Detection of Recently Discovered Human Polyomaviruses in a Longitudinal Kidney Transplant Cohort

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A large number of human polyomaviruses have been discovered in the last 7 years. However, little is known about the clinical impact on vulnerable immunosuppressed patient populations. Blood, urine, and respiratory swabs collected from a prospective, longitudinal adult kidney transplant cohort (n = 167) generally pre-operatively, at day 4, months 1, 3, and 6 posttransplant, and at BK viremic episodes within the first year were screened for 12 human polyomaviruses using real-time polymerase chain reaction. Newly discovered polyomaviruses were most commonly detected in the respiratory tract, with persistent shedding seen for up to 6 months posttransplant. Merkel cell polyomavirus was the most detection, but was not associated with clinical symptoms or subsequent development of skin cancer or other skin abnormalities. In contrast, KI polyomavirus was associated with respiratory disease in a subset of patients. Human polyomavirus 9, Malawi polyomavirus, and human polyomavirus 12 were not detected in any patient samples.

Abbreviations: BKPyV, BK polyomavirus; Ct, cycle threshold; EHV, Equid herpesvirus; HPyV12, Human polyomavirus 12; HPyV6, Human polyomavirus 6; HPyV7, Human polyomavirus 7; HPyV9, Human polyomavirus 9; JCPyV, JC polyomavirus; KIPyV, KI polyomavirus; KTx, kidney transplant; MCC, Merkel cell carcinoma; MCPyV, Merkel cell polyomavirus; MWPyV, Malawi polyomavirus; NTS, nose and throat swab; PCR, polymerase chain reaction; STLPyV, St. Louis polyomavirus; TSPyV, trichodysplasia spinulosa polyomavirus; WUPyV, WU polyomavirus

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Introduction

Human polyomaviruses BK (BKPyV) and JC (JCPyV) are ubiquitous within the human population. Childhood infections typically lead to asymptomatic latent infections in adulthood and cause disease only in immunosuppressed individuals. Immunosuppressive therapy needed after kidney transplantation (KTx) can lead to reactivation of BKPyV in KTx recipients, which in turn can lead to graft dysfunction and loss (1). In contrast, reactivation of JCPyV in KTx recipients occurs less frequently, and is typically, but not exclusively, asymptomatic in nature (2).

Over the last 7 years, a deluge of new human polyomaviruses (PyV) have been discovered, including the following: WUPyV and KIPyV in the respiratory tract of children, HPyV9 in the serum of KTx recipients, MWPyV and STLPyV in the feces of symptomatic and asymptomatic children, HPyV12 in liver and gastrointestinal tissues, HPyV6 and HPyV7 on healthy skin, TSPyV in hair and skin biopsies of a heart transplant patient suffering from trichodysplasia spinulosa, and MCPyV in the tumor tissues of Merkel cell carcinoma (MCC) patients (3–6). The nomenclature of the new PyVs has departed from that of BKPyV and JCPyV, which were named after the patient initials from whom they were originally isolated; WUPyV and KIPyV were named after the respiratory tract of children, HPyV9 in the serum of KTx recipients, MWPyV and STLPyV in the feces of symptomatic and asymptomatic children, HPyV12 in liver and gastrointestinal tissues, HPyV6 and HPyV7 on healthy skin, TSPyV in hair and skin biopsies of a heart transplant patient suffering from trichodysplasia spinulosa, and MCPyV in the tumor tissues of Merkel cell carcinoma (MCC) patients (3–6).
Of particular note are MCPyV, TSPyV, and HPyV7, all of which have been associated with skin cancer or other cutaneous abnormalities; MCPyV is strongly implicated as the etiologic agent of MCC (3,8), an aggressive skin cancer occurring primarily in the elderly and immunocompromised population. TSPyV has been associated with the rare follicular disease trichodysplasia spinulosa in predominantly solid organ transplant recipients (3,9), while HPyV7 has been recently linked to atypical pruritic rashes in lung transplant patients (10).

Overall, however, little is known about the clinical impacts of the new human polyomaviruses in vulnerable transplant populations, particularly KTx recipients (11–13). The aim of this study was to address this knowledge gap and investigate the clinical role these new polyomaviruses might play in a cohort of adult KTx recipients.

### Materials and Methods

#### Study population

The study cohort consisted of 167 prospectively recruited adult patients undergoing kidney transplantation surgery at the Princess Alexandra Hospital, Brisbane, Australia between August 2008 and December 2010. A subset of the cohort was used previously in a study describing the role of NR1I2 polymorphisms in BK viremia (14). The Metro South Human Research Ethics Committee approved this study (#HREC/08/QPAH/037). Patient immunosuppression was administered according to standard unit protocol. Specifically, induction therapy consisted of 20 mg intravenous basiliximab preoperatively and at day 4 postoperatively, and 500 mg intravenous methylprednisolone preoperatively and 12 h postoperatively. Oral tacrolimus was administered preoperatively at a dose of 0.075 mg/kg, and continued twice daily to achieve individualized target trough concentrations of approximately 6–10 μg/L over months 1–3 and 3–8 μg/L thereafter. Oral mycophenolate mofetil was initiated preoperatively at 1000 mg twice daily, and continued postoperatively with dosage adjustments as necessary for toxicity or rejection. Oral prednisolone was initiated postoperatively at a dose of 0.3 mg/kg ideal body weight once daily and maintained at this dose until 1 month posttransplant, after which it was tapered to a long-term maintenance dose of 5–7 mg by 6 months.

#### Sample collection

Whole blood, urine, and combined nose and throat flocked swabs (NTS) (Copan Diagnostics, Brescia, Italy) were collected from recipients pre-transplant, on day 4, and approximately at months 1, 3, and 6 posttransplant when possible. Additional sample collections were performed in patients who presented to outpatient clinics with BK viremia up to 1 year posttransplant. Blood and urine were also collected from living donors prior to transplant surgery.

#### Sample processing, nucleic acid extraction, and quality control

NTS samples were resuspended in 1 mL of phosphate-buffered saline, with all samples having their total nucleic acids extracted on the Qiagen QIAxtractor using the DX chemistry, as per manufacturer’s instructions (Qiagen, Chadstone Centre, Australia). In brief, 200 μL of sample was extracted through an automated silica membrane-based platform and eluted into 150 μL of DX elution buffer. Prior to extraction, each sample was spiked with 1 × 10^6 genome equivalents of Equid herpesvirus (EHV) culture (whole virus) for the purposes of monitoring extraction quality and presence of polymerase chain reaction (PCR) inhibitors. Extraction quality was monitored through an EHV real-time PCR (Table S1). Samples falling outside of the expected cycle threshold (Ct) value ± 3 Ct deviation were considered to have either failed extraction or contained PCR inhibitors, and were subsequently re-extracted.

#### Virus detection

A mixture of published and newly developed real-time PCR assays were used to screen for the recently discovered human polyomaviruses KPyV, WUPyV, MCPyV, HPyV7, TSPyV, HPyV9, MWPyV, STLPyV, and HPyV12 (4,5,9,15–18), as well as JCPyV and BKPyV (19,20) (Table S1). Assay specificity was ensured by in silico analyses against contrasting PyV genomes and the use of confirmatory assays. In most cases, samples were considered positive only if both the primary and confirmatory assays were positive. WUPyV, JCPyV, and BKPyV genomes have been well characterized, and therefore single detection assays could be used with high confidence of specificity. Quantification was performed using plasmid standards containing each assay target (Life Technologies, Scoresby, Australia). Assays targeting JCPyV, BKPyV, WUPyV, and MCPyV utilized the Qiagen Quantitect Probe PCR mix kit (Qiagen, Australia), while the remaining PyV assays (MCPyV, HPyV6,7,9,12, TSPyV, MWPyV, and STLPyV) used the Bioline SensiMix II Probe PCR mix kit (Bioline, Evelyne, Australia). Standard curves were retrospectively applied to pre-existing JCPyV and BKPyV detection data; therefore, calculated viral loads for the two viruses should only be taken as approximate values. For every assay, 8 pmol of each respective primer, 3.2 pmol of respective probe, and 2 μL of template were used in a final reaction volume of 20 μL. Cycling was performed with either a Rotorgene 6000 (Qiagen, Australia), or a LightCycler 480 (Roche Diagnostics, Castle Hill, Australia). All assays displayed a reproducible sensitivity of approximately 10 copies/reaction. Extended respiratory virus screening included the following: respiratory syncytial virus, influenza A and B viruses, parainfluenza 1, 2, and 3 viruses, human adenovirus, human metapneumovirus, human bocavirus, human coronavirus, 229E, HKU1, OC43, NL63, human rhinovirus, and cytomegalovirus by using previously reported real-time PCR assays (21,22).

#### Virus genome sequencing

Given the paucity of available whole genome sequences, a directed rolling circle amplification method (18) was used to amplify and sequence the whole genomes of STLPyV, TSPyV, HPyV6, and HPyV7.

#### Statistical analysis

A subgroup (n = 36) of the study cohort enrolled in an ongoing longitudinal skin cancer study was used to further investigate the role of MCPyV in skin cancer development. For inclusion in the skin cancer study, patients were required to be white race. The detection of MCPyV and development of skin cancer with 5 years posttransplant were compared in a 2 × 2 contingency table using a two-tailed Fisher’s exact test (http://www.graphpad.com/quickcalc/contingency1). The two-tailed Fisher’s exact test was also used to examine the significance of KIPyV association with respiratory disease, as compared with all other polyomavirus detections.

### Results

#### Sample cohort

Of the study cohort, 52 and 115 received kidneys from live and deceased donors, respectively. The majority (71%) were male and the age at transplantation ranged from 18 to 74 years (mean 48.5). In total, 507 blood, 338 urine, and 372 NTS samples were collected, with 3, 1, and 2 mean collections per patient, respectively.
Seven of the 10 PyVs tested for were detected in at least one sample, with only HPyV9, MWPyV, and HPyV12 not detected (Table 1). All samples were additionally screened using the HPyV9 confirmatory assay; however, no further positives were identified. Non-JCPyV/BKPyV polyomavirus detections were predominantly found in respiratory samples (Tables 1 and S2). Median time to first detection of each PyV occurred either at the time of transplant, or approximately 1 month later (Table 1). Viral loads were highest in the respiratory samples for all detected PyVs apart from TSPyV, which was only detected in one patient's blood. In contrast, both JCPyV and BKPyV were predominantly detected in urine and blood, with particularly high viral loads present within the urine (Tables 1 and S2). Co-detection of JCPyV or BKPyV with any of the other human polyomaviruses was rare and always involved one species occurring at very low viral loads (Table S2).

KIPyV had the highest NTS mean viral load of any PyV in this study, and compared to all the PyV detections, was the only one to be significantly associated (p < 0.0256) with respiratory disease. Of the seven patients who tested positive for KIPyV, three displayed respiratory symptoms (Table S3). Routine bacterial cultures and respiratory virus testing failed to identify any additional pathogens in these three patients. In two of the KIPyV-positive patients, KIPyV was detected in both pre- and posttransplant samples. Both had received extensive previous immunosuppression (Supplementary Material). In each of the two cases, viral loads increased 10- to 100-fold over time (Figure 1), coincident with the development of respiratory symptoms (Figure 1 and Table S3).

Six patients demonstrated persistent MCPyV shedding, which in all but one case was in the respiratory tract (Table S3). Routine bacterial cultures and respiratory virus testing failed to identify any additional pathogens in these three patients. In two of the KIPyV-positive patients, KIPyV was detected in both pre- and posttransplant samples. Both had received extensive previous immunosuppression (Supplementary Material). In each of the two cases, viral loads increased 10- to 100-fold over time (Figure 1), coincident with the development of respiratory symptoms (Figure 1 and Table S3).

Detections of HPyV6, TSPyV, and STLPyV were rare (Tables 1 and S1). In some cases persistence and increases in viral loads were observed during the periods of collection (Table S3). One PyV that had the highest NTS mean viral load of any PyV in this study, and compared to all the PyV detections, was the only one to be significantly associated (p < 0.0256) with respiratory disease. Of the seven patients who tested positive for KIPyV, three displayed respiratory symptoms (Table S3). Routine bacterial cultures and respiratory virus testing failed to identify any additional pathogens in these three patients. In two of the KIPyV-positive patients, KIPyV was detected in both pre- and posttransplant samples. Both had received extensive previous immunosuppression (Supplementary Material). In each of the two cases, viral loads increased 10- to 100-fold over time (Figure 1), coincident with the development of respiratory symptoms (Figure 1 and Table S3).

Hystory for 5 years posttransplant reviewed for the development of skin cancers or other abnormal skin pathologies. The 5-year skin pathology diagnosis for MCPyV, HPyV6, HPyV7, and TSPyV was 27.7%, 14.3%, 33.3%, and 1%, respectively. However, no discernable pattern between the development of skin cancers or other abnormal skin pathologies was associated with MCPyV, HPyV6, HPyV7, and TSPyV shedding, which in all but one case was in the respiratory tract (Figure S1). In all instances, MCPyV was present at the time of transplantation, and in some cases, it persisted for at least 7 months posttransplant. In one case, viral loads increased 10- to 100-fold over time (Figure 1), coincident with the development of respiratory symptoms (Figure 1 and Table S3).

Table 1: Polymavirus detection rates in patients' blood, urine, and nose and throat swabs, their mean viral loads measured as genomic copies per mL of sample, and months posttransplant for first detection

| Virus         | Pos. ps. (n = 167) | Blood Pos ps. | Mean viral load | Pos ps. | Urine Pos ps. | Mean viral load | NTS Mean viral load | Range (months) | First detection post-Tx |
|---------------|-------------------|---------------|-----------------|---------|---------------|-----------------|-------------------|-----------------|-------------------------|
| MCPyV         | 17 (10.2%)        | 3 (1.8%)      | 1.07E+04^2      | 2 (1.2%)| <LoQ          | 14 (8.4%)       | 3.06E+04          | 0–7             | 0.9 0                   |
| WUPyV         | 15 (9.0%)         | 2 (1.2%)      | <LoQ            | 4 (2.4%)| <LoQ          | 10 (6.0%)       | <LoQ              | 0–6.3           | 2.2 0.9                |
| HPyV6         | 7 (4.2%)          | 1 (0.6%)      | <LoQ            | 1 (0.6%)| <LoQ          | 5 (3.0%)        | 2.07E+02          | 0–7             | 1.4 0                   |
| KIPyV         | 7 (4.2%)          | 0             | <LoQ            | 1 (0.6%)| <LoQ          | 7 (4.2%)        | 7.86E+05          | 0–2.8           | 1 0.9                  |
| HPyV7         | 3 (1.8%)          | 0             | <LoQ            | 1 (0.6%)| <LoQ          | 2 (1.2%)        | <LoQ              | 0–1.2           | 1.4 1.4                |
| TSPyV         | 2 (1.2%)          | 1 (0.6%)      | 1.94E+05        | 0       | <LoQ          | 1 (0.6%)        | 8.03E+04          | 0.9             | 0.9 0.9                |
| STLPyV        | 1 (0.6%)          | 0             | <LoQ            | 0       | <LoQ          | 1 (0.6%)        | 8.03E+04          | 0.9             | 0.9 0.9                |
| HPyV9         | 0                 | -             | -               | -       | -             | -               | -                 | -               | -                       |
| MWPyV         | 0                 | -             | -               | -       | -             | -               | -                 | -               | -                       |
| HPyV12        | 0                 | -             | -               | -       | -             | -               | -                 | -               | -                       |
| JCPyV^1       | 24 (14.4%)        | 6 (3.6%)      | 2.96E+04^2      | 20 (12.0%)| 4.15E+06     | 1 (0.6%)        | <LoQ              | 0–3.4           | 1.1 0.9                |
| BKPyV^1       | 49 (29.3%)        | 21 (12.6%)    | 3.85E+06        | 45 (26.9%)| 5.51E+09     | 6 (3.6%)        | 1.98E+04          | 0–9.8           | 2.3 2.2                |

Tx, transplant; NTS, nose and throat swab; <LoQ, below limit of quantitation; MCPyV, Merkel cell polyomavirus; WUPyV, WU polyomavirus; HPyV, human polyomavirus; KIPyV, KI polyomavirus; TSPyV, trichodysplasia spinulosa polyomavirus; STLPyV, St. Louis polyomavirus; MWPyV, Malawi polyomavirus; JCPyV, JC polyomavirus; BKPyV, BK polyomavirus.

1Approximate viral load values.
2Single detection above limit of quantitation.
virus detection and skin disease or cancer load could be identified (Table 2). Within the secondary study sub-group, 16.6% (6/36) of the participants had at least one MCPyV detection, which constituted 33% (6/18) of all MCPyV-positive patients within the larger cohort. The 5-year skin cancer rate of the subgroup’s MCPyV-positive subjects was 66.6% (4/6), which did not significantly differ from the overall skin cancer rate of 69.4% (25/36). All cancers detected were keratinocytic cancer and no patient developed MCC during the period of follow-up.

**Virus genome sequencing**

Both TSPyV, one of the two STLPyV, two of the seven HPyV6, and none of the HPyV7 isolates were able to be
amplified and have their entire genome sequenced. Overall, each sequenced genome showed high homology to existing reference genomes (Table S4) and did not exhibit any unique alterations in their noncoding control regions (Table S4). Furthermore, the TSPyV genome within patient 127 was completely stable, despite active replication during the first month of immunosuppression posttransplant (Table S4).

**Discussion**

There has been increasing attention given to the pathogenicity of polyomaviruses posttransplantation. BKV infection is commonly detected in KTx recipients, and can portend graft dysfunction and nephropathy in cases of persistently high viremia (1). This study aimed to investigate whether the recently discovered human polyomaviruses were present and associated with disease in KTx populations. The two “traditional” human polyomaviruses, JCPyV and BKPyV, have been well characterized within the KTx population, and have thus been used in this study as an internal control and comparator to the other polyomaviruses being tested for. Indeed, the range of viral loads observed for both viruses within the urine and blood samples correlated well with previously reported values (12,23,24).

In line with previous findings, the initial detection of BKPyV in this study typically occurred approximately 2 months posttransplant (25), and contrasted with the majority of the new human polyomaviruses, which occurred in samples collected at, or around, the time of transplantation. This timing of primary detections suggests that most of these viruses occur as pre-existing persistent infections within KTx patients, or less frequently, are acquired soon after surgery as nosocomial infections. Furthermore, the lack of detections in any donor samples suggests that the PyVs are reactivating as a response to immunosuppressive therapy rather than being donor-derived. The BKPyV infection pattern of viremia occurring prior to viremia (25) was confirmed in this study, but was not observed for any of the new PyVs, indicating that the urogenital tract is not likely to be a primary site of infection for these viruses.

Of all the detected new human PyVs, only KIPyV was significantly associated with symptomatology within the first 6 months posttransplant. The persistent detection of KIPyV in the NTS samples, the increasing viral loads, and concurrent respiratory symptoms align with KIPyV’s possible involvement in respiratory disease (26). In the only other study to describe KIPyV in KTx patients, the detection of the virus in respiratory tract samples was concluded by the authors not to be associated with respiratory disease, as only 17% of cases presented with mild upper respiratory tract infection (11). However, in this study, 43% of cases were associated with respiratory tract disease, with no other pathogen detected. This suggests that virus reactivation and disease may be possible under certain circumstances, such as long-term pre-existing immunosuppression (e.g. patients 43 and 68 in this study); however, further study with larger numbers of cases is required to formalize any links between KIPyV and respiratory disease. Recently KIPyV was reported as the sole pathogen in the upper respiratory tract and lung tissue of a pediatric transplant patient who died of respiratory failure (27), which adds further evidence towards a contextual role of the virus in respiratory disease.

MCPyV, HPyV6, HPyV7, and TSPyV are thought to be skin-tropic viruses and have been associated with skin cancer (MCPyV) (8), or skin pathologies in immunosuppressed subjects (TSPyV, HPyV6, and HPyV7) (9,10,28). In this study, however, there were only a limited number of patients in whom these viruses were detected, and therefore it was difficult to discern associations with subsequent skin pathologies. Skin malignancies occur more often in fair-skinned people; therefore, a subpopulation of the cohort that was selected for light complexities was used to examine the MCPyV data in further detail. A number of MCPyV-positive patients in the subgroup went on to develop skin cancer, but the malignancy rates were not significantly different from those of the overall population. While the results are suggestive of a lack of association between MCPyV and skin malignancies, the small number of cases clearly require further studies with much larger sample sizes and longer posttransplant periods to adequately address any potential role of MCPyV in KTx patient skin pathology. The frequency of persistent MCPyV shedding in the respiratory tract is also of great interest, and should be a target of future investigation.

HPyV9, MWPyV, and HPyV12 were not detected within the study population. In contrast, a recently published study (29) described detection of HPyV9 at low loads in the sera of a KTx patient (29). This discrepancy is most likely explained by the previous study’s use of larger template volumes, which would have conferred a fivefold increase to the testing protocol’s effective sensitivity, and may have been sufficient to detect the reported very low viral loads.

In conclusion, the majority of the recently discovered human polyomaviruses can be detected in KTx patients within the first 6 months of transplantation, and occur primarily within the respiratory tract. Clinical symptoms or disease were not seen in association with most of these polyomaviruses. KIPyV was the exception, being associated with respiratory symptoms in a subset of KTx patients. MCPyV and other presumed skin-tropic PyVs were not associated with increased rates of posttransplant skin malignancies; however, the frequent shedding of MCPyV, a suspected oncogenic agent, requires further study to elucidate what role it may have in postoperative KTx disease.
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Disclosure

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

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New Polyomaviruses in Kidney Tx Patients

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Data S1: Supplementary Material.
Figure S1: Patients showing persistent polyomavirus shedding, in one or more concurrent sample types. Month of collection posttransplant, along with which sample type was collected and tested during that time period (B, blood; U, urine; N, nose and throat swab) is shown on the x-axis. Viral loads are shown as copies per mL of sample along the y-axis. Asterisks indicate viral loads below the assay’s quantitation capabilities, and have for illustration purposes been shown with a value of 3500.

Table S1: Primer and probe sequences for real-time PCRs used in the study. EHV was used solely for extraction quality control.

Table S2: Results of all study samples screened for 10 polyomaviruses stratified by subject, month of collection posttransplant, and sample type. Red = blood, yellow = urine, green = combined nose and throat swab. All detection shown as genomic copies per mL of sample.

Table S3: Polyomavirus-positive samples with relevant patient symptoms within 1 week period either side of sample collection.

Table S4: Overview of whole genome sequences amplified using dRCA.