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ORIGINAL ARTICLE

BK virus genotypes and humoral response in kidney transplant recipients with BKV associated nephropathy

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Abstract

Background: Among kidney transplant recipients (KTR) with BK virus associated nephropathy (BKVN), BKV genotypes' evolution and anti-BKV humoral response are not well established. We aim to analyze BKV replication and genetic evolution following transplantation, and characterize concomitant anti-BKV-VP1 humoral response.

Methods: We retrospectively analyzed 32 cases of biopsy-proven BKVN. Stored plasma and kidney biopsies were tested for BKV viral load, and VP1 sequencing performed on positive samples. BKV-VP1 genotype-specific neutralizing antibodies (NABs) titers were determined at transplantation and BKVN.

Results: At the time of BKVN diagnosis, BKV viral load was $8.2 \log_{10}$ IU/10⁶ cells and $5.4 \log_{10}$ IU/mL in kidney and plasma, respectively. VP1 sequencing identified the same BKV-subtype in both compartments in 31/32 cases. At the time of transplantation, 8/20 (40%) of biopsies tested positive for BKV detection, whereas concomitant BKV viremia was negative. VP1 sequencing identified a different subtype compared to BKVN in 5/6 of these samples. This was confirmed following transplantation: 8 patients had a BKV+ biopsy before BKV viremia, and VP1 sequencing identified a different subtype compared to BKVN in all of them. After the onset of BKV viremia and prior to BKVN diagnosis, the BKV subtype in BKV+ plasma and kidney biopsy was the same as the one isolated at BKVN. BKV-VP1 NABs titers were significantly higher at the time of BKVN compared to transplantation ($p = .0031$), with similar titers across genotypes.

Conclusion: Altogether, our data suggest that among some KTR with BKVN, the BKV genotype from the donor may not be responsible for BKVN pathogenesis.

KEYWORDS

BK virus, BKV associated nephropathy, kidney transplantation, genetics, humoral response

Abbreviations: ATG, anti-thymocyte globulins; BKV, BK virus; BKVN, BK virus associated nephropathy; BKV-VP1, BK virus viral protein 1 (major capsid protein); CI, confidence interval; CMV, cytomegalovirus; CNI, calcineurin inhibitors; c-PRA, calculated panel reactive antigen; DNA, deoxyribonucleic acid; DSA, donor-specific antibodies; eGFR, estimated glomerular filtration rate; HIV, human immunodeficiency virus; HLA, human leucocyte antigen; IC50, 50% inhibitory concentration; IHC, immunohistochemistry; IQR, interquartile range; IV, intra-venous; KB, kidney biopsy; KT, kidney transplantation; KTR, kidney transplant recipients; LTag, large T antigen; MFI, mean fluorescence intensity; MMF, mycophenolate mofetil; NABs, neutralizing antibodies; OD, optical density; OR, odd ratios; qPCR, quantitative polymerase chain reaction; SV40, simian polyomavirus 40; VL, viral load; VLP, viral-like particle.

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1 | INTRODUCTION

BK virus (BKV) is a small non-enveloped double-strand DNA virus of the *Polyomaviridae* family.¹ BKV is a ubiquitous pathogen whose natural acquisition occurs early during childhood, mainly through the respiratory tract.² Following primary infection, BKV establishes persistent infection predominantly in the renourinary tract, where it is distributed from the kidney to the bladder.³ BKV can be divided into four main genotypes (I to IV) and several subtypes according to the sequence of the major capsid protein VP1.⁴ VP1 is the only protein expressed at the surface of the virus, and contains the main epitopes targeted by anti-BKV T-cells and neutralizing antibodies (NABs).⁵ Seroprevalence in the adult population is high (above 80%),⁶ BKV-I subtype being largely predominant worldwide followed by genotype IV.⁷ BKV-I, II, and IV have been shown to act as different serotypes,⁸ suggesting that KTR who are infected with one genotype may be vulnerable to others especially after kidney transplantation (KT) under immunosuppressive treatments.⁹

Following transplantation, due to impaired cellular and humoral immunity, persistent BKV replication in urine can be followed by BKV viremia which is detected in 10%–20% of kidney transplant recipients (KTR).¹⁰ In the absence of any therapeutic intervention, 10%–50% of the viremic subjects progress to BKV associated interstitial nephropathy (BKVN) in a median delay of 2–6 weeks, which results in graft loss in 30%–65% of the cases after one year.¹¹ There is currently no specific antiviral drug available, and the mainstay of therapy for significant BKV replication and BKVN is reducing immunosuppressive drugs,¹² despite the risk of subsequent T-cell mediated and/or antibody mediated rejection.^{13,14}

Virological and immunological determinants of the progression from self-limited BKV viruria or viremia to BKVN in KTR remains poorly understood. In particular, the origin of the viral strain responsible for the BKVN is not well established. Due to the persistence of BKV in the renourinary tract and the high rate of BKVN among KTR, the kidney donor is thought to be the main source of viral transmission in recipients with BKV infection.¹⁵ However, previous studies have shown that up to 60% of the KTR with BKV viruria shed BKV strain(s) distinct from the donor.¹⁶ Identifying the BKV strain of the donor and recipient prior to transplantation could allow us to better understand the pathogenesis of post-transplant BKV infection, and help to develop genotype-specific monitoring and therapeutical strategies. Additionally, considerable debate exists regarding the importance of BKV humoral immunity in the modulation of the clinical course of BKV infection. Although pre-existing high BKV-specific antibody titers might have a protective role,¹⁷ a strong antibody response is not always associated with viral clearance or BKVN occurrence.¹⁸

To further address these questions, we conducted a retrospective study among KTR diagnosed with biopsy-proven BKVN to: (1) analyze the kinetics of BKV replication in plasma and kidney; (2) study the in vivo BKV genetic diversity in both compartments following transplantation; (3) quantify the anti-BKV-VP1 humoral response associated with BKV replication using NAB testing.

2 | MATERIALS AND METHODS

2.1 | Study design and population

We conducted a retrospective study in the kidney transplant center of the APHP (Assistance Publique des Hôpitaux de Paris)—Saint Louis Hospital (Paris, France). All KTR provided written consent for the use of their samples for research purpose at the time of registration on the transplant waiting list. The study protocol was approved by the APHP local ethics committee and conducted in accordance with Declaration of Helsinki guidelines.

KTR received induction therapy with anti-thymocyte globulins (ATG), except for patients with past long-term immunosuppression who received basiliximab (in combination with a 500 mg methylprednisolone pulse). Maintenance therapy consisted either in a dual therapy associating a calcineurin inhibitor (CNI: tacrolimus or ciclosporin A) and mycophenolic acid (MMF), or a triple therapy with the addition of corticosteroid for the other patients. Since 2005, all KTR in our center are screened for BKV viremia monthly for the first 6 months post-transplant, then every 3 months until month 24, and every 6 months thereafter. For each transplantation with a deceased donor, a biopsy of the donor kidney is done at bench surgery before graft implantation as a routine protocol to determine baseline graft histology. Protocol kidney biopsies are also performed on months 3 and 12 post-transplant, and in the additional following cases: de novo acute kidney injury or proteinuria, suspicion of acute rejection or BKVN.

All KTR with a biopsy proven diagnosis BKVN according to the ATS-IDCOP and Banff Working Group criteria^{19,20} from January 2016 to June 2020 were included (cases of BKVN). The histological diagnosis was made in case of the presence of the following items: viral cytopathic changes (with positive staining of simian polyomavirus 40 (SV40) large T antigen (LTAg) IHC), interstitial inflammation, tubular atrophy and/or interstitial fibrosis. All biopsies were reviewed by a senior referent pathologist.

For comparison of BKV viral load (VL) in baseline kidney biopsy (KB) samples, we randomly selected during the same period control KTR who were diagnosed with histologically proven post-transplant BKVN (but who could have BKV viremia). All patients were followed-up until December 2021.

2.2 | Data and sample collection

Clinical data were retrospectively collected from each patient's medical record using an electronic standard case report form. Recorded data included demographic features, transplantation specifics, and characteristics of BKVN for all cases and controls.

For patients diagnosed with BKVN, available stored plasma and frozen KB samples between transplantation and BKVN were retrieved from the Virology and Pathology department collections for the detection of BKV DNA and VP1-genotyping. For the control group, only the biopsies performed at the time of transplantation were collected.

For every frozen biopsy, 10- μ m-thick sections were obtained using a cryostat. One section was preserved to be re-examined by a senior pathologist (light microscopy analysis and IHC staining for SV40 LTAg).

2.3 | BK virus associated nephropathy deoxyribonucleic acid detection

Total DNA extraction was performed from 500 μ l of plasma or biopsy sample using the semiautomatic NucliSENSTM easyMAG system (Biomérieux SA, Marcy l'Etoile, France), and eluted into a final volume of 100 μ l. Quantitative real-time PCR for the detection of BKV DNA was performed on 15 μ l of eluted DNA using *artus*[®] BK Virus RG PCR Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instruction. The lower limit of detection of the assay was 195 copies/ml. To correct for the variable amount of DNA in biopsy specimens, each biopsy sample was subjected to simultaneous real-time PCR for albumin using an "in-house" protocol (see [Supporting information](#)). The ratio of BKV to albumin content was used as a measure of the number of viral copies per 10⁶ cells in the tissue.

2.4 | BK virus viral protein 1 Sanger sequencing

VP1 Sanger sequencing was performed on plasma and biopsy BKV-positive samples in an automatic sequencer Genetic Analyzer 3500xL (Applied Biosystems, Foster City, USA) (detailed protocol available in Supporting information). For each sample, a VP1 consensus sequence was built using Geneious Prime 2019.0.4 software, and aligned with 12 VP1 reference sequences retrieved from GenBank database (one for each BKV subtype). Maximum likelihood phylogenetic trees were constructed on Mega 7.0 software using the neighbor-joining method with 1000 bootstrap replicates,²¹ and evolutionary distances computed using the Tamura 3-parameter model.²²

2.5 | BK virus viral protein 1 antibody testing

Serum samples at the time of transplantation (day 0) and BKVN diagnosis were collected from the Immunology department collection, and tested for the presence of both BKV-VP1 genotype-specific NAb and IgG. First, neutralization assays were conducted using BKV virus-like particles (VLPs) that expressed the VP1 major capsid proteins of BKV genotypes I, II, III or IVc2, according to a methodology previously described^{23,24} (detailed protocol in Supporting information). For each serum, the neutralization titer was defined as the sample dilution that resulted in a 50% inhibition of VLP infectivity compared to the control condition without serum, and was expressed as log₁₀ IC50. A neutralization titer of 2.0 log₁₀ IC50 was set as the threshold for quantification of antibody-mediated neutralization. Second, day 0 serum samples were also tested for the presence of BKV-VP1 IgG against subtypes Ia, Ib2, II, and IV using a VLP-based ELISA assay²⁵ (detailed protocol in Supporting information).

2.6 | Statistical analysis

For the descriptive analysis, continuous variables are presented as medians and interquartile ranges (IQR) and categorical variables are presented as numbers and percentages. Comparison was done using two-sided Student's t-test for continuous variables, and Chi² tests for categorical ones (or Fischer's test if conditions were not present). All statistical tests were two-sided; *p*-values <.05 were considered to be significant. Statistical analysis was performed using PRISM software.

3 | RESULTS

3.1 | Characteristics of the study population

From January 2016 to June 2020, 578 KT were performed in APHP-Saint Louis hospital. During this period, 32 cases of biopsy-proven BKVN were identified among KTR, leading to a frequency of 5.5%. The control population consisted in 98 KTR, including 20 (20.4%) with post-transplant BKV viremia but none with BKVN. The median duration of follow-up for cases and controls was respectively 46 [36–68] and 34 [28–44] months (*p* <.001).

Table 1 shows the main characteristics of the study population. Among BKVN cases, 18 (56%) were males, and median age at the time of transplantation was 55 (IQR: 46–63) years. Twenty-nine (91%) received a first transplantation, including 3 (9%) with a combined allograft. Compared to BKVN cases, control patients received more frequently a kidney from a deceased donor (100% vs. 91%, *p* <.001), had a lower calculated panel reactive antigen (c-PRA) score (20 [0–54]) vs. 50 [9–88]), but a higher proportion of human leucocyte antigen (HLA) mismatch equal or superior to 3 (97% vs. 77%, *p* = .001). Induction therapy consisted in anti-thymocyte globulin administration in the majority of the patients with a higher proportion in controls (99% compared to 84% of cases, *p* = .003).

Among cases, viral nephropathy was diagnosed a median time of 12 (8.2–17.5) months after transplantation, and 2.4 (1.3–6.4) months after the onset of BK viremia. Median serum creatinine level at diagnosis was 197 (135–239) μ M, and diagnostic kidney biopsies showed BKVN stage A in 5 (18%) patients, B in 21 (78%) and C in 1 (4%) according to the ATS-IDCOP and Banff Working Group histological staging criteria.

3.2 | Kinetics of BK virus deoxyribonucleic acid detection from transplantation to BK virus associated nephropathy

Table 2 describes the kinetics of BKV replication in the KB and concomitant plasma from transplantation to BKVN in cases.

At the time transplantation, 40% (8/20) of baseline biopsies (corresponding to the donor kidney) tested positive for the detection of BKV DNA (median VL: 3.7 log₁₀IU/10⁶ cells, IQR: 3.5–4.4). Concomitant plasma was negative in all cases. As a comparison, only 2/98 (2%)

TABLE 1 Clinical characteristics of the 32 cases of BKV associated nephropathy

Variable	BKVN cases (N = 32) n (%); median [IQR]	Control KTR (N = 98) n (%); median [IQR]	p-Value
Demographic features			
Age at the time of TRANSPLANTATION (years)	55 [46–63]	55 [43–64]	.88
Male sex	18 (56)	60 (61)	.62
Country of origin			.43
Western Europe	17 (44)	33 (39)	
Caribbean	2 (6)	4 (5)	
Northern Africa/Middle East region	6 (19)	14 (16)	
Sub-Saharan Africa	4 (12)	24 (28)	
Asia	3 (9)	10 (12)	
Primary kidney disease			.80
Vascular and/or diabetes	9 (28)	33 (34)	
Chronic glomerulonephritis	6 (19)	22 (23)	
Interstitial nephropathy	5 (16)	11 (11)	
Other	12 (37)*	31 (32)	
Kidney transplantation specifics			
Duration of pre-transplantation dialysis (months)	37 [11–60]	48 [24–68]	.09
First transplantation	29 (91)	88 (88)	.74
Type of transplantation			.71
Isolated kidney transplantation	29 (91)	90 (93)	
Combined transplantation	3 (9)**	7 (7)	
Deceased donor	26 (81)	97 (100)	<.001
Cold ischemia time (hours)	13 [6–15]	14 [11–16.5]	.04
c-PRA score	50 [9–88]	20 [0–54]	.003
HLA mismatch			.001
< 3	7 (23)	3 (3)	
≥ 3	23 (77)	95 (97)	
Induction therapy			.003
ATG	27 (84)	97 (99)	
Anti-CD25	4 (13)	1 (1)	
Other [†]	3 (9)	0	

Characteristics of BKVN

Age at the time of diagnosis (years)	56 [47–64]
Median time between transplant and diagnosis (months)	12.0 [8.2–17.5]
Median time between first BK viremia and diagnosis (months)	2.4 [1.3–6.4]
Immunosuppressive regimen at BKVN diagnosis	
Triple therapy	29 (91)
Corticosteroid	29 (91)
Median dose (mg/day)	10 [8–10]
Mycophenolic acid	16 (50)
Azathioprine	13 (41)
Tacrolimus	19 (59)
Ciclosporin	12 (38)
m-TOR inhibitors	2 (6)

(Continues)

TABLE 1 (Continued)

Variable	BKVN cases (N = 32) n (%); median [IQR]	Control KTR (N = 98) n (%); median [IQR]	p-Value
Serum creatinine level at diagnosis ($\mu\text{mol/L}$)	197 [135–239]		
eGFR at diagnosis (ml/min/1.73 m^2)	31 [22–48]		
DSA (NA = 2)	6 (20)		
Histological classification of BKVN (NA = 5)			
Stage A	5 (18)		
Stage B	21 (78)		
Stage C	1 (4)		
BKV genotype			
BKV-I	16 (50) [‡]		
BKV-II	8 (25)		
BKV-III	1 (3)		
BKV-IV	7 (22)		

Abbreviations: ATG, anti-thymocyte globulin; BKVN, BK-virus associated nephropathy; c-PRA: calculated panel reactive antibody; DSA, donor-specific antibodies; eGFR, estimated glomerular filtration rate (using MDRD [modification of diet in renal disease]); IQR, interquartile range; TRANSPLANTATION, kidney transplantation; TRANSPLANTATIONR, kidney transplant recipients; NA: missing data.

*Undetermined (N = 8), polycystic kidney disease (N = 3), congenital nephropathy (N = 1).

**Kidney-pancreas (N = 1), kidney–liver (N = 2).

[†]Other induction therapy: rituximab (N = 1), anti-CD52 (N = 1), plasma exchange (N = 1).

[‡]BKV-I subtypes: BKV-Ia (N = 3), BKV-Ib1 (N = 4), BKV-Ib2 (N = 8), BKV-Ic (N = 1).

TABLE 2 Kinetics of BKV DNA detection in kidney biopsy and concomitant plasma samples from transplantation to BKVN diagnosis

Variable n (%); median [IQR]	Day of transplantation (N = 20)	Pre-BKVN		BKVN diagnosis (N = 32)
		Before BKV viremia (N = 16)	After BKV viremia (N = 8)	
Median delay from Transplantation (months)	0	3.0 [3.0–3.0]	3.5 [3.0–6.5]	12.0 [8.2–17.5]
BKV DNA positive biopsy samples	8/20 (40)	8/16 (50)	7/8 (87)	32/32 (100)
Median VL in BKV+ biopsy samples ($\log_{10}\text{IU}/10^6$ cells)	3.7 [3.5–4.4]	4.8 [3.3–6.2]	5.2 [4.4–7.3]	8.2 [6.3–8.9]
BKV+ biopsy samples with same genotype as BKVN biopsy	1/6 (17) [†]	0/8	7/7	Ref
BKV DNA positive plasma samples	None	None	8/8	32/32
Median VL in BKV+ plasma samples ($\log_{10}\text{IU/ml}$)	.	.	3.5 [3.4–4.1]	5.4 [4.3–6.0]
BKV+ plasma samples with same genotype as BKVN biopsy	.	.	8/8 (100)	31/32 (97)**

Abbreviations: BKVN, BK virus associated nephropathy; IQR, interquartile range; IU: international units; KB, kidney biopsy; KT, kidney transplantation; NA, not available; VL, viral load.

[†]BKV-subtype could not be determined for 2/8 patients with a BKV-positive kidney biopsy at day 0.

*All eight BKV-positive pre-BKVN biopsy samples with a negative concomitant plasma had a different genotype compared to the one in BKVN biopsy.

**In one patient, VP1 Sanger sequencing identified a different BKV-genotype at the time of BKVN diagnosis in kidney biopsy (BKV-Ic) and concomitant plasma (BKV-Ib1).

of baseline kidney biopsies in the control population were positive for BKV detection ($p < .0001$).

Pre-BKVN kidney biopsies were collected between transplantation and BKVN diagnosis for 24 of the 32 cases. More specifically, 16 KTR had a biopsy before the onset of BKV viremia in a median delay

of 3 [3–3] months post-transplant. BKV DNA was detected in 8/16 (50%) of these patients ($4.8 \log_{10}\text{IU}/10^6$ cells, IQR: 3.3–6.2). All BKV+ biopsy samples with negative concomitant plasma were re-examined by a senior pathologist and show no evidence of viral-induced lesions (normal light microscopy and negative IHC staining for SV40 LTag).

After the onset of BKV viremia, eight patients had a KB available for virological analysis, including 7/8 (87%) with positive detection of BKV DNA ($5.2 \log_{10}$ IU/10⁶ cells, IQR: 4.4–7.3).

At the time of BKVN diagnosis, BKV DNA was detected at high VLs both in KB ($8.2 \log_{10}$ IU/10⁶ cells, IQR: 6.3–8.9) and concomitant plasma ($5.4 \log_{10}$ IU/ml, IQR: 4.3–6.0).

3.3 | In vivo BKV genetic diversity in kidney biopsy and plasma from transplantation to BKVN diagnosis

The distribution of BKV genotypes identified at BKVN diagnosis in the plasma and KB is shown in Table 1. The most frequent genotypes detected were BKV-I (16/32, 50%), BKV-II (8/32, 25%) and BKV-IVc2 (7/32, 23%). VP1 sequencing identified the same BKV subtype in both compartments in all but one patient, for whom BKV-Ic was isolated in biopsy compared to BKV-Ib1 in concomitant plasma (Table 2).

On the day of transplantation, VP1 sequencing was obtained for six of the eight patients with a BKV-positive baseline biopsy. Among them, 5/6 had a different BKV-subtype compared to the one isolated in BKVN biopsy (Tables 2 and 3).

Following transplantation, before the onset of BKV viremia, eight patients had a BKV+ pre-BKVN KB. VP1 sequencing identified a different BKV-subtype compared to the one at BKVN diagnosis in all eight samples. In particular, 5/8 patients already had a BKV-positive biopsy at baseline, including two with the same BKV subtype (Patients #3 and #10) (Table 3). After the onset of BKV viremia and before BKVN, VP1 sequencing identified the same BKV subtype compared to the one isolated at BKVN both in BKV+ biopsies ($N = 7$, Table 2), and BKV+ plasma samples ($N = 19$). Overall, for the patients with several BKV-positive biopsies before and after the onset of viremia ($N = 11$), we observed a genotype switch in the kidney as illustrated for two patients in Figure 1.

3.4 | BK virus viral protein 1 antibody response from transplantation to BKVN

Among the 32 patients with BKVN, 30 had a serum sample available at baseline for anti-BKV antibody testing. Using a genotype-specific ELISA-VLP assay, we found that at the time of transplantation, all patients harbored anti-BKV VP1 IgG against at least one subtype (100% seroprevalence), and most frequently against three (16/30, 53%) or four (12/30, 40%) different subtypes (Figure 1A). In particular, 80% of BKVN cases had positive BKV-IgG against the BKVN subtype at transplantation (Table S1). Serum samples at transplantation were simultaneously tested for the presence of BKV-VP1 NAbs against all four main genotypes (I to IV) using VLP-based neutralization testing. Twenty-three (77%) recipients harbored BKV-VP1 NAbs against at least one subtype (Figure 1A). Half of the patients had detectable NAbs against the BKVN genotype, most of them at low titers (median \log_{10} IC₅₀ 2.0, IQR: 2.1–3.1) (Table S1). Median BKV-VP1 NAb titers were not statistically different between the different BKV-genotypes (Figure 2B).

At the time of BKVN diagnosis, BKV NAb titers were significantly higher compared to the day of transplantation (4.14, IQR: 3.76–4.60; $P = .003$) but were similar across all subtypes (Figure 2B). BKV VP1 NAb titer against the genotype isolated in BKVN biopsy was not statistically different compared to those directed against the other subtypes both at transplantation (2.81 [IQR: 2.65–3.21] versus 2.73 [2.49–3.07], non-significant) and BKVN (3.85 [3.63–4.88] versus 4.47 [3.76–4.88], non-significant) (Figure 2C).

4 | DISCUSSION

In this study, we describe the kinetics of BKV replication and genetic evolution in plasma and kidney of KTR with biopsy-proven BKVN, as well as concomitant anti-VP1 NAb response. These results provide new insights on the pathogenesis of BKVN following KT.

First, we show that at the time of BKVN diagnosis, VP1 sequencing identified the same BKV genotype both in plasma and KB in all but one case. BKV-I subtype was largely predominant (50% of the cases) followed by genotype IVc2 (25%), in accordance with data from the literature²⁶. Interestingly, we found a high prevalence of BKV-II subtype (25% of BKVN diagnostic samples), which had already been found in another recent study in Spain.²⁷ This may be due to the fact that almost a third of our study population originated from Africa, a region where the prevalence of BKV-II is higher compared to Europe, Northern America, or Asia.⁷ Moreover, VP1 sequencing identified the same BKV-subtype in all plasma samples, from the onset of BK viremia to BKVN. These results suggest that early VP1 sequencing of the BKV circulating strain in viremic KTR identifies the subtype further responsible for the viral nephropathy.

Second, we found that 40% (8/20) of the baseline biopsies (corresponding to the donor kidney) were BKV-positive among cases. This proportion was significantly higher compared to control KTR who did not develop post-transplant BKVN (2%, $p < .0001$). BKV DNA detection in baseline KB is not surprising as the reno-urinary tract is the main site of BKV latency following primary infection.¹ In another study, the virological analysis of frozen tissue sections from kidney biopsies of 69 KTR revealed that BKV DNA was detected in 5/57 (8.8%) at baseline, although none of them further developed BKVN.²⁸ The higher rate of BKV-positive biopsies at transplantation observed in our study population (40%) could be due to sampling bias, with higher proportion of medullary samples among BKVN cases, where BKV latency is known to be predominant. Alternatively, the donors' characteristics (age, cause of death, lymphocyte count) could explain this discrepancy. However, we were not able to collect the information regarding the donors' characteristics due to the retrospective design of the study (missing data). Further comparison of BKV detection in baseline biopsies of BKVN cases and matched controls (on both donor and recipient characteristics) could help us to clarify the results observed in our study.

Third, pre-BKVN biopsies performed between transplantation and BKVN tested positive for BKV detection in 15/24 (63%) patients, including eight before the onset of viremia. In a previous study

**TABLE 3** Results of BKV DNA detection and VP1 sequencing on available kidney biopsy samples and concomitant plasma between day 0 and BKVN diagnosis

Patient	Day 0 (KT)				Pre BKVN				BKVN diagnosis				
	Plasma		KB		Plasma		KB		Plasma		KB		
	BK VL (logU/I)	BKV subtype	BK VL (logU/I/ 10 ⁶ cells)	BKV subtype	BK VL (logU/I)	BKV subtype	BK VL (logU/I/ 10 ⁶ cells)	BKV subtype	BK VL (logU/I)	BKV subtype	BK VL (logU/I/ 10 ⁶ cells)	BKV subtype	
1									Time post-KT (months)	BK VL (logU/I)	BKV subtype	BK VL (logU/I/ 10 ⁶ cells)	BKV subtype
2									3	5,5	II	4,72	II
3	0	la	4,33	la	0		5,2	la	6	4,78	II	6,76	II
4					3,41	IVc2	6,27	IVc2	11	5,19	lb2	8,53	lb2
5									9	6,27	IVc2	8,63	IVc2
6					0		6,15	lb1	42	4,6	la	9,35	la
7									12	3,12	II	7,59	II
8	0	lb1	6,73	lb1	3,53	lb2	8,25	lb2	17	6,44	lb2	10,48	lb2
9					0		4,38	lb2	14	6,01	lb2	9,8	lb2
10	0	la	3,68	la	0		3,78	la	12	4,06	II	7,36	II
11	0	-	3,26	-	0		6,17	la	29	6,35	lb2	4,05	lb2
12	0	-	2,97	-	0		6,88	II	48	5,35	lb1	9,59	lb1
13	0	lb1	4,75	lb1	5,15	IVc2	9	IVc2	10	4,06	IVc2	9,48	IVc2
14									4	6,09	IVc2	5,67	IVc2
15	0		0		0		0		4	4,78	lb2	8,88	lb2
16	0		0		0		0		44	5,38	II	8,37	II
17					0		0		3	4,07	la	4,68	la
18	0	II	3,57	II	0		0		29	5,5	II	8,38	II
19	0		0		0		0		3	6,57	lb1	5,62	lb1
20					0		0		36	6,72	IVc2	8,9	IVc2
21	0		0		0		0		14	3,66	lb1	4,08	lc
22	0		0		0		0		11	4,26	lb2	5,36	lb2
23					2,79	II	3,42	II	5	6,22	lc	9,25	lc
24	0		0		0		0		14	4,88	II	7,89	II
					0		0		31	5,41	II	8,91	II

(Continues)

TABLE 3 (Continued)

Patient	Day 0 (KT)				Pre BKVN				BKVN diagnosis						
	Plasma		KB		Time post-KT (months)		Plasma		Time post-KT (months)		Plasma		KB		
	BK VL (logUI/l)	BKV subtype	BK VL (logUI/10 ⁶ cells)	BKV subtype	BK VL (logUI/l)	BKV subtype	BK VL (logUI/l)	BKV subtype	BK VL (logUI/l)	BKV subtype	BK VL (logUI/l)	BKV subtype	BK VL (logUI/10 ⁶ cells)	BKV subtype	
25	0		0		3	III	3,32	III	0		6	6,18	III	8,41	III
26	0		0		6	IVc2	3,4	IVc2	4,15	ND	14	5,74	IVc2	9,58	IVc2
27					3		0		2,59	IVc2	18	5,39	lb2	6,43	lb2
28	0		0		3		0		0		10	5,58	la	7,45	la
29	0		0		3		0		0		14	5,06	lb1	8,98	lb1
30	0		0		8	IVc2	4,48	IVc2	5,19	IVc2	12	3,96	IVc2	7,95	IVc2
31	0		3,64	IVc2	3		0		2,81	la	12	4,27	IVc2	5,87	IVc2
32	0		0		9	la	4	la	4,72	la	13	3,83	la	6,45	la

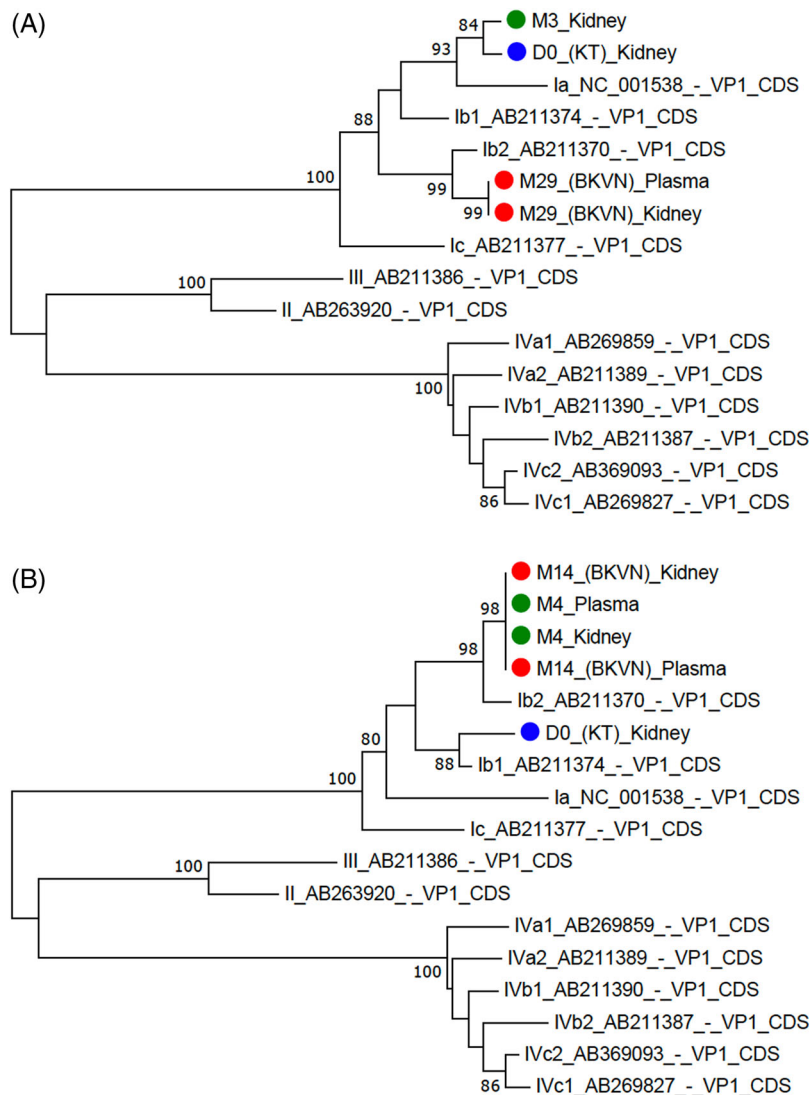
Notes: Blank cases for BKV DNA detection indicate missing samples. A hyphen (-) in BKV subtype column for BKV-positive samples (-) indicates that VP1 sequencing failed.

Abbreviations: BKV, BK virus; BKVN, BK virus associated nephropathy; IU: international units; KT, kidney transplantation; VL, viral load.

Samples with BKV-positive kidney biopsy and a negative concomitant plasma are highlighted in grey.



FIGURE 1 Maximum likelihood trees representing the BK virus (BKV)-subtypes identified in consecutive BKV-positive samples of plasma and kidney biopsy using VP1 Sanger sequencing. (A) Patient #3 and (B) Patient #8



including 21 KTR with BKVN, Randhawa et al. detected BKV DNA in 19/50 (38%) of the KB samples performed between transplantation and BKVN diagnosis, but no data were available regarding BKV detection in concomitant plasma.²⁹ In our study, all eight BKV-positive biopsy samples in KTR prior to the onset of viremia were normal on microscopic examination and had negative SV40 IHC staining, suggesting that BKV was present in a latent state. Moreover, VP1-sequencing of these biopsies systematically identified a different subtype compared to the one in BKVN biopsy. Taken together, our results show that among KTR with a BKV+ biopsy prior to the onset of viremia, the BKV subtype identified in the kidney is not always the one isolated at BKVN. In particular, in 5/6 patients, the BKV subtype isolated at day of transplantation in the kidney allograft (corresponding to the donor strain), was not the one responsible for BKVN. In KTR, the origin of the viral strain responsible for BKV infection remains a matter of debate. Based on epidemiological data and due to the persistence of BKV in the renourinary tract, the kidney donor is thought to be the main source of viral transmission in recipients with BKV infection following transplantation.³⁰ However, although a new viral strain may be

transmitted through the kidney allograft during transplantation, the latent recipient strain remains largely predominant in the bladder, the native kidneys, and ureters where BKV is distributed following primary infection.³ Recently, virological analysis of BKV-positive urine samples from pairs of living donors and kidney recipients have shown that up to 60% of the KTR with BK viruria shed BKV strain(s) distinct from the donor.¹⁶ We hypothesize that following transplantation, due to impaired cellular and humoral immunity, the BKV recipient strain whose reservoir remains largely predominant in the native ureters and bladder may reactivate, lead to BK viremia, and further colonize the allograft. Our hypothesis needs to be confirmed by the analysis of pre-transplant recipient (urine, native KB) and donor (urine) samples that were not available in our collections. Systematic screening of both donor and recipients for BKV infection (using plasma/urines/biopsy samples) could help us to unravel whether BKVN results from the reactivation of the latent recipient subtype, rather from transmission to the donor although both mechanisms may not be mutually exclusive.

Previous studies have showed that in KTR with active BKV replication, the increase of BKV antibody levels correlates with the

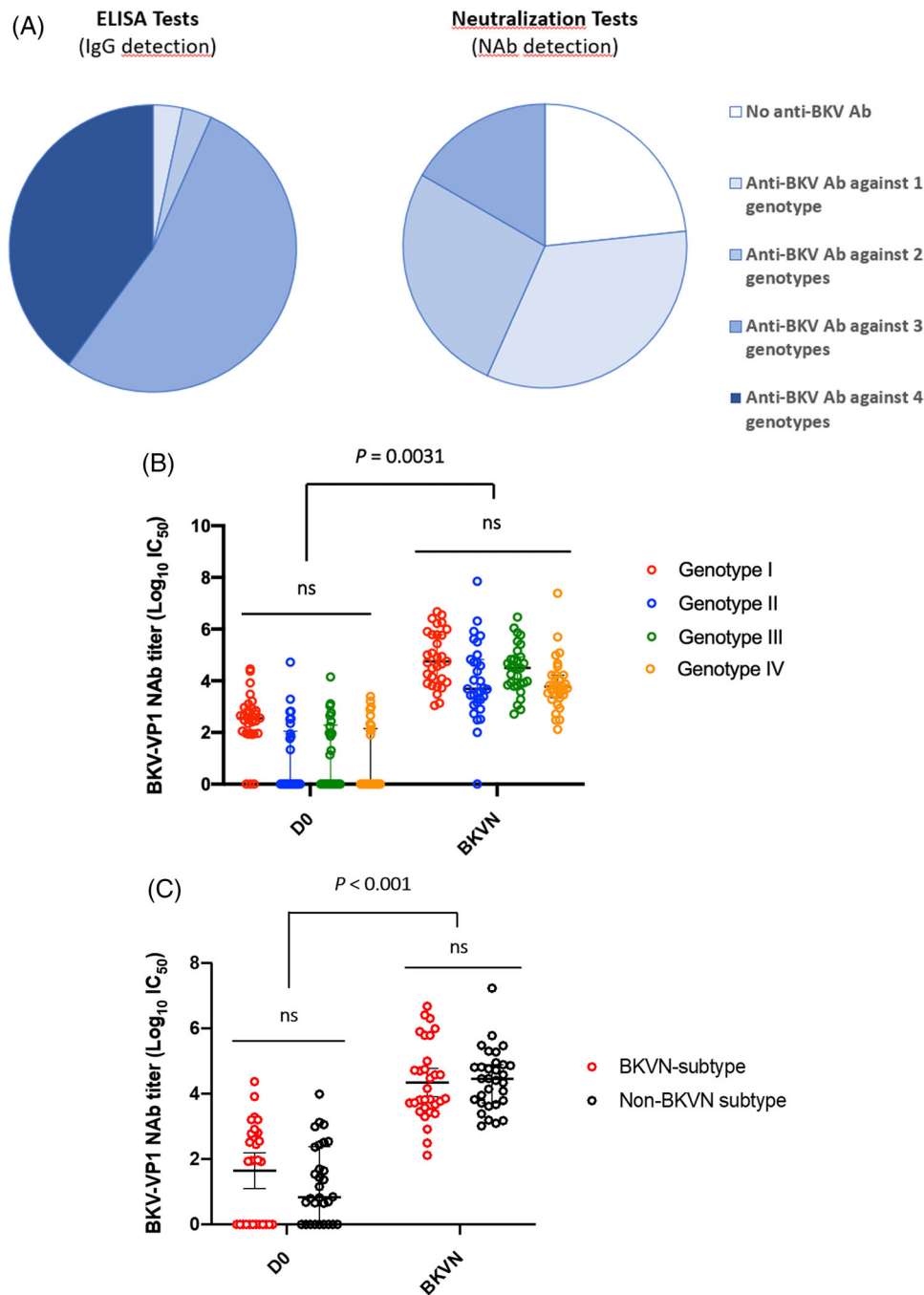


FIGURE 2 BK virus viral protein 1 (BKV-VP1) genotype specific antibody detection from the day of kidney transplantation to BKVN diagnosis. BKV VP1-genotype specific antibody titers against all four main genotypes were determined on available serum samples at the time of transplantation ($N = 30$) using ELISA- viral-like particle (VLP) or neutralization tests and BKVN diagnosis ($N = 31$) only with neutralization tests. All samples were tested with each genotype-specific VLP using both techniques. For neutralization testing, results are presented as $\text{log}_{10} \text{IC}_{50}$ (each dot representing the sample of one patient).

(A) Proportion of samples with multiple BKV-subtypes antibodies detected using neutralization or ELISA tests at the day of transplantation ($N = 30$).

(B) BKV-VP1 genotype-specific NAb titers for serum samples tested at the time of transplantation (D0, $N = 30$) and BKVN diagnosis ($N = 31$).

(C) Comparison of BKV-VP1 NAb titers against the BKVN-subtype compared to others in serum samples at the time of transplantation (D0, $N = 30$) and BKVN diagnosis ($N = 31$).



intensity of infection. BKV-VP1 IgG titers is higher in patients with BK viremia compared to those with self-limited viruria.^{31,32} However, little data are available regarding the kinetics of BKV Nab titers in KTR with biopsy-proven BKVN. In order to explain the genetic variability observed in the kidney using Sanger sequencing, we hypothesized that the reactivation of BKV subtype responsible for BKVN may be due to an impaired genotype-specific NAb response, as previously suggested.²⁴ At the time of transplantation, BKV seroprevalence in our population was 100% based on ELISA testing. However, only 77% of the patients harbored detectable BKV Nabs against at least one genotype, all of them at low titers. These results are concordant with previous studies that identified patients with NAb titers below 4 log₁₀ IC50 at-risk for BKV infection following transplantation.¹⁷ Following transplantation, we observed a strong increase of NAb titers against all BKV-subtypes in our cohort, without any difference across genotypes. This is probably due to cross-reactivity of BKV NAb following the immune activation triggered by persistent viral replication, as the different BKV genotypes share a large number of epitopes targeted by NAb, especially in the BC loop.⁹ Our results suggest that in patients with BKVN, pre-transplant screening of BKV-VP1 genotype-specific NAb cannot help to predict the risk of BKV infection with one genotype or another. The rise of BKV NAb titers is not the only effector of viral clearance in all patients, and immunosuppression tapering which allows restoration of BKV cellular immune response is also a key factor in the control of BKV replication.^{33,34}

Our study has some limits. First, the small number of patients identified limits the power. However, biopsy-proven BKVN is a rare complication following transplantation, and no study has included as much cases of BKVN for virological and immunological analysis. Second, due to its retrospective design, we were not able to provide a detailed longitudinal analysis for all cases due to missing samples. In particular, no urine samples were available for virological analysis as urine screening is not routinely performed in our center in accordance with recent international guidelines.¹⁰ If BKVN results at least in part from the reactivation of the recipient strain, we can hypothesize that KTR with detectable BKV in urine prior to transplant, the BKV strain could be the same as the one responsible for the BKVN. This needs to be further confirmed in a prospective study. Third, BK genotyping data are based on VP1 Sanger sequencing which is able to detect only the major BKV subtypes in a defined sample. The sensitivity of this technique does not allow us to discriminate some subtypes, nor to detect the presence of minority variants. As a consequence, we cannot exclude that the subtype identified at day 0 (donor strain) is still present in the kidney at BKVN diagnosis. Ongoing whole genome sequencing analysis will further help to answer the question. Finally, regarding the BKV Nab response, we cannot exclude a humoral escape to specific variants as suggested recently by Peretti et al.²⁴ Neutralizing testing using VLPs with BKV strains isolated in patients with persistent BK viremia may help to answer this question.

In conclusion, we provide a detailed virological and immunological analysis of BKVN pathogenesis in a cohort of 32 KTR. Early VP1 sequencing of BKV+ plasma samples at the onset of viremia identifies the strain further responsible for the viral nephropathy. Sequential analysis of BKV+ kidney biopsies from the day of transplantation to

BKVN shows that in a subset of patients, the genotype identified early post-transplant in the kidney is different compared to the genotype identified at the time of BKVN, suggesting that the donor strain did not solely contribute to the pathogenesis of BKVN in these KTR. BKV-VP1 NAb response in KTR with BKVN is strong and cross-reactive. The role and monitoring of BKV NAb titer during BKVN needs to be further evaluated.

AUTHOR CONTRIBUTIONS

Julien Gras, Constance Delaugerre, and Jean Michel Molina designed the study. Julien Gras and Marie Noëlle Peraldi collected clinical data. Julien Gras and Lucie Bonnet-Madin performed neutralizing antibody testing. Etienne Brochot performed BKPyV-IgG ELISA testing. Julien Gras and Marie Laure Nere performed quantitative PCR and Sanger sequencing. Julien Gras and Maud Salmona performed sequencing analysis. Jérôme Verine performed pathological re-examination of kidney biopsies. Julien Gras, Jean Michel Molina, and Constance Delaugerre wrote the main manuscript and prepared figures. All authors reviewed the paper.

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CONFLICT OF INTEREST

All authors declare no conflicts of interest for this work.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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