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Protocols

Development of a urinary antigen test for BK Polyomavirus to help clinicians in patients' follow-up

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ABSTRACT

BK polyomavirus (BKPyV) is responsible for hemorrhagic cystitis and nephropathy in bone marrow- and kidney transplant recipients, respectively. Monitoring BKPyV DNAuria and/or DNAemia by quantitative molecular tests is thus a common practice in the management of these patients. However, critical issues have been raised regarding these methods and the detection of viral genomes by positive PCR is not always associated with true viral replication. Identification of novel biomarkers and development of new assays are thus needed to help clinicians in patients' follow-up and enable timely and beneficial therapeutic interventions. Here, we describe the development of a urinary lateral flow assay that specifically detects the major BKPyV VP1 protein in a few minutes. This assay was characterized using cell culture-produced virus, pseudoviruses as well as VP1 pentamers representing the four BKPyV genotypes, diluted in urine matrix. The limit of detection was around 1 µg/mL of purified VP1 pentamers. This assay also enabled to detect BKPyV VP1 in frozen urine from transplant patients with high DNAuria. Clinical validation studies are now needed to evaluate the performance of this assay in real life and its relevance for interventions.

1. Introduction

BK polyomavirus (BKPyV) is a ubiquitous virus with a prevalence of around 90 % in the adult population worldwide (François et al., 2022). Four BKPyV genotypes further divided in subtypes exist with varying prevalence in different world regions: 80, 15 and 5 % for genotype I, IV and II/III, respectively (Morel et al., 2017). The primary infection occurs during childhood, probably via the aero-digestive route, then the virus may diffuse hematogenously and subsequently persist in the renal epithelium.

Although generally silent in immunocompetent individuals, BKPyV can be pathogenic in persons with immunosuppression, particularly in bone marrow- and kidney- transplant recipients. Indeed, BKPyV-associated haemorrhagic cystitis (BKPyV-HC) occurs in 8 %-25 % of paediatric and 7 %-54 % of adult recipients undergoing allogeneic haematopoietic stem cell transplant (HSCT), which leads to higher

mortality and prolonged hospitalization (Cesaro et al., 2018). Besides, 1–10 % of kidney transplant recipients (KTR) experience BKPyV-associated nephropathy (BKPyVAN), which can lead to graft failure and return to dialysis (Kotton et al., 2024). No effective antiviral treatment that are specific to BKPyV exists but BKPyVAN in KTR can be preemptively avoided by reducing the immunosuppressive treatment though this entails significant risks of graft rejection (Parajuli et al., 2018). In contrast, such therapeutic intervention has no benefit in HSCT patients because of the risk of worsening the donor versus host alloreactivity and the severity of graft-versus-host disease (Cesaro et al., 2018).

Monitoring BKPyV is thus a common practice in the management of bone marrow- and kidney- transplant recipients (Cesaro et al., 2018; Kotton et al., 2024) and currently, the determination of BKPyV DNAuria and/or DNAemia by quantitative molecular tests are the key diagnostic tools in clinical virology laboratories. However, critical issues have been

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raised regarding these methods (Kotton et al., 2024), and importantly the detection of viral genomes by PCR is not always associated with true viral replication. Moreover, as demonstrated by Leuzinger et al (Leuzinger et al., 2019), in plasma samples, PCR detects genome which is largely free and not encapsidated and thus does not objectively indicate viral replication (hence use of the term DNAemia and not viremia). For BKPyV nephropathy, nucleic acid tests have a poor positive predictive value but an excellent negative predictive value (Kotton et al., 2024). Urinary PyV-Haufen test and quantification of BKPyV VP1 messenger RNA has been evaluated but is either difficult to implement in a routine laboratory activity and not suitable for screening (33477927, 12829934). It is therefore important to identify new biomarkers and develop new assays in order to overcome these issues and enable timely and beneficial therapeutic interventions.

In this study, we developed a qualitative lateral flow assay (LFA) that specifically detects the presence of the BKPyV capsid protein VP1. We also investigated its applicability as a new diagnostic method to detect BKPyV replication from urine samples.

2. Materials and methods

2.1. Production of cell culture virus, pseudoviruses, virus-like particles and capsomers

Cell culture virus (Dunlop strain; genotype Ia) was produced in Vero cells (CCL-81), as described previously (Dugan et al., 2007). Pseudoviruses were produced and purified on iodixanol gradient, as previously described (Pastrana et al., 2013). BKPyV VP1 pentamers of different subtypes (Ia, Ib2, II, III and IV) were produced using a BL21 bacterial strain transformed with a pET-21a-d plasmid containing the VP1 sequence fused to glutathione S-transferase. The JCPyV VP1 sequence was also used to produce control capsomers. The capsomers were purified on Glutathione Sepharose 4B resin (GE Healthcare).

2.2. Monoclonal antibody production and selection

Anti-BKPyV VP1 Monoclonal antibodies (mAbs) were generated by fusion of splenocytes harvested from mice immunized with BKPyV VLPs. Stable hybridomas were then cloned by limiting dilution and screened by ELISA for reactivity against BKPyV VLPs representing the four genotypes. Among the specific mAbs, the F6 antibody was selected for its binding and affinity capacities.

2.3. BKPyV VP1 antigen capture ELISA

A sandwich ELISA was developed by coating microtiter plates with the F6 mAb and detecting the captured antigen with biotin-conjugated F6 mAb. The microtiter plates were coated overnight at 4°C with 50 ng of F6 mAb per well in 0.05 M carbonate buffer (pH 9.6). Wells were washed once with phosphate-buffered saline containing 0.5 % (wt./vol) Tween 20 before blocking for 2 h at 37°C with 200 µL of phosphate-buffered saline containing 2 % (wt./vol) fetal bovine serum to minimize non-specific binding. Samples (urine, cell culture virus, pseudoviruses or capsomers) were mixed with an equal volume of pre-treatment buffer. Fifty microliters of pretreated samples were added and incubated for 60 min at room temperature. After washing three times, the plates were incubated at room temperature for one hour with biotin-conjugated F6 mAb (2 mg/L; 100 µL per well) and then washed three times with phosphate-buffered saline containing 0.5 % (wt./vol) Tween 20. Subsequently, 100 µL of streptavidin-horseradish peroxidase substrate solution was added and incubated for 15 min at room temperature. The reaction was interrupted by adding the same volume of 2 M H₂SO₄. Absorbance was measured at 450 nm on a microplate reader. The limit of detection (LOD) was set at 0.4 based on results obtained with 18 BKPyV PCR-negative urine samples and considering the mean + 2 standard deviations of the optical densities.

2.4. Development of the lateral flow strip

For antigen capture, the F6 mAb was covalently coupled to red-stained nano-act cellulose beads (Asahi Kasei®) at a ratio of 0.1 before deposition on the conjugate pad at a ratio of 0.2 % dry extract. For binding detection, the F6 mAb (2 µg per strip) was applied to the test area on a nitrocellulose membrane (HF+180; Millipore®). Thus, the test line indicates a positive result for the sandwich immunoassay reaction between BKPyV VP1 antigen and the F6 mAb pairs. Anti-mouse antibody (1 µg per strip) was applied to the control zone. The lateral flow system was created by overlapping a sample pad, a conjugate pad, a nitrocellulose membrane and an absorbent pad.

2.5. Lateral flow assay (LFA) procedure

To perform the LFA, 25 µL of samples (urine, cell culture virus, pseudoviruses or capsomers) were added to 25 µL Tris-HCl. Pretreatment buffer (50 µL) was then added. The mixture was incubated at room temperature for 10 min before application to the sample window on the test strip. The readout could be determined visually after 10–15 min. BKPyV-positive samples result in the formation of a red-colored band on both the test and control lines, whereas negative samples produce a red-colored band only on the control line. To quantify band intensity, the test strip was scanned and analyzed on a Qiagen ESEQuant Flex LF Reader (Qiagen, Hilden, Germany). The LOD was set to an intensity of 10 based on analysis of eighteen BKPyV PCR-negative urine samples from kidney-transplant patients. Above this value, the result was considered as positive.

2.6. Quantification and genotyping of experimental samples

Viral DNA was extracted from 200 µL of total urine or cell culture media using a NucliSENS easyMAG system (bioMérieux). The extracted DNA was recovered in 50 µL elution buffer and frozen at –80°C before use. BKPyV DNA was amplified using a quantitative real-time PCR assay kit that targets the large T antigen gene (RealStar® BKV PCR Kit), as previously described (Descamps et al., 2015). BKPyV genotype was determined by sequencing the BKPyV virus typing and grouping region, located in the VP1 sequence, as described previously (Morel et al., 2017).

3. Ethics

This retrospective study using patient samples was approved by the local institutional review board (reference: PI2022_843_0079). The study was conducted in accordance with the French national reference methodology (MR-004) and was recorded in Amiens University Medical Center's registry of data-processing activities, in accordance with Article 30 of the European Union's General Data Protection Regulation.

4. Results

4.1. Production of monoclonal antibodies to specifically detect BKPyV VP1 antigen

Anti-BKPyV VP1 mAbs were generated as described in the materials and methods section. The F6 mAb (IgG1 isotype) was selected for its binding and affinity capacities and further characterized in a capture ELISA assay. First, a stock of BKPyV (Dunlop strain; genotype Ia; 11.73 log₁₀ viral genome copies/mL (c/mL); 10⁶ FFU/mL) produced in Vero cells was serially two-fold diluted in pooled BKPyV-negative urine (Fig. 1A). Saturating optical densities were observed up to 10 log₁₀ c/mL and then a progressive decrease to reach the LOD around 8.5 log₁₀ c/mL (~10⁴ FFU/mL). We also evaluated the capacity of the capture ELISA assay to detect pseudoviruses (*i.e.* viral capsids incorporating a reporter plasmid) or capsomers (*i.e.* VP1 pentamers), representing the different

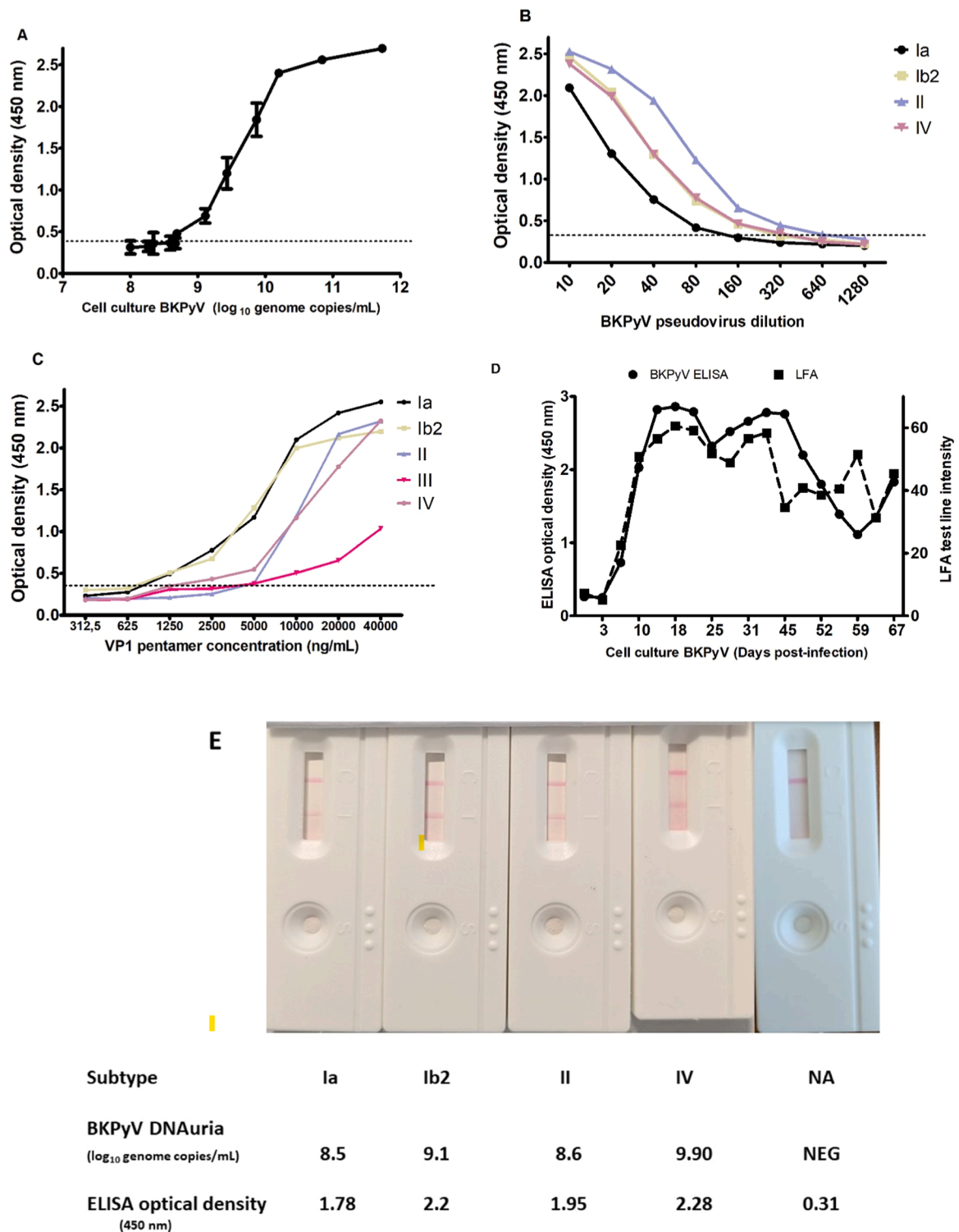


Fig. 1. Detection of BKPyV VP1 in urine by capture ELISA and LFA. (A) BKPyV viral stock (Dunlop strain, genotype Ia) produced in Vero cells was serially two-fold diluted in BKPyV negative urine. The presence of BKPyV VP1 was detected in each sample by capture ELISA. Data are presented as mean \pm SD of three independent experiments. The dotted line represents the positivity threshold defined for the ELISA assay. (B) Pseudoviruses representing the different BKPyV subtypes were produced in HEK293TT cells and serially two-fold diluted in BKPyV negative urine. The presence of BKPyV VP1 was detected in each sample by capture ELISA. Data are presented as mean of three independent experiments (The standard deviation is not shown to avoid overloading the figure). (C) Capsomers representing the different BKPyV subtypes were produced in bacteria and purified before serial two-fold dilution in BKPyV negative urine. The presence of BKPyV VP1 was detected in each sample by capture ELISA. Data are presented as mean of three independent experiments (The standard deviation is not shown to avoid overloading the figure). (D) Vero cells were infected with BKPyV (Dunlop strain) at an MOI of 1. Supernatants were collected at each passage and the presence of BKPyV VP1 was assessed by capture ELISA and LFA. ELISA results are shown as optical densities measured at 450 nm. LFA results are shown as test line intensities Measured by Qiagen ESEQuant Flex LF Reader (Qiagen, Hilden, Germany). (E) The presence of BKPyV VP1 was detected by LFA in urine samples from kidney-transplant patients with high BKPyV DNAuria and different BKPyV subtypes.

genotypes and diluted in a BKPyV-negative urine matrix (Figs. 1B and 1C). The LOD was exceeded for pseudoviruses representing all genotypes up to a 160-fold dilution (Fig. 1B). Positive results were also observed above 5 µg/mL of capsomers for all subtypes and even 1.25 µg/mL for subtypes Ia and Ib2 (Fig. 1C). Importantly, the F6 mAb showed no cross-reactivity with the JC virus (JCPyV), a related polyomavirus frequently found in patients' urine samples, when performing capture ELISA with cell culture produced JCPyV or JCPyV capsomers (data not shown).

4.2. Detection of BKPyV VP1 antigen by lateral flow assay (LFA)

To qualitatively detect BKPyV VP1 antigens and avoid the need for a multi-step ELISA technique, we developed a rapid screening test in the form of an immunochromatographic strip using the F6 mAb. The samples used in the capture ELISA were also assessed in LFA and the intensities of the test bands were read on a Qiagen ESEQuant Flex LF Reader in order to objectively quantify the results (Table 1). Setting the band test intensity threshold at 10, the qualitative results were identical to those obtained with the capture ELISA. Similar results were also obtained in both assays in terms of sensitivity and specificity, with no detectable test band for JCPyV samples. These data demonstrate that it is possible to detect BKPyV VP1 antigen in a urine matrix using LFA. To further characterize our LFA, we evaluated BKPyV production in cell culture over a period of 2 months. At each cell passage, the presence of VP1 in cell supernatants was assessed using LFA and capture ELISA as control. As shown in Fig. 1D, both assays gave very similar results. VP1 antigen was detectable from day 3 post-infection and reached a plateau around day 10, followed by a slow decline from day 40. More interestingly, we also tested our LFA using frozen BKPyV PCR-positive urine samples from patients having received a kidney or bone marrow transplant. These samples were obtained from patients with either described haemorrhagic cystitis or detection of urinary BKPyV DNA without documented nephropathy at the time. Positive signals were obtained using several samples containing BKPyV of the four main viral subtypes

with elevated DNAuria (Fig. 1E). PCR-negative urine samples were also included to evaluate the performance of our LFA, all of which returned negative results. Altogether, our results demonstrate that the presence of BKPyV VP1 antigen in urine samples can be detected very quickly and easily by LFA.

5. Discussion

In this study, we describe the development of a LFA that enables to specifically detect the presence of BKPyV VP1 antigen in urine. This assay detects all BKPyV genotypes, though with slightly lower reactivity for subtype III which is not very prevalent worldwide. In contrast, it does not cross-react with JCPyV, which is frequently found in urine from transplant patients (Demey et al., 2024).

More than 80 % of HSCT patients develop high-level BKPyV DNAuria but only 5 %–20 % develop BKPyV-HC. Thus, high-level BKPyV DNAuria is not specific for BKPyV-HC and increased risk for BKPyV-HC is only associated to DNAuria > 7 log₁₀ c/mL. JCPyV, Adenovirus, herpes simplex virus, cytomegalovirus and other infectious or non-infectious aetiologies may also cause HC, and must be excluded for appropriate diagnosis and management. The LFA described in this study will enable us to investigate whether the detection of BKPyV VP1 antigen in the urine of HSCT patients is more strongly associated with BKPyV-HC than high level DNAuria. If so it could allow clinicians to easily and rapidly diagnose BKPyV-HC.

In KTR, high-level BKPyV DNAuria precedes DNAemia on average by approximately 6 weeks (range, 1–12 weeks). Thus, BKPyV-DNAuria > 10⁷ c/mL associated with undetectable DNAemia points to a “possible” BKPyVAN. Besides, BKPyV-DNAemia > 10³ c/mL sustained for more than two weeks and BKPyV DNAemia > 10⁴ c/mL are respectively associated with “probable” and “presumptive” BKPyVAN (Kotton et al., 2024). BKPyVAN can only be proven by specific immunohistochemistry on a biopsy. Critical issues have been raised regarding BKPyV molecular tests, such as underquantification or false-negative results because of viral sequence variability as well as false-positive

Table 1
Mean±SD LFA test band intensity as measured by the ESEQuant Flex LF reader.

| | Viral load (Log ₁₀ viral genome copies/mL) | | | | | | | | | | | | |
|--------------------|---|--------------|------------|--------------|--------------|------------|--------------|--------------|------------|--------------|--------------|-----------|-----------|
| | 11.7 | 10.8 | 10.5 | 10.2 | 9.9 | 9.4 | 9.1 | 8.7 | 8.7 | 8.6 | 8.3 | 8.3 | 8.0 |
| Cell culture BKPyV | 40.7 ± 2.4 | 41.5 ± 4.3 | | 57.0 ± 3.0 | 22.9 ± 1.9 | 23.0 ± 4.2 | 17.9 ± 2.2 | 15.3 ± 0.9 | 11.8 ± 1.0 | 6.0 ± 1.1 | 6.3 ± 0.5 | 6.5 ± 0.8 | 6.3 ± 0.3 |
| Cell culture JCPyV | | | 6.7 ± 0.31 | | | | | | | | | | |
| | dilution | | | | | | | | | | | | |
| | 10 | 20 | | 40 | 80 | | 160 | 320 | | 640 | 1280 | | |
| Pseudovirus (Ia) | 23.12 ± 6.89 | 51.32 ± 0.31 | | 21.59 ± 1.19 | 25.08 ± 1.95 | | 19.13 ± 3.25 | 14.92 ± 1.4 | | 13.15 ± 0.44 | 6.93 ± 0.16 | | |
| Pseudovirus (Ib2) | 32.87 ± 0.11 | 64.45 ± 4.87 | | 44.02 ± 1.43 | 33.97 ± 2.45 | | 23.45 ± 0.43 | 18.22 ± 1.83 | | 15.88 ± 3.56 | 12.57 ± 0.13 | | |
| Pseudovirus (II) | 63.21 ± 1.81 | 40.23 ± 2.05 | | 43.87 ± 5.19 | 36.25 ± 1.06 | | 27.36 ± 2.77 | 17.89 ± 3.08 | | 14.55 ± 0.24 | 12.55 ± 0.04 | | |
| Pseudovirus (IV) | 59.35 ± 1.24 | 46.90 ± 2.34 | | 49.54 ± 0.21 | 39.63 ± 2.95 | | 25.97 ± 3.85 | 22.09 ± 1.04 | | 18.94 ± 0.49 | 10.54 ± 0.45 | | |
| | Concentration (ng/mL) | | | | | | | | | | | | |
| | 40000 | 20000 | | 10000 | 5000 | | 2500 | 1250 | | 625 | 312.5 | | |
| Capsomer (Ia) | 82.45 ± 1.33 | 62.43 ± 2.21 | | 60.61 ± 5.37 | 52.8 ± 3.15 | | 48.43 ± 3.23 | 40.14 ± 1.12 | | 15.63 ± 3.97 | 8.63 ± 1.38 | | |
| Capsomer (Ib2) | 45.44 ± 3.09 | 39.69 ± 0.91 | | 34.75 ± 0.03 | 18.47 ± 0.95 | | 12.75 ± 0.24 | 12.81 ± 0.22 | | 9.52 ± 0.56 | 8.68 ± 0.11 | | |
| Capsomer (II) | 72.67 ± 0.1 | 61.58 ± 1.63 | | 45.25 ± 2.75 | 24.19 ± 1.93 | | 22.78 ± 0.2 | 15.35 ± 0.51 | | 8.60 ± 0.42 | 7.60 ± 0.06 | | |
| Capsomer (III) | 53.57 ± 2.03 | 37.79 ± 1.18 | | 30.39 ± 4.42 | 16.44 ± 0.41 | | 18.42 ± 0.77 | 15.75 ± 0.05 | | 6.14 ± 1.15 | 8.37 ± 0.35 | | |
| Capsomer (IV) | 74.17 ± 1.08 | 59.21 ± 3.24 | | 52.82 ± 0.08 | 41.21 ± 6.09 | | 27.53 ± 2.72 | 23.87 ± 1.91 | | 5.24 ± 0.75 | 7.97 ± 0.48 | | |
| Capsomer (JCPyV) | 9.61 ± 0.41 | | | | | | | | | | | | |

results because of cross-detection of sequences conserved across other polyomaviruses such as JCPyV (Kotton et al., 2024). More importantly, the detection of viral genomes by PCR is not always associated with true viral replication, with high proportions of non-encapsidated BKPyV genomes which may lead to unnecessary immunosuppression reduction responsible for increased risk of graft rejection (Leuzinger et al., 2019, 2023). Therefore, it will be of major interest to investigate whether the detection of BKPyV VP1 antigen in KTR urine enables to better predict true BKPyV replication. This could permit timely therapeutic interventions and avoid unnecessary immunosuppressive reduction. From the kinetic data in cell culture, we observed that the antigenic test was positive a few hours after PCR. This is not surprising since, as with other viruses, PCR will always be more sensitive.

If LFA proves effective in the management of HSCT patients and/or KTR, it would offer many advantages as compared to molecular tests. In addition to easy and quick handling, the use of urine samples instead of plasma can offer a practical advantage in pediatric patients. LFA could also be a cost effective BKPyV screening method for transplant programs in resource-limited settings. With the development of research programmes for antivirals, immunoglobulins or T-cells therapy against BKPyV, we need a range of tools to assess the performance of these future new therapeutic solutions. The main limitation of this new tool is that it can currently only be used for studies on BKPyV *in vitro*. It must subsequently demonstrate its capacity and non-inferiority in the detection of BKPyV in patient urine. The test has also not been developed for blood samples.

In conclusion, we demonstrated that LFA enables to easily and quickly detect BKPyV VP1 antigen in frozen urine samples from KTR and HSCT patients. Funding are currently being sought for a multi-center study to test our LFA in real conditions and to evaluate the potential benefit of BKPyV VP1 antigen detection as compared to molecular testing, using fresh urine samples collected from patients following kidney or bone marrow transplants.

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CRedit authorship contribution statement

Castelain Sandrine: Writing – review & editing, Supervision. **Gaboriaud Pauline:** Writing – review & editing, Investigation, Formal analysis. **Helle François:** Writing – review & editing, Writing – original draft, Validation, Supervision, Conceptualization. **Collet Louison:** Writing – review & editing, Validation. **Aubry Aurélien:** Writing – review & editing, Validation, Methodology. **Morel Virginie:** Validation, Methodology. **Descamps Véronique:** Investigation, Data curation.

Brochot Etienne: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Investigation, Formal analysis, Conceptualization. **Touzé Antoine:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **Demey Baptiste:** Writing – review & editing, Investigation, Formal analysis. **Fourdinier Ophélie:** Writing – original draft, Investigation, Formal analysis, Data curation.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Etienne Brochot, Pauline Gaboriaud and Antoine Touzé reports a relationship with Spydiag (biotechnology start-up) that includes: board membership. The other authors declare that they have no competing interests.

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