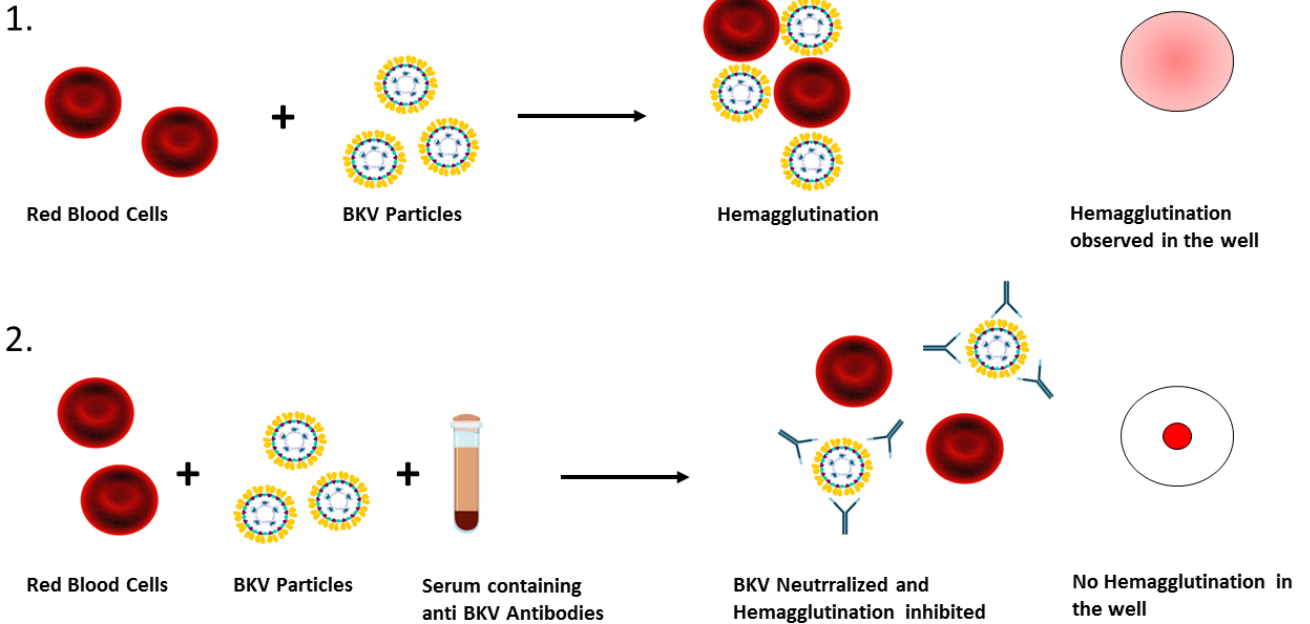
3.4. Hemagglutination inhibition assays

Many laboratories have used hemagglutination inhibition assays (HIAs) to measure the antibody titers to BKPyV because of the rapidity and ease with which they can be performed. However, HIAs are less sensitive and less accurate than enzyme immunoassays. Experiments with the HIA have shown that greatly differing anti-BKPyV titers and anti-JCPyV antibodies were obtained in individual sera, thus overcoming the cross-reactivity problem expected for JCV and BKPyV [125]. It is noteworthy that the HIA is technically demanding and cannot differentiate between different BKPyV serotypes.

A. ELISA using VLPs:

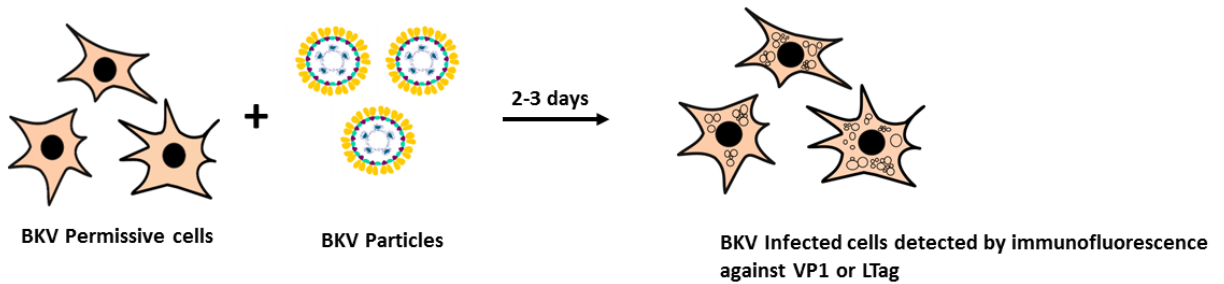


B. HIA:

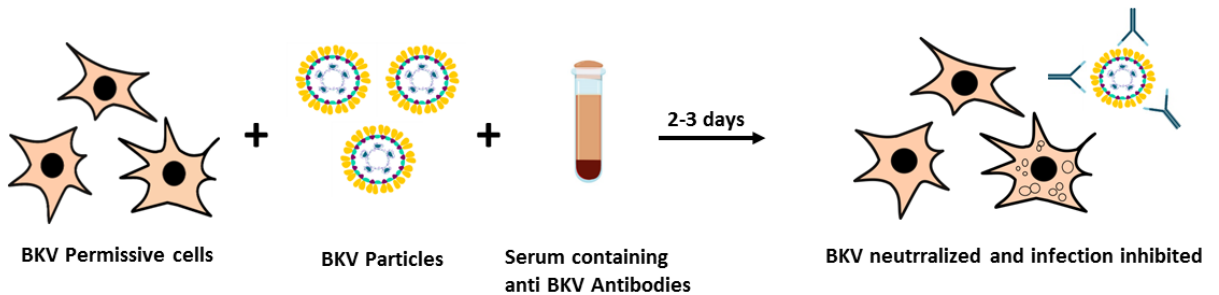


C. NA:

1.



2.



D. Multiplex Assay:

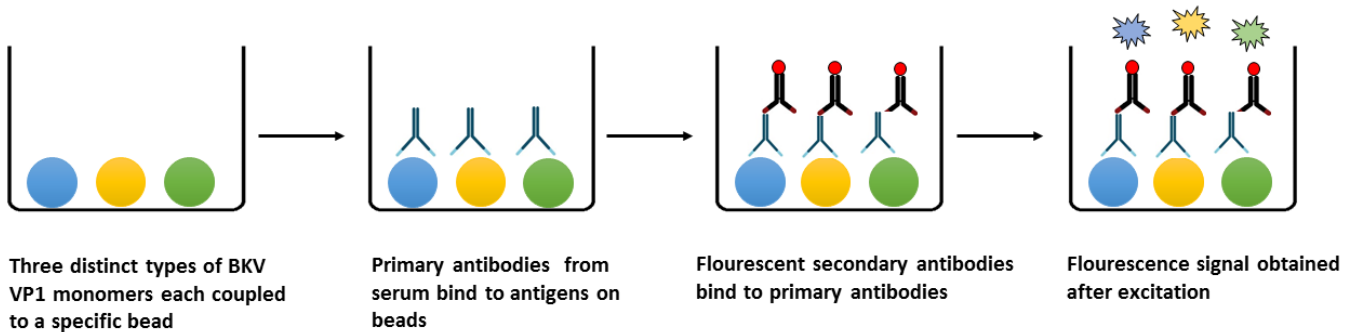


Figure 16: Illustration of the different techniques utilized in BKPyV serology assessment.

- ELISA technique using BKPyV VP1 VLPs as antigens (A).
- Hemagglutination inhibition assay (HIA): Red blood cells agglutination is induced by interaction with BKPyV particles (B1). Hemagglutination is inhibited by serum antibodies (B2).
- Neutralization inhibition assay (NIA): BKPyV permissive cells are infected by BKPyV particles, and the infection is measured by immunofluorescence against BKPyV proteins (C1). Serum antibodies neutralize BKPyV particles decreasing the number of BKPyV infected cells (C2).
- Multiplex immunoassay utilizing different beads coupled to distinct antigens and immobilized to reaction wells. (D).

4. Clinical studies of BKPyV serology

4.1. BKPyV serology studies available in literature

In Table 7, we provide an overview of the research studies conducted on BKPyV serostatus in kidney transplant donors and recipients. In 2017, Wunderink et al. established that donor pre-transplant BKPyV seroreactivity best predicted the occurrence of a manifest BKPyV infection in renal allograft recipients. The researchers found a strong correlation between donor BKPyV serostatus on the one hand and the development of post-transplantation BKPyV viremia and BKPyVAN on the other. These findings strongly suggest that the kidney allograft has an important role in the BKPyVAN development, since it acts as a vector for transmitting BKPyV to the recipient. Consequently, it is assumed that the intensity of the donor's BKPyV seroreactivity corresponds to the infectious BKPyV load in the kidney allograft, which in turn is correlated with the BKPyV infection risk in the recipient. In contrast, the recipient's BKPyV seroreactivity might reflect his/her overall anti-BKPyV immunity status. Thus, it may be relevant to assess the post-transplantation BKPyV infection risk by assaying for anti-BKPyV IgGs prior to kidney transplantation [5]. Similarly, Solis et al. found that patients who received a kidney graft from donors with elevated BKPyV-neutralizing antibody titers became positive for BKPyV DNA. The researchers also found that the recipient's pre-transplantation titer of neutralizing antibodies against donor-specific BKPyV strains determined the BKPyV replication risk. Solis et al. suggested that physicians must take account the individual BKPyV risks when choosing immunosuppression strategies and monitoring patients after transplantation. Along with the recipient's BKPyV DNA load, the neutralizing antibodies titer against the replicating strain is a valuable disease progression marker [139]. Similarly, many studies found that a positive donor BKPyV serostatus is associated with post transplantation BKPyV infection [140–142]. In contrast, Abend et al. reported that BKPyV viremia was not significantly correlated with the recipient's serostatus. This might have been because the anti-BKPyV antibodies levels were too low to provide protection in a transplantation context (i.e., with suppressed cellular immunity and elevated viral loads). Abend et al. suggested that BKPyV viremia may be due to a donor-virus-derived infection, and thus that it may be possible to identify recipients at a clinical BKPyV infection risk by measuring the donor' serostatus [117]. On the other hand, Hirsch et al. proposed that the high-risk group to develop BKPyV infection after transplantation is not the seropositive donor and seronegative recipient transplant

combination [77]. In view of these findings, we call on the scientific community to strive to (i) develop clear guidelines for assessing BKPyV serostatus, (ii) define quantitative cut-offs, and (iii) develop standard assay controls and reference samples. This will be the first step on the road to faithfully analyzing, comparing, and exploiting data on BKPyV serostatus and, ultimately, implementing these findings in clinical practice.

Table 7: An overview of the different research studies pertaining to the involvement of pre-transplant BKPyV serology testing in post-transplantation BKPyV infection.

Authors	Year	Number of Patients	Type of Assay and BKPyV Antigen	Conclusions from the Study
Solis et al. [139]	2018	168 KTR + 69 donors	Neutralization assay using pseudovirion system (BKPyV genotypes I, II, and IV)	Recipients with high neutralizing antibody titer have a lower risk for developing BKPyV viremia
Abend et al. [117]	2016	116 donor-recipient pairs	Neutralization inhibition assay using BKPyV particles (serotypes I, II, III, and IV) VLP-based ELISA to detect antibodies against BKPyV serotype I	Donor with significant serum neutralizing activity is associated with elevated risk for BKPyV viremia regardless of recipient serostatus
Wunderink et al. [5]	2016	407 donor-recipient pairs	Luminex assay detecting IgG reactivity against BKPyV Ib1 VP1 protein. <i>n</i> = 396 reanalyzed by VP1 VLPs-based ELISA to detect antibodies against BKPyV genotype Ib2	Donor BKPyV IgG levels were strongly associated with the occurrence of recipient viremia and BKPyVAN
Sood et al. [102]	2013	192 adult and 11 pediatric donor-recipient pairs	BKPyV VLPs-based ELISA to detect human IgG Antibodies	Infection was highest in the Donor+/Recipient- group and lowest in the Donor-/Recipient- group
Ali et al. [143]	2011	36 pediatric KTRs +donors	BKPyV VP1 VLPs-based indirect ELISA to detect human IgG antibodies	Low BKPyV serostatus in children is associated with a high risk of post-transplantation BKPyV viremia, particularly in the context of donor with high BKPyV serostatus
Bijol et al. [144]	2010	45 pediatric KTRs	BKPyV VLPs-based ELISA to detect human IgG antibodies	Positive recipient BKPyV serostatus did not confer protection to BKPyV after transplantation
Bohl et al. [91]	2008	87 KTRs	BKPyV VP1 VLPs-based ELISA to detect human IgG Antibodies	Pre-transplant seropositivity did not protect against sustained BKPyV viremia but it might mitigate the severity of infection
Bohl et al. [140]	2005	142 recipients and 84 donors	BKPyV VP1 VLPs-based ELISA to detect human IgG Antibodies	BKPyV infection in the recipient was strongly associated with a positive BKPyV donor antibody status
Smith et al. [145]	2004	173 pediatric KTRs	BKPyV VP1 VLPs-based indirect ELISA to detect human IgG Antibodies	Recipient seronegativity for BKPyV was significantly associated with the development of BKPyVAN
Hirsch et al. [77]	2002	77 KTRs	Hemagglutination inhibition assay	The high-risk group is not the seropositive donor and seronegative recipient transplant combination
Flegstad et al. [146]	1991	10 KTRs	Neutralization inhibition assay Hemagglutination inhibition assay IgG, IgA, and IgM ELISA	Positive recipient BKPyV serostatus did not confer protection to BKPyV after transplantation Children with BK nephritis demonstrated lower pretransplant antibodies levels when compared to control groups (no infection)
Andrews et al. [147]	1988	496 donor-recipient pairs	Hemagglutination inhibition assay	A sero-positive donor increased the rate of primary and reactivation infections with BKPyV

4.2. BKPyV serology studies limitations

Cohort size: Some studies included a relatively small cohort with less than 150 recipients [77,91,102,117,140,143,144]. In studies with smaller sample sizes, outliers can skew the data, and it is more difficult to find a statistically significant result. The interpretation of results with larger sample sizes is more precise and reliable. Such studies have more accurate mean values, increased estimate confidence, and decreased uncertainty. Our study included a large study cohort of 329 recipients and 222 donors, giving us more confidence in its results.

Donor-recipient pairs: Some studies did not investigate the pre-transplant donor's serostatus role in BKPyV infection [77,91,144–146]. This is despite reports that a seropositive donor increased the reactivation infection rate [140,147]. We investigated both donors and recipients' serostatus to assess each as a risk factor for BKPyV reactivation. In addition, we assessed the recipient-donor pair's seroreactivity as a marker for BKPyV infection.

BKPyV serotypes analyzed: Only two studies so far have analyzed BKPyV pre-transplant serology of three or more serotypes, and compared it to BKPyV infection [117,139]. Others have analyzed only Ib2 serology and compared it to BKPyV infection development. But, a recipient's sero-positivity against one BKPyV serotype may not protect him/her against viremia development by another serotype. Similarly, a sero-positive donor may not be the infection source if a different BKPyV serotype is replicated in the recipient. A recipient's seropositivity might have been concluded to be non-protective because it reflected immunity against a different serotype than the one propagated in the post-transplant infection.

Assay normalization and cutoff definitions: Many of the studies published so far were not clear about their cutoff or seropositivity definitions. Moreover, some studies used a reference sample from a patient known to be BKPyV positive for inter-plate normalization. Inter-plate normalization is crucial to ensure that results are reliable for comparison. In addition, it allows the calculation of a "corrected" assay value for each patient. Moreover, a normalization well that can be universal between different laboratories is a must for any commercial ELISA assay. We used for the first time a commercial BKPyV VP1 antibody (3B2) to normalize our ELISA experiments.

Choice of immune assay: ELISA is a highly sensitive and specific and inexpensive technique once setup that can be easily implemented as a routine serology test in clinics. Many diagnostic clinical tests such as Epstein Barr virus (EBV) or CMV serology are actually ELISA-based tests. Small sample volumes are required for ELISA measurements, and rapid results can be obtained with this technique. Some of the previous studies assessed serology with hemagglutination inhibition assay (HIA), multiplex assay, or neutralization inhibition assay (NIA) [113]. HIA risks false-negative calls in the low antibody context and is less sensitive than ELISA. In addition, HIA is technically demanding and can't differentiate between different antibody classes. The multiplex immune assay requires specialized equipment and analysis software, which are not available in most clinical settings. Regarding NIA, it can only be used with pseudovirions (PsV) or viruses that can be grown. In addition, it involves cell culture, and is very technically demanding and time-consuming. This makes it less suitable for clinical routine tests.

II. OBJECTIVES

BKPyV associated nephropathy (BKPyVAN) can lead to renal nephropathy and even graft rejection, constituting a real threat to the renal transplantation procedures success [76]. It is managed by reducing immunosuppression, which may increase the acute rejection risk and may be unsuitable for some patients [79]. Several risk factors have been proposed for BKPyV reactivation post-transplantation such as high donor [5] or low recipient IgG titer [139]. However, there exist no clear guidelines for BKPyV serology assessment or sero-positivity definition [113]. Additional studies are needed to validate the BKPyV serology as a marker for BKPyV reactivation. Moreover, a standard serology assay development may allow the faithful analyses and comparison of BKPyV serology research findings. Implementing such an assay in clinical practice allows patient stratification into high or low-reactivation risk according to the BKPyV pre-transplant serostatus. This may reduce the BKPyV post transplantation infection risk.

This study's main objective is assessing the impact of pre-graft serology on the risk of BKPyV infection reactivation post-renal transplantation.

We aimed to:

- Evaluate if the donor, recipient or donor-recipient pair serostatus is implicated in the post-transplantation BKPyV infection risk.
- Develop and validate a standardized BKPyV ELISA technique with comparable results between laboratories.
- Explore the relevance of serology assessment against at least four BKPyV serotypes in obtaining more accurate results concerning the BKPyV serology and activation correlation.

The results of this work were the subject of an article submitted to Clinical Microbiology and Infection Journal:

The Impact of Pre-Graft Serology on the Risk of BKPyV Infection Reactivation Post-Renal Transplantation

Fatima Dakroub, Antoine Touzé, Fadi Abdel Sater, Toni Fiore, Virginie Morel, François Helle, Catherine François, Gabriel Choukroun, Claire Presne, Nicolas Guillaume, Gilles Duverlie, Sandrine Castelain, Haidar Akl and Etienne Brochot.

This research work and its publication are available in parts III (Results) and VII (Annexes) respectively.

III. RESULTS

The Impact of Pre-Graft Serology on the Risk of BKPyV Infection Reactivation Post-Renal Transplantation

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Conflict of interest: All authors have no conflict of interest to declare.

Abstract

Background: BK polyomavirus associated nephropathy (BKPyVAN), is a troublesome disease induced by BK polyomavirus (BKPyV) reactivation in immunocompromised renal graft recipients. BKPyVAN can progress to graft dysfunction and has no current treatment, making immunosuppression reduction the only management choice. Thus, predictive BKPyV infection reactivation markers are needed for high-risk patient identification.

Methods: we conducted a retrospective study to assess the correlation between the BKPyV pre-transplant serostatus and post-transplant BKPyV infection incidence. Sera from 329 recipients and 222 matched donors were tested for anti-BKV antibodies against four BKPyV serotypes by a VLPs- based IgG ELISA, and BKPyV DNA load was monitored for at least 1-year post transplantation.

Results: 80 (24%) recipients were viruric and 59 (18%) recipients were viremic post transplantation. An elevated BKPyV viremia risk was observed for recipients who had a mean antibody titer for all serotypes ≤ 400 before transplantation (odd ratio [OR], 5.58; 95% confidence interval [CI], 2.60-11.79; $P < 0.0001$). In addition, kidney recipients from donors with a mean BKPyV antibody titer ≤ 400 had a lower BKPyV viremia risk (OR, 0.47; CI, 0.21-0.95; $P = 0.055$). Furthermore, a lower mean antibody titer in donors was associated with a late onset of BKPyV viremia (> 4 months).

Conclusions: Both donor and recipient mean BKPyV antibody titer may serve as a predictive tool to manage clinical BKPyV infection by identification of patients at high reactivation risk.

Keywords: BKPyV; BKPyV associated nephropathy; BKPyV reactivation; BKVPyV serostatus; BKPyV seroprevalence; serological technique; BKPyV virus serology; kidney transplantation

Abbreviations: BKTGR, BK virus typing and grouping region ; BKPyVAN, BKPyV associated nephropathy; BKPyV, BK polyomavirus; BMI, body mass index; *D-/R+*, Negative donor positive recipient; *D-/R-*, negative donor negative recipient; *D+/R+*, positive donor positive recipient; *D+/R-*, positive donor negative recipient; CI, Confidence interval; c/ml, Copies per milliliter; CNI, calcineurin inhibitor; DNA, Deoxyribonucleic acid; ELISA, Enzyme linked immunosorbent assay; FBS, Fetal bovine serum; GFP, Green fluorescent protein; HRP, Horse radish peroxidase; IgG, Immunoglobulin G; KTRs, Kidney transplant recipients; LB, Lenox Broth; mAb ,

Monoclonal antibody; OD, Optical density; OR, Odds ratio; PCR, Polymerase chain reaction; PBS, Phosphate buffered saline; rATG, rabbit antithymocyte globulin; SD, Standard deviation; VLPs, Virus like particles; VP1, viral capsid protein 1; X^2 test, *Chi-Square* test;

A. Context

The BK polyomavirus (BKPv) is a DNA virus that belongs to the *polyomaviridae* family. BKPv genotypes I, II, III, and IV behave as five distinct serotypes [15] that are extremely prevalent amongst the general population. The primary BKPv infection is usually asymptomatic in immunocompetent individuals. However, potent immunosuppressive therapy places renal graft recipients at risk for BKPv reactivation and viremia progression [72,148]. The infection persistence can lead to BKPv associated nephropathy, usually associated with graft rejection [149]. To limit the patients' progression to BKVAN, current guidelines implicate renal recipients in regular BKPv viruria and viremia monitoring after the transplantation procedure [150,151]. Still, around 8% of renal transplant patients develop BKPvVAN, of which the majority experience graft dysfunction and loss [152]. Since only limited studies exist on anti-viral treatment against BKPv [153,154], BKPvVAN management is based on judicious immunosuppression decrease and acute rejection monitoring [155]. However, studies demonstrate that BKPvVAN risk is not fully eliminated by viremia screening [156], and that the immunosuppression reduction strategy is not suitable for all patients (i), and can increase the acute rejection risk (ii) [157]. Several risk factors have been proposed for post transplantation BKPv reactivation such as male sex, older age and potent immunosuppression [158]. Still, it is relevant to identify additional factors that allow better high-risk patient stratification. Two early post-transplantation BKPv replication risk factors have been proposed: A high BKPv antibody titer in the donor and a low titer in the recipient [111]. However, a limited number of studies assess this hypothesis: only twelve studies, of which two use the unreliable hemagglutination inhibition method for serostatus determination. In addition, some of these studies include a relatively low number of patients and others do not include the donors in their cohort. It is also worth to mention the contradictory nature of their results, with some being in favor of the previous hypothesis and others against it. We summarized these studies and their characteristics in table 2 of a review that we published recently [113]. Wunderink et al demonstrated a strong correlation between post-transplantation BKPv infection and pre-transplantation BKPv IgG levels. The authors measured the IgG titer using both LUMINEX assay and ELISA, but detected antibodies only against the Ib BKPv serotype [5]. By measuring BKPv Ia, II and IV neutralizing antibodies, a 2018 study by Solis et al supported a high recipient

antibody titer protective role. However, the authors used the more technically demanding and time consuming neutralization assay, which is less suitable for clinical measurements [139]. We aimed to evaluate the mentioned hypothesis by assessing the BKPyV serostatus against four different serotypes of the virus: Ib2, Ia, IV and II. It's because we believe that a positive pre-transplantation antibody titer against one serotype may not grant a recipient protection against infection with another serotype. Moreover, it is important to inspect whether the pre-graft serology for four BKPyV serotypes has an additional interest in the post-transplantation BKPyV infection risk. In this retrospective study, we assessed the total BKPyV serostatus of 329 kidney transplant recipients (KTRs) and 222 matched donors. Then, we correlated it with BKPyV post transplantation viremia incidence using for the first time a commercial antibody for ELISA inter-plate normalization. Besides standardizing our technique, we adopted a well-proposed definition for our positivity cutoff and sero-positive patients.

B. Materials and methods

1. Study design and cohort

In this retrospective study, adult renal graft recipients who underwent transplantation between January 2013 and May 2018 at the Amiens University Medical Center were included. In total, 329 recipients were included and 21 were excluded based on the following criteria: transplantation failure (i), death during the first year (ii), following up outside Amiens University Medical Center (iii), lack of samples (iv) and return to dialysis (v). Pre-transplantation sera samples from 222 matched donors were collected and included in the cohort (Fig 22). The Amiens University Medical Center's institutional review board approved this study.

2. Immunosuppression protocols

The induction treatment for all patients included corticosteroids (SOLU-MEDROL®; Pfizer) and antibody induction. The latter consisted of either basiliximab (Simulect®; Novartis Pharmaceuticals Corp.) or rabbit antithymocyte globulin (rATG; Genzyme) according to immunologic risk assessment. The maintenance immunosuppression protocol was composed of a calcineurin inhibitor (CNI), with most patients receiving tacrolimus (PROGRAF®; Astellas Pharma Inc.). The others —particularly those at a high risk for tacrolimus induced toxicity—

received cyclosporine A (NEORAL®; Novartis Pharmaceuticals Corp.). The CNI treatment was combined with the anti-metabolite mycophenolate mofetil (CELLCEPT®; Genentech) or with everolimus (Certican®; Novartis Pharmaceuticals Corp.). Most patients received corticosteroids maintenance (Cortancyl®; Sanofi) that was halted between days seven and eight for eligible patients.

3. Virus-like particles (VLPs) synthesis:

Recombinant baculoviruses containing either Ia VP1 or IV VP1 expression plasmids were used to transduce SF21 insect cells cultured in Grace's Insect Medium 1X (Gibco). Virus-like particles representing serotypes Ia and IV were harvested from SF21 cells 48 to 72 hours post-transduction. A mammalian system was utilized for the serotypes Ib2 and II VLPs generation using VP1 plasmids with a green fluorescent protein (GFP) marker plasmid. The plasmids were amplified by transformation into E.coli bacteria (NEB 5-alpha, New England Biolabs) using the heat shock method (ice, 30 min; 42°C, 30 s; ice, 5 min). After allowing resistance genes expression, E.coli cells were inoculated into selection petri plates (VWR) prepared with Lenox Broth (LB) medium (Sigma) and an antibiotic. The antibiotic used was either ampicillin (Thermofischer) or zerocin (Invitrogen) according to each plasmid's type. One culture-forming unit was retrieved and grown for plasmid extraction. DNA extraction was performed using a plasmid extraction kit (NucleoBond® Xtra Midi/Maxi) according to the manufacturer's instructions. 293TT cells were cultured using Dulbecco's modified eagle medium (Gibco DMEM (1X) + GlutaMAX™). 293 TT cells were then transfected with the plasmids using a Polyplus transfection® kit containing a jet OPTIMUS transfection reagent (according to the manufacturer's instructions). Cells containing the expressed VLPs were harvested 48 hours post-transfection and stored at -80°C. The mammalian and insect expression systems are summarized in Figures 17 and 18 respectively.

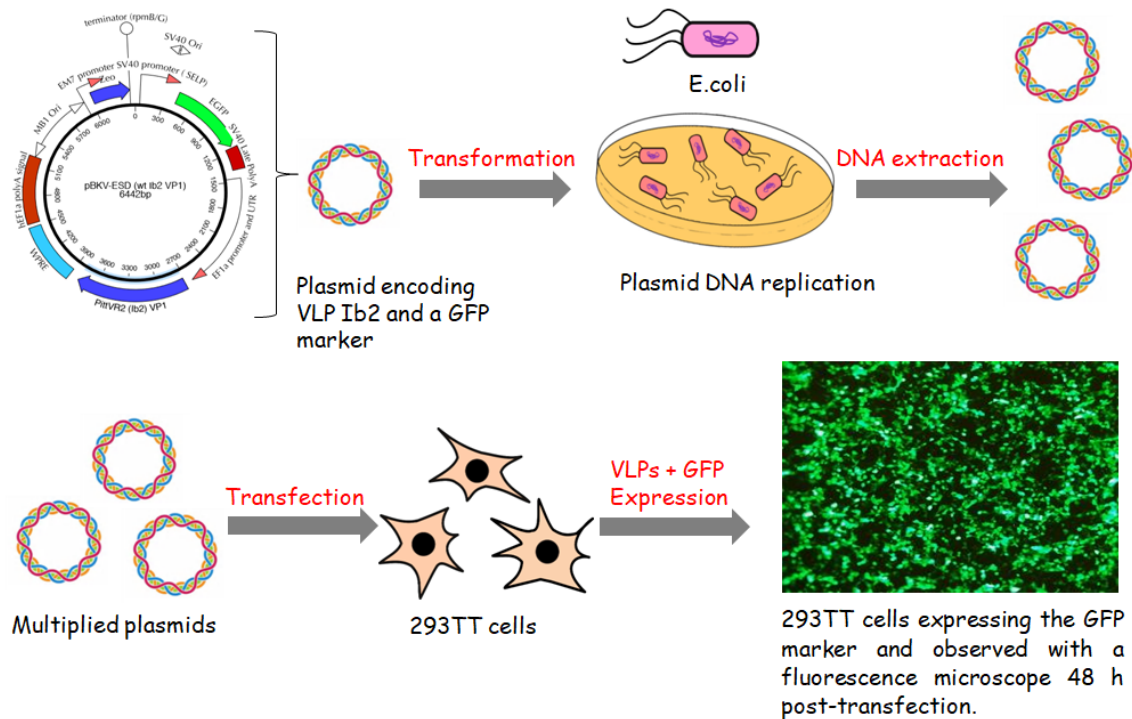


Figure 17: Ib2 VLPs production using a mammalian expression system.

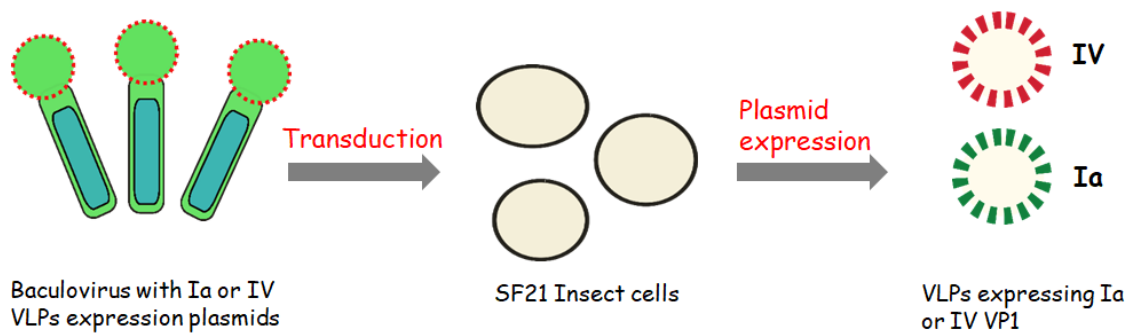


Figure 18: Ia and IV VLPs production using an insect expression system.

4. VLPs extraction and purification:

The purification method was the same for VLPs produced in the insect or the mammalian system. The plasma membrane was lysed by incubating the cell pellet with a solution containing NP40 (Tergitol, Sigma) and a protease inhibitor (Thermoscientific pierce mini-tablets) for 30 min on ice. Then, the nucleus containing the VLPs was sedimented by centrifugation (2000 rpm, 20 min, 4°C). The nucleus lysis buffer was prepared using phosphate buffered saline (PBS) 1X (Dulbecco w/o Ca²⁺ & w/o Mg²⁺), Sodium Chloride 5M (Sigma), plasmid safe (Epicenter) and added to the lysate. After mixing well, the cell lysate was sonicated (at amplitude 80) and centrifuged (10000g, 10 min, 4°C). The supernatant was

passed on an iodixanol (VISIPAQUE™ 320 mgI/ml) gradient (six concentrations ranging between 20 and 45%). The gradient was ultra-centrifuged for 24 hours at 32,000 rpm (Ultracentrifuge (Sw32) Model: Beckman Coulter Optima L-100 XP). Gradient fractions were tested for VLPs using ELISA. The fractions containing VLPs were collected and stored at -20°C. VLP purification is illustrated in Figure 19.

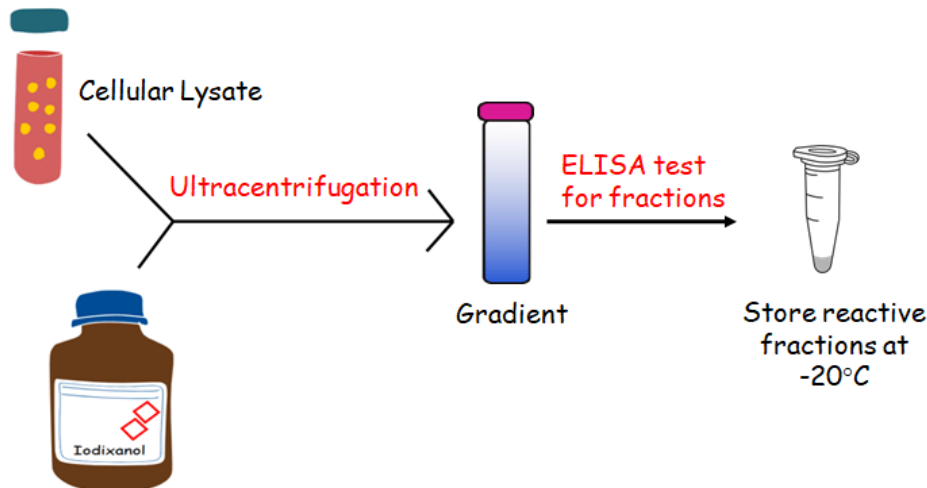
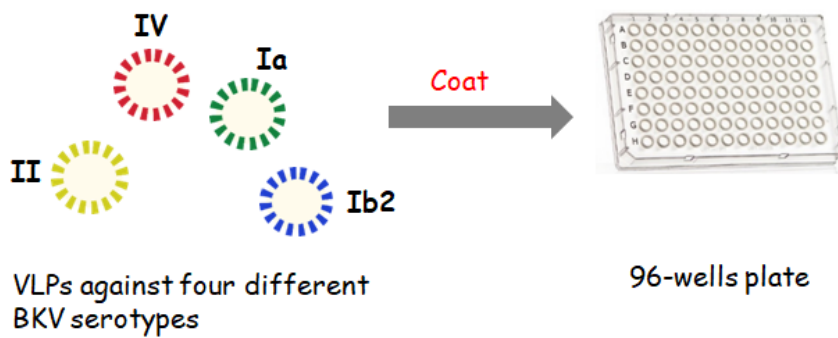


Figure 19: Overview of the VLP purification process.

5. Enzyme-linked immunosorbent assay

For plate coating, BKPv VP1 VLPs were diluted to 1 µg/mL and 100 µl were added to each well of a 96-well polystyrene flat-bottomed Polysorp plate (Nunc immune-plate, Thermo Scientific) and incubated overnight at 4°C. Patients' serum samples were stored at -80 °C after collection, and diluted by 1/100 before being added to the plate. After the plate was blocked with a fetal bovine serum (FBS, Gibco) solution then washed, 200 µl of each sample was added to the first well and diluted by two-fold dilution. A commercial anti-BKPv VP1 monoclonal antibody (3B2, Sigma-Aldrich) was diluted and added to normalization wells, while negative control wells contained only PBS 1X (Dulbecco w/o Ca²⁺ & w/o Mg²⁺). After incubation for 1 hour, the plate was washed and a secondary IgG Fc specific antibody coupled to horse radish peroxidase (HRP) (Southern Biotech) was added to the wells (anti-human to sample wells and anti-mouse to normalization wells). After incubation and washing, o-phenylenediaminedihydrochloride (Sigma) and hydrogen peroxide (Sigma) were added to each well and incubated at room temperature in the dark for 30 min. The reaction was halted by 100 µl of 1M sulfuric acid, and the optical density (OD) was read at 492 nm by an ELISA spectrophotometer (TECAN® Sunrise).

VLP production for plate coating:



Anti-VP1 antibody assessment:

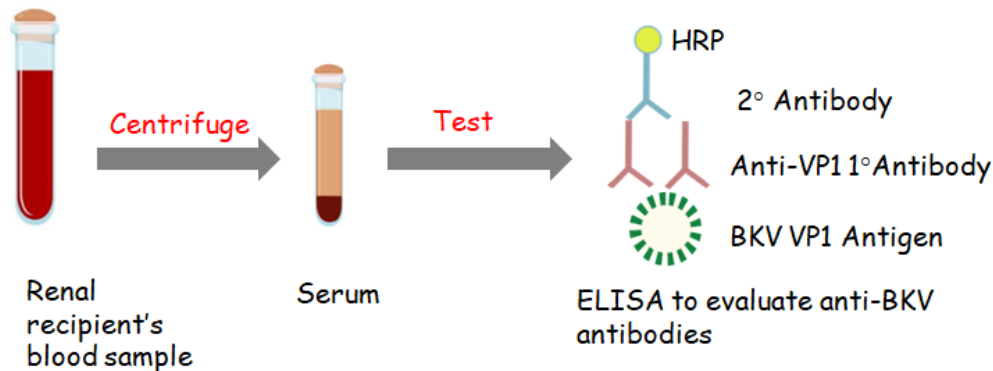


Figure 20: Technical concept of the BKV VP1 VLPs-based ELISA.

6. ELISA normalization and cutoff determination

Each plate included normalization wells (n=3 per plate) in which 3B2 commercial anti-BKPyV antibody (Sigma) was diluted (1/5000) and then added. The antibody dilution was always the same for all plates. Only plates in which the normalization wells produced a mean OD between 0.7 and 1.5 were accepted to assure inter-plate standardization. The OD of the negative control subtracted the OD of a given sample. Then, the normalization well's OD divided it to give a corrected OD for each sample. If the corrected OD at a given titer was greater than 110% cutoff, the sample was positive at that titer.

For cutoff determination, we determined the mean OD of 18 serum samples from children aged between 15 months and 2 years plus two standard deviation. A sample was considered seropositive if it's OD was positive (> 110% cutoff) at titers ≥ 200 .

7. BKPyV viremia screening and BKPyV genotyping

Viremia and viruria were assessed monthly for the first six months after transplantation and then at months 9 and 12 using a quantitative real time PCR kit (RealStar Altona). After viral load determination, positive samples were sequenced based on a fragment called the BKPyV virus typing and grouping region (BKTGR) [41].

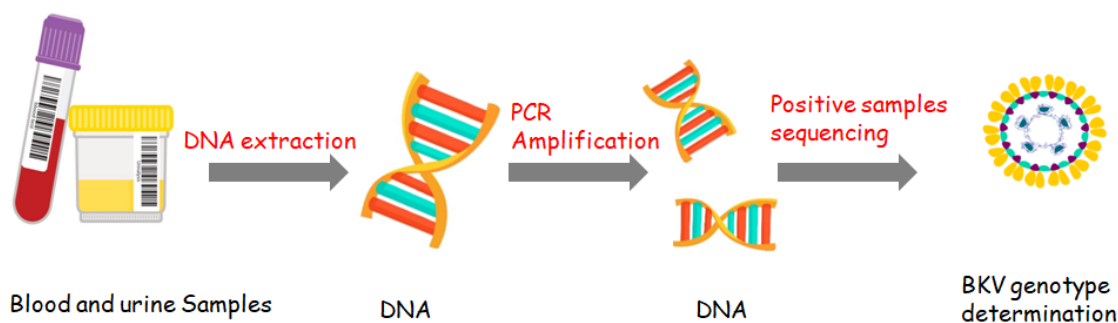


Figure 21: BKPyV DNA load screening and genotyping.

8. Statistical analyses

Data analysis was performed using *GraphPad Prism* version 5 for Windows (*GraphPad* Software, La Jolla, California, USA, www.graphpad.com). *Kolmogorov–Smirnov test* was used to examine if variables were normally distributed. Mann-Witney U test was used to compare two independent groups. The *Chi-Square* test (χ^2 test) and Fisher's exact test were used to determine if there was a significant relationship between categorical variables (BKPyV infection and the other categorical variables). Odds ratio and its 95 % Confidence interval were used to report the association strength between BKV infection and each variable. A probability value of less than 0.05 was regarded as statistically significant.

C. Results

1. Viremia in the studied population

In this retrospective study, 329 recipients and 222 matched-donor samples were available after the exclusion of 38 unfitting samples (according to the mentioned criteria) (Fig 22).

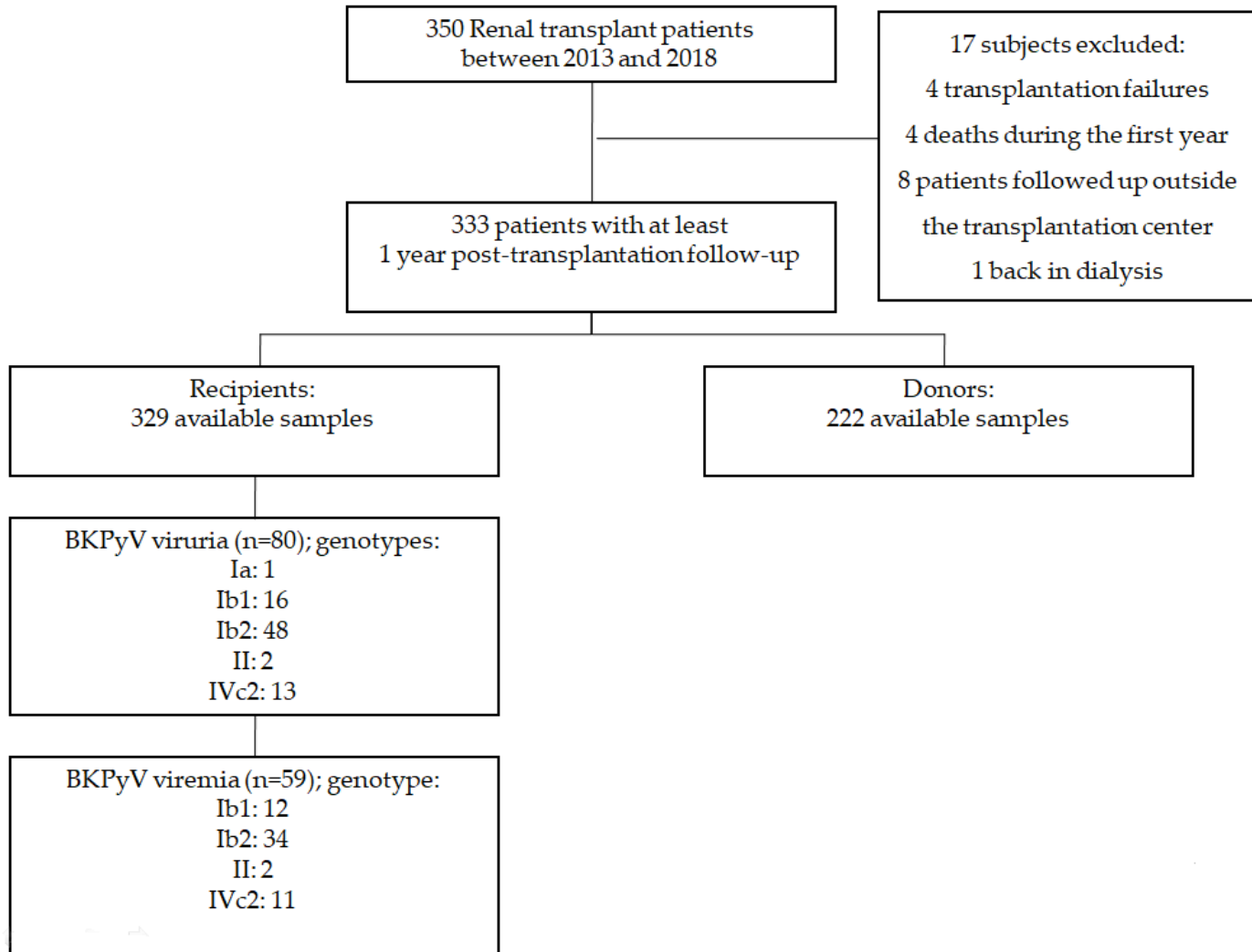


Figure 22: BkPyV post-transplant infection prevalence and genotypic distribution amongst the study's recipient cohort.

BkPyV viremia and viruria were measured until 1-year post-transplantation. A total of 80 recipients developed BkPyV viruria (Table 8). The majority of patients replicated BkPyV in their urine in the first 3 months after transplantation. Most of the patients became viremic between two to four months after transplantation (68%) (Fig 23). The BkPyV Ib2 genotype [34(58%)] was detected in the majority of the 59 recipients who progressed to viremia. Viremia level was considered high and as presumptive nephropathy in recipients with viremia > 4 log copies/milliliter (c/ml).

Table 8: Primary detection kinetics of post-transplantation viruria (n=80) and viremia (n=59) amongst the studied KTRs (n=329).

	BKPyV Infection	
	New viruria cases (n)	New viremia cases (n)
< 1 Month	18	-
Month 1	7	3
Month 2	12	9
Month 3	17	20
Month 4	9	10
Months 5-8	5	8
Months 9-11	2	2
Month 12	10	7

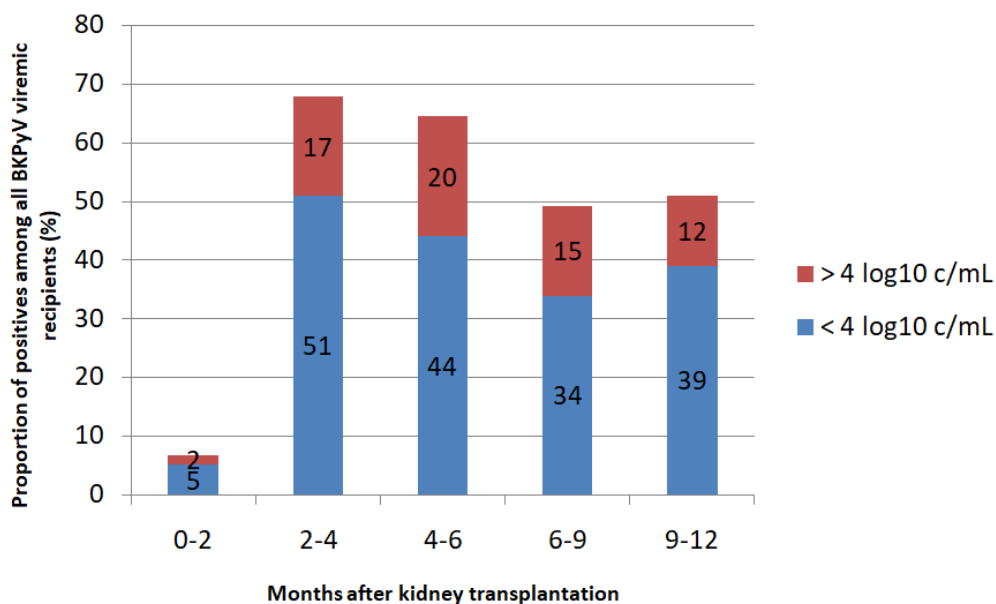


Figure 23: BKPyV viremia characteristics and detection kinetics in recipients with BKPyV viremia (n =59).

c/mL, copies per milliliter.

BKPyV seroprevalence in our cohort (n=551) was 75.6%, 82.55, 76.5% and 64.9% for serotypes Ib2, Ia, IV and II respectively. In addition, most of the recipients (72.6%) were sero-positive for all the studied BKVPyV serotypes (Ib2, Ia, IV and II), while only 0.9% were sero-negative for the 4 serotypes (Table 9). Moreover, 16.6% of donors were sero-positive for 4 serotypes, a percentage similar to that of donors who were sero-negative for all serotypes (14.8%).

Table 9: Number of recipients and donors having an IgG titer < 200 with 0, 1, 2, 3 or 4 BKPyV serotypes.

IgG titer < 200	Recipients		Donors	
	Total (n=329)	P+ (n=59)	Total (n=222)	P+ (n=35)
0 serotype	239	38	37	2
1 serotype	61	11	51	13
2 serotypes	22	7	67	10
3 serotypes	4	2	34	7
4 serotypes	3	1	33	3

BKPyV viremia incidence during follow-up was compared with specific Donor, recipient and transplantation characteristics. Despite the significance lack, some evident trends were observed for several characteristics. Viremia was more common among recipients with blood groups incompatible donors (17% vs 11%; p value, 0.19) (Table 10). Similar proportions of patients whose donors were males or old (> 50 years) were observed in the viremia and no viremia groups. Table 11 shows that viremic and non-viremic recipient's significant differences were not observed regarding listed recipient baseline characteristics, including the underlying immunosuppressive regime. Although non-significant, a recipient's older age and greater cold ischemia time were associated with viremic patients. Regarding induction immunosuppression, we found that thymoglobulin treatment was associated with BKPyV viremia (54% vs 44%; p value, 0.14). Moreover, we observed that tacrolimus maintenance increased BKPyV viremia risk (76% vs 67%; p value, 0.16).

Table 10: Donor characteristics sorted for BKPyV viremia among 329 renal transplant recipients in the first-year post-transplantation.

¹ Data from two non-viremic patients' donors were missing.

² Age data of one viremic patient's donor was missing.

³ Cross match data was missing for four non-viremic patients.

⁴ A rhesus negative recipient with a rhesus positive donor was always considered blood groups incompatible regardless of the ABO classification.

⁵ Three viremic patients' blood group compatibility data were missing.

	Recipients with BKPyV Viremia n=59	Recipients without BKPyV viremia n=270	p value
Donor characteristics			
Male sex	32 (54%)	152 (56%) ¹	0.72
Age > 50 years	36 (61%) ²	160 (59%) ¹	0.37
Cardiovascular Arrest	18 (30.5%)	80 (30%) ¹	0.92
Vasoactive Drugs	48 (81%)	229 (85%)	0.5
Cross match	2 (4%)	7 (3%) ³	0.74
Anti-DSA Antibodies	4 (7%)	15 (5.5%)	0.7
Blood Group			
Incompatible ⁴	10 (17%)	29 (11%) ⁵	0.19

Table 11: Recipient and transplantation characteristics sorted for BKPyV viremia among 329 renal transplant recipients in the first-year post-transplantation.

¹ No BKPyV viremia's n total differed according to missing data: two patients' BMI; one patient's transplantation number; two and four patients' infection and transfusion data respectively; one non-viremic recipient's induction mAb; maintenance immunosuppression of nine recipients;

² A recipient was considered overweight if BMI > 25 Kg/m².

³ Dialysis and its duration data were missing for one non-viremic patient.

⁵ Cold ischemia time data was missing for three non-viremic patients.

⁶ Proliferation inhibitors data was missing for one viremic patient.

BMI, body mass index; mAb, monoclonal Antibody.

	BKPyV Viremia (n=59)	No BKPyV Viremia (n/total) ¹	P value
Recipient characteristics			
Male sex	42 (71%)	182/270 (67%)	0.57
Age > 50 years	37 (63%)	146/270 (54%)	0.22
BMI > 25 (Kg/m ²) ²	32 (54.2%)	145/268 (54%)	0.98
Dialysis pre-transplantation	57 (97%)	255/269 (95%) ³	0.55
Dialysis time, months, median (range)	30 (1-92)	30 (24-395) ³	0.657
First transplantation	50 (85%)	235/269(87%)	0.19
Cold ischemia time, min, median (range)	900 (36-1536)	816 (19-2413) ⁵	0.34
Transplantation complications			
Nosocomial Infection	19 (32%)	96/268 (36%)	0.59
Transfusion	9 (15%)	38/266 (14%)	0.84
Induction immunosuppression with mAb			
Basiliximab	27 (46%)	151/269 (56%)	0.14
Thymoglobulins	32 (54%)	118/269 (44%)	0.14
Maintenance immunosuppression			
Calcineurin inhibitors			
Tacrolimus	45 (76%)	179/267 (67%)	0.16
Cyclosporine A	14 (24%)	87/267 (32.5%)	0.18
Corticosteroids	50 (85%)	229/266 (86%)	0.78
Proliferation inhibitors			
Mycophenolate mofetil (MMF)	57/58 (98%) ⁶	255/268 (95%)	0.28
Everolimus	1/58 (2%)	6/268 (2%)	1

2. Post-transplantation viremia incidence in recipients according to pre-transplantation serostatus:

Donor serology analysis independent of recipient serology and vice versa demonstrated an association between the high donor IgG pre-transplantation titer and the post-transplantation infection risk (Table 12). A significant risk of Ib1 and Ib2 viremia development was observed in recipients whose donors were seropositive in collective Ia and Ib2 serology analysis (OR, 2.85; CI, 1.04 to 7.79; P=0.04). In addition, a lower infection risk was observed in seropositive recipients analyzed for Ib2 serology (OR, 0.69; CI, 0.31 to 1.55; P=0.37) compared to seronegative recipients. Although non-significant, the Ia, collective Ia and Ib2, II and IV serology analysis against BKPyV viremia development also showed a decreased infection risk with a seropositive recipient.

The infection risk can be masked when analyzing a specific BKPyV serotype serology against general viremia development. The Ib2 serology analysis against total BKPyV development for donors revealed that a recipient with a seropositive donor is around two times more likely to develop viremia (OR, 2.3, CI, 1.02, 5.18; p=0.043). Ib2 serology analysis against Ib1 and Ib2 viremia development revealed a much higher risk for infection (OR, 7.5; CI, 2.19 to 25.73; P= 0.0002).

Table 12: Post-transplantation BKPyV viremia incidence in recipients according to pre-transplantation BKPyV serostatus for serotypes Ib2, Ia, collective Ia and Ib2, IV and II.

Pre-transplant Serology	Serostatus	Total n(%)	No BKPyV Viremia n (%)	BKPyV Viremia n (%)	p value	Odds ratio (CI; 95%)	BKPyV Viremia			p value	Odds ratio (CI; 95%)
							with I serotype n (%)	with IV serotype n (%)	with II serotype n (%)		
Ib2 serotype	R+	290 (88.1%)	240 (83%)	50 (17%)	0.37	0.69	37 (13%)			0.082	0.4875
	R-	39 (11.9%)	30 (77%)	9 (23%)		(0.31 to 1.55)	9 (23%)				(0.21 to 1.10)
	D+	130 (59%)	104 (80%)	26 (20%)	0.043	2.3	25 (20%)			0.0002	7.516
	D-	92 (41%)	83 (90%)	9 (10%)		(1.02 to 5.18)	3 (3%)				(2.19 to 25.73)
Ia serotype	R+	305 (92.7%)	253 (83%)	52 (17%)	0.163	0.499	40 (13%)			0.11	0.44
	R-	24 (7.3%)	17 (71%)	7 (29%)		(0.19 to 1.26)	6 (25%)				(0.16 to 1.2)
	D+	150 (67.6%)	125 (83%)	25 (17%)	0.595	1.24	20 (13%)			0.62	1.24
	D-	72 (32.4%)	62 (86%)	10 (14%)		(0.56 to 2.74)	8 (11%)				(0.51 to 2.97)
Serotypes Ia and Ib2	R+	311 (94.5%)	258 (83%)	53 (17%)	0.088	0.4109	41 (13%)			0.16	0.4746
	R-	18 (5.5%)	12 (67%)	6 (33.3%)		(0.14 to 1.14)	5 (28%)				(0.16 to 1.34)
	D+	137 (62%)	113 (82.5%)	24 (17.5%)	0.36	1.42	23 (17%)			0.04	2.85
	D-	85 (38%)	74 (87%)	11 (13%)		(0.66 to 3.09)	5 (6%)				(1.04 to 7.79)
IV serotype	R+	286 (87%)	239 (83%)	47 (16%)	0.07	0.508		10 (3%)		1	1.48
	R-	43 (13%)	31 (74%)	12 (28%)		(0.24 to 1.06)		1 (2%)			(0.18 to 11.87)
	D+	137 (62%)	120 (87.5%)	17 (12.5%)	0.084	0.52		4 (3%)		1	0.855
	D-	85 (38%)	67 (79%)	18 (21%)		(0.25 to 1.09)		3 (3%)			

II										
serotype	R+	306 (93%)	254 (83%)	52 (17%)	0.105	0.467		2 (1%)	1	0.385
	R-	23 (7%)	16 (70%)	7 (30%)		(0.18 to 1.19)		0		(0.017 to 8.27)
	D+	52 (23.4%)	46 (88.5%)	6(11.5%)	0.339	0.63		0		
	D-	170(6.6%)	141 (83%)	29 (17%)		(0.24 to 1.62)		0	Nd	Nd

In addition, analyzing the total (mean of IgG titer against serotypes Ib2, Ia, IV, and II) BKPyV serology (Table 13) also revealed that the D+/R- group had the greatest BKPyV incidence. In addition, it showed that the viremia incidence was higher in seropositive donors than in seronegative donors (17.9% vs 12.7%) and that this trend was inverted in recipients (17% vs 37.5%).

Table 13: Post-transplantation BKPyV viremia incidence in recipients according to pre-transplantation total BKPyV serostatus.

BKPyV-specific IgG antibody titer \geq 200 were considered as positive and $<$ 200 as negative. A recipient's total BKPyV serostatus in this study represents the mean of IgG titer against serotypes Ib2, Ia, IV, and II.

Total BKPyV Serology	Serostatus	BKPyV Viremia	No BKPyV Viremia	P value
	R+ (n=313)	53 (17%)	260 (83%)	0.059
	R- (n=16)	6 (37.5%)	10 (62.5%)	
	Total	59		
	D+ (n=128)	23 (17.9%)	105 (81 %)	Nd
	D- (n=94)	12 (12.7)	82 (87.3%)	
	Total	35		
	D-/R+ (n=91)	12 (13.1%)	79 (86.9%)	Nd
	D-/R- (n=3)	0 (0%)	3 (100%)	
	D+/R+ (n=121)	21 (17.3%)	100 (82.7%)	
	D+/R- (n=7)	2 (28.5%)	5 (71.4%)	
	Total	35		

We then analyzed the post-transplantation BKPyV viremia incidence in recipients according to pre-transplantation BKPyV Serostatus of the donor-recipient pairs (Table 14).

These pairs were divided into four categories: D-/R+, D-/R-, D+/R+ and D+/R-.

It is impressive that the collective Ia and Ib2 analysis revealed a complete protection from infection in the R+/D- pair [0/82(0%)].

Table 14: Incidence of post-transplantation BKPyV viremia or serotype specific BKPyV viremia in recipients according to four different pre- transplantation BKPyV-specific antibody groups: D-/R+, D-/R-, D+/R+, and D+/R-.

BKPyV-specific IgG antibody titer \geq 200 were considered as positive and $<$ 200 as negative.

Pre-transplant Serology	Serostatus	Total n (%)	No BKPyV Viremia n (%)	BKPyV Viremia n (%)	P value	Viremia			P value
						with the I serotype n (%)	with the IV serotype n (%)	with the II serotype n (%)	
Ib2 serotype	D-/R+	81 (36%)	74 (91%)	7 (9%)	0.15	1 (1%)			0.0024
	D-/R-	11 (5%)	9 (82%)	2 (18%)		2 (17%)			
	D+/R+	114 (51%)	92 (81%)	22 (20%)		21 (19%)			
	D+/R-	16 (6.7%)	12 (75%)	4 (25%)		4 (27%)			
Ia serotype	D-/R+	67 (30.2%)	58 (87%)	9 (13%)	0.367	7 (10%)			0.47
	D-/R-	5 (2.2%)	4 (80%)	1 (20%)		1 (20%)			
	D+/R+	138 (62.2%)	117 (85%)	21 (15%)		17 (12%)			
	D+/R-	12 (5.4%)	8 (67%)	4 (33%)		3 (25%)			
Serotype Ia and Ib2	D-/R+	82 (37%)	72 (88%)	10 (12%)	0.59	5 (7%)			0.16
	D-/R-	3 (1%)	2 (67%)	1 (33%)		0 (0%)			
	D+/R+	126 (57%)	105 (83%)	21 (17%)		20 (16%)			
	D+/R-	8 (73%)	11 (5%)	3 (27%)		3 (27%)			

IV serotype	D-/R+	74 (33%)	61 (82%)	13 (17.5%)	0.03	3 (4%)	0.8
	D-/R-	11 (5%)	6 (54.5%)	5 (45%)		0 (0%)	
	D+/R+	119 (53%)	105 (88%)	14 (12%)		4 (3%)	
	D+/R-	18 (8%)	15 (83%)	3 (17%)		0 (0%)	
II serotype	D-/R+	159 (71.6%)	134 (84%)	25 (16%)	0.219	0 (0%)	Nd
	D-/R-	11 (5%)	7 (64%)	4 (36%)		0 (0%)	
	D+/R+	50 (22.5%)	44 (88%)	6 (12%)		0 (0%)	
	D+/R-	2 (0.9%)	2 (100%)	0 (0%)		0 (0%)	

When considering serotype IV serology, the highest viremia incidence was reported for the D-/R- (sero-negative donor, sero-negative recipient) group (45%) (Table 14). It was significantly higher than that of the D+/R- group (17%). However, analyzing IV serology solely against IV viremia development showed that the D-/R- and the D+/R- groups didn't replicate BkPyV serotype IV (3/15). This result difference between the two analyses' approaches stresses the importance of serostatus assessment for each BkPyV serotype. This allows a wider study perspective and supports the ability to drive more accurate conclusions.

The donor's serostatus implication in the BkPyV reactivation risk is notable when assessing Ib2 serology. Incidence of BkPyV viremia in general, and Ib viremia was always higher in sero-positive donors (Fig 24). Similarly, Ib2 pre-transplantation serology analysis showed that the lowest likelihood of Ib1 and Ib2 viremia development was in the D-/R+ group (1%), whilst the highest likelihood was in the D+/R- group (27%) with P=0.0024 (Table 14, Fig 25).

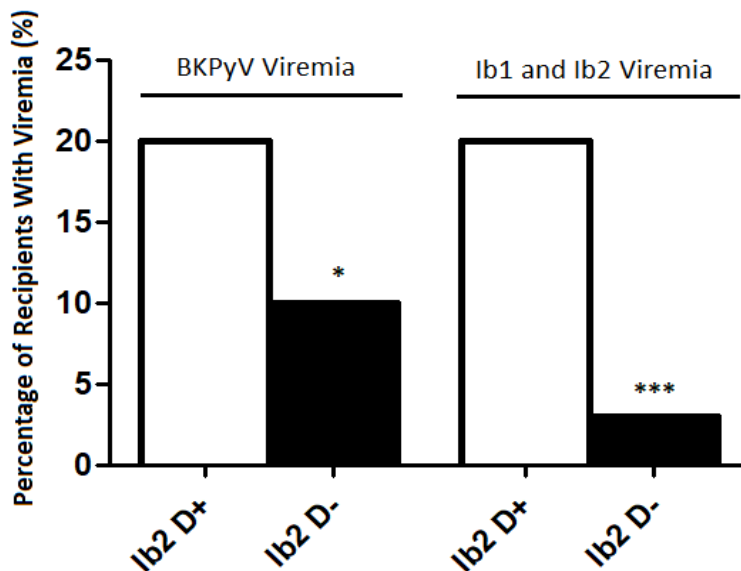


Figure 24: Percentage of viremic recipients according to the pre-transplantation donor Serostatus.

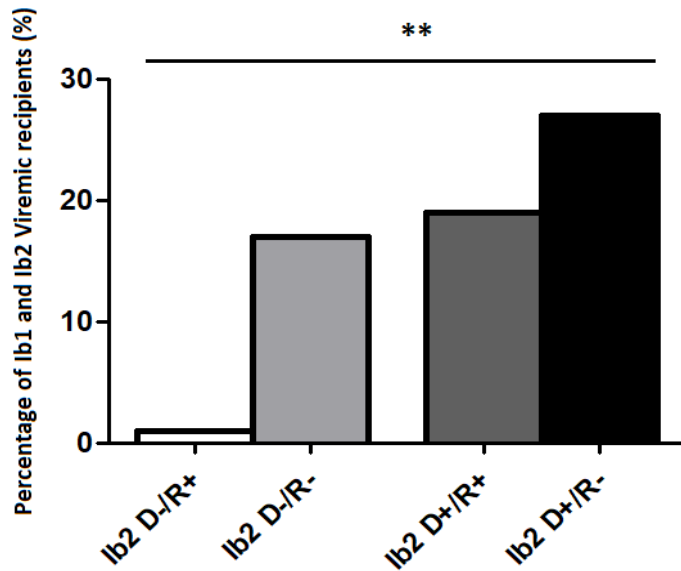


Figure 25: Percentage of Ib1 and Ib2 viremic recipients according to the donor and recipient pairs' Ib2 pre-transplantation serostatus.

3. BKPyV antibody titers and post-transplant viremia incidence:

BKPyV infection reactivation risk was evaluated in the first-year post transplantation according to donor or recipient pre-transplantation BKPyV IgG titer (Table 15). A higher viremia risk was determined in recipients with a lower total BKV serostatus (BKPyV \leq 800: OR, 3.11; 33.3% vs 13.8%; P=0.0006 and BKPyV \leq 400: OR, 5.58; 48.8% vs 14.5%; P<0.0001). A similar trend was observed when analyzing the recipient pre-transplant serostatus for each serotype: Ib2 \leq 800 (OR, 2.03; P=0.016), II \leq 400 (OR, 3.64; P=0.0002), and IV \leq 400 (OR, 2.62; P=0.0039). The lowest viremia incidence was observed in recipients whose donors had low Ib2 IgG levels (Ib2 \leq 400: OR, 0.39; P=0.01). A similar but not statistically significant trend was observed in recipients whose donors had low total BKPyV IgG titer (BKV \leq 400: OR, 0.477, P=0.055). Thus, a high viremia risk can be predicted by two factors: a high donor's and a low recipient's IgG titer. However, the IgG titer appears to be much more important in the recipient than in the donor in predicting the viremia risk in the post-transplant period.

Table 15: Recipient BKPyV viremia post-transplantation risk based on pre-transplantation BKPyV specific antibody titer.

	Titers	BKPyV Infection n=59	No BKPyV infection n=270	P value	Odds ratio	95% CI
Recipients						
Total BKPyV	≤ 1600 (n=148)	32 (22%)	116 (78%)	0.148	1.57	0.89 to 2.77
	> 1600 (n=181)	27 (15%)	154 (85%)			
	≤ 800 (n=69)	23 (33%)	46 (66%)	0.0006	3.11	1.68 to 5.73
	> 800 (n=260)	36 (14%)	224 (86%)			
	≤ 400 (n=33)	16 (49%)	17 (51%)	P< 0.0001	5.58	2.6 to 11.79
	> 400 (n=296)	43 (15%)	253 (85%)			
Serotype Ib2	≤ 1600 (n=180)	40 (25%)	140 (75%)	0.03	1.995	1.07 to 3.54
	> 1600 (n=149)	19 (13%)	130 (87%)			
	≤ 800 (n=116)	29 (25%)	87 (75%)	0.016	2.03	1.14 to 3.59
	> 800 (n=213)	30 (14%)	183 (86%)			
Serotype Ia	≤ 400 (n=125)	29 (23%)	96 (77%)	0.055	1.75	0.99 to 3.092
	> 400 (n=204)	30 (15%)	174 (85%)			
Serotype II	≤ 400 (n=54)	17 (32%)	37 (68%)	0.0002	3.64	1.887 to 7.04
	> 400 (n=275)	42 (15%)	233 (85%)			
Serotype IV	≤ 400 (n=68)	21 (31%)	47 (69%)	0.0039	2.62	1.41 to 4.86
	> 400 (n=261)	38 (15%)	223 (85%)			
Donors						
Total BKPyV	≤ 1600 (n=218)	34 (16%)	184 (84%)	0.61	0.55	0.05 to 5.48
	> 1600 (n=4)	1 (25%)	3 (75%)			
	≤ 400 (n=165)	21 (13%)	144 (87%)	0.055	0.477	0.21 to 0.95
	> 400 (n=57)	14 (24.5%)	43 (75.5%)			
Serotype Ib2	≤ 1600 (n=204)	30 (15%)	174 (85%)	0.15	0.44	0.14 to 1.34
	> 1600 (n=18)	5 (28%)	13 (72%)			
	≤ 400 (n=138)	15 (11%)	123 (89%)	0.01	0.39	0.18 to 0.81
	> 400 (n=84)	20 (24%)	64 (76%)			

Analytical performance of the proposed titer thresholds was summarized in Table 16. According to Youden's indices, antibody titers do not make it possible to discriminate in pre-graft but must be associated in 2nd intention with the categories (example R+/D+).

Table 16: Analytical performance of the proposed titer thresholds.

	Sensitivity	Specificity	VPP	VPN	Youden's index
Recipient BKPyV at threshold 1600	54%	57%	21.60%	85%	0.11
Recipient BKPyV at threshold 800	39%	83%	33.30%	86%	0.22
Recipient BKPyV at threshold 400	27%	94%	48.50%	85.50%	0.21
Recipient Ib2 at threshold 1600	68%	48%	22%	87.20%	0.16
Recipient Ib2 at threshold 800	49%	68%	25%	86%	0.17
Recipient Ia at threshold 400	49%	64.50%	23%	85.30%	0.14
Recipient II at threshold 400	29%	86%	31.50%	85%	0.15
Recipient IV at threshold 400	35.50%	83%	31%	85.40%	0.20
Donor BKPyV threshold at 1600	3%	98.40%	25%	84.40%	0
Donor BKPyV threshold at 400	40%	77%	24.50%	87.30%	0.17
Donor Ib2 threshold at 1600	14.30%	93%	28%	85.30%	0.10
Donor Ib2 threshold at 400	57%	66%	24%	89%	0.23

BKPyV infection reactivation risk was then analyzed according to the serotype of the reactivated virus. Ib2 or Ia serology was analyzed against Ib1 and Ib2 viremia development. Similarly, IV IgG titer thresholds were analyzed according to viremia development by serotype IV (Table 17). It is very notable how this change in analysis strategy can influence the significance of the obtained risk.

Table 17: Recipient BKPyV serotype specific viremia post-transplantation risk based on pre-transplantation BKPyV specific antibody titer.

Recipients	Titer	BKPyV Infection n=46	No BKPyV infection n=283	P value	Odds Ratio	95% CI
Serotype Ib2	≤ 1600 (n=180)	40 (22%)	140 (78%)	P< 0.0001	6.8	2.7 to 16.5
	> 1600 (n=149)	6 (4%)	143 (96%)			
	≤ 800 (n=116)	32 (28%)	84 (72%)	P< 0.0001	5.4	2.7 to 10.6
	> 800 (n=213)	14 (7%)	199 (93%)			
Serotype Ia	≤ 400 (n=125)	23 (18%)	102 (82%)	0.07	1.77	0.94 to 3.32
	> 400 (n=204)	23 (11%)	181 (89%)			
Serotype IV		n=11	n=318	0.01	4.95	1.46 to 16.7
	≤ 400 (n=68)	6 (9%)	62 (91%)			
	> 400 (n=261)	5 (2%)	256 (98%)			
Donors						
Serotype Ib2		n=28	n=194	0.36	2.59	0.3 to 20.2
	≤ 1600 (n=204)	27 (13%)	177 (87%)			
	> 1600 (n=18)	1 (6%)	17 (94%)			
	≤ 400 (n=138)	15 (11%)	123 (89%)			
	> 400 (n=84)	13 (15.5%)	71 (84.5%)	0.3	0.66	0.29 to 1.47

Significantly high odds ratios were obtained with lower recipient titers for serotype Ib2 (Ib2 ≤ 1600; OR, 6.8; CI, 2.7 to 16.5; P< 0.0001; Ib2 ≤ 800; OR, 5.4; CI, 2.7 to 10.6; P< 0.0001) (Table 17). Similar Ib2 serology analysis against total viremia (Table 15) may have masked the impact of BKPyV recipient serology on viremia development risk (Ib2 ≤ 1600; OR, 1.99; CI, 1.07 to 3.54; P=0.03; Ib2 ≤ 800; OR, 2.03; CI, 1.14 to 3.59; P=0.016). Although significant, these OR ratios were notably lower than those obtained with specific serotype analysis. Similarly, serotype IV recipient analysis against IV viremia solely (IV ≤ 400; OR, 4.95; CI, 1.46 to 16.7; P=0.01) revealed an elevated infection risk compared to analysis with general viremia (IV ≤ 400; OR, 2.62; CI, 1.41 to 4.86; P=0.0039). This shows that the serological titer seems interesting to evaluate, especially in the recipient.

To further validate the pre-transplantation IgG titer's association with viremia incidence, we determined both the recipients and donors' pre-transplant IgG titer average for viremic and non-viremic recipients (Fig 26). For serotypes Ib2, II and IV, viremic patients had significantly lower recipient pre-transplant IgG titer (Ib2, 1183; II, 2836; IV, 1700) compared to non-viremic patients (Ib2, 1548; II, 4010; IV, 2056) (Fig 26A). In contrary, a higher pre-transplantation donor Ib2 IgG titer (589) was significantly associated with viremia development compared to lower donor Ib2 IgG titer (357.7) in non-viremic patients (Fig 26B). Hence, it is relevant to consider the total pre-transplant BKPyV titer as a predictive tool for post-transplant viremia occurrence.

In addition, we determined the recipients and donors IgG titer average of each serotype for viremic and non-viremic recipients who specifically propagated the serotype in consideration (Fig 27). It was revealed that the association between a higher donor serotype Ib2 IgG titer and Ib1 and Ib2 viremia development was highly significant (P value < 0.001) (Fig 27B). Although analysis of Ib2 with general BKPyV viremia revealed a similar decrease of donor IgG titer in non-viremic patients, the significance of this association was partially masked (P value < 0.01) (Fig 26B). The latter comparison reflects the importance of serotype specific serology analysis with serotype-specific viremia compared to general viremia analysis. Moreover, collective Ia and Ib2 serology also revealed a highly significant association between viremia development and high donor IgG titer (P value < 0.001) (Fig 27C).

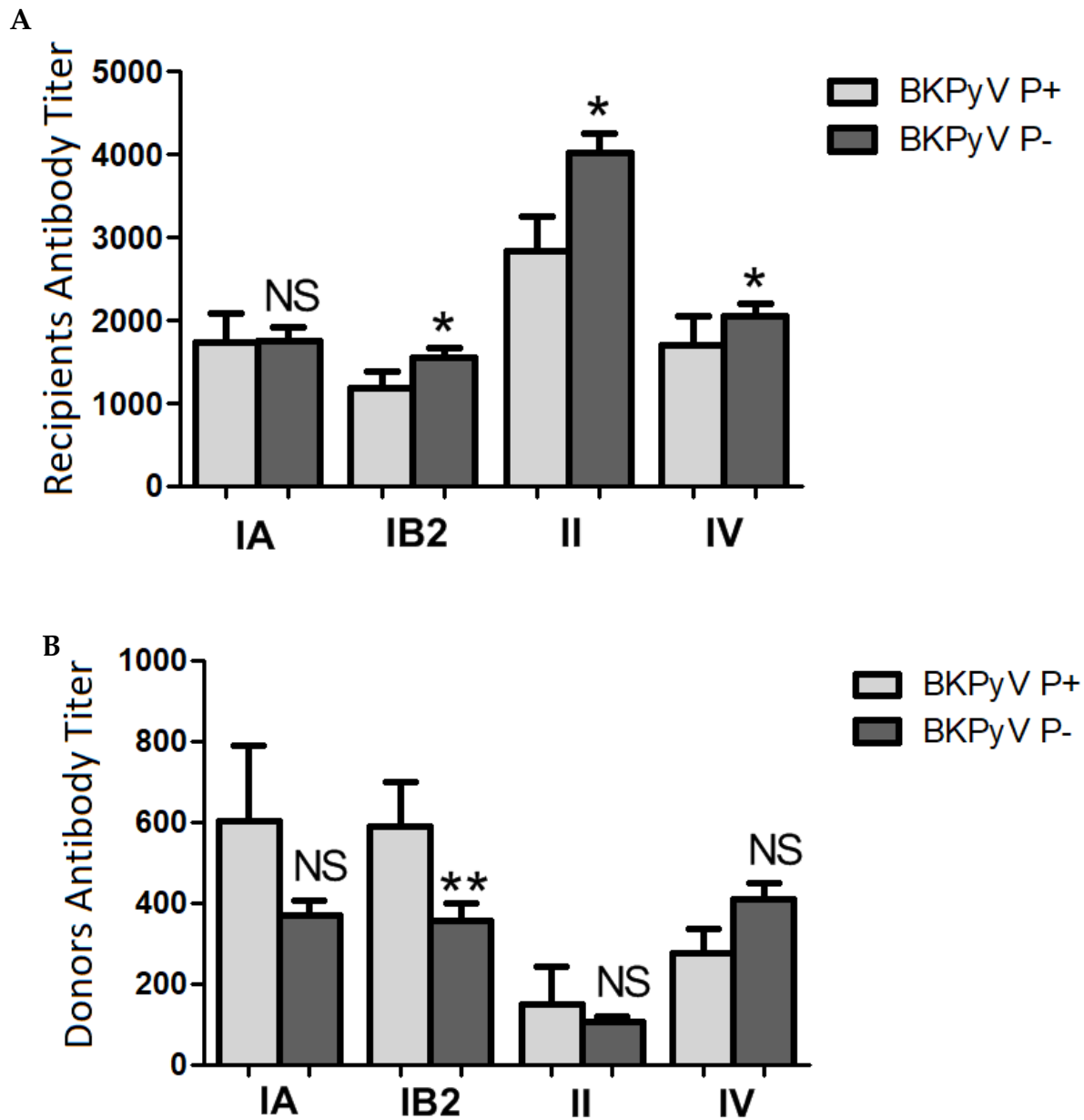
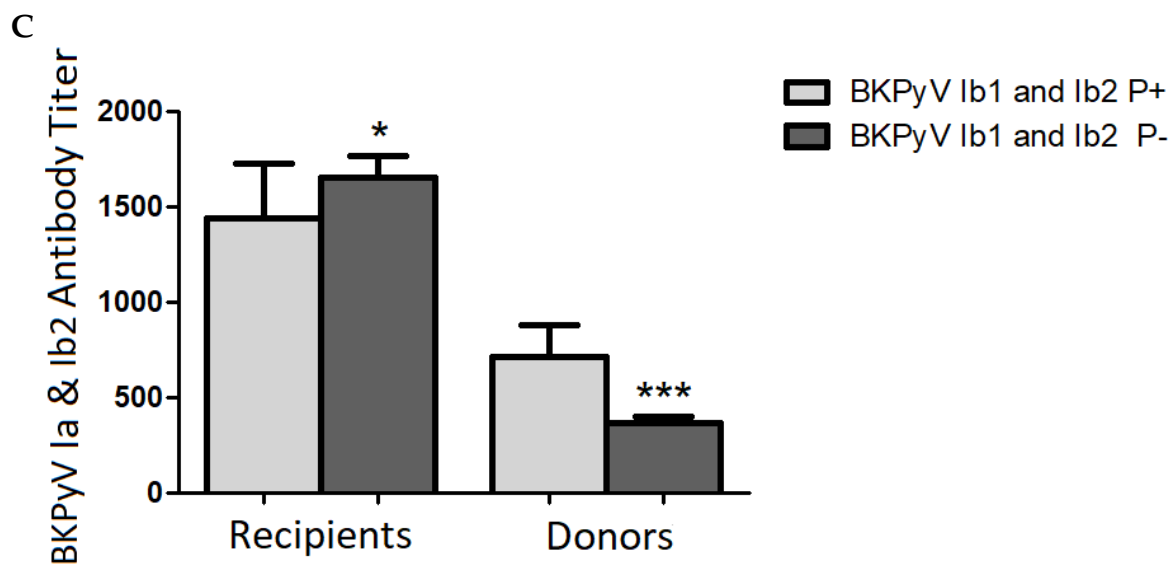
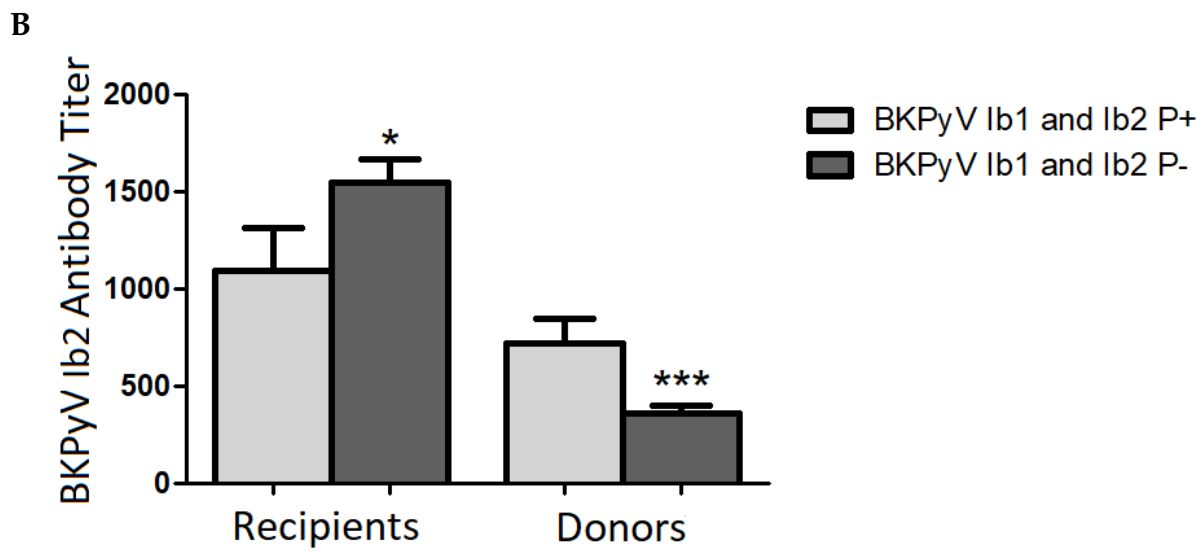
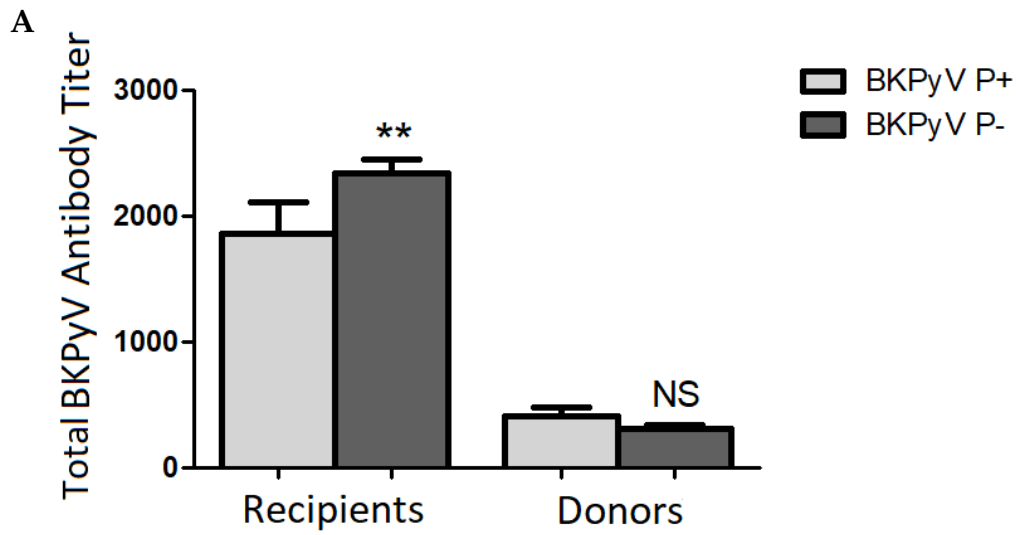


Figure 26: Mean of recipients (A) or donors' (B) antibody titer for BKPyV serotypes Ia, Ib2, II, and IV, according to post-transplant viremia development in recipients.

*P value < 0.05, significant difference; **P value < 0.01, significant difference; NS P value > 0.05, No significant difference. Some values were not plotted due to the y-axis log2 scale presentation. BKPyV P+, BK polyomavirus plasma positive; BKPyV P-, BK polyomavirus plasma negative.



D

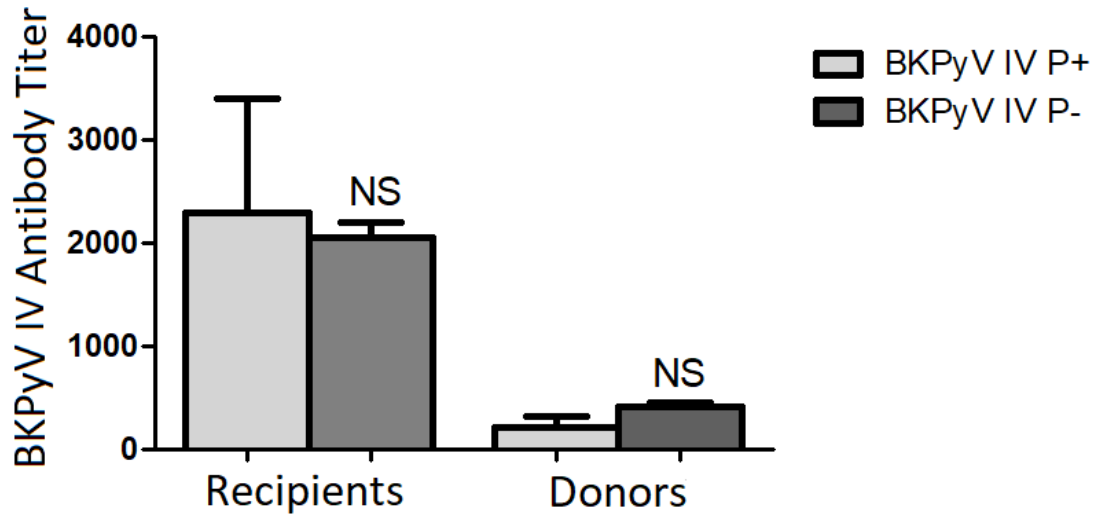
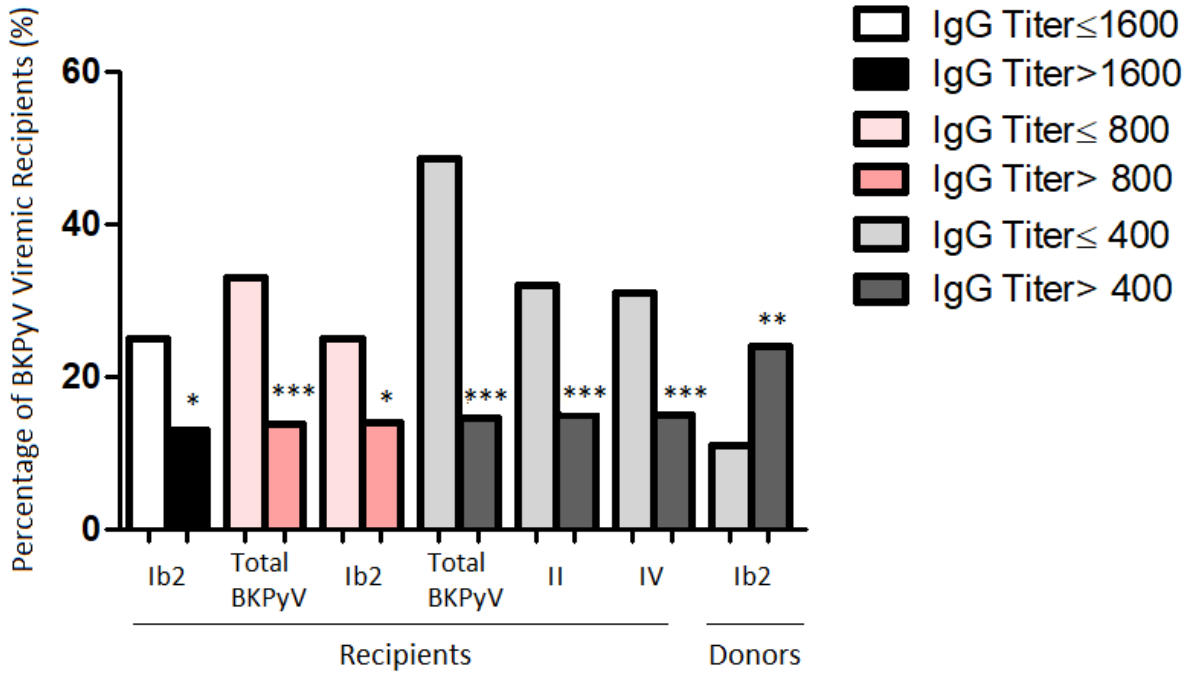


Figure 27: Mean of recipients or donors' antibody titer for BKPyV (A) or serotypes Ib2 (B), collective Ia and Ib2 (C) and IV (D), according to serotype- specific post-transplant viremia development in recipients.

*P value < 0.05, significant difference; **P value < 0.01, significant difference; ***P value < 0.001, significant difference; NS P value > 0.05, No significant difference. Some values were not plotted due to the y-axis log2 scale presentation.

It is evident from our work that the pre-transplantation anti-BKPyV antibody titer is significantly associated with BKPyV viremia development after transplantation. A high donor antibody titer and a low recipient antibody titer both represent risk factors for BKPyV infection reactivation. We summarized our significant results regarding the effect of different titer thresholds on the infection incidence in the recipients in figure 28.

A



B

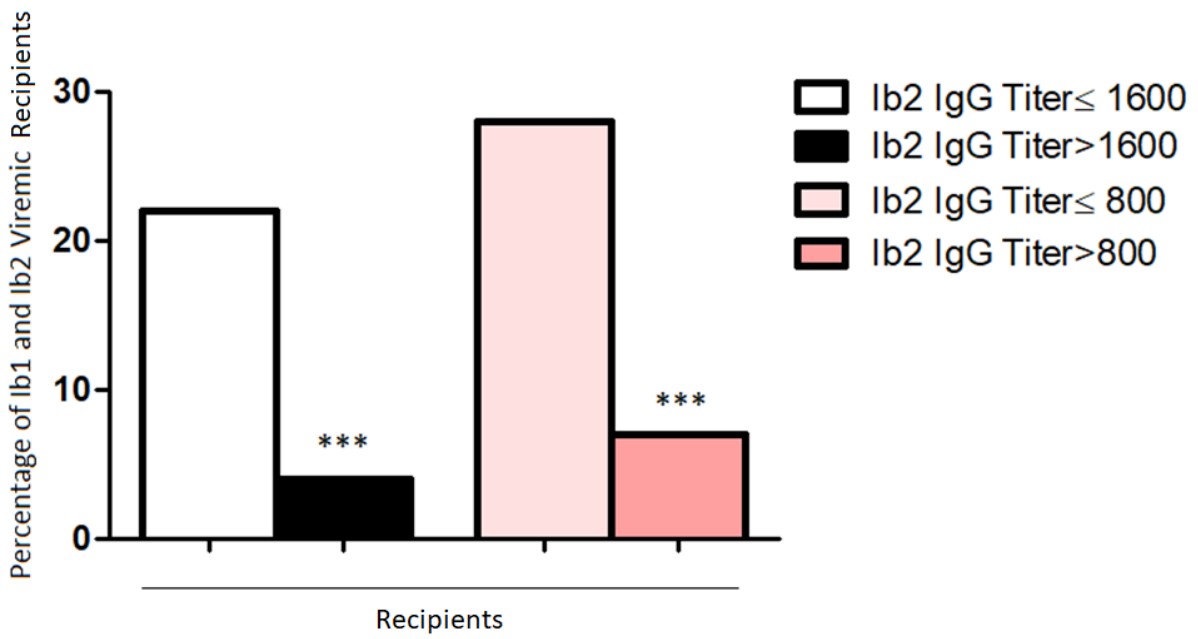


Figure 28: Percentage of recipients with total BKPyV viremia (A) or Ib1 and Ib2 viremia (B) according to different BKPyV IgG titer thresholds.

4. Viremia level according to the antibody titer

Assessment of high-level viremia ($> 4 \log_{10}$ c/ml; defined as presumptive nephropathy development) risk was performed based on recipients or donors mean IgG titer for serotypes Ia, Ib2, II, and IV (Fig 29). The average donors' antibody titer for serotypes II and IV was significantly higher in patients with viremia $> 4 \log_{10}$ c/ml (II, 109; IV, 374.8) compared to those with low-level viremia (II, 36.36; IV, 163.6) (Fig 29A). Although non-significant, the same trend was obtained for serotypes Ia and Ib2. In contrast, patients with viremia $> 4 \log_{10}$ c/ml demonstrated a non-significant lower recipient IgG titer for all serotypes (Fig 29B). Considering the BKPyV serology, it also showed that a lower recipient's IgG titer was associated with presumptive nephropathy (viremia $> 4 \log_{10}$ c/ml) risk (Fig 29C), but the observed association was not significant. Moreover, the serotype-specific analysis (matching serotypes for serology and viremia) revealed a similar trend (Fig 30). Although non-significant, lower recipient IgG titer were always associated with viremic patients presenting viremia $> 4 \log_{10}$. In contrary, higher donor Ib2 IgG titer was significantly correlated with higher Ib1 and Ib2 viremia level (Fig 30A).

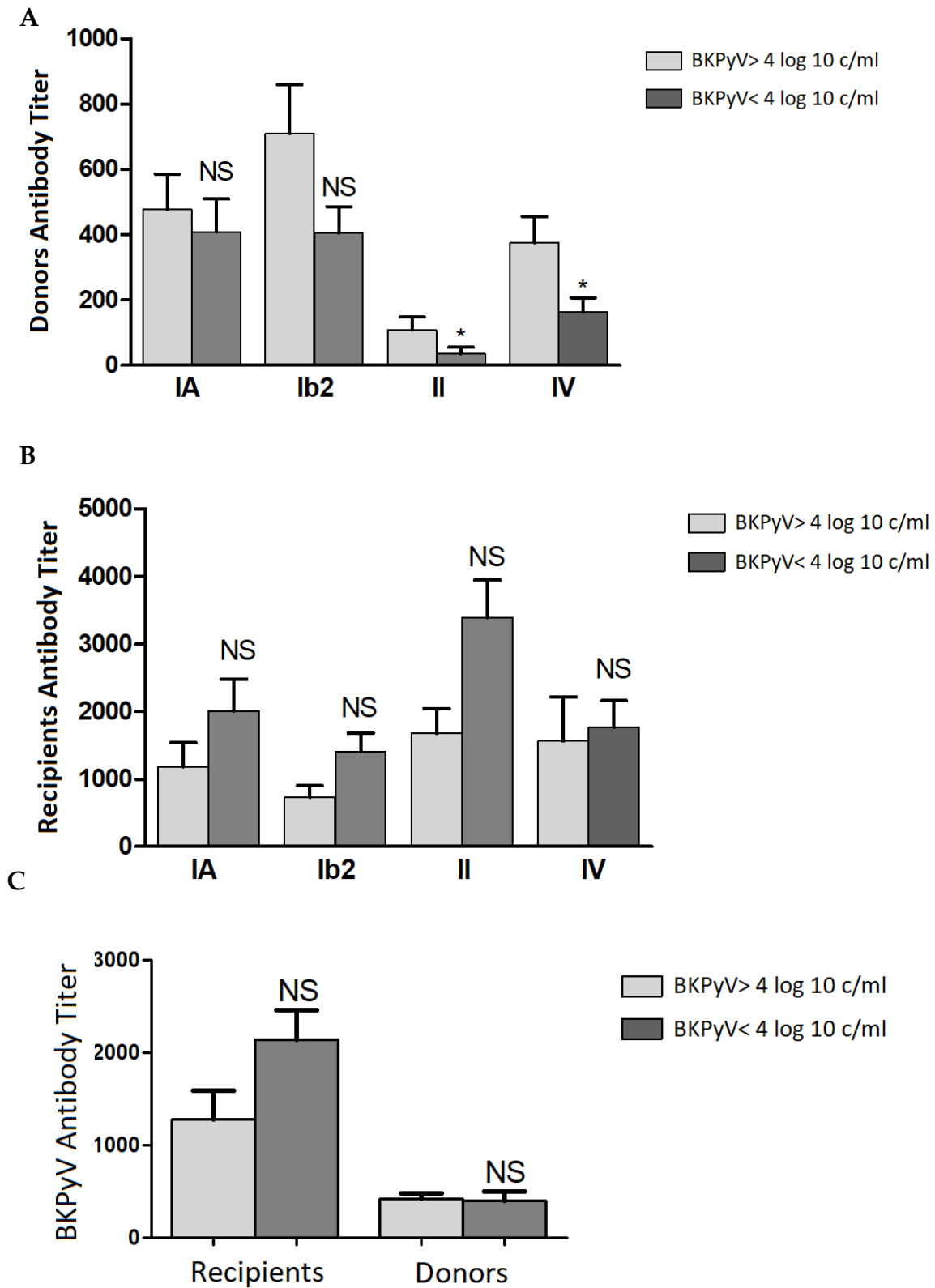
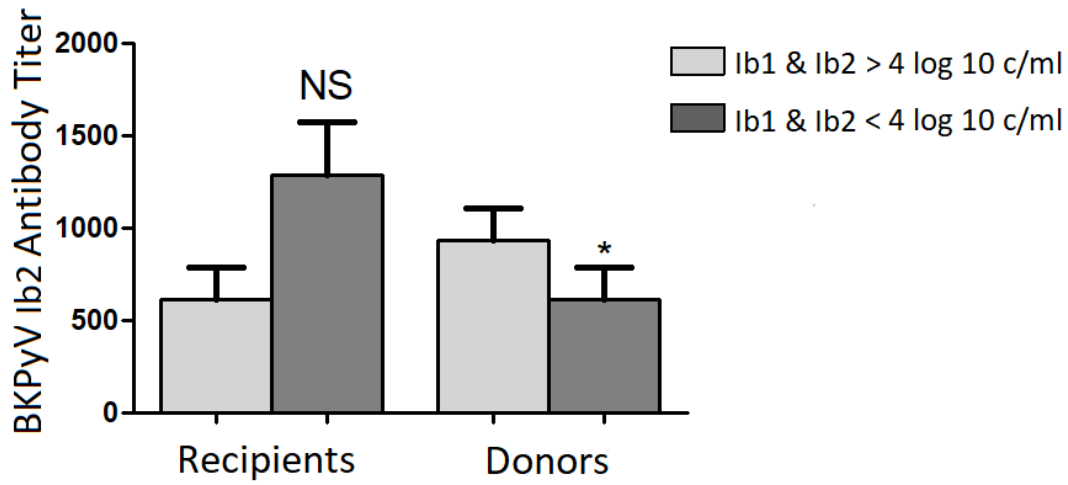


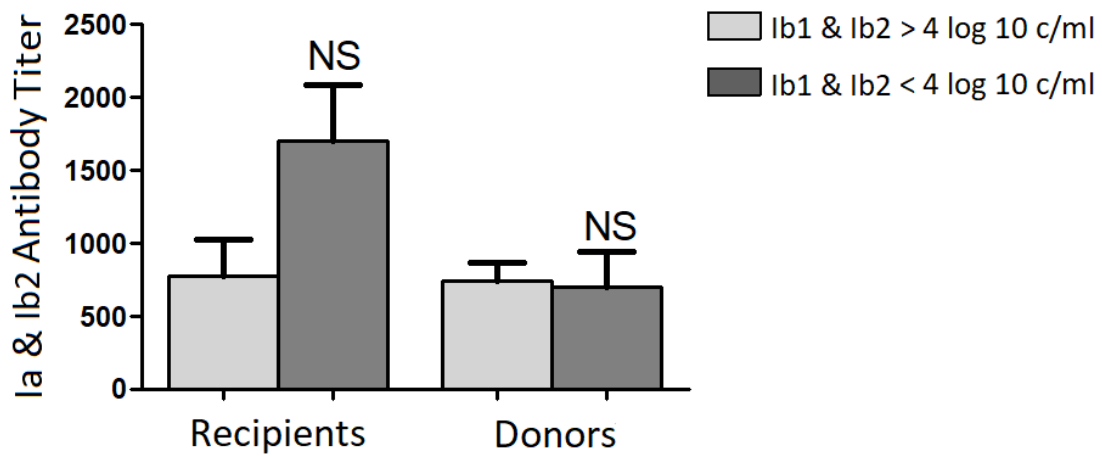
Figure 29: Mean donors' (A) or recipients' (B) BKPyV antibody titer levels for BKPyV serotypes Ia, Ib2, II, and IV or that of the total BKPyV IgG titer (C) in recipients with viremia levels BKPyV < 4log 10 or BKPyV > 4log 10 (presumptive nephropathy).

*P value < 0.05, significant difference; NS P value > 0.05, No significant difference.

A



B



C

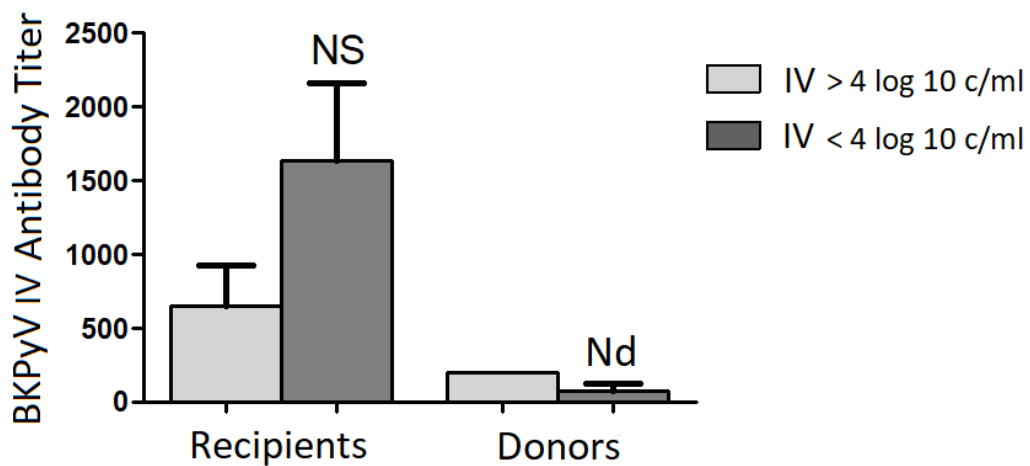


Figure 30: Mean donors' or recipients' I b2 (A), or collective I a and I b2 (B) or IV (C) antibody titer levels in recipients according to the serotype-specific viremia intensity.

*P value < 0.05, significant difference; NS P value > 0.05, No significant difference; Nd statistics can't be determined.

Categorizing recipients according to donor and recipient pairs' serostatus reveals that recipients with Ib2 sero-positive donors are more likely to develop high level Ib1 and Ib2 viremia (Fig 31). Actually, 50% of Ib2 D+/R- and 33% of Ib2 D+/R+ viremic patients had high level Ib1 and Ib2 viremia. All recipients with Ib2 sero-negative donors had low-level viremia only (Fig 31 A). Similarly, only serotype IV D+/R+ recipients developed serotype IV viremia > 4 log₁₀ c/ml, further supporting that a sero-positive donor increased the high-level viremia risk (Fig 31 B). Consequently, a high donor serostatus may not only predict viremia risk, but also the infection's severity.

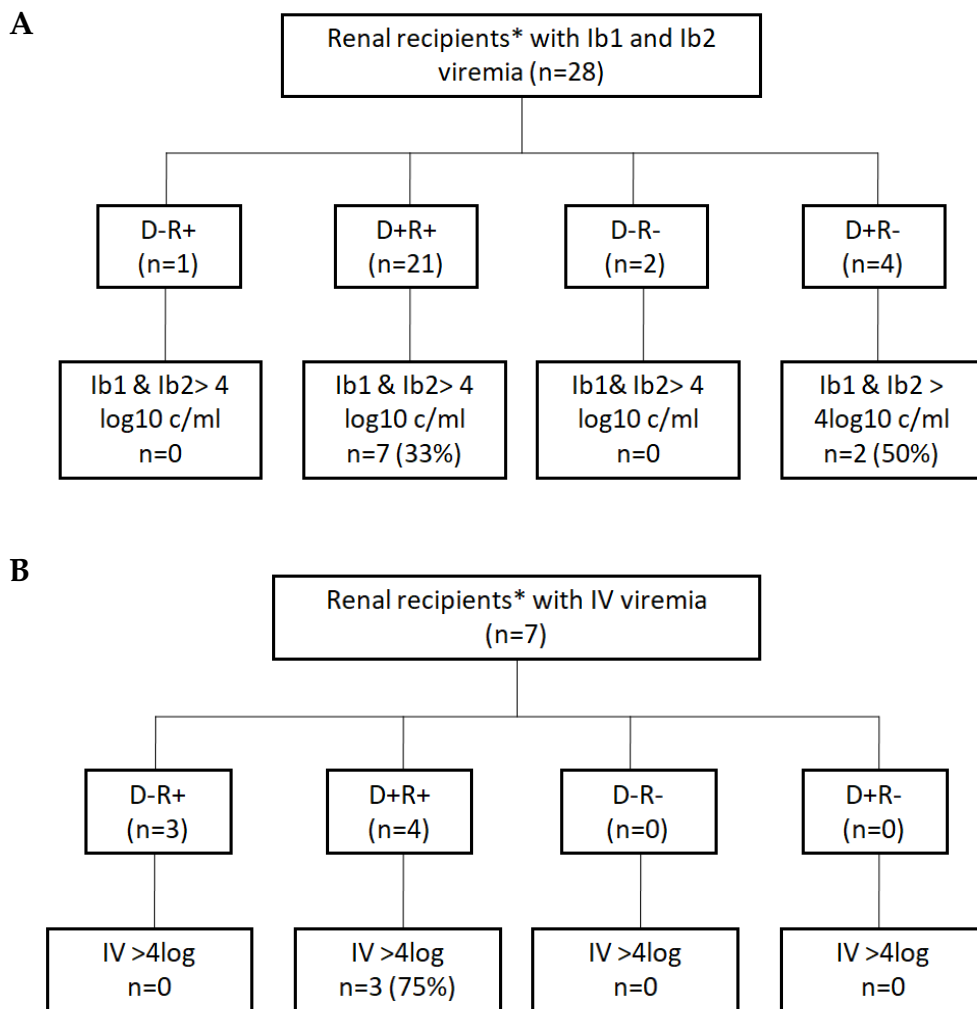


Figure 31: Renal recipients* distribution based on: the pre-transplantation donor and recipient BKPyV serotype Ib2 sero-status and viremia (A), and serotype IV sero-status and viremia (B).

*Only recipients whose donors were included in the study.

5. BKPyV antibody titer levels and late viremia onset:

We investigated whether the pre-transplantation IgG titer had an effect on the viremia temporal onset (Fig 32). For each serotype (Ia, Ib2, II, and IV), we determined the recipient and donors' IgG titer mean for patients who developed viremia at different post-transplantation time points. After analyzing the donors' Ia and Ib2 IgG titers, we found that a decreasing antibody titer was associated with later viremia development (Fig 32A). This association was only significant for serotype Ib2 (1-2 months, IgG titer=900; 3 months, IgG titer=807; 4-6 months, IgG titer=188.8). For all serotypes, we observed that patients who developed viremia more than 4 months post transplantation had higher IgG titer compared to those with viremia at months 1, 2 and 3. But only results for serotype II were significant (Fig 32B). Although not statistically significant, the total BKPyV IgG titer analysis showed that later viremia onset (>month 4) was associated with a lower donor but higher recipient titer (Fig 32C).

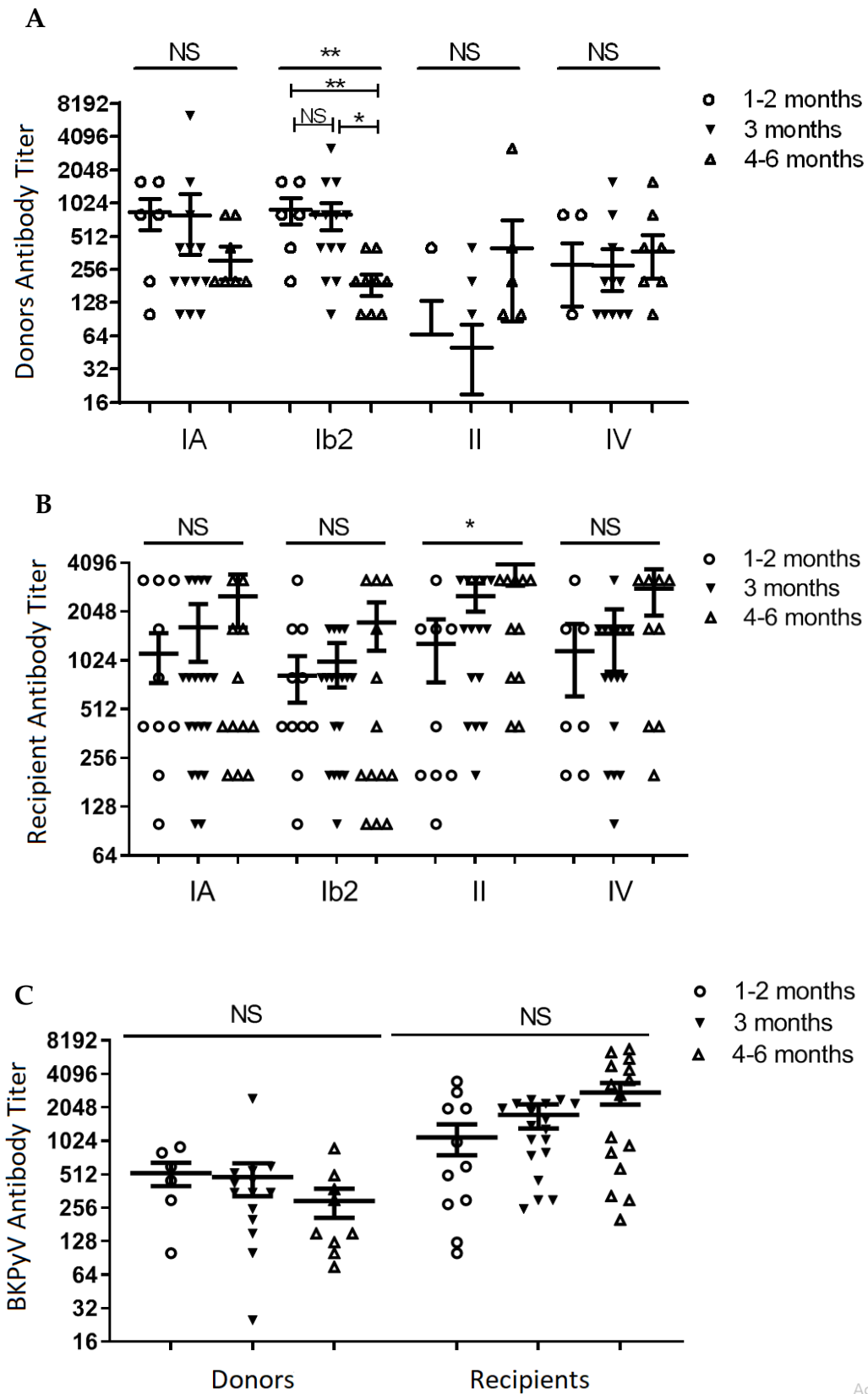


Figure 32: Post-transplant BKPyV viremia kinetics according to the pre-transplant recipients (A) or donors (B) BKPyV antibody titer mean for each serotype, or that of the total BKPyV IgG titer (C).

*P value<0.05, significant difference; ** P value<0.01, significant difference; NS P value>0.05, No significant difference. Some values were not plotted due to the y-axis log2 scale presentation.

6. Onset of BKPyV viremia:

To explore further the observed correlation between pre-graft BKPyV serology and BKPyV infection, Kaplan Meier curves were plotted (Fig 33). They presented the viremic recipients' percentage in the first-year post-transplantation based on the donor/recipient pairs' serology. BKPyV viremia occurred at a shorter time in the Ib2 D+/R- group compared with the other groups (Fig 33A). Both groups with sero-negative donors (D-/R- and D-/R+) showed a delayed viremia onset compared to groups with a sero-positive donor (D+/R- and D+/R+). Similarly, the total BKPyV D+/R- group had a shorter BKPyV viremia onset time compared with the other groups (Fig 33B).

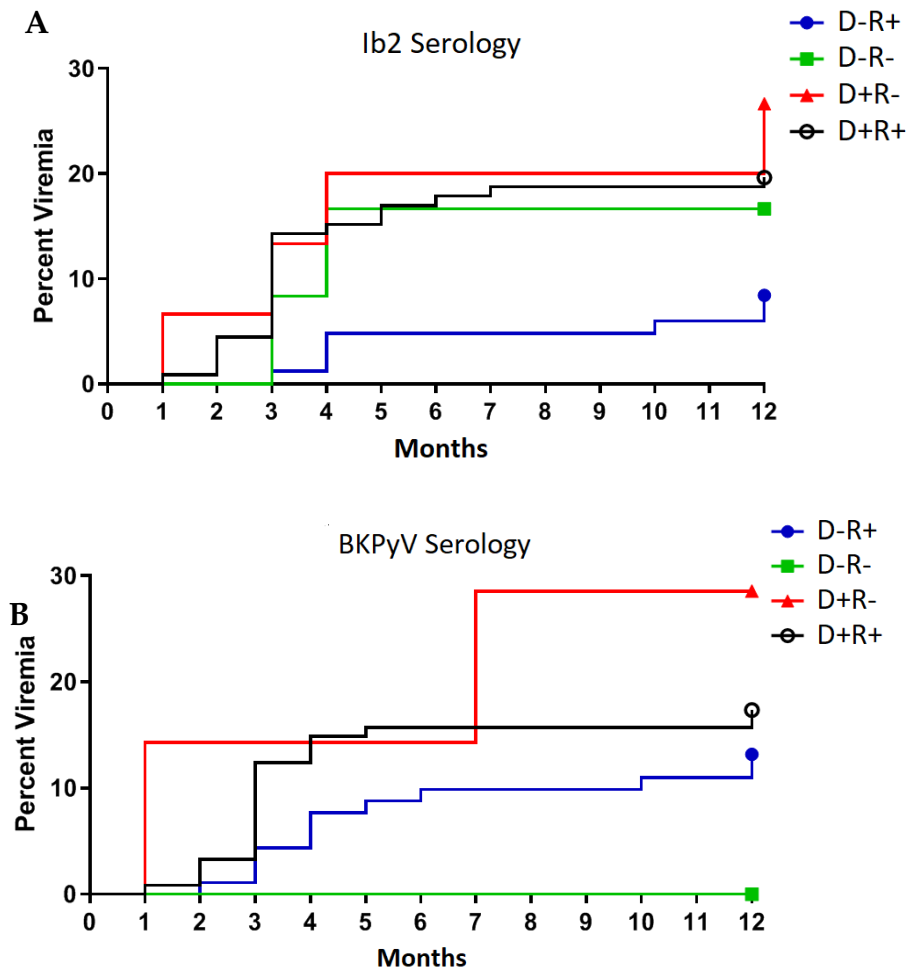


Figure 33: Kaplan-Meier curves showing BKPyV viremia percentage during the first year post-transplantation according to four distinct pre-transplantation Ib2-specific (A) or BKPyV-specific (B) IgG groups: D-/R+, D-/R-, D+/R+ and D+/R-.

IV. DISCUSSION

BKPyVAN is a troublesome disease induced by potent immunosuppressive regimes post-renal transplantation [79]. With around 1 to 10% incidence in KTRs, it is a major renal allograft dysfunction cause that can lead to renal nephropathy and even graft rejection [76]. BKPyVAN is managed by reducing immunosuppression, which may increase the acute rejection risk and may be unsuitable for all patients [79]. It is relevant to identify and validate new BKPyV reactivation risk factors, since BKPyVAN currently lacks an efficient treatment. A high donor IgG titer and a low recipient IgG titer have been identified as risk factors for BKPyV infection post-transplantation [111]. Validating these two risk factors is complicated due to the absence of an agency-approved and commercially accessible BKPyV immune-assay [112]. In addition, there are currently no clear guidelines for BKPyV serology assessment or sero-positivity definition [113].

In this retrospective study, we used ELISA to measure pre-transplant serum antibodies against four different BKPyV serotypes. We chose ELISA because it is less technically and time demanding than the neutralization assay, and cheaper than the LUMINEX multiplex immune assay which requires expensive machinery and products. Moreover, we used for the first time a commercial BKPyV VP1 antibody for inter-plate normalization. Hence, it is easier to implement an ELISA assay as a routine pre-transplant assay in clinics where the normalization well can be universal between independent laboratories.

Only 0.9% of recipients in our cohort (n=551) were sero-negative to the four studied serotypes. This supports our theory that the total BKPyV sero-prevalence is around 99%. The BKPyV seroprevalence was lower among donors compared to recipients. This can be explained by the fact that the renal recipients in our cohort were patients who have suffered from kidney-related diseases for notable time durations. This may have made them more susceptible to BKPyV infection. Conceivably, recipients may have experienced more intense primary infections or undetected reactivations when still in their immunocompetent state. Thus, recipients are more likely to have higher IgG titers in their serum than donors.

After transplantation, 59 (18%) of our recipients developed BKPyV viremia, a result similar to recent studies [117,139]. The least serotype propagated in viremic recipients was II (only 3.3%). This raises questions about the virulence difference between different BKPyV

serotypes. It also raises questions about the renal cells' permissiveness to BKPyV serotype II. BKPyV has a very high tropism, and BKPyV serotype II may have targeted other cells during the primary infection. This explains the serotype II sero-prevalence level of 64.9% in our cohort.

Similar to Smith et al [145], we demonstrated that post-transplant BKPyV viremia development was significantly associated with a sero-negative recipient. These findings contradict earlier studies which suggested that a positive recipient serostatus didn't confer protection against BKPyV infection [91, 144]. In agreement with previous studies [117, 140], we confirmed that a seropositive donor is strongly associated with BKPyV infection

Hirsch et al had previously proposed that the D+/R- group is not the highest risk group [77], but they used the unreliable HIA for BKPyV titer assessment. We, however, demonstrated that the lowest Ib1 and Ib2 viremia development likelihood was in the D-/R+ group (1%) and the highest in the D+/R- group (27%). Sood et al also demonstrated that the D+/R- group had the highest infection incidence [102], but proposed that the lowest incidence was in the D-/R- group. We believe that this donor-recipient pairs' categorization (D/R) is insufficient for all patients' stratification. For example, in our cohort of 329 patients, 83 patients were D-/R+ and 15 were D+/R- for serotype Ib2. This leaves the rest (n=231), without a classification strategy into a low or high-risk group. An alternative criterion taking into account these 'in-between' patients is needed to aid in BKPyV serology-based decision making before transplantation.

We also showed that a higher donor's IgG titer was associated with presumptive nephropathy development. A higher donor IgG may reflect a higher BKPyV infection degree, consequently a higher number of infected cells in the renal graft. This explains the increased infection intensity in recipients with high-titer donors. Similarly, Wunderink et al had proposed that BKPyVAN occurrence was strongly associated with a high donor's IgG levels [5].

Regarding viremia onset, a higher recipient's IgG titer probably reflected a stronger anti-BKPyV humoral immunity that could have delayed viremia development after transplantation. In contrary, a highly sero-reactive donor may have transmitted BKPyV through the graft to the recipient. The latter replicates BKPyV early after transplantation. Recipients with a later viremia onset may have contracted the infection from a source other than the donor.

We also highlighted the importance of IgG measurement against most BKPyV serotypes (i), and BKPyV genotyping after infection reactivation (ii). Results' discrepancies were obtained when analyzing a serotype's serology against total or serotype-specific viremia development.

For example, Ib2 serology analysis against total viremia resulted in a lower odds ratio than that from analysis with Ib1 and Ib2 viremia. Hence, single-serotype analysis masked the impact of BKPyV recipient serology on viremia development risk.

Many previous studies analyzed only Ib2 serotype serology against viremia developed with all BKPyV serotypes. A recipient's Ib2 sero-positivity may not protect him/her against viremia development by another BKPyV sero-type. Similarly, a Ib2 sero-positive donor may not be the infection source if the developed infection is by a different BKPyV serotype.

ELISA cross-reactivity may have contributed to the study's limitations, so we started our serum titration with a 1/100 dilution. The measurement of both non-neutralizing and neutralizing BKPyV antibodies represented another limitation. However, a titer value that might be determined by ELISA as a BKPyV reactivation risk represents the titer obtained from total antibody measurement. If the technique becomes a routine clinical test, the total antibody will be measured and assessed according to literature recommendations.

Despite the large size of our cohort, our results were complicated sometimes by certain subgroup analysis. In total, only two and eleven patients developed serotypes II and IV viremia respectively. Statistical power and precision are decreased by this drastic decrease in a subgroup's sample size compared to that of the total cohort.

In conclusion, we demonstrated that the recipients, the donors and the recipient-donor pair's serostatus represent a marker for BKPyV reactivation. Moreover, the donor's serostatus is a risk factor for this reactivation's extent and severity. We also demonstrated the increased value of serology assessment against multiple BKPyV serotypes, compared to the more common single BKPyV serology measurement.

Moreover, we highlighted the importance of determining the BKPyV IgG titer for serotypes Ib2, Ia, IV and II. These variables can be used as an additional asset to categorize patients into high and low risk recipients, especially alongside the insufficient current categorical classification into D-/R+, D+/R-, D+/R+ and D+/R-.

Ultimately, we proposed a reliable normalization step for the BKPyV IgG ELISA test and clear definitions for cutoffs and sero-positivity.

V. CONCLUSION

BKPyV reactivation in renal graft recipients can lead to grave complications such as nephropathy and graft loss. The current absence of specific and effective anti-BKPyV treatments places immunosuppression reduction or modulation as the sole choice for BKPyV infection management. The problem is that therapeutic immunosuppression reduction represents a risk factor for acute rejection. In addition, it is an unsuitable therapy choice for many patients. It is therefore necessary to develop alternative strategies for BKPyV infection management in KTRs. Patient stratification into low and high-risk groups based on BKPyV pre-transplantation serology may decrease the BKPyV incidence. It provides clinicians with a tool to customize immunosuppression regimes according to the assessed reactivation risk. Moreover, high-risk patients may be subject to closer and more frequent BKPyV screening, in an attempt to prevent the infection's progression into BKPyVAN. However, routine BKPyV pre-transplant serology implementation into clinics is complicated by many factors like guidelines absence and cutoff definitions.

We confirmed the correlation between the BKPyV reactivation risk post-transplantation and each of the following:

- ❖ The recipient pre-transplantation serostatus
- ❖ The donor pre-transplantation Serostatus
- ❖ The donor-recipient pairs pre-transplantation Serostatus

We demonstrated that the D+/R- group represented the high-risk group for infection, while the D-/R+ group was the low risk group.

In addition, we determined that the donor serostatus can be a marker for BKPyV infection severity after transplantation. High donor pre-transplant IgG titer was associated significantly with higher viremia levels and presumptive nephropathy development.

Moreover, we identified a relationship between pre-transplant BKPyV serology and the BKPyV viremia temporal onset. Lower donor titers were associated with later viremia onset refuting the assumption that a sero-positive donor is always the infection source.

We also developed a standardized ELISA technique with a commercial anti-BKPyV antibody that is effective, fast and relatively inexpensive for clinical utilization. Moreover, this technique encompassed clear cutoff and sero-positivity definitions.

Moreover, we showed the importance of the BKPyV serotype-specific serology assessment and correlation with the relevant viremia development. It reflected that the truth about BKPyV serology-infection correlation could have been masked in previous studies that only assessed the BKPyV Ib2 serotype serostatus.

In the light of this study:

- ❖ We call for the acceleration of BKPyV serostatus screening implementation as a pre-transplant routine test in transplantation centers. This allows clinicians to closely monitor at risk patients who should: be subject to earlier and more regular BKPyV screening (i), and receive customized immunosuppression regimes (ii).
- ❖ The development of a multivalent BKPyV vaccine that can be administered at a young age may prove efficient in increasing the R+ profile among the general population.

VI. SUMMARY IN FRENCH

L'impact de la sérologie pré-greffe sur le risque de réactivation du BKPyV après une transplantation rénale

Abstract

Contexte : La néphropathie associée au virus BK (BKPyVAN) est une pathologie observée chez les receveurs d'une greffe rénale suite à une réactivation du virus BK (BKPyV). BKPyVAN peut évoluer vers un dysfonctionnement de la greffe et n'a actuellement aucun traitement, rendant la réduction de l'immunosuppression comme seul choix thérapeutique. Cependant, cette réduction s'avère inadaptée ou non applicable conduisant à une augmentation du risque de rejet aigu. Ainsi, des marqueurs prédictifs en pré-greffe de réactivation de l'infection à BKPyV sont nécessaires pour l'identification des patients à haut risque.

Méthodes : nous avons mené une étude rétrospective pour évaluer la corrélation entre le statut sérologique du BKPyV en pré-transplantation et l'incidence de l'infection par BKPyV en post-transplantation. Des sérums de 329 receveurs et 222 donneurs appariés ont été testés pour les anticorps anti-BKPyV contre quatre sérotypes de BKPyV par un test IgG ELISA à base de VLPs, la charge virale du BKPyV a été surveillée pendant au moins 1 an après la transplantation par PCR.

Résultats : 80 (24%) receveurs étaient viruriques et 59 (18%) receveurs étaient virémiques en post-transplantation. Un risque élevé de virémie à BKPyV a été observé pour les receveurs qui avaient un titre moyen d'anticorps pour tous les sérotypes ≤ 400 avant la transplantation (Odds ratio [OR], 5.58; intervalle de confiance à 95% [IC], 2.60-11.79 ; $P < 0.0001$). De plus, les receveurs de reins à partir de donneurs avec un titre moyen d'anticorps anti- BKPyV ≤ 400 avaient un risque de virémie à BKPyV plus faible (OR, 0.47; CI, 0.21-0.95 ; $P = 0.055$). Une tendance similaire a été observée lors de l'analyse indépendante de chaque sérotype. De plus, un titre moyen d'anticorps plus faible chez les donneurs était associé à un début tardif de virémie à BKPyV (> 4 mois).

Conclusions : Le titre moyen d'anticorps anti-BKPyV du donneur et du receveur peut servir d'outil prédictif pour gérer l'infection clinique avec BKPyV, en identifiant les patients à haut risque de réactivation du virus.

Mots-clés : virus BK ; néphropathie associée au BKPyV ; réactivation du BKPyV ; statut sérologique du BKPyV ; séoprévalence du BKPyV ; technique sérologique ; sérologie du virus BK ; transplantation rénale

Introduction

Le polyomavirus BK (BKPyV) est un virus à ADN appartenant à la famille des *Polyomaviridae*. Les génotypes I (2 sérotypes), II, III et IV de BKPyV se comportent comme cinq sérotypes distincts [15] qui sont extrêmement répandus dans la population générale. L'infection primaire par BKPyV est généralement asymptomatique chez les individus immunocompétents. Cependant, un traitement immunosuppresseur puissant expose les greffés rénaux à un risque de réactivation du BKPyV et de progression de la virémie [72, 148]. La persistance de l'infection peut conduire à une néphropathie associée au BKPyV, généralement associée à un rejet de greffe [149]. Pour limiter la progression des patients vers la BKPyVAN, les recommandations actuelles impliquent chez les receveurs un suivi régulier de la virurie et de la virémie à BKPyV après la procédure de transplantation [150, 151]. Pourtant, environ 8% des patients transplantés rénaux développent la BKPyVAN, dont la majorité souffre d'un dysfonctionnement et d'une perte de greffe [152]. Comme il n'existe que des études limitées sur le traitement antiviral contre le BKPyV [153,154], la prise en charge de la BKPyVAN repose sur une diminution judicieuse de l'immunosuppression et une surveillance du rejet aigu [155]. Cependant, des études démontrent que le risque de la BKPyVAN n'est pas totalement éliminé par le dépistage de la virémie [156], et que la stratégie de réduction de l'immunosuppression ne convient pas à tous les patients (i), et peut augmenter le risque de rejet aigu (ii) [157]. Plusieurs facteurs de risque ont été proposés pour la réactivation du BKPyV en post-transplantation tels que le sexe masculin, l'âge avancé et l'immunosuppression puissante [158]. Néanmoins, il est important d'identifier des facteurs supplémentaires qui permettent une meilleure stratification des patients à haut risque. Deux facteurs de risque de réplication précoce du BKPyV en post-transplantation ont été proposés : un titre élevé d'anticorps anti-BKPyV chez le donneur et un titre bas chez le

receveur [111]. Cependant, un nombre limité d'études évaluent cette hypothèse : seulement douze études, dont deux utilisent la méthode non robuste d'inhibition de l'hémagglutination pour la détermination de statut sérologique. De plus, certaines de ces études incluent un nombre relativement faible de patients et d'autres n'incluent pas les donneurs dans leur cohorte. Il convient également de mentionner la contradiction dans leurs résultats, dont certains étant en faveur de l'hypothèse précédente et d'autres contre. Nous avons résumé ces études et leurs caractéristiques dans le tableau 2 d'une revue que nous avons publiée récemment [113]. Wunderink et al ont démontré une forte corrélation entre l'infection par le BKPyV en post-transplantation et les niveaux d'IgG anti-BKPyV en pré-transplantation. Les auteurs ont mesuré le titre d'IgG en utilisant à la fois le test LUMINEX et ELISA, mais n'ont évalué que des anticorps contre le sérotype Ib de BKPyV [5]. En mesurant les anticorps neutralisants anti- BKPyV Ia, II et IV, une étude réalisée en 2018 par Solis et al a soutenu le rôle protecteur de titre d'anticorps élevé chez le receveur. Cependant, les auteurs ont utilisé le test de neutralisation, plus exigeant techniquement, long à mettre en place, lequel est moins adapté aux mesures cliniques [139]. Nous avons cherché à évaluer l'hypothèse mentionnée en évaluant le statut sérologique du BKPyV par rapport à quatre sérotypes différents du virus : Ib2, Ia, IV et II. Nous émettons l'hypothèse qu'un titre d'anticorps en pré-transplantation positif contre un sérotype peut ne pas conférer à un receveur une protection contre une infection par un autre sérotype. De plus, il est important de vérifier si la sérologie en pré-greffe pour quatre sérotypes du BKPyV présente un intérêt supplémentaire pour le risque d'infection par BKPyV en post-transplantation. Dans cette étude rétrospective, nous avons évalué le statut sérologique total du BKPyV de 329 greffés de rein (KTR) et de 222 donneurs appariés. Ensuite, nous l'avons corrélée avec l'incidence de virémie à BKPyV en post-transplantation en utilisant pour la première fois un anticorps commercial pour la normalisation inter-plaque. En plus de standardiser notre technique, nous avons adopté une définition pour notre seuil de positivité des patients séropositifs.

Discussion

La BKPyVAN est une maladie incommode induite par de puissants régimes immunosuppresseurs en post-transplantation rénale [79]. Avec une incidence d'environ 1 à 10% chez les greffés de reins, il s'agit d'une cause majeure de dysfonctionnement des allogreffes rénales qui peut entraîner une néphropathie rénale et même un rejet de

greffe [76]. La BKPyVAN est gérée en réduisant l'immunosuppression, ce qui peut augmenter le risque de rejet aigu et peut ne pas convenir à tous les patients [79]. Il est pertinent d'identifier et de valider des nouveaux facteurs de risque de réactivation de BKPyV, puisque BKPyVAN manque actuellement d'un traitement efficace. Un titre d'IgG élevé de donneur et un titre d'IgG bas de receveur ont été identifiés comme facteurs de risque d'infection par BKPyV après la transplantation [111].

La validation de ces deux facteurs de risque est compliquée en raison de l'absence d'un test immunitaire pour le BKPyV commercialement accessible et approuvé par les différentes sociétés savantes [112]. De plus, il n'y a actuellement pas de directives claires pour évaluer les sérologies de BKPyV ou définir la séropositivité [113]. Certaines études ont associé la séropositivité de receveur avec un risque plus faible de réactivation de l'infection par BKPyV [139,145], tandis que d'autres études contredisaient cette conclusion [91,146]. Hirsch et al ont proposé que le groupe D +/R- n'est pas le groupe à haut risque [77], mais ils ont utilisé le test HIA non fiable pour l'évaluation du titre des anticorps anti-BKPyV. Au contraire, Sood et al ont utilisé le test ELISA-VLP pour démontrer que le groupe R-/D + avait la plus forte incidence d'infection par BKPyV [102]. Cette contradiction claire dans les résultats de la littérature nécessite des études supplémentaires pour examiner la corrélation entre la sérologie et l'infection par BKPyV.

Choix des essais immunologiques pour la mise en œuvre clinique

Dans cette étude rétrospective, nous avons utilisé le test ELISA pour mesurer les anticorps sériques contre 4 sérotypes différents de BKPyV en pré-transplantation : Ib2, Ia, IV et II. Nous avons choisi la technique ELISA car celle-ci est moins exigeante techniquement par rapport aux tests de neutralisation, qui nécessite une culture cellulaire. Il est également moins cher que le test immunitaire multiplex LUMINEX, qui nécessite des machines et des produits coûteux. Par conséquent, il est plus facile de mettre en place le test ELISA en tant que test de routine avant la transplantation dans les cliniques, ce qui représente l'objectif des études sérologiques de BKPyV. De plus, nous avons utilisé pour la première fois un anticorps commercial anti-VP1 BKPyV pour normaliser nos expériences. C'était une étape critique pour la normalisation inter-plaques. De plus, elle ouvre la voie au développement d'un test ELISA commercial, où le puit de normalisation peut être universel entre les laboratoires indépendants.

Épidémiologie du BKPyV en pré et post transplantation

La séroprévalence du BKPyV dans notre cohorte (n = 551) était de 75.6%, 82.55%, 76.5% et 64.9% pour les sérotypes Ib2, Ia, IV et II respectivement. Seulement 0.9% des receveurs étaient séronégatifs pour les quatre sérotypes étudiés. Cela confirme notre théorie selon laquelle la séroprévalence totale du BKPyV est d'environ 99%. De plus, 16.6% des donneurs étaient séropositifs pour les 4 sérotypes, un pourcentage similaire à celui des donneurs séronégatifs pour tous les sérotypes (14.8%). La séroprévalence du BKPyV était plus faible chez les donneurs que chez les receveurs. Cela peut s'expliquer par le fait que les receveurs rénaux dans notre cohorte étaient des patients souffrant de maladies rénales pendant des durées de temps notables. Cela peut les rendre encore plus vulnérables à l'infection par le BKPyV, qui a un tropisme rénal. Vraisemblablement, les receveurs peuvent avoir subi des infections primaires plus intenses ou des réactivations non détectées lorsqu'ils sont encore dans leur état immunocompétent. Ainsi, les receveurs sont plus susceptibles d'avoir des titres d'IgG plus élevés dans leur sérum que les donneurs. Ces titres élevés diminuent le risque d'obtenir des résultats faussement négatifs chez les receveurs séropositifs.

Après la transplantation, 59 (18%) de nos receveurs ont développé une virémie à BKPyV, un résultat similaire aux études récentes [117, 139]. Le sérotype le moins identifié sur les souches virales chez les receveurs virémiques était le sérotype II (seulement 3,3%). Cette observation impose des questions sur la différence de virulence entre les différents sérotypes de BKPyV. Cela également pose des questions sur la permissivité des cellules rénales pour le sérotype II du BKPyV. Le BKPyV a un tropisme très élevé et le sérotype II du BKPyV peut avoir ciblé d'autres cellules au cours de la primo-infection. Ceci explique le niveau de séroprévalence de 64.9% pour le sérotype II dans notre cohorte.

Sérologie des receveurs et des donneurs et risque de virémie à BKPyV

Les « odds ratios » de notre étude ont confirmé plusieurs associations significatives entre les titres d'IgG bas de receveur et élevés de donneur avec le développement de la virémie (tableaux 15 et 17). En déterminant le titre moyen d'IgG pour chaque sérotype étudié, nous avons confirmé de nouveau la corrélation entre le statut sérologique en pré-transplantation et la virémie. L'analyse sérologique totale du BKPyV a démontré qu'un titre d'IgG faible du receveur était impliqué dans le développement de la virémie du BKPyV. De plus, l'analyse sérologique du sérotype Ib2 et collective des Ia et Ib2 a révélé qu'un

un titre d'IgG élevé du donneur et plus faible du receveur étaient significativement corrélés avec le développement de virémie à Ib1 et Ib2.

Sérologie des couples receveur et donneur et risque de virémie à BKPyV

Dans le tableau 14, nous avons démontré que la probabilité la plus faible de développement de virémie à Ib1 et Ib2 était dans le groupe D-/R+ (1%). En revanche, la probabilité la plus élevée était dans le groupe D+/R- (27%) ($p = 0.0024$). Bien que non significative, une observation similaire a été faite pour le sérotype Ia (D-/R+, 10% vs D+/R-, 25%; $P = 0.47$). Nous avons noté l'absence de tout patient virémique pour le sérotype IV ou II dans les catégories D+/R-. Nous pensons cependant que la catégorisation de ces paires donneur-receveur est insuffisante pour la stratification de tous les patients. Par exemple, dans notre cohorte de 329 patients, 83 et 15 patients étaient respectivement D-/R+ et D+/R- pour le sérotype Ib2 (tableau 14). Ainsi, 231 patients sont sans stratégie de classification dans un groupe à risque faible ou élevé. Un critère alternatif prenant en compte ces patients « intermédiaires » est nécessaire pour aider à la prise de décision basée sur la sérologie de BKPyV avant la transplantation.

Sérologie du donneur et risque présomptif de néphropathie

L'évaluation de la virémie de haut niveau ($> 4 \log_{10}$ c/ml ; définie comme le développement d'une néphropathie présomptive) a montré qu'un titre d'IgG plus élevé du donneur était associé à une néphropathie présomptive. Un titre d'IgG faible d'un receveur était également associé à un risque de néphropathie présomptive plus élevé, mais le résultat n'était pas statistiquement significatif. Un titre d'IgG plus élevé de donneur peut refléter un degré d'infection par BKPyV plus élevé, par conséquent un nombre plus élevé de cellules infectées dans le greffon. Cela explique l'augmentation de l'intensité de l'infection chez les receveurs ayant des donneurs à titre élevé.

Apparition de la virémie en relation avec la sérologie du BKPyV

En ce qui concerne le début de la virémie, le titre d'IgG d'un receveur élevé reflétait probablement une immunité humorale anti-BKPyV plus forte qui aurait pu retarder le développement de la virémie après la transplantation. En revanche, un donneur fortement séropositif peut avoir transmis le BKPyV par la greffe au receveur. Ce dernier réplique le BKPyV tôt après la transplantation. Les receveurs avec un début de virémie plus tardif peuvent avoir contracté l'infection à partir d'une source autre que le donneur.

Évaluation de la sérologie et de la virémie à BKPyV spécifique au sérotype

Nous avons également souligné l'importance de la mesure des IgG contre la plupart des sérotypes du BKPyV (i) et du génotypage du BKPyV après la réactivation de l'infection (ii). Les divergences des résultats ont été obtenues lors de l'analyse de la sérologie d'un sérotype par rapport au développement d'une virémie spécifique au sérotype. Par exemple, le tableau 12 montre que pour la sérologie collective de la et Ib2, un donneur séropositif a démontré une légère augmentation du risque de virémie qui n'est pas significative (OR, 1.42; IC, 0.66 à 3.09; P = 0.36). Au contraire, l'analyse du développement d'une virémie uniquement à Ib1 et Ib2 a révélé un risque plus élevé et qui est significatif (OR, 2.85; IC, 1.04 à 7.79; P = 0.04).

De même, l'analyse de la sérologie IV uniquement contre le développement de la virémie à IV a montré que les groupes D-/R- et D+/R- ne répliquaient pas le sérotype IV du BKPyV (D-/R-, 0% ; D+/R-, 0%) (Tableau 14). La même analyse mais contre la virémie sans distinguer les sérotypes du BKPyV avait montré un résultat radicalement différent (D-/R-, 45% ; D+/R-, 17%). De même, le résultat de l'analyse de la sérologie II avec la virémie spécifique du sérotype II a montré une différence complète avec les résultats de l'analyse générale de la virémie à BKPyV. Aucun des patients n'avait en fait d'infection par le BKPyV sérotype II (0%).

L'analyse sérologique uniquement pour le sérotype Ib2 contre la virémie totale (tableau 15) peut avoir masqué l'impact de la sérologie des receveurs du BKPyV sur le risque de développement de la virémie (Ib2 ≤ 1600 ; OR, 1.99; IC, 1.07 à 3.54; P=0.03 ; Ib2 ≤ 800 ; OR, 2.03; CI, 1.14 à 3.59; P=0.016). Bien que significatifs, ces ratios OR étaient notamment inférieurs à ceux obtenus avec une analyse de sérotype spécifique. Des « odds ratios » significativement élevés ont été obtenus avec des titres de receveurs inférieurs pour le sérotype Ib2 analysés par rapport au risque de développement de virémie à Ib1 et Ib2 (Ib2 ≤ 1600 ; OR, 6.8; IC, 2.7 à 16.5; P<0.0001 ; Ib2 ≤ 800 ; OR, 5.4; IC, 2.7 à 10.6; P< 0.0001) (Tableau 17). De même, l'analyse des receveurs de sérotype IV contre la virémie IV uniquement (IV ≤ 400 ; OR, 4.95; IC, 1.46 à 16.7; P=0.01) a révélé un risque d'infection élevé par rapport à l'analyse avec la virémie générale (IV ≤ 400 ; OR, 2.62; IC, 1.41 à 4.86; P =0.0039).

Il a été révélé que l'association entre un titre d'IgG de sérotype Ib2 plus élevé et le développement de la virémie à Ib1 et Ib2 était hautement significative (valeur p< 0.001)

La figure 29A montre une corrélation significative entre un titre d'IgG de donneur élevé et le développement d'une virémie pour les sérotypes II et IV, mais pas pour la et Ib2. La figure 30A, au contraire, révèle que cette dernière corrélation est significative pour le sérotype Ib2 en

considérant seulement les virémies à Ib1 et Ib2. L'importance de cette corrélation significative a donc été masquée par le manque d'analyse spécifique au sérotype.

De nombreuses études précédentes analysaient uniquement la sérologie du sérotype Ib2 contre la virémie développée avec tous les sérotypes du BKPyV. La séropositivité à Ib2 d'un receveur ne peut le protéger contre le développement de la virémie à un autre sérotype du BKPyV. De même, un donneur séropositif pour Ib2 peut ne pas être la source d'infection si l'infection est développée par un sérotype différent du BKPyV.

Répondre aux limites de l'étude

La réactivité croisée d'ELISA peut avoir contribué aux limites de l'étude, nous avons donc commencé notre titrage sérique avec une dilution 1/100. La mesure des anticorps anti-BKPyV non neutralisants et neutralisants représentait une autre limitation. Cependant, une valeur de titre qui pourrait être déterminée par ELISA comme un risque de réactivation de BKPyV représente le titre obtenu à partir de la mesure totale des anticorps. Si la technique devient un test clinique de routine, les anticorps totaux seront mesurés et évalués conformément aux recommandations de la littérature.

Malgré la grande taille de notre cohorte, nos résultats étaient parfois compliqués par certaines analyses de sous-groupes. Au total, seuls deux et onze patients ont développé respectivement une virémie des sérotypes II et IV. Sur les 11 patients virémiques pour le sérotype IV du BKPyV, seuls sept étaient éligibles pour la catégorisation des paires donneur-receveur. Lors de l'analyse du développement de néphropathie présomptive, seuls trois patients avaient une virémie $IV > 4 \log_{10} \text{ c/ml}$. La puissance et la précision statistiques sont diminuées par cette diminution drastique de la taille de l'échantillon d'un sous-groupe (patients virémiques à sérotype IV) par rapport à celle de la cohorte totale. De plus, aucun des patients virémiques à sérotype II ($n = 2$) ne présentait d'échantillons de donneurs, ce qui compliquait davantage le traitement et les analyses des données.

Résumé

En conclusion, nous avons démontré que le statut sérologique des receveurs, donneurs et des couples receveur-donneur représentent un marqueur de la réactivation du BKPyV. De plus, le statut sérologique du donneur est un facteur de risque pour l'ampleur et la gravité de cette réactivation. Nous avons également démontré la valeur accrue de l'évaluation sérologique de plusieurs sérotypes du BKPyV, par rapport à la mesure la plus courante de sérologie du BKPyV basée uniquement sur le sérotype Ib2.

De plus, nous avons souligné l'importance de déterminer le titre d'IgG anti-BKPyV pour les sérotypes Ib2, Ia, IV et II. Ces variables peuvent être utilisées comme un outil d'évaluation supplémentaire pour classer les patients en receveurs à haut et à faible risque, en particulier parallèlement à la classification catégorielle actuelle insuffisante en D-/R+, D+/R-, D+/R+ et D+/R-. Cela permet aux cliniciens de suivre de près les patients à risque qui devraient : être soumis à un dépistage plus précoce et plus régulier du BKPyV (i), et recevoir des régimes d'immunosuppression personnalisés (ii).

Finalement, nous avons proposé une étape de normalisation fiable pour le test ELISA des IgG du BKPyV et des définitions claires pour les seuils (cutoffs) et la séropositivité. À la lumière de cette étude, et en étoffant notre cohorte pour des analyses de sous-groupes, nous espérons proposer une classification en pré-greffe du niveau de risque de répllication du BKPyV pour peut-être implémenter cette technique dans le bilan avant la réalisation de la greffe.

Conclusions

La réactivation du BKPyV chez les receveurs de greffe rénale peut entraîner de graves complications telles que la néphropathie et la perte du greffon. L'absence actuelle de traitements anti-BKPyV spécifiques et efficaces laisse la réduction ou la modulation de l'immunosuppression comme le seul choix pour la gestion de l'infection par le BKPyV. Le problème est que la réduction de l'immunosuppression thérapeutique représente un facteur de risque de rejet aigu. De plus, c'est un choix thérapeutique inapproprié pour de nombreux patients. Il est donc nécessaire de développer des stratégies alternatives pour la gestion de l'infection par BKPyV chez les greffés de rein (KTR). La stratification des patients en groupes à faible et à haut risque sur la base de la sérologie du BKPyV en pré-transplantation peut diminuer l'incidence du BKPyV. Elle fournit aux cliniciens un outil pour personnaliser les régimes d'immunosuppression en fonction du risque de réactivation évalué. De plus, les patients à haut risque peuvent être soumis à un dépistage plus rapproché et fréquent du BKPyV, dans le but de prévenir la progression de l'infection en BKPyVAN. Cependant, la mise en œuvre systématique de la sérologie du BKPyV en pré-transplantation dans les cliniques est compliquée par de nombreux facteurs tels que l'absence de lignes directrices et les définitions de seuil (cutoff).

Dans notre étude, nous avons identifié des titres d'IgG du donneur et du receveur en pré-transplantation comme marqueur de réactivation du BKPyV.

De plus, nous avons confirmé la corrélation entre le risque de réactivation du BKPyV après la transplantation et chacun des facteurs suivants :

- ❖ Le statut sérologique du receveur avant la transplantation
- ❖ Le statut sérologique du donneur en pré-transplantation
- ❖ Le statut sérologique des paires donneur-receveur en pré-transplantation

Nous avons démontré que le groupe D+/R- représentait le groupe à haut risque d'infection, tandis que le groupe D-/R+ était le groupe à faible risque.

De plus, nous avons déterminé que le statut sérologique du donneur peut être un marqueur de la gravité de l'infection par BKPyV après la transplantation. Un titre d'IgG élevé chez le donneur en pré-transplantation était associé de manière significative à des taux de virémie plus élevés et à un développement présomptif de la néphropathie.

De plus, nous avons identifié une relation entre la sérologie du BKPyV en pré-transplantation et l'apparition temporelle de la virémie à BKPyV. Des titres plus faibles des donneurs ont été associés à un début de virémie retardé réfutant l'hypothèse selon laquelle un donneur séropositif est toujours la source d'infection.

Nous avons également développé une technique ELISA standardisée avec un anticorps anti-BKPyV commercial qui est efficace, rapide et relativement peu coûteux pour une utilisation clinique. De plus, cette technique englobe des « cutoffs » et des définitions de séropositivité claires.

Enfin, nous avons montré l'importance de l'évaluation sérologique spécifique des sérotypes du BKPyV et sa corrélation avec le développement de la virémie concernée. Elle a révélé que la corrélation sérologie-infection BKPyV aurait pu être masquée dans des études précédentes qui évaluaient uniquement le statut sérologique vis-à-vis du sérotypeIb2 du BKPyV.

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VIII. ANNEXES

Review

Pre-Transplantation Assessment of BK Virus Serostatus: Significance, Current Methods, and Obstacles

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Abstract: The immunosuppression required for graft tolerance in kidney transplant patients can trigger latent BK polyomavirus (BKPyV) reactivation, and the infection can progress to nephropathy and graft rejection. It has been suggested that pre-transplantation BKPyV serostatus in donors and recipients is a predictive marker for post-transplantation BKPyV replication. The fact that research laboratories have used many different assay techniques to determine BKPyV serostatus complicates these data analysis. Even studies based on the same technique differed in their standard controls choice, the antigenic structure type used for detection, and the cut-off for seropositivity. Here, we review the different BKPyV VP1 antigens types used for detection and consider the various BKPyV serostatus assay techniques’ advantages and disadvantages. Lastly, we highlight the obstacles in the implementation of a consensual BKPyV serologic assay in clinics (e.g., the guidelines absence in this field).

Keywords: BK virus; serological technique; BK virus serology; kidney transplantation

1. Introduction

The best treatment for patients with end-stage renal disease is kidney transplantation. However, technically successful transplantations can be complicated by renal dysfunction episodes in the following months [1]. There are many reasons for this renal dysfunction: Failure to control opportunistic infections, the antiviral and immunosuppressant drugs’ nephrotoxicity, and both acute and chronic immune-mediated graft rejection. The guidelines on the kidney transplant recipients treatment suggest that the immune mediated graft rejection can be mitigated by intensive immunosuppressant treatment in the immediate post-transplantation period [2]. The immunosuppression required for the graft function maintenance increases the risk of viral infections in kidney recipients [3]. A common condition in immunosuppressed individuals is the BKPyV reactivation [4]. Even though we still lack specific anti-BKPyV treatments, there are no methods for reliably predicting the onset of BKPyV-associated infectious complications. However, it has been postulated that the kidney allograft is the infection source; consequently, the donor’s BKPyV seroreactivity may reflect the subsequent BKPyV load in the recipient. Conversely, the recipient’s seroreactivity reflects his/her immune status against BKPyV. Hence, BKPyV serostatus is a valuable tool for predicting the BKPyV-associated disease occurrence after transplantation [5]. Here, we review and compare the different assay techniques used to assess

BKPyV seroreactivity. We also consider the clinical BKPyV infection management as a function of the patient's BKPyV serostatus. Lastly, we discuss the obstacles in the routine BKPyV serostatus assessment in a clinical setting.

2. Virology and Epidemiology of BKPyV

The BKPyV is a *Polyomaviridae* family member. It is a non-enveloped virus with a diameter of 45 nm and a ~5 kb double-stranded DNA genome. Four major subtypes (I to IV) have been described, with subtype I being the most prevalent worldwide [6]. These subtypes have been further divided into subgroups, and it has been shown that genotypes I, II, III, and IV behave as fully distinct serotypes [7]. The viral capsid's outer surface is composed of 72 VP1 pentamers arranged in a T = 7 d icosahedral structure stabilized by calcium cations and disulfide bonds. The viral proteins VP2 and VP3 reside at the capsid's inner part. DNA binding is mediated by the VP1 N-terminal domain, which lies inside the virion. A copy of VP2 or VP3 interacts with a VP1 pentamer through hydrophobic interactions [8]. After a primary BKPyV infection (which usually occurs during childhood), the virus becomes latent in the kidneys and the urinary tract. It can be reactivated in an immunosuppression context, leading in many cases to the virus particles excretion in the urine. It has been reported however, that occasional BKV excretion in the urine was detected in healthy adults and children as well [9]. An important risk factor for manifesting polyomavirus renal graft infection after transplantation is high dose immunosuppressive therapy [10]. BKPyV can also induce other diseases in immunocompromised patients (e.g., hemorrhagic cystitis in bone marrow transplant recipients and in cyclophosphamide-treated cancer patients). The guidelines for these conditions recommend regular BKPyV replication monitoring and immunosuppressant dose adjustment for patients with high viral loads [2].

3. Immune Control of BKPyV

Both humoral and cellular immune responses are involved in the BKPyV infection control. In a cohort of renal recipients with BKPyVAN, Hariharan et al. observed significantly higher BKPyV-specific antibody titers in subjects after BKPyVAN resolution as compared to titers at BKPyVAN diagnosis time. The authors suggest that BKPyV-specific antibody titers are associated with viral clearance [11], but the increase in IgG levels can also be linked to viral replication. It has been suggested that BKPyV-specific T-cells play a dual role in the BKPyV infection control in renal transplantation patients. The reduction in immunotherapy leads to the cellular immunity restoration, which may succeed in inhibiting the infection and preventing BKVAN. On the other hand, if it fails to achieve viral clearance, the T-cell mediated immune response may add insult to the injury by homing T-cells to the graft and causing damage to the graft cells [12]. Only limited data exist on the innate immunity involvement in the BKPyV infection. However, a study by Womer et al. demonstrates lower levels of peripheral blood dendritic cells in patients with BKVAN compared to renal recipients with stable graft function. These cells are responsible for antigen presentation and T-cell activation [13].

4. BKPyV-Associated Nephropathy

Post-transplantation immunosuppression may lead to tBKPyV replication reactivation, which in turn may result in BK virus-associated nephropathy (BKPyVAN). This disease is a major renal allograft dysfunction cause (with a 1–10% incidence in kidney transplant patients) and can sometimes progress to interstitial nephritis with ureteric stricture and stenosis [14]. Several candidate biomarkers for BKPyV replication have been identified, such as decoy cells detection in the urine and BKPyV DNA load in urine and plasma [15]. Although BKPyVAN can appear as early as 1 month after transplantation, some cases are not detected until more than 80 months after the procedure. The viral reactivation is asymptomatic, and the infection is often only revealed by kidney failure. Despite a significant increase in clinical awareness and a better understanding of BKPyV infections, BKPyVAN still poses a real problem for kidney transplant patients [16]. The only available treatment strategy for BKPyVAN seeks

to reduce virus replication while avoiding graft rejection; this corresponds to a timely level reduction of immunosuppression and (in some cases) antiviral therapy initiation [14]. Although a partial immune function restoration controls BKPyV replication, it increases the risk of the allograft immune rejection. There is a real need for controlled studies to find safe and effective treatment for BKVAN, especially for those in whom immunosuppression reduction is not possible [17].

5. Current Clinical Approaches for Assessing BKPyV Serology

Two risk factors for early post-transplantation BKPyV replication have been identified: A low BKPyV antibody titer in the recipient, and a high titer in the donor [18]. It has therefore been hypothesized that a single BKPyV serostatus assessment before transplantation can predict the post-transplantation BKPyV replication risk [5]. Despite these findings, a standardized, commercially available, regulatory-agency-approved assay for anti-BKPyV antibodies is not available [19]. More sensitive, standardized immunoassays would facilitate the donor/recipient immune status assessment and thus enable the clinician to more closely monitor patients with a high predicted viral replication risk [20]. Around the world, thousands of patients are on organ transplant waiting lists, and transplantation is becoming a major financial burden in the developed world [21]. Consequently, it is essential to improve BKPyV serologic assays and donor–recipient BKPyV seroreactivity matching with a view to increasing the kidney graft survival rate. To achieve this objective, the most cost-effective strategies for BKPyV screening in different patient populations must be determined—as noted in the Kidney Disease: Improving Global Outcomes guidelines [2].

Most serological assays detect antibodies against the immunodominant BKPyV capsid protein VP1; including enzyme-linked immunosorbent assays (ELISAs), neutralization assays, multiplex immunoassays, and hemagglutination inhibition assays. Most serologic assays detect the immunodominant BKPyV capsid protein VP1 (the virus’s major surface protein) [22]. Cost-effective strategies for BKPyV screening have been sought in various patient populations [23]. It is known that systemic BKPyV infections induce strong, stable, prolonged antibody responses against viral structural proteins. Thus, past BKPyV infections can be detected with high sensitivity by measuring the anti-VP1 antibodies accumulation. In contrast, antibodies against the large T-antigen (LT) are infrequent and have low titers—making them unsuitable infection markers in most cases [24]. The low antibody response against LT might be due to poor immune accessibility and/or poor recognition; the latter is thought to be due to the similarity between the LT functional domains and that of cellular proteins [25].

5.1. VP1 Antigens Used in BKPyV Serologic Assays

Although all serologic assays reviewed here detect anti-BKPyV VP1 antibodies, they differ regarding the target antigens. Furthermore, several different VP1 antigen types can be detected. Below, we briefly describe the VP1 antigens that have been incorporated into the serologic assays developed by research laboratories.

5.1.1. Virus-Like Particles

Virus-like particles (VLPs) are most commonly generated from VP1 structural proteins, but VLPs with both VP1 and VP2/VP3 proteins have been synthesized. Although VLPs resemble native virions assembled into capsids (comprising 72 capsomers with a T = 7 symmetry), they do not contain viral genetic material. They can be used for diagnostic antigens for detecting serum specific antibodies against BKPyV VP1. The VLPs’ structure, transduction efficiency, and tropism are similar to those of native virions, except for the fact that VLPs do not undergo post-translational modification [26]. BK virus VLPs can encapsidate DNA fragments derived from the cells in which they were produced; consequently, the VLPs in each production batch contain VLPs with differing densities, depending on the incorporated DNA amount and size [27]. The VLPs quantity and quality can be affected by many factors, including the used production system type and the purification method. Virus-like particles can be produced in insect cells giving them the advantage of being free of mammalian pathogens;

however, the yields are rather low, with a high cost and risk of contamination with enveloped baculovirus particles and host DNA [28]. Yeast production systems have the advantage of producing safe, DNA-free VLPs, which makes them perfect to produce VLP vaccines. In fact, a study found that recombinant VLPs synthesized in yeast and used in an ELISA for human polyomaviruses have many advantages in ease of production, protein yield, and cost terms [26]. The 293TT mammalian cell line is most commonly used for VP1 VLPs synthesis because it allows authentic assembly and folding of recombinant proteins. Still, the production costs in the mammalian system are high, yields are low, and the cells are vulnerable to infection with mammalian pathogens [28]. One must also consider the VLPs' purity and integrity prior to the use in immunoassays. In fact, VLPs can be coupled to biotin for use in ELISAs. VLPs can be treated with the EZ-Link Sulfo-NHS-LC biotinylation kit and then bound to streptavidin plates, after which a sample diluent is added. Kardas et al. reported that standard polyomavirus VP1 VLPs and biotinylated VLPs did not differ significantly with regard to assay variability at the population level [29]. The VLP profile may vary even when the same production, purification, and quantification methods are applied. It is important to assess each batch's quality by ensuring that the VLPs' hemagglutination activity and immunogenicity make them suitable for serologic assays [27]. After production, SDS-PAGE can be used to confirm that the VLP batch has a major protein band at ~40 kDa, and thus can be qualified for use in ELISAs [30]. It is known that native VLPs and denatured VLPs have different antigenic epitopes; denatured VLPs react less efficiently with BKPyV-positive human serum. BK virus VLPs are stable at relatively high pH values, which enables them to be used in conventional ELISAs [27]. These VLPs are therefore the best tools for detecting BKPyV seroreactivity and have also been extremely valuable in BKPyV epidemiological studies.

5.1.2. Pseudovirions and Native Virion

The term "pseudovirion" (PsV) is used to describe synthetic viruses produced by the plasmid transfection of genes encoding capsid proteins and artificial genetic material used as a reporter. Although PsVs are similar to native virions in many ways (e.g., their behavior within cells), these synthetic viruses cannot replicate and do not propagate infection in cell cultures or *in vivo*. Hence, PsVs have become common tools for studying cellular entry and neutralization, and might be valuable in the future as vaccine vehicles or gene transfer tools [31]. Pastrana et al. generated pseudovirions by co-transfecting BKPyV capsid protein expression plasmids coding for VP1, VP2, and VP3 with a reporter plasmid encoding luciferase into 293TT cells. The cells were suspended and lysed 48 h post-transfection. The lysate was incubated overnight to allow capsid maturation, and then clarified. Ultracentrifugation using an iodixanol gradient was then used to purify the pseudovirions from the clarified supernatant [32]. Pseudovirions are mainly used in serum neutralization assays, where they contain a luciferase or green fluorescent protein reporter plasmid [33].

Apart from PsVs and VLPs, native virus particles can also be used as antigens in immunoassays. Native BKPyV particles are usually grown in HEK, Vero, or 293TT cells, harvested, purified, and quantified prior to their use in serologic assays [34]. It is also noteworthy that only the subtype Ia BKPyV (Dunlop or Gardner strain) can be propagated easily in culture, which means that the use of whole-virion antigens is not practical when the measurement of antibodies against different BKPyV serotype strains is required.

5.1.3. Soluble VP1 Proteins

Both recombinant and synthetic soluble VP1 proteins have been used as antigens in ELISAs. In a computer-assisted analysis of the late viral region, Pirtrobon et al. produced two specific, synthetic BKPyV VP1 peptides with a stable secondary structure. The synthetic peptides were incorporated in ELISAs that could detect anti-BKPyV antibodies in the absence of cross-reactivity with other small DNA tumor viruses [35]. The use of uniform, well-defined synthetic peptides with a high epitope density advantageously limits inter- and intra-assay variability and increases sensitivity. However, cross-reactivity can still be a problem, since synthetic peptides may not be able to bind specifically

enough to the target antibodies; the short peptides may have a different conformation when compared with full-length VP1 molecules assembled into capsomers during VLP synthesis [36]. Transfecting *E. coli* with pGEX VP1 plasmids produced VP1 pentamers; the resulting VP1 protein is fused to an N-terminal glutathione S-transferase (GST). After affinity purification on glutathione resin, the fusion proteins can be bound to 96-well polystyrene plates (using a casein-glutathione conjugate) and used in a capture ELISA [37]. Alternatively, the VP1-GST fusion proteins can be directly affinity-purified on polystyrene beads for use in a multiplex immunoassay [24].

5.2. Assay Techniques for BKPyV Seroreactivity

Four different techniques can be used to evaluate seroreactivity to BK virus. The techniques' respective advantages and disadvantages are summarized in Table 1.

Table 1. Advantages and disadvantages of assay techniques for BK polyomavirus (BKPyV) seroreactivity.

Technique Requirement	Advantages	Disadvantages	Time
Enzyme Immunoassay	Small quantities of sample required Versatile and customizable Inexpensive once set up	Can only measure one analyte at a time Cross-reactivity Relatively expensive initial investment Time consuming Elevated risk of error when testing a large number of samples	2 days (if wells are coated with antigen overnight)
Hemagglutination Inhibition Assay	Highly specific	Technically demanding Cannot distinguish between antibody classes Requires either intact virions or VLPs	1 day
Multiplex Assay	Simultaneous detection of multiple antigens High speed and dynamic range Customizable Reduced workflow	Expensive especially if a small number of antigens is analyzed Specialized equipment and analysis software are not available in most clinical settings Lack of normalization	Around 2 days (if beads are prepared in advance)
Neutralization Inhibition Assay	Highly Specific Measures neutralizing antibodies	Can only be used with PsV or viruses that can be grown Technically demanding Very time-consuming	Around 5 days

5.2.1. Enzyme Immunoassays

Enzyme-linked immunosorption is a rapid, high-throughput, sensitive, and highly reproducible method for antibody detection. Furthermore, colorimetric, chemiluminescent, or fluorescence ELISAs typically have a broad dynamic range [34]. The ELISA plates can be coated with any of the above-mentioned BKPyV antigens' types. Kean et al. studied several human polyomaviruses and found that a VP1 pentamer-based ELISA performed better than the more common VLP-based ELISA. The casein-glutathione conjugate used to capture the GST-VP1 capsomers on Polysorp 96-well plates fully exposed the bound capsomers to the serum sample and facilitated all the VP1-reactive antibodies measurement [37]. However, Bodaghi et al. reported that ELISAs with VP1 VLPs as coating antigens are more specific and sensitive than those with VP1 monomers or pentamers. Furthermore, the researchers' denaturation experiments experimentally confirmed the antigen's three-dimensional structure importance [25]. In the absence of standardized ELISAs for BKPyV, research and clinical laboratories have developed their own in-house ELISAs using various antigens, protocols, and standards. This complicated the comparison of BKPyV ELISA serology results between one lab and another, especially in the absence of guidelines on quantitative cut-offs. As mentioned above, the BKPyV VP1 antigen can be used in different forms. Even labs that use the same type of antigen (VLPs, for example) can differ regarding the antigen production and purification methods and the final concentration used to coat wells. Another variable is the reference material used to optimize the assay, which may differ from one lab to another. For example, the negative control is a blank well in some studies [29,38] and a bovine serum albumin-coated well in others [39,40]. Bodaghi et al. used an SF9 extract as a negative control [25], while Abend et al. used human anti-chicken lysozyme IgG [23]. Similarly, the normalization well composition may vary, and some labs even skip this step. Inter-plate normalization usually involves diluting an internal reference serum close to 1 optical density. Hence, the absence of a standardized, commercially available antibody prevents labs from

using the same identical normalization step. In addition to technical variables, the cut-off for positivity can be set differently in each laboratory. A clear BKPyV seropositive sample definition is currently lacking, and each laboratory uses its own in-house method to determine the cut-off. In summary, inter-ELISA variability is caused by differences in the reference material (normalization antibodies and negative controls), the VP1 antigen's type and concentration, the experimental protocol, the cut-off, and the seropositivity definition. A growing body of evidence suggests that pre-transplantation testing for BKPyV exposure can help to predict the occurrence of BKPyV-associated diseases after transplantation. Despite that, there are currently no consensus guidelines on an ELISA technique that healthcare institutions could use to determine the BKPyV serostatus of kidney or bone marrow transplant recipients. It will be difficult (but not impossible) to implement a technique that can be universally applied for pre-transplantation BKPyV serology assessments in a clinical setting. In the light of research performed over the last decade (i.e., strong evidence of a relationship between pre-transplantation BKPyV serology and post-transplantation BKPyVAN), it is now more important than ever to develop a standard ELISA for pre-transplantation BKPyV serostatus.

5.2.2. Multiplex Immunoassays

ELISA and other conventional serologic assays measure the presence of serum antibodies against a single antigen per well. In contrast, multiplex technologies enable the production of arrays of sensors—each of which provides its own unique detection signal. Multiple antigens can be measured simply by placing the sample in contact with the array [41]. Protein–protein interactions have been explored in multiplexed planar and suspension arrays, both of which requiring pre-purified proteins [42]. In a multiplex suspension array, a template (e.g., a micro well) is filled with different sensing elements in solution [41]. One of the best suspension array examples that efficiently detects antiviral antibodies in serum is the Luminex Multi-Analyte Profiling[®] (xMAP[®]) technology, in which indicator molecules are covalently attached to 5.6- μm polystyrene bead sensor elements. The beads have an internal color code that is obtained by filling them with different proportions of two or three spectrally distinct fluorochromes—resulting in an array of at least 500 separate bead sets [42]. Thus, the difference in the internal classification dye quantity in each microsphere results in a unique emission profiles generation, even though these same-sized beads have similar emission requirements [43]. Luminex indirect serologic assays have been extensively validated for the detection of antibodies against several polyomaviruses types [44–46] including BKPyV [5,47,48]. The BKPyV VP1 protein was expressed in *Escherichia coli* as a fusion protein with GST, and then affinity-purified using Luminex beads coupled to glutathione-casein. The modified beads could be used directly for the detection of anti-BKPyV antibodies [42]. When Luminex beads are used in serologic assays, non-specific background binding is a major drawback; human sera may contain antibodies that bind directly to the beads. Serum panels vary in the proportion of these sera, which frequently exceeds 5%. Using SeroMap beads (rather than xMAP[®] beads) to minimize binding to heterophilic serum antibodies only partially solves the problem, so the sera pretreatment with background inhibitors was recently suggested [49]. Furthermore, seroepidemiologic studies require many samples to be tested for several analytes in a rapid, sensitive, specific manner. This kind of analysis is facilitated by multiplex assay formats. Hence, if one seeks to detect anti-BKPyV antibodies against several viral serotypes, multiplex technology will be a time saver. This technique allows the simultaneous analysis of each serum sample against all the BKPyV serotypes at once. Furthermore, multiplex technology minimizes the experimental variability associated with conventional serology methods because multiple data points are obtained from a single measurement. The technique's requirement for a very low sample volume also maximizes data collection. In contrast, multiplex technology may offer fewer advantages in a clinical setting; the costly, specialized equipment and analytical software are unlikely to be available in all hospital laboratories. Compared with epidemiological studies, the number of subjects to be assessed at a given time point in a hospital or a transplant center is much lower. This means that the cost of performing these assays will be higher than for conventional serologic tests (e.g., ELISAs). Furthermore, it is harder to define a clear cut-off in

multiplex assays, since the result for each sample is usually expressed as mean fluorescence intensity. Lastly, it is noteworthy that multiplex assays use soluble VP1 proteins (rather than VLPs); this may constitute a slight drawback because many studies have suggested that the conformational structure of VP1 inside VLPs offers more specificity and sensitivity than that of VP1 monomers or capsomers.

5.2.3. Neutralization Inhibition Assays

A neutralization inhibition assay for BKPyV serology has been reported in the literature. In general, serum samples are serially diluted, pre-incubated with PsVs or native virions, added to seeded cells, and then incubated for a period of at least 48 or 72 h. The cell lysate is then analyzed: The greater the neutralizing antibodies titer in the serum is, the lower is the PsV-transduced or virion-infected cells' number and thus the weaker is the signal [33]. Solis et al. synthesized three different PsV types and then measured the antibody titers against BKPyV in the sera of 156 kidney transplant recipients at six different time points. The researchers demonstrated that this technique could quantify antibody titers in many samples [50]. This technique's greatest drawback is probably the need for cell culture—making it time-consuming, technically demanding and therefore unsuitable for clinical measurements. Furthermore, there is no standard method for a reliable neutralization inhibition assay so far, and as in the ELISA case, the seropositivity definition differs from one lab to another. Other variables include the BKPyV antigen type and the cell type used in the assay. For instance, RPTEC [23] and 293TT cells [7,32] have both been used to determine BKPyV serostatus.

5.2.4. Hemagglutination Inhibition Assays

Many laboratories have used hemagglutination inhibition assays (HIAs) to measure the antibody titers to BKPyV because of the rapidity and ease with which they can be performed. However, HIAs are less sensitive and less accurate than enzyme immunoassays. Experiments with the HIA have shown that greatly differing anti-BKPyV titers and anti-JCV antibodies were obtained in individual sera, thus overcoming the cross-reactivity problem expected for JCV and BKPyV [34]. It is noteworthy that the HIA is technically demanding and cannot differentiate between different BKPyV serotypes.

5.3. Clinical Studies of BKPyV Serology

In Table 2, we provide an overview of the research studies conducted on BKPyV serostatus in kidney transplant donors and recipients. In 2017, Wunderink et al. established that donor pre-transplant BKPyV seroreactivity best predicted the occurrence of a manifest BKPyV infection in renal allograft recipients. The researchers found a strong correlation between donor BKPyV serostatus on the one hand and the development of post-transplantation BKPyV viremia and BKPyVAN on the other. These findings strongly suggest that the kidney allograft has an important role in the BKPyVAN development, since it acts as a vector for transmitting BKPyV to the recipient. Consequently, it is assumed that the intensity of the donor's BKPyV seroreactivity corresponds to the infectious BKPyV load in the kidney allograft, which in turn is correlated with the BKPyV infection risk in the recipient. In contrast, the recipient's BKPyV seroreactivity might reflect his/her overall anti-BKPyV immunity status. Thus, it may be relevant to assess the post-transplantation BKPyV infection risk by assaying for anti-BKPyV IgGs prior to kidney transplantation [5]. Similarly, Solis et al. found that patients who received a kidney graft from donors with elevated BKPyV-neutralizing antibody titers became positive for BKPyV DNA. The researchers also found that the recipient's pre-transplantation titer of neutralizing antibodies against donor-specific BKPyV strains determined the BKPyV replication risk. Solis et al. suggested that physicians must take account the individual BKPyV risks when choosing immunosuppression strategies and monitoring patients after transplantation. Along with the recipient's BKPyV DNA load, the neutralizing antibodies titer against the replicating strain is a valuable disease progression marker [50]. Similarly, many studies found that a positive donor BKV serostatus is associated with post-transplantation BKV infection [51–53]. In contrast, Abend et al. reported that BKPyV viremia was not significantly correlated with the recipient's serostatus.

This might have been because the anti-BKPyV antibodies levels were too low to provide protection in a transplantation context (i.e., with suppressed cellular immunity and elevated viral loads). Abend et al. suggested that BKPyV viremia may be due to a donor-virus-derived infection, and thus that it may be possible to identify recipients at a clinical BKPyV infection risk by measuring the donor's serostatus [23]. On the other hand, Hirsch et al. proposed that the high-risk group to develop BKV infection after transplantation is not the seropositive donor and seronegative recipient transplant combination [15]. In view of these findings, we call on the scientific community to strive to (i) develop clear guidelines for assessing BKPyV serostatus, (ii) define quantitative cut-offs, and (iii) develop standard assay controls and reference samples. This will be the first step on the road to faithfully analyzing, comparing, and exploiting data on BKPyV serostatus and, ultimately, implementing these findings in clinical practice.

Table 2. An overview of the different research studies pertaining to the involvement of pre-transplant BKPyV serology testing in post-transplantation BKPyV infection.

Authors	Year	Number of Patients	Type of Assay and BKV Antigen	Conclusions from the Study
Solis et al. [50]	2018	168 KTR + 69 donors	Neutralization assay using pseudovirion system (BKPyV genotypes I, II, and IV)	Recipients with high neutralizing antibody titer have a lower risk for developing BKPyV viremia
Abend et al. [23]	2016	116 donor-recipient pairs	Neutralization inhibition assay using BKPyV particles (serotypes I, II, III, and IV) VLP-based ELISA to detect antibodies against BKPyV serotype I	Donor with significant serum neutralizing activity is associated with elevated risk for BKPyV viremia regardless of recipient serostatus
Wunderink et al. [5]	2016	407 donor-recipient pairs	Luminex assay detecting IgG reactivity against BKPyV I b1 VP1 protein. <i>n</i> = 396 reanalyzed by VP1 VLPs-based ELISA to detect antibodies against BKPyV genotype Ib2	Donor BKPyV IgG levels were strongly associated with the occurrence of recipient viremia and BKPyVAN
Sood et al. [54]	2013	192 adult and 11 pediatric donor-recipient pairs	BKPyV VLPs-based ELISA to detect human IgG Antibodies	Infection was highest in the Donor+/Recipient- group and lowest in the Donor-/Recipient- group
Ali et al. [52]	2011	36 pediatric KTRs + donors	BKPyV VP1 VLPs-based indirect ELISA to detect human IgG antibodies	Low BKPyV serostatus in children is associated with a high risk of post-transplantation BKPyV viremia, particularly in the context of donor with high BKPyV serostatus
Bijol et al. [55]	2010	45 pediatric KTRs	BKPyV VLPs-based ELISA to detect human IgG antibodies	Positive recipient BKPyV serostatus did not confer protection to BKV after transplantation
Bohl et al. [56]	2008	87 KTRs	BKPyV VP1 VLPs-based ELISA to detect human IgG Antibodies	Pre-transplant seropositivity did not protect against sustained BKPyV viremia but it might mitigate the severity of infection
Bohl et al. [51]	2005	142 recipients and 84 donors	BKPyV VP1 VLPs-based ELISA to detect human IgG Antibodies	BKPyV infection in the recipient was strongly associated with a positive BKPyV donor antibody status
Smith et al. [57]	2004	173 pediatric KTRs	BKPyV VP1 VLPs-based indirect ELISA to detect human IgG Antibodies	Recipient seronegativity for BKPyV was significantly associated with the development of BKPyVAN
Hirsch et al. [15]	2002	77 KTRs	Hemagglutination inhibition assay	The high-risk group is not the seropositive donor and seronegative recipient transplant combination
Flegstad et al. [58]	1991	10 KTRs	Neutralization inhibition assay Hemagglutination inhibition assay IgG, IgA, and IgM ELISA	Positive recipient BKPyV serostatus did not confer protection to BKPyV after transplantation Children with BK nephritis demonstrated lower pretransplant antibodies levels when compared to control groups (no infection)
Andrews et al. [53]	1988	496 donor-recipient pairs	Hemagglutination inhibition assay	A seropositive donor increased the rate of primary and reactivation infections with BKPyV

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The impact of pre-graft serology on the risk of BKPyV infection reactivation post-renal transplantation

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Key Words:	BKPyV, BKPyV virus serology, kidney transplantation, BKVPyV serostatus, serological technique
Abstract:	Objectives: BK polyomavirus associated nephropathy (BKPyVAN), is a troublesome disease induced by BK polyomavirus (BKPyV) reactivation in immunocompromised renal graft recipients. BKPyVAN can progress to graft dysfunction and has no treatment really effective, making immunosuppression reduction the only management choice. Thus predictive BKPyV infection reactivation markers are needed for high-risk patient identification. Methods: we conducted a retrospective study to assess the correlation between the BKPyV pre-transplant serostatus and post transplant BKPyV infection incidence. Sera from 329 recipients and 222 matched donors

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	<p>were tested for anti-BKV antibodies against four BKPyV serotypes by a VLPs- based IgG ELISA, and BKPyV DNA load was monitored for at least 1 year post transplantation.</p> <p>Results: 80 (24%) recipients were viruric and 59 (18%) recipients were viremic post transplantation. In the pre-transplant period, the probability of developing viremia for serotype I was 1% in the D-/R+ group versus 27% in the D+/R- group (P=0.0009). An elevated BKPyV viremia risk was observed for recipients who had a mean antibody titer for all serotypes ≤ 400 before transplantation (odd ratio [OR], 5.58; 95% confidence interval [CI], 2.60-11.79; P<0.0001). In addition, kidney recipients from donors with a mean BKPyV antibody titer ≤ 400 had a lower BKPyV viremia risk (OR, 0.47; CI, 0.21-0.95; P=0.055). Furthermore, a lower mean antibody titer in donors was associated with a late onset of BKPyV viremia (>4 months).</p> <p>Conclusions: Both donor and recipient mean BKPyV antibody titer may serve as a predictive tool to manage clinical BKPyV infection by identification of patients at high reactivation risk.</p>

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6 **reactivation post-renal transplantation**
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Abstract

Objectives: BK polyomavirus associated nephropathy (BKPyVAN), is a troublesome disease induced by BK polyomavirus (BKPyV) reactivation in immunocompromised renal graft recipients. BKPyVAN can progress to graft dysfunction and has no treatment really effective, making immunosuppression reduction the only management choice. Thus predictive BKPyV infection reactivation markers are needed for high-risk patient identification.

Methods: we conducted a retrospective study to assess the correlation between the BKPyV pre-transplant serostatus and post-transplant BKPyV infection incidence. Sera from 329 recipients and 222 matched donors were tested for anti-BKV antibodies against four BKPyV serotypes by a VLPs- based IgG ELISA, and BKPyV DNA load was monitored for at least 1 year post transplantation.

Results: 80 (24%) recipients were viruric and 59 (18%) recipients were viremic post transplantation. In the pre-transplant period, the probability of developing viremia for serotype I was 1% in the D-/R+ group versus 27% in the D+/R- group (P=0.0009). An elevated BKPyV viremia risk was observed for recipients who had a mean antibody titer for all serotypes ≤ 400 before transplantation (odd ratio [OR], 5.58; 95% confidence interval [CI], 2.60-11.79; P<0.0001). In addition, kidney recipients from donors with a mean BKPyV antibody titer ≤ 400 had a lower BKPyV viremia risk (OR, 0.47; CI, 0.21-0.95; P=0.055). Furthermore, a lower mean antibody titer in donors was associated with a late onset of BKPyV viremia (>4 months).

Conclusions: Both donor and recipient mean BKPyV antibody titer may serve as a predictive tool to manage clinical BKPyV infection by identification of patients at high reactivation risk.

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58 **Keywords:** BKPyV; BKVPyV serostatus; BKPyV seroprevalence; serological technique; BKPyV
59 virus serology; kidney transplantation
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61 **Abbreviations:** BKTGR, BK virus typing and grouping region ; BKPyVAN, BKPyV associated
62 nephropathy; BKPyV, BK polyomavirus; CI, Confidence interval; c/ml, Copies per milliliter;
63 CNI, calcineurin inhibitor; DNA, Deoxyribonucleic acid; ELISA, Enzyme linked immunosorbent
64 assay; FBS, Fetal bovine serum; GFP, Green fluorescent protein; HRP, Horse radish peroxidase;
65 IgG, Immunoglobulin G; KTRs, Kidney transplant recipients; OD, Optical density; OR, Odds
66 ratio; PCR, Polymerase chain reaction; PBS, Phosphate buffered saline; rATG, rabbit
67 antithymocyte globulin; SD, Standard deviation; VLPs, Virus like particles; VP1, viral capsid
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Introduction

The BK polyomavirus (BKPyV) is a DNA virus that belongs to the *polyomaviridae* family. BKPyV genotypes I, II, III, and IV behave as four distinct serotypes (1) that are extremely prevalent amongst the general population. Potent immunosuppressive therapy places renal graft recipients at risk for BKPyV reactivation (2,3). The infection persistence can lead to BKPyV associated nephropathy, usually associated with graft rejection (4,5). Current guidelines implicate renal recipients in regular BKPyV viruria and viremia monitoring after the transplantation procedure (6,7). Still, around 8% of renal transplant patients develop BKPyVAN, of which the majority experience graft dysfunction and loss (8). Since only limited studies exist on anti-viral treatment against BKPyV (9,10), BKPyVAN management is based on judicious immunosuppression decrease and acute rejection monitoring (11). However, studies demonstrate that BKPyVAN risk is not fully eliminated by viremia screening (12), and that the immunosuppression reduction strategy is not suitable for all patients (i), and can increase the acute rejection risk (ii) (13,14). Several risk factors have been proposed for post transplantation BKPyV reactivation such as male sex and potent immunosuppression (15). Still, it is relevant to identify additional pre-transplantation factors that allow better high-risk patient stratification. A high BKPyV antibody titer in the donor and a low titer in the recipient have been proposed as BKPyV reactivation risk factors (16). However, only twelve studies assessed this hypothesis. Some used the unreliable hemagglutination inhibition method for serostatus determination. In addition, others included a relatively small cohort and others do not include the donors in their cohort. It is also worth to mention the contradictory nature of their results, with some being in

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3 102 favor of the previous hypothesis and others against it. We summarized these studies and their
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5 103 characteristics in a review that we published recently (17). Wunderink et al demonstrated a
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8 104 strong correlation between post-transplantation BKPyV infection and pre-transplantation
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11 105 BKPyV IgG levels. The authors solely detected antibodies against the Ib BKPyV serotype (18). A
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13 106 2018 study by Solis et al supported a high recipient antibody titer protective role. However, the
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15 107 authors used the more technically demanding and time consuming neutralization assay (19). It
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18 108 is important to inspect whether the pre-graft serology for four BKPyV serotypes has an
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20 109 additional interest in the post-transplantation BKPyV infection risk. We aimed to evaluate the
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23 110 mentioned hypothesis by assessing the BKPyV serostatus against four different serotypes of the
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25 111 virus: Ib2, Ia, IV and II. In this retrospective study, we assessed the total BKPyV serostatus of 329
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28 112 kidney transplant recipients (KTRs) and 222 matched donors. Then, we correlated it with
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30 113 BKPyV post transplantation viremia incidence using for the first time a commercial antibody for
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33 114 ELISA inter plate normalization.

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Methods

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126 Study Design and Cohort

127 In this retrospective study, adult renal graft recipients who underwent transplantation between
128 January 2013 and May 2018 at the Amiens University Medical Center were included. In total,
129 329 recipients were included and 21 were excluded based on the following criteria:
130 transplantation failure (i), death during the first year (ii), following up outside Amiens
131 University Medical Center (iii), lack of samples (iv) and return to dialysis (v). Pre-
132 transplantation sera samples from 222 matched donors were collected and included in the
133 cohort (Fig S1). The Amiens University Medical Center's institutional review board approved
134 this study. This project was conducted in accordance with the reference methodology (MR-004
135 France) in accordance with Article 30 of the GDPR.

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137 Immunosuppression Protocols

138 The induction treatment for all patients included IV methylprednisolone and either basiliximab
139 (Novartis Pharmaceuticals Corp.) or rabbit antithymocyte globulin (rATG; Genzyme). The
140 maintenance immunosuppression protocol was composed of a calcineurin inhibitor (CNI), with
141 most patients receiving tacrolimus (PROGRAF®; Astellas Pharma Inc.). The others received
142 cyclosporine A (NEORAL®; Novartis Pharmaceuticals Corp.). The CNI treatment was combined
143 with the anti-metabolite mycophenolate mofetil (CELLCEPT®; Genentech) or with everolimus
144 (Certican®; Novartis Pharmaceuticals Corp.). Most patients received corticosteroids
145 maintenance (Cortancyl®; Sanofi) that was halted later.

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147 **Virus-Like Particles (VLPs) synthesis:**

148 Recombinant baculoviruses containing either Ia VP1 or IV VP1 expression plasmids were used
149 to transduce SF21 insect cells, which produced virus-like particles representing serotypes Ia and
150 IV. A mammalian system was utilized for the serotypes Ib2 and II VLPs generation using VP1
151 plasmids with a green fluorescent protein (GFP) marker plasmid. Briefly, the plasmids were
152 amplified by transformation into E.coli bacteria (NEB 5-alpha, New England Biolabs) then
153 extracted using a plasmid extraction kit (NucleoBond® Xtra Midi/Maxi). 293TT cells were then
154 transfected with the plasmids, and the expressed VLPs were harvested 48 hours post-
155 transfection. The purification method was the same for VLPs produced in the insect or the
156 mammalian system. Following plasma membrane and nucleus lysis, the cell lysate was
157 sonicated and passed on an iodixanol (VISIPAQUE™ 320 mgI/ml) gradient, then ultra
158 centrifuged for 24 hours at 32,000 rpm. Fractions containing the VLPs were collected, quantified
159 and stored at -20°C.

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161 **Enzyme Linked Immunosorbent Assay**

162 For plate coating, BKPyV VP1 VLPs were diluted to 1µg/mL and 100 µl were added to each well
163 of a 96-well polystyrene flat-bottomed Polysorp plate (NunC immune-plate, Thermo Scientific)
164 and incubated overnight at 4°C. Patients' serum samples were stored at -80 °C after collection,
165 and diluted by 1/100 before being added to the plate. After the plate was blocked with a fetal
166 bovine serum (FBS, Gibco) solution then washed, sample was added to the first well and diluted
167 by two-fold dilution. A commercial anti-BKPyV VP1 monoclonal antibody (clone 3B2, Sigma-

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3 168 Aldrich) was diluted and added to normalization wells, while negative control wells contained
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5 169 only PBS 1X (Dulbecco w/o Ca²⁺ & w/o Mg²⁺). After incubation for 1 hour, the plate was washed
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8 170 and a secondary IgG Fc specific antibody coupled to horse radish peroxidase (HRP) (Southern
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11 171 Biotech) was added to the wells (anti-human to sample wells and anti-mouse to normalization
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13 172 wells). After incubation and washing, o-phenylenediaminedihydrochloride and hydrogen
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15 173 peroxide were added to each well and incubated at room temperature in the dark for 30 min.
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18 174 The reaction was halted by 100 µl of 1M sulfuric acid, and the optical density (OD) was read at
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21 175 492 nm by an ELISA spectrophotometer.
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24 25 177 **ELISA Normalization and Cutoff Determination**

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28 178 Each plate included normalization wells (n=3 per plate) in which 3B2 commercial anti-BKPyV
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30 179 antibody was diluted (1/5000) and then added. The antibody dilution was always the same for
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33 180 all plates. Only plates in which the normalization wells produced a mean OD between 0.7 and
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35 181 1.5 were accepted to assure inter-plate standardization. The OD of a given sample was
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38 182 subtracted by the OD of the negative control well. Then, it was divided by the normalization
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40 183 well's OD to give a corrected OD for each sample. If the corrected OD at a given titer was
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43 184 greater than 10% cutoff, the sample was positive at that titer.

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45 185 For cutoff determination, we determined the mean OD of 18 serum samples from children aged
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48 186 between 15 months and 2 years plus two standard deviation.

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190 **BKPyV Viremia Screening and BKV genotyping**

191 Viremia and viruria were assessed monthly for the first six months after transplantation and
192 then at months 9 and 12 using a quantitative real time PCR kit (RealStar Altona). After viral load
193 determination, positive samples were sequenced based on a fragment called the BKPyV virus
194 typing and grouping region (BKTGR) (20).

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196 **Statistical Analyses**

197 Data analysis was performed using *GraphPad Prism* version 5 for Windows (*GraphPad Software*,
198 La Jolla, California, USA, www.graphpad.com). Kolmogorov–Smirnov test was used to examine
199 if variables were normally distributed. Mann-Witney U test was used to compare two
200 independent groups. The Chi-Square test (X^2 test) and Fisher’s exact test were used to determine
201 if there was a significant relationship between categorical variables (BKPyV infection and the
202 other categorical variables). Odds ratio and its 95 % Confidence interval were used to report the
203 association strength between BKV infection and each variable. A probability value of less than
204 0.05 was regarded as statistically significant.

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Results

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212 **Viremia in the studied population**

213 In this retrospective study, 329 recipients and 222 matched-donor samples were available after
214 the exclusion of 17 unfitting samples (according to the mentioned criteria) (Fig S1). A total of 80
215 recipients developed BKPyV viruria. The BKPyV Ib2 genotype [34 (58%)] was detected in the
216 majority of the 59 recipients who progressed to viremia. Most of the patients became viremic
217 between two to four months after transplantation (68%) (Fig S2). BKPyV seroprevalence in our
218 cohort (n=551) was 75.6%, 82.55, 76.5% and 64.9% for serotypes Ib2, Ia, IV and II respectively. In
219 addition, only 1.2% were sero-negative for the 4 serotypes (Table S1).

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221 **Post-transplantation Viremia Incidence in Recipients According to Pre-transplantation**

222 **Serostatus**

223 We first compared the serotype-specific pre-transplant serology to viremia development
224 regardless of the replicating virus serotype (Table 1). When considering serotype IV serology,
225 the highest viremia incidence was reported for the D-/R- (sero-negative donor, sero-negative
226 recipient) group (45%). However, analyzing IV serology solely against IV viremia development
227 showed that the D-/R- and the D+/R- groups didn't replicate BKPyV serotype IV (3/15). This
228 result difference between the two analyses' approaches stresses the importance of serostatus
229 assessment for each BKPyV serotype. Ib2 pre-transplantation serology analysis showed that the
230 lowest likelihood of Ib1 and Ib2 viremia development was in the D-/R+ group (1%), whilst the

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3 231 highest likelihood was in the D+/R- group (27%) with P=0.0009 (Table 1). The number of patients
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5 232 with serotype IV infection was insufficient for proper statistics determination.
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9 233 Moreover, donor serology analysis independent of recipient serology and vice versa
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11 234 demonstrated similar findings in the Ib2 serology category (Table 2). A significant risk of Ib2
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13 235 viremia development was observed in recipients whose donors were seropositive compared to
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15 236 those with seronegative donors (OR, 7.5; CI, 2.19 to 25.73; P= 0.0002).
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22 238 **Association of the BKPyV Infection Risk with the Pre-graft antibody titer in Donors and**
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24 239 **Recipients:**
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26 240 BKPyV infection reactivation risk was evaluated in the first year post transplantation according
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28 241 to pre-transplantation BKPyV IgG titer (Table 3). A higher viremia risk was determined in
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30 242 recipients with a lower total BKV serostatus (BKPyV \leq 800: OR, 3.11; 33.3% vs 13.8%; P=0.0006
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32 243 and BKPyV \leq 400: OR, 5.58; 48.8% vs 14.5%; P<0.0001). A similar trend was observed when
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34 244 analyzing the recipient pre-transplant serostatus for each serotype: Ib2 \leq 800 (OR, 2.03; P=0.016),
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36 245 II \leq 400 (OR, 3.64; P=0.0002), and IV \leq 400 (OR, 2.62; P=0.0039). The lowest viremia incidence was
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38 246 observed in recipients whose donors had low Ib2 IgG levels (Ib2 \leq 400: OR, 0.39; P=0.01). Thus, a
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40 247 high viremia risk can be predicted by two factors: a high donor's and a low recipient's IgG titer.
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252 **Viremia Level According to the Antibody Titer**

253 Assessment of high-level viremia ($>4 \log_{10}$ c/ml; defined as presumptive nephropathy
254 development) risk was performed based on recipients or donors mean IgG titer for serotypes Ia,
255 Ib2, II, and IV (Fig S3). The average donors antibody titer for serotypes II and IV was
256 significantly higher in patients with viremia $>4 \log_{10}$ c/ml (II, 109; IV, 374.8) compared to those
257 with low-level viremia (II, 36.36; IV, 163.6) (Fig S3A). In contrast, recipients with viremia $>4 \log_{10}$
258 c/ml demonstrated a non-significant lower recipient IgG titer for all serotypes and a reverse
259 trend is observed among donors (Fig S3B). Categorizing recipients according to donor and
260 recipient pairs' serostatus (Fig S4) reveals that 50% of Ib2 R-/D+ viremic patients had high level
261 Ib1 and Ib2 viremia. All recipients with Ib2 sero-negative donors had low-level viremia only.
262 Similarly, only serotype IV R+/D+ recipients developed serotype IV viremia $> 4 \log_{10}$ c/ml.
263 Consequently, a high donor serostatus may not only predict viremia risk, but also the infection's
264 severity.

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266 **BKV Antibody Titer Levels and Late Viremia Onset:**

267 We investigated whether the pre-transplantation IgG titer had an effect on the viremia temporal
268 onset (Fig 1). For each serotype (Ia, Ib2, II, and IV), we determined the recipient and donors' IgG
269 titer mean for patients who developed viremia at different post-transplantation time points.
270 Analyzing the donors' Ia and Ib2 IgG titers revealed that a decreasing antibody titer was
271 associated with later viremia development (Fig 1A). This association was only significant for
272 serotype Ib2 (1-2 months, IgG titer=900; 3 months, IgG titer=807; 4-6 months, IgG titer=188.8). For

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3 273 all serotypes, we observed that patients who developed viremia more than 4 months post
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5 274 transplantation had higher IgG titer compared to those with earlier viremia. Only results for
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8 275 serotype II were significant (Fig 1B). Although not statistically significant, the total BKPyV IgG
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11 276 titer analysis showed that later viremia onset (>month 4) was associated with a lower donor but
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13 277 higher recipient titer (Fig 1C).

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18 279 **Onset of BKPyV Viremia:**

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20 280 To explore further the observed correlation between pre-graft BKPyV serology and BKPyV
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23 281 infection, Kaplan Meier curves were plotted (Fig 2). BKPyV viremia occurred at a shorter time in
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25 282 the Ib2 D+/R- group compared with the other groups (Fig 2A). Both groups with sero-negative
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28 283 donors (D-/R- and D-/R+) showed a delayed viremia onset compared to groups with a sero-
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30 284 positive donor (D+/R- and D+/R+). Similarly, the total BKPyV D+/R- group had a shorter BKPyV
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32 285 viremia onset time compared with the other groups (Fig 2B).

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Discussion

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303 BKPyVAN is a troublesome disease induced by potent immunosuppressive regimes post-renal

304 transplantation (21). It is relevant to identify and validate new BKPyV reactivation risk factors,

305 since BKPyVAN currently lacks an efficient treatment. A high donor IgG titer and a low

306 recipient IgG titer has been identified as risk factors for BKPyV infection post-transplantation

307 (16). Validating these two risk factors is complicated due to the absence of an agency-approved

308 and commercially accessible BKPyV immune-assay (22). In addition, there are currently no clear

309 guidelines for BKPyV serology assessment or sero-positivity definition (17). Some studies

310 associated the recipient's sero-positivity with a lower risk for BKPyV infection reactivation

311 (19,23), while other studies concluded contradicted that finding (24–26). Hirsch et al proposed

312 that the D+/R- group is not the highest risk group (4), but they used the unreliable HIA for

313 BKPyV titer assessment. In contrary, Sood et al utilized VLP ELISA to demonstrate that the R-

314 /D+ group had the highest BKPyV infection incidence (27). This clear contradiction in literature

315 findings calls for additional studies to investigate the BKPyV serology and infection correlation.

316 In this retrospective study, we used ELISA to measure pre-transplant serum antibodies against 4

317 different BKPyV serotypes: Ib2, Ia, IV, and II. We chose ELISA because it is less technically and

318 time-demanding than other assays. It is also cheaper than the LUMINEX multiplex immune

319 assay, which requires expensive machinery and products. Hence, it is easier to implement

320 ELISA as a routine pre-transplant assay in clinics, which represents the ultimate goal of BKPyV

321 serology studies. Moreover, we used for the first time a commercial BKPyV VP1 antibody to

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3 322 normalize our experiments. This was a critical step for inter-plate normalization. In addition, it
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5 323 paves the way in front of a commercial ELISA assay development, where the normalization well
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8 324 can be universal between independent laboratories.
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11 325 Regarding BKPyV epidemiology, each serotype exhibited a sero-prevalence of around 75%.
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13 326 Only 1% of recipients were sero-negative to the four studied serotypes. This supports our theory
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15 327 that the total BKPyV sero-prevalence is around 99%. After transplantation, 59 (18%) of our
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17 328 recipients developed BKPyV viremia, which is similar to recent studies (19,28). The least
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19 329 serotype propagated in viremic recipients was II (only 3.3%). This raises questions about the
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21 330 virulence difference between different BKPyV serotypes. It also raises questions about the renal
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23 331 cells permissiveness to BKPyV serotype II. BKPyV has a very high tropism, and BKPyV serotype
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25 332 II may have targeted other cells during the primary infection. This explains the II serotype sero-
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27 333 prevalence level of 64.9% in our cohort.
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33 334 We also highlighted the importance of IgG measurement against most BKPyV serotypes (i), and
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35 335 BKPyV genotyping after infection reactivation (ii). Results' discrepancies were obtained when
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37 336 analyzing a serotype's serology against total or serotype-specific viremia development. Many
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39 337 previous studies analyzed only Ib2 serotype serology against viremia developed with all BKPyV
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41 338 serotypes. A recipient's Ib2 sero-positivity may not protect him/her against viremia
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43 339 development by another BKPyV sero-type. Similarly, a Ib2 sero-positive donor may not be the
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45 340 infection source if the developed infection is by a different BKPyV serotype.
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50 341 Our study's odds ratios confirmed the lower recipient and higher donor serostatus association
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52 342 with viremia development. By determining the mean IgG titer for each studied serotype, we
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54 343 confirmed again the pre-transplant serostatus- viremia correlation. In addition, we
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3 344 demonstrated that a higher donor mean titer for serotypes II and IV was significantly associated
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5 345 with higher viremia levels of $>4\log_{10}$ c/ml (Fig 1). A higher donor IgG may reflect a higher
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8 346 BKPyV infection degree, consequently a higher number of infected cells in the renal graft. This
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11 347 explains the increased infection intensity in recipients with high-titer donors.
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13 348 Regarding viremia onset, a higher recipient's IgG titer probably reflects a stronger anti-BKPyV
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15 349 humoral immunity that could have delayed viremia development. In contrary, a highly sero-
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18 350 reactive donor may have transmitted BKPyV through the graft to the recipient. The latter
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21 351 replicates BKPyV early after transplantation. Recipients with a later viremia onset may have
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23 352 contracted the infection from a source other than the donor.
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25 353 ELISA cross-reactivity may contribute to the study's limitations, so we started our serum
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28 354 titration with a 1/100 dilution. The measurement of both non-neutralizing and neutralizing
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31 355 BKPyV antibodies represents another limitation. However, a titer value that might be
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33 356 determined by ELISA as a BKPyV reactivation risk represents the titer obtained from total
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35 357 antibody measurement. If the technique becomes a routine clinical test, the total antibody will be
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38 358 measured and assessed according to literature recommendations.
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40 359 In conclusion, we demonstrated that both the recipients and donors serostatus represent a
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43 360 marker for BKPyV reactivation. Moreover, the donors' serostatus is a risk factor for this
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45 361 reactivation extent and severity. We highlighted the importance of determining the BKPyV titer
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48 362 mean for serotypes Ib2, Ia, IV and II. This variable can be used as an additional asset to
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50 363 categorize patients into high and low risk recipients. This allows clinicians to closely monitor at
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53 364 risk patients who should: be subject to earlier and more regular BKPyV screening (i), and receive
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55 365 customized immunosuppression regimes (ii). Ultimately, we proposed a reliable normalization
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3 366 step for the BKPyV IgG ELISA test and clear definitions for cutoffs and sero-positivity. In the
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6 367 light of this study, we call for the acceleration of BKPyV serostatus screening implementation as
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8 368 a pre-transplant routine test in transplantation centers.

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21 374 Maryland, for providing Ib2 and II VP1 expression plasmids.

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Figure legends

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482 **Figure 1:** Post-transplant BKPyV viremia kinetics according to the pre-transplant recipients (A)
483 or donors (B) BKPyV antibody titer mean for each serotype, or that of the total BKPyV IgG titer
484 (C). *P value<0.05 significant difference; ** PVALUE<0.01 significant difference; NS
485 PVALUE>0.05 No significant difference. Some values were not plotted due to the y-axis log2
486 scale presentation.

487
488 **Figure 2:** Kaplan-Meier curves showing BKPyV viremia percentage during the first year post-
489 transplantation according to four distinct pre-transplantation Ib2-specific (A) or BKPyV-specific
490 (B) IgG groups: R+/D-, R-/D-, R+/D+ and R-/D+

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492 **Figure S1:** BKV post-transplant infection prevalence and genotypic distribution amongst the
493 study's recipient cohort

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495 **Figure S2:** BKPyV viremia characteristics and detection kinetics in recipients with BKPyV
496 viremia (n =59). c/mL, copies per milliliter.

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498 **Figure S3:** Mean donors' (A) or recipients' (B) BKPyV antibody titer levels for BKPyV serotypes
499 Ia, Ib2, II, and IV in recipients with viremia levels BKPyV< 4log 10 or BKPyV> 4log 10 (high level
500 viremia). *P value<0.05 significant difference; NS PVALUE>0.05 No significant difference.

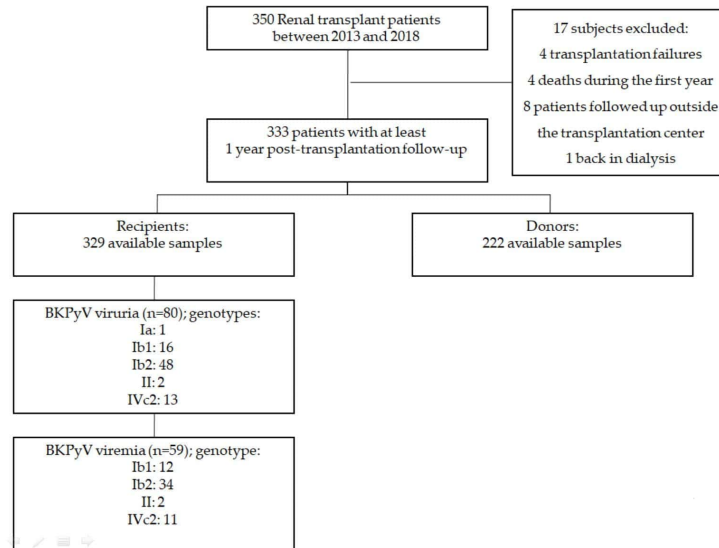
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502 **Figure S4:** Renal recipients*' distribution based on: the pre-transplantation donor and recipient
503 BKV serotype Ib2 sero-status and BKPyV serotype Ib1 and Ib2 viremia (A), and the pre-
504 transplantation donor and recipient BKPyV serotype IV sero-status and BKV serotype IV
505 viremia (B), including BKPyV viremia >4log.

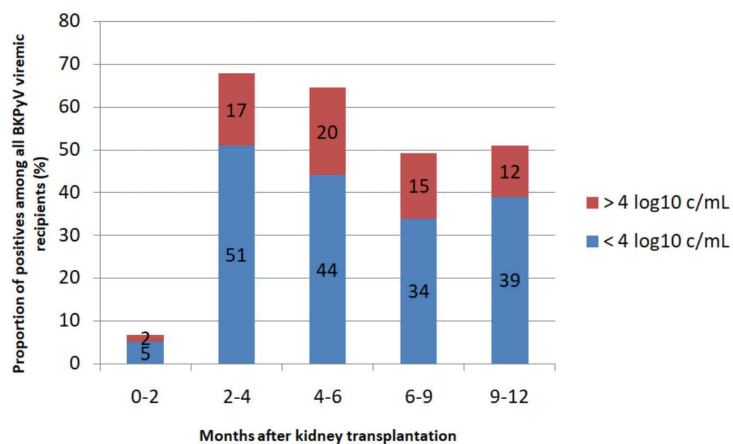
506 *Only recipients whose donors were included in the study.

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IgG titer<200	Recipients		Donors	
	Total (n=329)	P+ (n=59)	Total (n=222)	P+ (n=35)
0 serotype	239	38	37	2
1 serotype	61	11	51	13
2 serotypes	22	7	67	10
3 serotypes	4	2	34	7
4 serotypes	3	1	33	3

Table S1: Number of recipients and donors having an IgG titer<200 with 0, 1, 2, 3 or 4 BKPyV serotypes.

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Pre-transplant Serology	Serostatus	Total n (%)	No BKV Viremia n (%)	BKV Viremia n (%)	P value	Viremia			P value
						with the I serotype n (%)	with the IV serotype n (%)	with the II serotype n (%)	
Ib2 serotype	D-/R+	83 (37.4%)	76 (92%)	7 (8%)	0.112	1 (1%)			0.0009
	D-/R-	12 (5.4%)	10 (83%)	2 (17%)		2 (17%)			
	D+/R+	112 (50.5%)	90 (80%)	22 (20%)		21 (19%)			
	D+/R-	15 (6.7%)	11 (73%)	4 (27%)		4 (27%)			
Ia serotype	D-/R+	67 (30.2%)	58 (87%)	9 (13%)	0.367	7 (10%)			0.5292
	D-/R-	5 (2.2%)	4 (80%)	1 (20%)		1 (20%)			
	D+/R+	138 (62.2%)	117 (85%)	21 (15%)		17 (12%)			
	D+/R-	12 (5.4%)	8 (67%)	4 (33%)		3 (25%)			
IV serotype	D-/R+	76 (34.2%)	61 (80%)	15 (20%)	0.012		3 (4%)		Nd
	D-/R-	11 (5%)	6 (55%)	5 (45%)		0 (0%)			
	D+/R+	117 (52.7%)	105 (90%)	12 (10%)		4 (3%)			
	D+/R-	18 (8.1%)	15 (83%)	3 (17%)		0 (0%)			
II serotype	D-/R+	159 (71.6%)	134 (84%)	25 (16%)	0.219			0 (0%)	Nd
	D-/R-	11 (5%)	7 (64%)	4 (36%)		0 (0%)			
	D+/R+	50 (22.5%)	44 (88%)	6 (12%)		0 (0%)			
	D+/R-	2 (0.9%)	2 (100%)	0 (0%)		0 (0%)			

Table 1: Incidence of post-transplantation BKPyV viremia or serotype specific BKPyV viremia in recipients according to four different pre-transplantation BKPyV-specific antibody groups: D-/R+, D-/R-, D+/R+, and D+/R-. BKPyV-specific IgG antibody titer \geq 200 were considered as positive and <200 as negative.

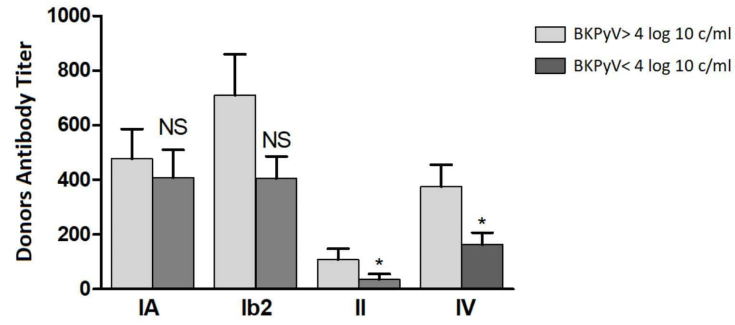
Pre-transplant Serology	Serostatus	Total n (%)	No BKV Viremia n (%)	BKV Viremia n (%)	P value	Odds ratio (CI; 95%)	Viremia			P value	Odds ratio (CI; 95%)
							with the I serotype n (%)	with the IV serotype n (%)	with the II serotype n (%)		
Ib2 serotype	R+	290 (88.1%)	240 (83%)	50 (17%)	0.372	1.44 (0.64-3.22)	37 (13%)			0.082	0.49 (0.21-1.10)
	R-	39 (11.9%)	30 (77%)	9 (23%)		2.46 (1.09 to 5.53)	9 (23%)				7.516 (2.19 to 25.73)
	D+	127 (57.2%)	101 (80%)	26 (20%)	0.026		25 (20%)			0.0002	
	D-	95 (42.8%)	86 (91%)	9 (9%)			3 (3%)				
Ia serotype	R+	305 (92.7%)	253 (83%)	52 (17%)	0.163	0.499 (0.19 to 1.26)	40 (13%)			0.106	2.208 (0.82 to 5.89)
	R-	24 (7.3%)	17 (71%)	7 (29%)		1.24 (0.56 to 2.74)	6 (25%)				0.812 (0.33 to 1.94)
	D+	150 (67.6%)	125 (83%)	25 (17%)	0.595		20 (13%)			0.646	
	D-	72 (32.4%)	62 (86%)	10 (14%)			8 (11%)				
IV serotype	R+	287 (87.2%)	239 (83%)	48 (17%)	0.135	0.566 (0.26 to 1.20)		10 (3%)		1	1.48 (0.18 to 11.87)
	R-	42 (12.3%)	31 (74%)	11 (26%)		0.418 (0.20 to 0.87)	1 (2%)				0.855
	D+	135 (60.8%)	120 (89%)	15 (11%)	0.0178		4 (3%)			1	
	D-	87 (39.2%)	67 (77%)	20 (23%)			3 (3%)				
II serotype	R+	306 (93%)	254 (83%)	52 (17%)	0.105	0.467 (0.18 to 1.19)			2 (1%)	1	0.385 (0.017 to 8.27)
	R-	23 (7%)	16 (70%)	7 (30%)		0.63 (0.24 to 1.62)	0				Nd
	D+	52 (23.4%)	46 (88.5%)	6 (11.5%)	0.339		0		0	Nd	Nd
	D-	170 (6.6%)	141 (83%)	29 (17%)			0				

Table 2: Post-transplantation BKPyV viremia incidence in recipients according to pre-transplantation BKPyV serostatus for serotypes Ib2, Ia, IV, and II. BKPyV-specific IgG antibody titer \geq 200 were considered as positive and <200 as negative.

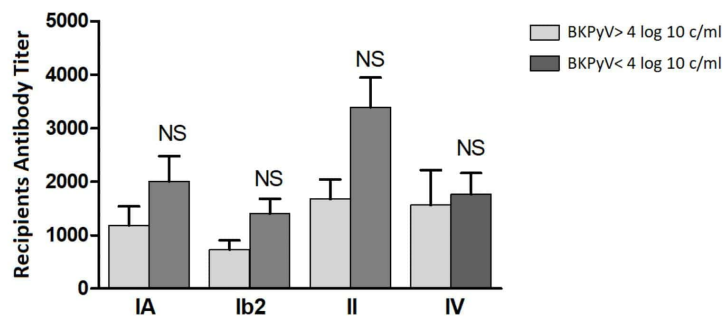
	Titers	BKPyV infection	No infection	P value	Odds Ratio	95 % CI	
		n=59	n=270				
Recipients		≤1600	32 (22%)	116 (78%)	0.148	1.57	0.89 to 2.77
		>1600	27 (15%)	154 (85%)			
	BKPyV	≤800	23 (33%)	46 (66%)	0.0006	3.11	1.68 to 5.73
		>800	36 (14%)	224 (86%)			
		≤400	16 (49%)	17 (51%)	P<0.0001	5.58	2.6 to 11.79
		>400	43 (15%)	253 (85%)			
	Serotype Ib2	≤1600	40 (25%)	140 (75%)	0.03	1.995	1.07 to 3.54
		>1600	19 (13%)	130 (87%)			
		≤800	29 (25%)	87 (75%)	0.016	2.03	1.14 to 3.59
		>800	30 (14%)	183 (86%)			
	Serotype Ia	≤400	29 (23%)	96 (77%)	0.055	1.75	0.99 to 3.092
		>400	30 (15%)	174 (85%)			
	Serotype II	≤400	17 (32%)	37 (68%)	0.0002	3.64	1.887 to 7.04
		>400	42 (15%)	233 (85%)			
Serotype IV	≤400	21 (31%)	47 (69%)	0.0039	2.62	1.41 to 4.86	
	>400	38(15%)	223 (85%)				
Donors			n=35	n=187			
		≤1600	34 (16%)	184 (84%)	0.61	0.55	0.05 to 5.48
	>1600	1 (25%)	3 (75%)				
	BKPyV	≤400	21 (13%)	144 (87%)	0.055	0.477	0.21 to 0.95
		>400	14 (25%)	43 (75%)			
		≤1600	30 (15%)	174 (85%)	0.15	0.44	0.14 to 1.34
		>1600	5 (28%)	13 (72%)			
	Serotype Ib2	≤400	15 (11%)	123 (89%)	0.01	0.39	0.18 to 0.81
>400		20 (24%)	64 (34%)				

Table 3: Recipient BKPyV viremia post-transplantation risk based on pre-transplantation BKPyV-specific antibody titer

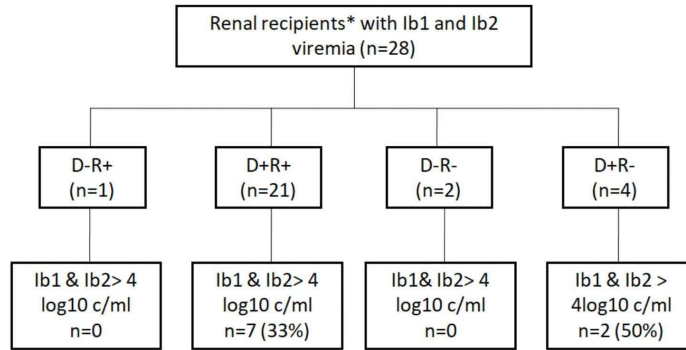
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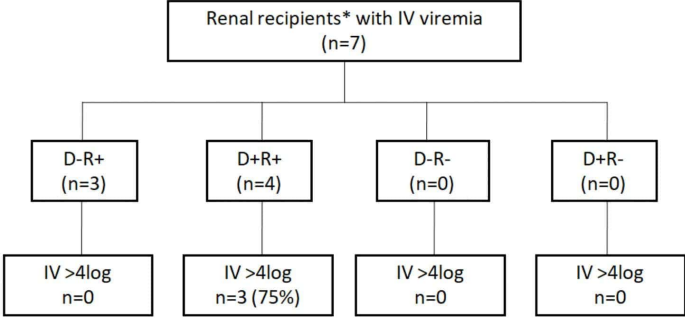
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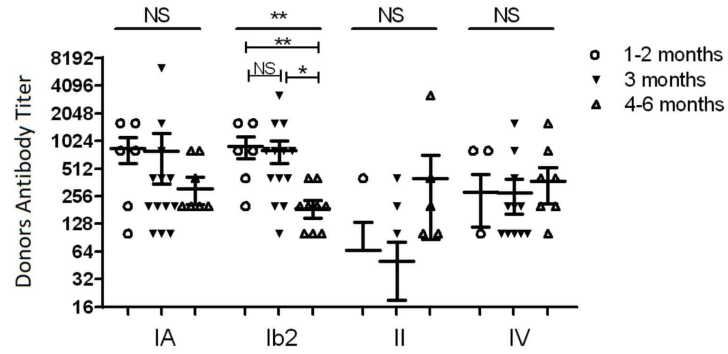
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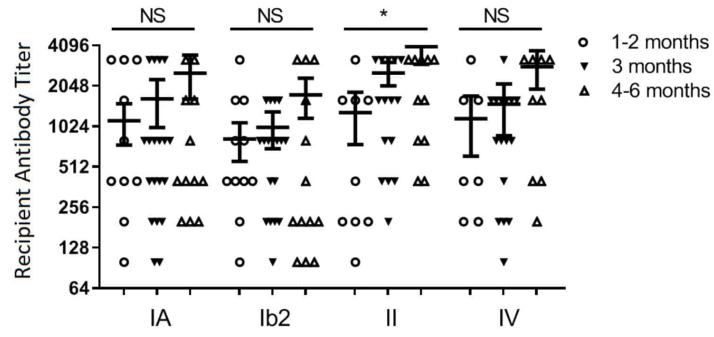
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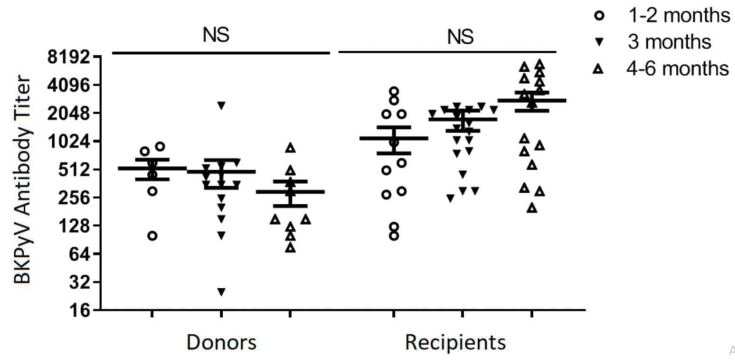
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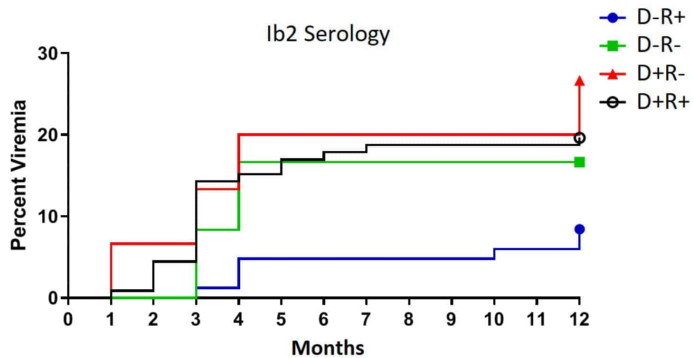


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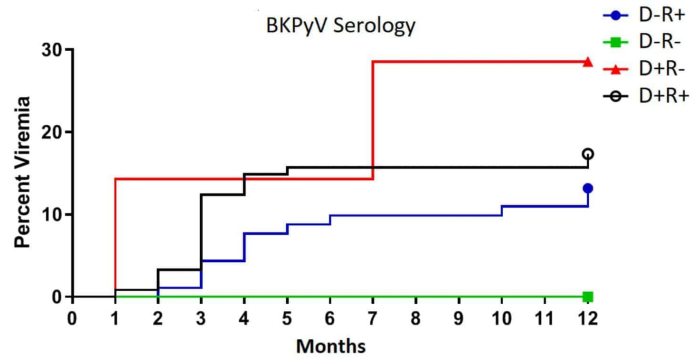


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Article

Indoleamine 2,3-Dioxygenase Is Involved in Interferon Gamma's Anti-BKPyV Activity in Renal Cells

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Abstract: Reactivation of BK polyomavirus (BKPyV) infection is frequently increasing in transplant recipients treated with potent immunosuppressants and highlights the importance of immune system components in controlling viral reactivation. However, the immune response to BKPyV in general and the role of antiviral cytokines in infection control in particular are poorly understood. Here, we investigated the efficacy of interferons (IFN) alpha, lambda and gamma with regard to the BKPyV multiplication in Vero cells. Treatment with IFN-gamma inhibited the expression of the viral protein VP1 in a dose-dependent manner and decreased the expression of early and late viral transcripts. Viral inhibition by IFN-gamma was confirmed in human cells (Caki-1 cells and renal proximal tubular epithelial cells). One of the IFN-stimulated genes most strongly induced by IFN-gamma was the coding for the enzyme indoleamine 2,3 dioxygenase (IDO), which is known to limit viral replication and regulates the host immune system. The antiviral activity induced by IFN-gamma could be reversed by the addition of an IDO inhibitor, indicating that IDO has a specific role in anti-BKPyV activity. A better understanding of the action mechanism of these IFN-gamma-induced antiviral proteins might facilitate the development of novel therapeutic strategies.

Keywords: BK virus; interferon; indoleamine-2,3-dioxygenase; immunity

1. Introduction

The ubiquitous BK polyomavirus (BKPyV) is involved in the development of nephropathy after kidney transplantation. The emergence of this BKPyV-associated nephropathy (BKVAN) [1] appears to be correlated with the clinical introduction of more powerful immunosuppressants [2]. In view of the growing number of immunocompromised or immunosuppressed patients, BKPyV is an increasingly important public health issue. Following an asymptomatic primary infection, the virus settles in the urinary tract and establishes a persistent, subclinical infection there. BKPyV-associated nephropathy is characterized by high-level BKPyV replication in the transplanted kidney's proximal tubular epithelial cells, which results in cell lysis and denudation of the tubular monolayer. The influx of inflammatory cells into the interstitium leads to tubular atrophy, interstitial fibrosis [3], and thus

impaired renal function. BKVAN affects 1% to 10% of kidney transplant patients in the first two years after transplantation [2,4]. Since an effective antiviral drug is not available, the most common treatment approach involves decreasing immunosuppressive therapy to reduce the risk of BKVAN.

Interferons (IFNs) are key antiviral cytokines that act in the body's first-line defense against infection [5]. The pro-inflammatory cytokine IFN-gamma is the only member of the type II interferon family. It is produced by innate immune cells (natural killer (NK) and antigen presenting cells) and T-cells during the adaptive immune response. Interferon-gamma activates the Janus kinase/signal transducer and transcription activator (JAK-STAT) pathway and thus allows the transcription of IFN-stimulated genes (ISGs)—many of which are involved in the fight against viral infections. Moreover, this antiviral cytokine stimulates the maturation of T and B lymphocytes and increases antibody production. It also enhances the expression of human leukocyte antigen class I and II molecules by macrophages and activates neutrophils and NK cells. However, IFN-gamma's role in controlling BKPyV infection is poorly documented. Only one study has demonstrated the effect of IFN-gamma on BKPyV [6]. Various IFN-gamma-regulated antiviral mechanisms can be induced in target cells. Interferon-gamma is a potent activator of indoleamine 2,3-dioxygenase (IDO, an enzyme involved in tryptophan catabolism). It is thought that IDO contributes to the host's innate defenses and regulates the immune response. Since many microbial organisms depend on tryptophan, it has been suggested that the degradation of this amino acid by IDO-expressing innate immune cells is the prime IDO-mediated mechanism of defense against infections [7]. The enzyme's possible involvement in the control of viral infections has been evidenced by in vitro experiments in which cytomegalovirus (CMV) replication was inhibited by treatment with IFN-gamma [8]. Subsequently, viruses such as herpes simplex virus type 2 [9], measles virus [10] and vaccinia virus [11] have been found to be susceptible to the depletion tryptophan by IDO.

Here, we analyzed the possible role of IDO secreted by IFN-gamma-stimulated cells. Our results suggest that IDO has an antiviral activity on BKPyV in vitro in Caki-1 and RPTE cells.

2. Materials and Methods

2.1. Cell Cultures

The monkey-derived Vero cell line and the human Caki-1 cell line were obtained from the ATCC (ATCC-CCL-81 and ATCC-HTB-46, respectively) and cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum. Human renal proximal tubular epithelial cells immortalized with pLXSN-hTERT retroviral transfection (RPTE/TERT1 cells) were obtained from Evercyte and maintained in renal epithelial cell basal medium (REGM CC-3191, Lonza) supplemented with human epidermal growth factor, 0.5% fetal bovine serum, hydrocortisone, epinephrine, insulin, triiodothyronine, transferrin and GA-1000 (REGM SingleQuots™ Supplement Pack, CC-4127, Lonza). All cells were cultured in a humidified environment with 5% CO₂ at 37 °C.

2.2. Antibodies and Reagents

The rabbit recombinant monoclonal anti-IDO antibody (ab211017) was purchased from Abcam. The monoclonal antibody (mAb) for the detection of STAT-1 expression was purchased from Santa Cruz (C-136) and the mAb for the detection of phospho-STAT1 (Tyr701) expression was obtained from Cell Signaling (D4A7). The 3B2 monoclonal anti-BKPyV VP1 antibody, the rabbit anti-mouse IgG (whole molecule) peroxidase-labelled antibody and the mouse anti-rabbit IgG (whole molecule) peroxidase-labelled antibody were obtained from Calbiochem. Alexa 260 Fluor Plus 488-conjugated goat anti-Mouse IgG (H + L) was purchased from Thermo Fisher. Recombinant human IFN-gamma was purchased from Roche (100,000 IU; Switzerland) and recombinant human IFN-alpha and IFN-lambda 1 were obtained from PBL Assay Science. Pyridone-6 and the pharmacological IDO inhibitor IDO5L were obtained from Calbiochem and Fisher Scientific, respectively.

2.3. BKPyV Production

The BKV-pUC19 plasmid (kindly provided by W.J. Atwood, Brown University, USA) was used to produce the BKPyV. The plasmid was obtained from pBKv (34-2) (Dunlop strain, genotype I), as described previously [12], and digested with BamHI (New England Biolabs; 2 U for every 1 µg of DNA) for 4 h at 37 °C to separate the BKPyV genome from the backbone. The DNA was then incubated at 65 °C (to inactivate BamHI) and was transfected into Vero cells using lipofectamine (Invitrogen), according to the manufacturer's instructions. Cells were cultured until a cytopathic effect was observed (after 4 weeks, typically). Next, the BKPyV was amplified by successive infections of naive cells at a multiplicity of infection (MOI) of one every four days. Extracellular and intracellular viral particles were harvested, extracted with chloroform [13] and filtered at 0.45 µm. The titers of viral stocks were determined by the 50% tissue culture infective dose immunofluorescence method (see below).

2.4. Cell Viability Assay

Vero cells were treated with different IFN concentrations in the presence of BKPyV for 72 h. The percentage of viable cells was measured using the CellTiter-glo[®] luminescent/cell viability assay (Promega, Madison, WI, USA), as described previously [14].

2.5. Immunofluorescence Staining

Cells were washed with PBS, fixed with paraformaldehyde (3.7% in PBS) and permeabilized with Triton-X100 (0.5% in cytoskeleton buffer: 10 mM PIPES, 300 mM sucrose, 100 mM NaCl, 3 mM MgCl₂, 1 mM EGTA). Infected cells were detected by immunofluorescence staining of VP1. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Immunostained cells were observed with a Zeiss Axio Vert.A1 microscope equipped with Colibri 7 LED 298 illumination (Zeiss, Oberkochen, Germany). Fluorescent signals were acquired with an AxioCam 305 color camera (Zeiss). The percentage of infected cells was automatically determined using the QuantIF macro in ImageJ (version 1.52e and Java version 8; freely available online at MDPI.com) [15].

2.6. Western-Blot

Total cell proteins were harvested 3 days after infection using radioimmunoprecipitation assay buffer (20 mM HEPES KOH pH 7.4; 150 mM NaCl; 5 mM EDTA; 0.1% SDS; 1% DOC and 1% Triton X-100). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Proteins of interest were identified using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Burlington, MA, USA), as recommended by the manufacturer. The primary antibodies were diluted in PBS containing 0.1% Tween, 5% bovine serum albumin and 0.05% azide, and the secondary antibodies were diluted in PBS containing 0.1% Tween and 5% dried milk.

2.7. Real-Time PCR

Total cellular RNA was extracted using a RNeasy Mini kit and the "Animal tissues and cells" protocol from Qiagen, according to the manufacturer's instructions. Total RNA was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit from Life Technologies. The RNA was amplified with TaqMan Universal Master Mix on an ABI 7900HT Sequence Detection System (Life Technologies, Carlsbad, CA, USA) using primers and probe sets for VP1, *IDO1* and the housekeeping gene *GAPDH*. Inhibition of BKPyV was normalized against the results for *GAPDH* and expressed according to the comparative Ct method as the fold induction [16].

2.8. Kymurenine Assay

One hundred and fifty microliters of culture supernatant were transferred to a 96-well U-bottom plate. Ten microliters of 30% trichloroacetic acid (Sigma-Aldrich, St-Louis, MO, USA) were added to each well and the plates were incubated for 30 min at 50 °C. The samples were then centrifuged at

2000× g for 10 min at room temperature. Next, 100 µL of supernatant were harvested and mixed with 100 µL of freshly prepared Ehrlich's reagent (2% 4-dimethylaminobenzaldehyde in glacial acetic acid; Fisher Scientific, Hampton, NH, USA). Absorbance at 492 nm was measured using a microplate reader (Tecan, Mannedorf, Switzerland).

2.9. Statistical Analysis

Data were expressed as the mean ± standard deviation (SD). Conditions were compared in a two-way analysis of variance with Bonferroni's correction different. The threshold for statistical significance was set to $p < 0.05$.

3. Results

3.1. Interferon-Gamma Inhibits BKPvV Multiplication more Potently than IFN-Alpha and IFN-Lambda 1

To determine the antiviral efficacy of the various types of IFN (IFN-alpha, IFN-lambda 1 or IFN-gamma), we measured the expression of the BKPvV structural protein VP1 in the presence of increasing concentrations of each IFN. To allow the induction of ISGs, Vero cells were incubated with IFN for 6 h before infection. As shown in Figure 1A, VP1 expression was only slightly inhibited by 100 and 1000 IU/mL of IFN-alpha but was strongly inhibited by 10 IU/mL of IFN-gamma. The expression of VP1 was unaffected by IFN-lambda. We also investigated viral RNA transcription by measuring the levels of mRNA for the early large T antigen (LTag) and the late structural protein VP1 three days post-infection. As shown in Figure 1B, early and late viral mRNA were inhibited in much the same manner as VP1 protein expression. The effect of IFN-gamma was not associated with changes in cell viability (Figure 1C). Interferon-lambda had a weak inhibitory effect on viral mRNA on Vero cells. In conclusion, IFN-gamma was the most potent antiviral IFN with regard to the inhibition of BKPvV multiplication.

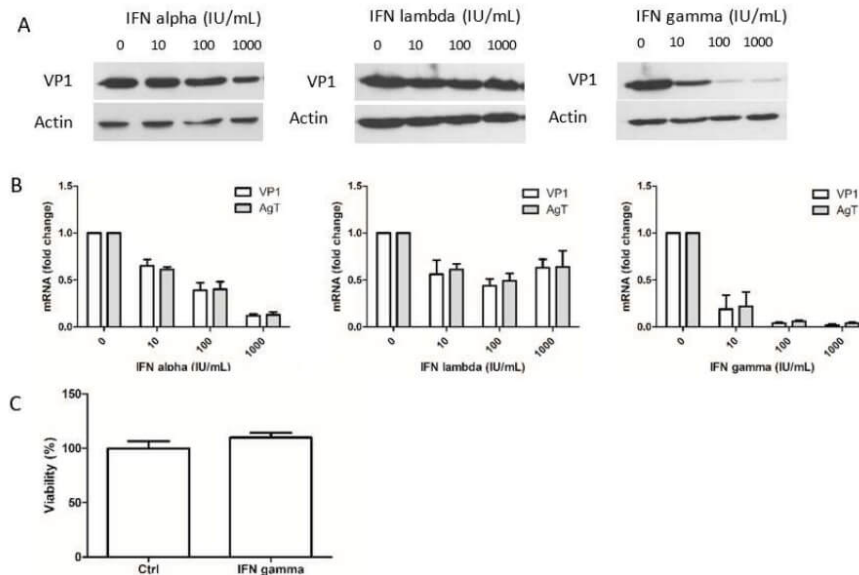


Figure 1. Effects of the three types of interferons (IFN) on BKPvV multiplication. (A) Vero cells were pre-incubated for 6 h with different concentrations of IFN-alpha, lambda or gamma and then inoculated for 3 h with BKPvV (multiplicity of infection, MOI = 0.5). Total cellular proteins were extracted 72 h post-

infection. The IFN was present throughout the experiment. VP1 and actin proteins were detected using Western blotting with specific antibodies. (B) Levels of LTag and VP1 transcripts were determined by qPCR. The results are the mean of three independent experiments performed in duplicate. (C) The absence of cytotoxicity in the presence of IFN-gamma was confirmed by the results of a cell viability assay.

3.2. The Jak-Stat Pathway Is Involved in the Antiviral Effect of IFNs on BKPyV Infection

The three types of IFN have a common signaling pathway. The Janus kinase/signal transducers and activators of transcription (JAK-STAT) pathway mainly comprises STAT1 and STAT2 heterodimers for IFN-alpha and lambda, and mainly STAT1 homodimers for IFN-gamma. As the three types of IFN had different effects on BKPyV infection, we investigated the JAK-STAT pathway's role in this context. First, we measured STAT1's phosphorylation status in Vero cells treated with 1000 IU/mL of the various types of IFN. As shown in Figure 2A, we detected brief STAT1 phosphorylation with IFN-alpha, prolonged phosphorylation with IFN-gamma and no phosphorylation with IFN-lambda. Under these conditions, the presence of BKPyV did not modify STAT1 phosphorylation. We also investigated the expression of STAT1 protein (encoded by an ISG). As shown in Figure 2B, the expression of STAT1 in Vero cells was weakly induced by IFN-alpha, strongly induced with 10 IU/mL of IFN-gamma and not induced by IFN-lambda. This confirmed that ISG induction was correlated to both Stat1 phosphorylation and IFN's antiviral effects. The JAK-STAT pathway's involvement was also confirmed by the fact that the JAK inhibitor pyridone-6 restored BKPyV's infection of Vero cells in the presence of IFN-gamma (Figure 2C). We conclude that IFN-gamma has a potent antiviral effect on BKPyV infection and is associated with a particular STAT1 phosphorylation profile.

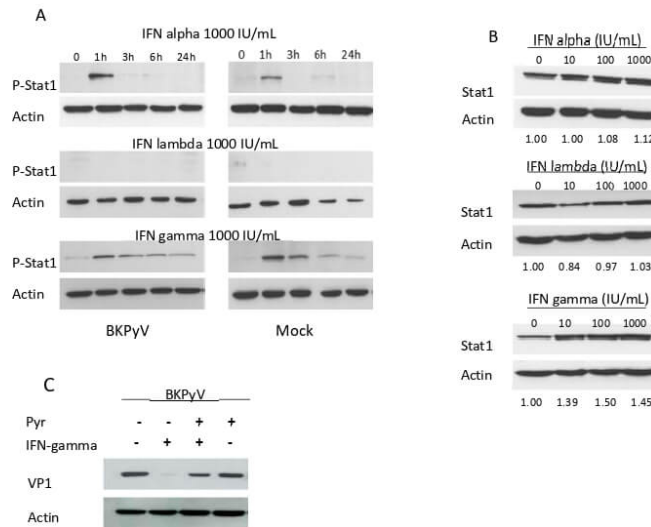


Figure 2. The Janus kinase/signal transducers and activators of transcription (JAK-STAT) pathway is involved in IFN's effect on BKPyV infection. (A) Vero cells were inoculated for 3 h with BKPyV at a MOI of 0.5. Twenty-four hours post-infection, the cells were incubated with 1000 IU/mL of IFN-alpha, -lambda or -gamma. Total cellular proteins were harvested 0, 1, 3, 6 and 24 h after IFN treatment. Phospho-Stat1 (Y 701) and actin proteins were detected by Western blotting. (B) Vero cells were pre-incubated for 6 h with different IFNs concentrations and then inoculated for 3 h with BKPyV at a

MOI of 0.5. Total cellular proteins were harvested 72 h after infection. The IFN was present throughout the experiment. STAT1 and actin proteins were detected by Western blotting. (C) Vero cells were incubated 4 h with 10 IU/mL IFN-gamma in the presence or absence of 10 μ M pyridone-6 and then infected for 3 h with BKPyV at a MOI of 0.5. The IFN and pyridone-6 were present throughout the experiment. Three days after infection, total cellular proteins were extracted and VP1 and actin were detected by Western blotting.

To find out which proteins might be involved in IFN-gamma's antiviral effect on BKPyV, we analyzed the Vero cells' transcriptomic study with a low-density array. Nine of the 64 genes studied had an induction profile that resembled that of the antiviral effect: moderate induction with IFN-alpha, poor induction with IFN-lambda and a strong induction by IFN-gamma (data not shown). Among the ISGs strongly induced by IFN gamma and poorly induced by IFN alpha, we focused on that coding for IDO—an intracellular enzyme involved in the rate-limiting step in tryptophan catabolism via the kynurenine pathway and which is reportedly active against many viruses [8,9,11,17–20]. Furthermore, IDO's immunomodulatory role makes it a promising candidate for combatting BKPyV infections.

3.3. The Antiviral Activity of IFN-Gamma (BKPyV Infection of Caki-1 Cells)

To determine whether the observed antiviral effect is specific to monkey-derived Vero cells, we chose to investigate the role of IDO in human renal cell lines (Caki-1 and RPTE/TERT1 cells). In order to avoid poor compatibility between the plasmid encoding the human *IDO1* gene and the monkey cells (which might compromise IDO's activation), we first determined whether IFN-gamma had an antiviral effect in cultures of human Caki-1 cells. To this end, Caki-1 cells were stimulated with various IFN-gamma concentrations and infected with BKPyV. Three days post-infection, total cellular proteins were harvested and VP1 expression was measured by Western blotting. Treatment with IFN-gamma was associated with significantly lower VP1 expression (relative to control experiments in the absence of IFN) in a dose-dependent manner (Figure 3A). Furthermore, an increase in the IFN dose was associated with a fall in the percentage of infected cells (Figure 3B,C). Since we had observed an influence of IFN on viral protein expression, we next determined whether IFN-gamma inhibited the production of BKPyV transcripts. Total RNA of infected cells was collected 72 h after infection. The samples were analyzed in a real-time quantitative-PCR assay for VP1 mRNA. As expected, the level of transcription inhibition was similar to that seen for VP1 protein expression 72 h after infection (Figure 3D).

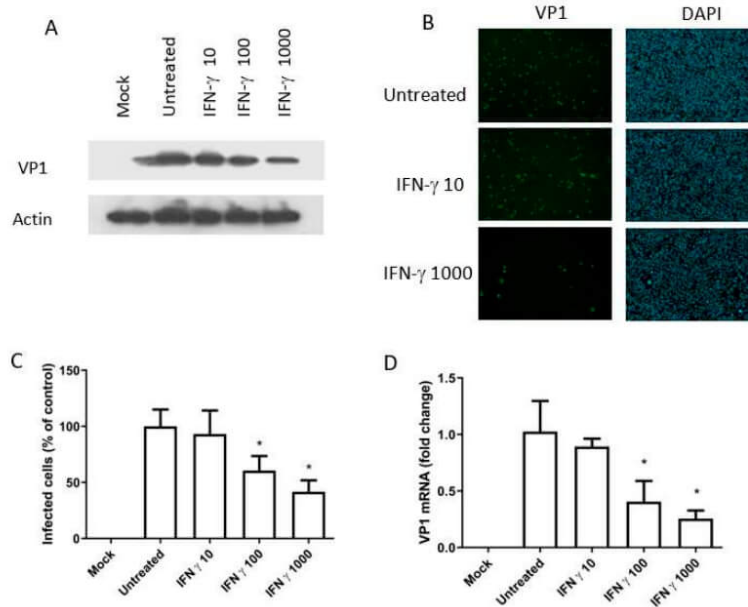


Figure 3. Interferon-gamma has dose-dependent activity against BKPv in cultures of Caki-1 cells. Caki cells were treated 6 h before BKPv infection (MOI: 0.5) with 10, 100 or 1000 IU/mL IFN-gamma (A) Total cellular proteins were extracted 72 h after infection. VP1 and actin were detected by Western blotting. (B) VP1 expression was analyzed using immunofluorescence. The cells were fixed three days post-infection and stained with monoclonal anti-BKPv VP1 antibody (in green). The DNA was stained with DAPI (in blue). Magnification $\times 400$. (C) The percentage of infected cells was calculated from (B) by comparison with the DAPI staining. (D) mRNA levels of VP1 were measured using real-time quantitative RT-PCR and normalized against *GAPDH*. Each bar represents the average of three independent experiments analyzed in triplicate. “Mock” corresponds to non-infected cells and “Untreated” corresponds to cells infected in the absence of IFN. * $p < 0.05$.

3.4. Expression of IDO in Caki-1 Cells

We next determined whether stimulation with IFN-gamma induced the expression of IDO in Caki-1 cells. To this end, Caki-1 cells were incubated with various IFN-gamma concentrations (ranging from 10 to 1000 IU/mL). We first monitored *IDO1* mRNA levels using a real-time quantitative RT-PCR (Figure 4A). Cells stimulated with IFN-gamma upregulated *IDO1* mRNA in a dose-dependent manner. In contrast, *IDO1* mRNA was not induced without IFN-gamma stimulation or by the virus. The expression of IDO was checked by Western blotting (Figure 4B). IDO was detected 24 h after stimulation with 100 and 1000 IU/mL IFN-gamma but not at all with 10 IU/mL IFN-gamma—even 72 h post-stimulation. These results show that IFN-gamma stimulated IDO expression in Caki-1 cells. We also measured IDO’s enzymatic activity by quantifying the production of the catabolite kynurenine at three different times after IFN-treatment (24, 48 and 72 h) and after transfection with a plasmid carrying the human *IDO1* gene. As shown in Figure 4C, our observation of dose-dependent kynurenine production confirmed that IDO is active in Caki-1 cells. The fact that IDO5L was able to block kynurenine production also confirmed that the IDO in Caki-1 cells was functional.

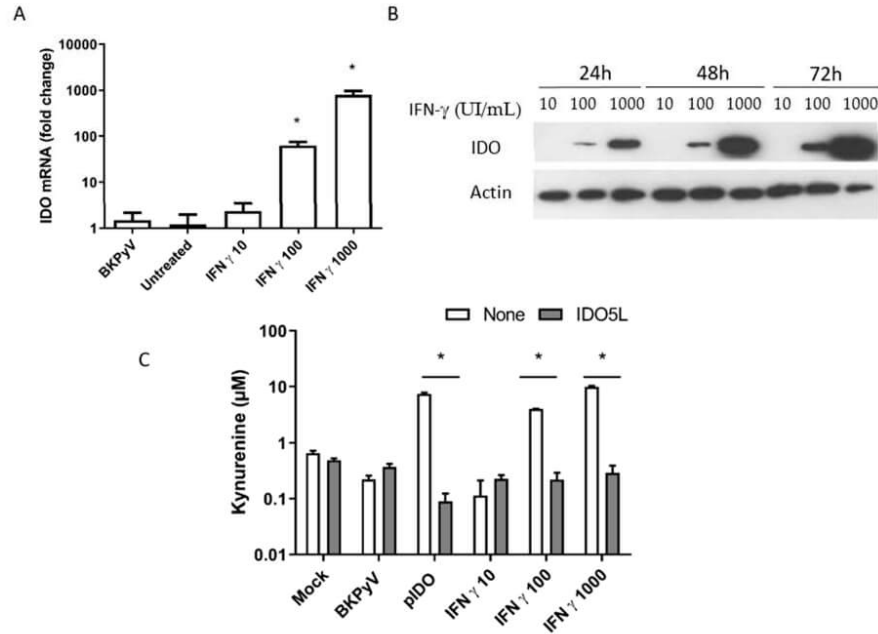


Figure 4. IFN-gamma promotes the over-expression of functionally active indoleamine 2,3-dioxygenase (IDO) in Caki-1 cells. (A) Caki cells were treated with IFN-gamma or transfected with a plasmid carrying the human *IDO1* gene. Three days after IFN treatment or transfection, the level of *IDO1* mRNA was measured using a quantitative RT-PCR assay. (B) For IDO protein expression, proteins were collected 24, 48 or 72 h after IFN treatment. The Western blot shows the IDO expression after treatment with 10, 100 or 1000 IU/mL of IFN. The results in A and C represent the mean ± SD of three independent experiments performed in triplicate. “Mock” corresponds to non-infected cells and “Untreated” corresponds to cells infected in the absence of IFN. * $p < 0.05$. (C) IDO’s functional activity and its inhibition by IDO5L at 50 μM were determined by measuring kynurenine accumulation in Ehrlich’s colorimetric reaction.

3.5. The Antiviral Effect of IDO (BKPyV Infection of Caki-1 Cells)

To assess IDO’s effects on BKPyV infection, we transfected Caki-1 cells with a plasmid encoding human *IDO1* and then infected them with BKPyV. Seventy-two hours later, we observed low VP1 expression (relative to a control experiment) on a Western blot; this confirmed IDO’s inhibitory effect on the BKPyV infection (Figure 5A, lane 10). The fact that this low expression was reversed by the addition of the IDO inhibitor IDO5L suggested that the effect was specific for IDO (Figure 5A, lane 5). To determine IDO’s role in the antiviral effect of IFN-gamma, we treated infected Caki-1 cells with IFN-gamma in the presence of IDO5L. As shown in Figure 5A (lanes 2, 3 and 4 vs. lanes 7, 8 and 9), IDO5L partially rescued the BKPyV infection. The action of IDO5L accounted for 40% of IFN-gamma’s antiviral activity (Figure 5B).

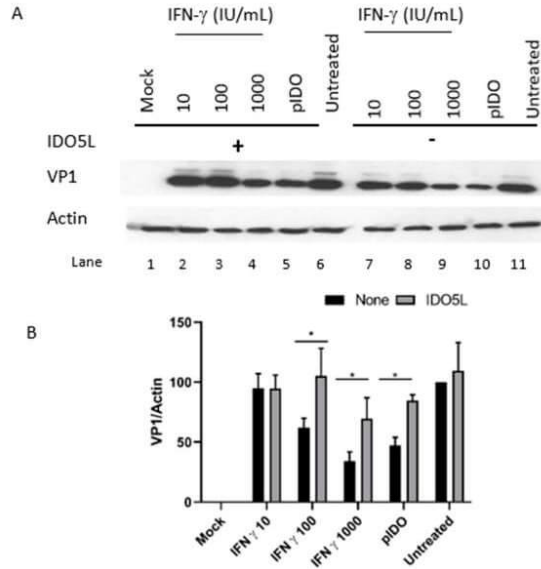


Figure 5. The effect of IDO on BKPvV infection. Caki cells were transfected with a plasmid carrying the human *IDO1* gene (lanes 5 and 10) or treated with various concentrations of IFN gamma (lanes 2, 3 and 4, and lanes 7, 8 and 9). IDO5L was added to 6 samples (lanes 1 to 6). The cells were then infected 24 h post-transfection, and total proteins were harvested 3 days post-infection. (A) VP1 and actin were detected by Western blotting. (B) Densitometric analysis of VP1 (normalized against actin). The results in B corresponds to the mean \pm SD of three independent protein samples per condition. “Mock” corresponds to non-infected cells and “Untreated” corresponds to cells infected in the absence of IFN. * $p < 0.05$.

3.6. Antiviral Effect of IFN-Gamma and IDO in RPTE/TERT1 Cells

To confirm these results, we also performed the same experiments on RPTE/TERT1 cells. We observed the rapid induction of IDO after IFN-gamma treatment of RPTE/TERT1 cells (Figure 6A). VP1 expression was strongly reduced after cell transfection with the plasmid carrying the human *IDO1* gene (Figure 6B,C). Moreover, IFN-gamma’s antiviral effect was partially restored by the presence of IDO inhibitor IDO5L (Figure 6B,C), suggesting that IDO contributes to the effect.

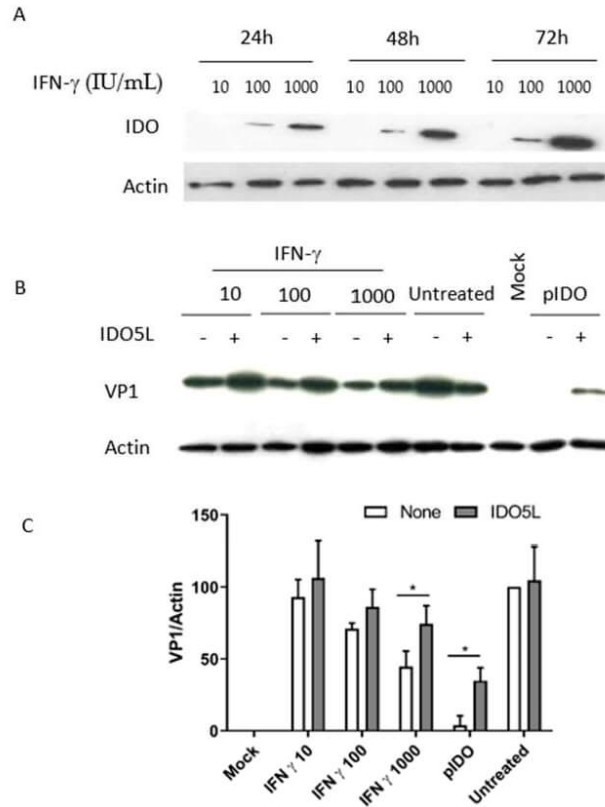


Figure 6. Antiviral effect of IFN-gamma and IDO with pLXSN-hTERT retroviral transfection (RPTE/TERT1) cells. RPTE/TERT1 cells were treated with IFN-gamma and collected after 24, 48 or 72 h. (A) Levels of IDO and actin expression were monitored by Western blotting. Infected cells were treated with 10, 100 and 1000 IU/mL IFN-gamma or transfected with a plasmid encoding human *IDO1*. IDO5L was added at the same time as IFN treatment or transfection. Total cellular proteins were harvested 3 days after infection. (B) VP1 and actin were detected by Western blotting. (C) Densitometric analysis of VP1, normalized against actin. The results in C represent the mean \pm SD of three independent protein samples per condition. “Mock” corresponds to non-infected cells and “Untreated” corresponds to cells infected in the absence of IFN. * $p < 0.05$.

4. Discussion

Our results demonstrated that BKPyV’s sensitivity to IFN depended on the type of cytokine. The first surprising finding was the moderate inhibitory effect of IFN-alpha on BKPyV infection. A low level of VP1 protein expression was only observed with 100 IU/mL of IFN-alpha, the VP1 mRNA expression was 40% of the positive control value at this concentration. There are two possible explanations for this low degree of sensitivity to type I IFN, which has also been described for human papillomavirus (HPV) [21]. Firstly, the IFN response (via ISG induction) might not be effective for DNA viruses. *Papillomaviridae* and *Polyomaviridae* have a common replication strategy; they use the host cell’s machinery (rather than viral polymerases) for multiplication. Secondly, these viruses might have developed ways of evading IFN’s antiviral effect. We showed here that IFN-lambda had only a small effect on the levels of early and late viral transcripts in cultures of Vero cells. Again, the same

result has been described for HPV [21]. We found that type II IFN had a strong antiviral effect from a concentration of 10 IU/mL upwards, as described previously [6]. Furthermore, we confirmed that levels of VP1 and LTag viral mRNA were low when Vero, Caki-1 and RPTE/TERT1 cells were exposed to IFN-gamma.

Assetta et al. showed that BKPyV's infection of RPTE cells was sensitive to a low concentration (10 IU/mL) of IFN beta [22]. We did not observe this effect with IFN alpha. Type I IFN alpha and beta have the same receptor (IFNAR1) and similar main activities but are not functionally redundant. Indeed, IFN beta's affinity for IFNAR1 is 50 times higher than that of IFN alpha, which explains the associated anti-proliferative activity [23]. This anti-proliferative activity might be important for IFN-beta's antiviral effect, since cell proliferation is a key event in BKPyV multiplication.

In view of these results, we investigated Stat1 phosphorylation. In line with the results for antiviral activity, we found that Stat1 was briefly phosphorylated after IFN-alpha treatment and lastingly phosphorylated with IFN-gamma treatment. We did not observe any Stat1 phosphorylation with IFN-lambda. These results suggest that prolonged Stat1 phosphorylation is necessary for IFN-gamma's induction of an antiviral state.

Our present results highlighted the antiviral role of IFN-gamma in the context of BKPyV infection. Several clinical studies attest to the importance of IFN-gamma in the clearance of BKPyV infections [24–26]. However, IFN-gamma has also harmful effects, such as the promotion of graft rejection. We therefore sought to address IFN-gamma's mechanism of action in this context and identify potentially involved IFN-induced proteins. Since many IFN-induced proteins could potentially be involved in this effect, we focused our attention on IDO. This enzyme was a good candidate because (i) it has already been described as being active against various viruses [8,9,11,17–20], and (ii) its immunomodulatory effect might be of value in the avoidance of graft rejection [27]. By studying two human cell lines (Caki-1 and RPTE/TERT1 cells), we demonstrated that IDO inhibits BKPyV multiplication. Moreover, the addition of an IDO inhibitor partially restored the viral infection. The antiviral contribution of IDO has been well characterized for the human respiratory syncytial virus [28]. The enzyme's primary physiological function is to catalyze the degradation of tryptophan, which decreases the availability of this essential amino acid and produces various metabolites (including kynurenine). Tryptophan availability is fundamental for the replication of intracellular pathogens, and so this modulation appears to be IDO's main antiviral mechanism. However, IDO's role as an antimicrobial factor in vivo is subject to debate. In fact, IDO's biological functions and ultimate effects (either antimicrobial or immunoregulatory) are believed to depend on the type of pathogen [29]. For example, mice models of infection have shown that IDO facilitates the elimination of *Toxoplasma gondii* but suppresses the elimination of *Leishmania major* [29]. Indeed, a genetic deficiency in IFN-gamma or IRF1 (in IFN-gamma/or IRF1/mice) and the pharmacological inhibition of IDO leads to massive mortality in mice a few days after acute infection with *Toxoplasma gondii*. In contrast, in an in vivo model of HSV1 infection, inhibition of IDO activity does not affect viral replication, virulence or induction of latency [29]. The activity of IDO also impacts the immune system. Indeed, a decrease in tryptophan levels and an increase in kynurenine levels both lead to the suppression of T cell function and thus generate an immunosuppressive microenvironment around cells that display IDO activity [29]. This immunomodulatory effect might be very valuable in the context of transplantation by both generating an antiviral effect and protecting the graft from rejection. Nevertheless, T cell inhibition might also impede the BKPyV-specific response that is thought to be particularly effective for viral clearance [25–27]. Furthermore, immunomodulation by IDO is also involved in decreased the anti-tumor defenses, which might be dangerous in transplant recipients. These considerations emphasize the importance of developing an in vivo model (such as an IDO/mouse) in which to study the true impact of IDO on BKPyV.

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An Epidemiological Study to Determine the BKPyV Sero-Prevalence and Genotypic Distribution in the Lebanese Population

BK polyomavirus (BKPyV) is a small, non-enveloped virus with a covalently closed and circular double stranded DNA genome surrounded by an icosahedral capsid. The term "BK" originated from a patient's initials, in which it was first detected in 1971. BKPyV causes asymptomatic infection reaching a sero-prevalence of 99% in adults, and then persists in the urothelium and renal cells in a latent state. In immunocompromised kidney transplant recipients, BKPyV can cause BKPyV associated nephropathy (BKPyVAN) which poses a real threat to graft survival. It is recognized as an early event that occurs within the first year after transplantation, and patients are only detected when they experience renal insufficiency. The BKPyVAN incidence increased in the previous years and the only treatment for this condition is by decreasing immunosuppression medication. We aim to perform an epidemiological study to determine the BKPyV sero-prevalence and genotypic distribution in the Lebanese population, and then compare the obtained genotypic and serological data with that of the French population. We collected a total of 151 serum and 100 urine samples from patients at the Saint George's Hospital after filling questionnaires. After the production of Ib2 BKPyV virus like particles (VLPs), they were used in ELISA tests to determine the BKPyV IgG levels in the patients' sera. Among the studied patients, 84 (56%) were males. 58 (38%) were elderly and 25 (17%) were cancer patients treated with chemotherapy. We extracted DNA from urine specimens, which will be amplified using real time PCR against the VP1 protein. The BKPyV genotypic distribution will be determined by sequencing of the positive urine samples.

Key words: BKV, BKV Seroreactivity, BKVAN, BKV Infection

Abstract

BK polyomavirus associated nephropathy (BKPyVAN), is a troublesome disease induced by BK polyomavirus (BKPyV) reactivation in immunocompromised renal graft recipients. BKPyVAN can progress to graft dysfunction and has no current treatment, making immunosuppression reduction the only management choice. Thus, predictive BKPyV infection reactivation markers are needed for high-risk patient identification. We conducted a retrospective study to assess the correlation between the BKPyV pre-transplant serostatus and post-transplant BKPyV infection incidence. Sera from 329 recipients and 222 matched donors were tested for anti-BKV antibodies against four BKPyV serotypes by a VLPs- based IgG ELISA, and BKPyV DNA load was monitored for at least 1-year post transplantation. 80 (24%) recipients were viruric and 59 (18%) recipients were viremic post transplantation. An elevated BKPyV viremia risk was observed for recipients who had a mean antibody titer for all serotypes ≤ 400 before transplantation (odd ratio [OR], 5.58; 95% confidence interval [CI], 2.60-11.79; $P < 0.0001$). In addition, kidney recipients from donors with a mean BKPyV antibody titer ≤ 400 had a lower BKPyV viremia risk (OR, 0.47; CI, 0.21-0.95; $P = 0.055$). Both donor and recipient mean BKPyV antibody titer may serve as a predictive tool to manage clinical BKPyV infection by identification of patients at high reactivation risk. In addition, a high donor's pre-transplant BKPyV antibody titer may predict the severity of the BKPyV infection in the recipient after transplantation.

Keywords: BKPyV; BKPyV associated nephropathy; BKPyV reactivation; BKVPyV serostatus; BKPyV seroprevalence; serological technique; BKPyV virus serology; kidney transplantation

Résumé

La néphropathie associée au virus BK (BKPyVAN) est une pathologie observée chez les receveurs d'une greffe rénale suite à une réactivation du virus BK (BKPyV). BKPyVAN peut évoluer vers un dysfonctionnement de la greffe et n'a actuellement aucun traitement, rendant la réduction de l'immunosuppression comme seul choix thérapeutique. Cependant, cette réduction s'avère inadaptée ou non applicable conduisant à une augmentation du risque de rejet aigu. Ainsi, des marqueurs prédictifs en pré-greffe de réactivation de l'infection à BKPyV sont nécessaires pour l'identification des patients à haut risque. Nous avons mené une étude rétrospective pour évaluer la corrélation entre le statut sérologique du BKPyV en pré-transplantation et l'incidence de l'infection par BKPyV en post-transplantation. Des sérums de 329 receveurs et 222 donneurs appariés ont été testés pour les anticorps anti-BKPyV contre quatre sérotypes de BKPyV par un test IgG ELISA à base de VLPs, la charge virale du BKPyV a été surveillée pendant au moins 1 an après la transplantation par PCR. 80 (24%) receveurs étaient viruriques et 59 (18%) receveurs étaient virémiques en post-transplantation. Un risque élevé de virémie à BKPyV a été observé pour les receveurs qui avaient un titre moyen d'anticorps pour tous les sérotypes ≤ 400 avant la transplantation (Odds ratio [OR], 5.58; intervalle de confiance à 95% [IC], 2.60-11.79 ; $P < 0.0001$). De plus, les receveurs de reins à partir de donneurs avec un titre moyen d'anticorps anti- BKPyV ≤ 400 avaient un risque de virémie à BKPyV plus faible (OR, 0.47; CI, 0.21-0.95 ; $P = 0.055$). Le titre moyen d'anticorps anti-BKPyV du donneur et du receveur peut servir d'outil prédictif pour gérer l'infection clinique avec BKPyV, en identifiant les patients à haut risque de réactivation du virus.

Mots-clés : virus BK; néphropathie associée au BKPyV; réactivation du BKPyV; statut sérologique du BKPyV; séroprevalence du BKPyV; technique sérologique; sérologie du virus BK; transplantation rénale

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