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The role of the DE and EF loop of BKPyV VP1 in the serological cross-reactivity between subtypes

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ABSTRACT

BK virus (BKPyV) is a causative agent of BKPyV-associated nephropathy and graft rejections in kidney transplant patients. It establishes persistent infection in the kidneys, which can lead to reactivation in an immunosuppressed state or transmission to kidney recipients. Complications in the case of donor-derived infections can be caused by differences between the four known BKPyV subtypes, as prior infection with one subtype does not guarantee protection against *de novo* infection with other subtypes. The recipient and donor pretransplant serotyping is not routinely performed since simple ELISA tests employing antigens derived from the major viral capsid protein 1 (VP1) are hindered by the high cross-reactivity of anti-VP1 antibodies against all subtypes. Identifying subtype-specific epitopes in VP1 could lead to the design of specific antigens and the improvement of serodiagnostics for kidney transplantation. We aimed to study the surface residues responsible for the interactions with the subtype-specific antibodies by focusing on the DE and EF loops of VP1, which have only a small number of distinct amino acid differences between the most common subtypes, BKPyV-I and BKPyV-IV.

We designed two mutant virus-like particles (VLPs): we introduced BKPyV-I characteristic amino acid residues (either H139N in the DE loop or D175E and I178V changes in the EF loop) into the base sequence of a BKPyV-IV VP1. This way, we created BKPyV-IV mutant VLPs with the sequence of either the BKPyV-I DE loop or the BKPyV-I EF loop. These mutants were then used as competing antigens in an antigen competition assay with a panel of patient sera, and changes in antibody reactivity were assessed by ELISA.

We found that the changes introduced into the BKPyV-IV VP1 EF loop restrict antibody recognition in most samples and that converting the BKPyV-IV DE loop into its BKPyV-I equivalent attracts anti-VP1 BKPyV-I antibodies. Although our results did not lead to the discovery of a subtype-specific epitope on the VP1, they suggested that the arrangement of the EF loop in VP1 might dictate the mode of interaction between virus and anti-VP1 antibodies in general and that the interactions between the antibodies and the viral capsid might be very complex. Consequently, an antigen competition assay as an assay to distinguish between BKPyV serotypes might prove difficult to interpret.

1. Introduction

Since its discovery in 1971 (Gardner et al., 1971), the BK virus (BKPyV) has been intensively investigated as a causative agent of BKPyV-associated nephropathy (BKPyVAN) and graft rejections in kidney transplant (KTx) patients (Purighalla et al., 1995; Randhawa et al., 1999). Although the seroprevalence is very high, the virus usually causes asymptomatic infections. However, it persists in the kidneys and can reactivate in a state of immunosuppression (Chesters et al., 1983; Heritage et al., 1981) or be introduced to a patient during transplantation through the infected donor kidney (Schmitt et al., 2014).

The BKPyV isolates are grouped into four subtypes (I-IV) using genotyping and serological methods. Historically, genotypes were defined solely on the nucleotide sequence of the segment of a late gene that encodes the major capsid protein, VP1 (Jin, 1993; Jin and Gibson, 1996). Later, four distinct serotypes that correlate with genotype classification were confirmed by neutralization assay with reporter pseudoviruses based on the VP1 of several primary isolates (Pastrana et al., 2013). The capsids are composed of 72 pentamers of VP1. Therefore, antigens in the form of virus-like particles (VLPs) or pseudoviruses are

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preferred for serotyping since they allow the detection of antibodies targeted to the quaternary structure of VP1 on the capsid surface. The four surface loops (BC, DE, EF, and HI) of VP1 are exposed on the exterior of the capsid. The major differences between the VP1 sequences of the BKPyV subtypes are clustered in the hypervariable region of the BC loop (amino acids 61–83) (Jin et al., 1993). The BC loop is a frequently studied region since it contains the receptor binding site (Neu et al., 2013) and accumulates mutations during the selection of virus variants (McIlroy et al., 2020). Additionally, several amino acid changes, mostly in the BC and EF loop and less frequently in the DE loop, were identified in the VP1 sequence of BKPyV in the urine of patients with BKPyVAN (Tremolada et al., 2010).

The existence of different subtypes can cause problems in the case of donor-derived infections: even if the patient has encountered the infection prior to the transplantation, their antibodies can be non-neutralizing if a different subtype is introduced. A recent report confirms that knowledge of BKPyV seropositivity with respect to the serotype is relevant for appropriate post-transplant management of patients at risk (François et al., 2022). The serological distinction between the subtypes is currently believed to be possible only by neutralization assay as the serum's non-neutralizing antibodies are usually considered widely cross-reacting. While neutralization-based assays are more specific and can eliminate cross-reactivity between different strains of BKPyV (Pastrana et al., 2013), they are too elaborate and time-consuming for use in clinical settings. Cross-reactivity between the subtypes can also be decreased in antigen competition assay by pre-incubation with soluble antigen (Hejtmánková et al., 2019; Wunderink et al., 2019), which is less inconvenient than elaborate neutralization assays. Unbound VP1 antigen of different subtypes is added to a serum dilution series and competes with either plate-bound (e.g., in ELISA setting) or bead-bound VP1 to detect subtype-specific antibodies targeting the bound antigen. The results of these studies suggest that pre-incubation of the serum with competing antigens can even eliminate the cross-reactivity between the subtypes.

Originally, this study aimed to identify the residues in the surface loop of the VP1 protein of BKPyV responsible for the interactions with the subtype-specific antibodies. Since the variability of the BC loop region across all genotypes is very high, we reasoned that selecting subtype-specific epitope in this area would be difficult. Therefore, we focused on the DE and EF loops with only a small number of distinct amino acid differences between the most common subtypes, BKPyV-I and BKPyV-IV.

To explore the role of the DE and EF loops in serological cross-reactivity, we designed two mutant VLPs. We chose a BKPyV-IV to form the base sequence into which we introduced amino acid residues from the corresponding position of the BKPyV-I sequence. The resulting mutants (BKPyV-IV-DEmut and BKPyV-IV-EFmut) were used as competing antigens in an antigen competition assay tested in an ELISA setting with a panel of patient sera and with control plasma sample from healthy subjects, Cytotect CP. Cytotect CP represents commercially available immunoglobulin preparations from the plasma of donors that are known to contain neutralizing antibodies against major subtypes of BKPyV (Barten and Zuckermann, 2018; Randhawa et al., 2015). We argued that targeting an important epitope would change the reactivity with BKPyV-IV specific antibodies or even cause reactivity with BKPyV-I specific antibodies. We expected that there could be differences between the reduction of reactivity after pre-incubation of sera with these mutants. This could point us to where exactly these antibodies are targeted. Thus, their use in antigen competition assay could bring valuable information about the target of subtype-specific and cross-reacting antibodies against BKPyV.

2. Methods and materials

2.1. Serum samples

The anonymized plasma samples (T01-T14) from KTx patients used as a primary antibody (1:200) in ELISA assay were kindly donated by Vidia Ltd. The urine and blood samples pre-characterization (Table 1) by quantitative real-time PCR (for BKPyV DNA detection) and the restriction fragment length polymorphism analysis of the variable region of BKPyV VP1 (for genotypes I-IV analysis) was carried out by Dr. Roubalová at Vidia Ltd. as described previously (Hejtmánková et al., 2019). Additionally, Cytotect CP (Biotest) was also used as a primary antibody (1:2000) in the ELISA assay (sample T15, Table 1).

2.2. Production and isolation of VLPs

VLPs were produced using plasmid encoding for the major capsid protein VP1 of four different variants. Plasmid (plaw/BKV-D VP1, kind gift from Christopher Buck) coding structural VP1 of BKPyV-I subtype (GenBank accession number 6GG0_1), further addressed as pBKPyV-I-VP1, and plasmid (pwB, Addgene) coding VP1 of BKPyV-IV subtype (GenBank accession number BAG84476), addressed as pBKPyV-IV-VP1, were used in this study. pBKPyV-IV-VP1 was constructed from pwB2b. pwB2b (Schowalter et al., 2011) was a gift from Christopher Buck (Addgene plasmid # 32,094). The BKV-IV sequence was cloned into by phGf (Addgene, plasmid #22,516) to generate a vector expressing BKPyV-IV VP1 and GFP from separate promoters. In addition, the start codon from GFP was mutated to obtain a construct that expresses VP1 only. pBKPyV-IV-VP1 was used for both site-directed mutagenesis to produce two variants of mutant VLPs, and pBKPyV-I-VP1 was used for VLP production directly.

Site-directed mutagenesis was used to introduce mutations in the VP1 gene of BKPyV-IV in the DE and EF loop region using the Q5 Site-Directed Mutagenesis Kit (NEB) according to the manufacturer's instructions with two sets of primers:

- **mut-BKPyV-IV-EF-Asp-Ille-Fw**: 5′ ACC ATC ACA CCC AAG AAC CCA ACC - 3′ (Tm 65 °C);
- **mut-BKPyV-IV-EF-Asp-Ille-Rv**: 5′ GCC ATC GGG ATA CTT GGT GCG ATA G - 3′ (Tm 63 °C);
- **mut-BKPyV-IV-DE-His-Fw**: 5′ GGT GCA CGA GCA CGG - 3′ (Tm 77 °C);
- **mut-BKPyV-IV-DE-His-Rv**: 5′ TTT TGG CTT CCG GCG TGC AGA TTC- 3′ (Tm 72 °C)

The cycle was set as follows: 1 × 98 °C for 3 mins, 25 cycles of 98 °C

Table 1
Overview of the pre-characterized blood and urine samples from KTx patients.

sample	viremia	viruria
T01	subtype BKPyV-IV	subtype BKPyV-IV
T02	negative	subtype BKPyV-IV
T03	negative	subtype BKPyV-IV
T04	negative	positive (unknown) [a)]
T05	negative	not available
T06	negative	positive (unknown) [a)]
T07	positive (unknown) [a)]	subtype BKPyV-I
T08	negative	subtype BKPyV-I
T09	negative	subtype BKPyV-I
T10	negative	negative
T11	positive (unknown) [a)]	positive (mix) [a)]
T12	negative	subtype BKPyV-I
T13	negative	subtype BKPyV-I
T14	negative	negative
T15 (Cytotect CP)	not relevant	not relevant

[a] Genotype in some samples could not be determined, or co-infection with more BKPyV genotypes was observed (mix).

for 10 s, 65 °C 20 s, 72 °C (for DE loop mutation) / 65 °C (for EF loop mutation) for 3.5 mins, then 1 × 72 °C 2 mins. The nucleotide changes in the relevant region of the mutated plasmids were verified by sequencing.

VLPs assembled from VP1 protein were produced using a mammalian expression system. The human embryonic kidney-derived cells 293TT were kindly provided by John Schiller and Christopher Buck (National Institutes of Health, Bethesda, MA, USA). The cells were maintained at 37 °C in a 5% CO_2 humidified incubator and cultured in Dulbecco's modified Eagle medium (DMEM, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal calf serum (Gibco, Thermo Fisher Scientific, Inc., Inc., Waltham, MA, USA), 1% Glutamax I (Invitrogen, Thermo Fisher Scientific, Inc.), 1% non-essential amino acids (Invitrogen, Thermo Fisher Scientific, Inc.) and 250 μg/mL hygromycin B (Invivogen, Toulouse, France). 293TT cells were transfected using TurboFect™ Transfection Reagent (Thermo Fisher Scientific) with 1) plasmids carrying VP1 gene of BKPyV-I and BKPyV-IV and 2) plasmids carrying VP1 gene of BKV-IV subtype with specific mutations in DE loop (N139H) and EF loop (E175D and V178I), further called BKPyV-IV-DEmut and BKPyV-IV-EFmut. Cells were harvested 48 h post-transfection. The isolation protocol that includes purification by ultracentrifugation through an Optiprep gradient has been previously described elsewhere (Buck et al., 2004).

2.3. Characterization of VLPs

After the purification, the samples were characterized using SDS-PAGE to check the presence of VP1 protein (results not shown), and VLPs were analyzed by JEOL JEM-1011 transmission electron microscope. The samples (7 μl) were placed on parafilm and adsorbed on the carbon-coated formvar copper grids (Electron microscopy Sciences, Hatfield, PA, USA) for 10 min. Grids were then washed twice on two drops of redistilled H_2O (100 μl each) and contrasted on two drops (50 μl each) of 2% (w/v) solution of phosphotungstic acid (pH 7.3). A protein concentration of selected fractions was measured using either a Qubit Fluorometer or a standard Bradford protein assay.

2.4. ELISA for sera characterization

ELISA assays were carried out in 96-well half-area microplates (Greiner Bio-one) coated with 50 ng/well of VLPs in phosphate-buffered saline (PBS) and incubated at 4 °C overnight. Between each step, the plate was washed 4 times with PBS + 0.1% Tween-20 (v/v). The plate was blocked with 100 μl/well of 5% milk in PBS at room temperature (RT) for 2 h. Human sera (300 - 500 μl) were diluted in 5% milk in PBS (final dilution 1:200) and incubated on the plate for 1 hour at RT. Then, 50 μl of diluted secondary antibody conjugated with horseradish peroxidase, Peroxidase-conjugated AffiniPure Goat Anti-Human IgG, Fcγ Fragment Specific (Jackson ImmunoResearch Europe Ltd, # 109–035–008) for human sera (diluted 1:4000), was added to each well and incubated at RT for 30 min. Solution with ABTS substrate (0.3 mg/ml ABTS, 9.2 M citrate buffer pH = 4, 0.025% H_2O_2) was added, 50 μl/well, and incubated at RT for 30 min. Absorbance was measured at 415 nm with the Epoch™ Microplate Spectrophotometer (BioTek). Samples were run in quadruplicates. The cut-offs were calculated as the mean absorbance values of unreactive serum samples plus two standard deviations using 7 BKPyV-unreactive sera from healthy adults.

2.5. Antigen competition assay

The diluted human sera (1:100) were pre-incubated with either control or mutant VLPs in a final concentration of 5 μg/ml on a shaker at 4 °C overnight. Samples were further diluted (final dilution 1:200) and tested in ELISAs as described above.

2.6. Statistical analysis

Due to the small sample size (quadruplicates), determining the distribution of the variable (absorbance value for each competing antigen) was important for choosing an appropriate statistical method. Consequently, the Shapiro-Wilk test was performed. Based on the outcomes (reported in Table S2), we decided to use either a one-way ANOVA with Welch and Brown and Forsythe test with Tamhane's T2 multiple comparisons test or Kruskal-Wallis with Dunn's post hoc test, as indicated in Table S2. Statistical analysis was performed using GraphPad Prism v. 8.0.1.

3. Results and discussion

3.1. The design and preparation of VLPs

We designed two mutant VP1 sequences and prepared VLPs consisting of either of these VP1 proteins: we introduced a one-point mutation (N139H) in the DE loop or two one-point mutations (E175D and V178I) in the EF loop of VP1 protein of BKPyV-IV, introducing the respective amino acids from the sequence of BKPyV-I, thus creating BKPyV-IV mutants with either the DE loop (BKPyV-IV-DEmut) or the EF loop (BKPyV-IV-EFmut) surface amino acids identical with that of BKPyV-I (Fig. 1). Please note that mutation in DE loop of VP1 changed charge of amino acid at the given position, while the mutation in EF loop did not (Fig. 1C). We hypothesized that mutation at the position 139 in DE loop might represent a target of subtype I specific antibodies since it was identified as the only major mutation in subtype I (outside BC loop) in a recent study that focused on the accumulation of mutation of VP1 in KTx patients with prolonged viruria (McIlroy et al., 2020).

We first created plasmids carrying the corresponding genes for the altered VP1 sequence by site-directed mutagenesis. The four types of VLPs (two mutant VLPs variants - BKPyV-IV-DEmut and BKPyV-IV-EFmut, and control BKPyV-I and BKPyV-IV) were produced by transfection of the 293TT cells and purified through an Optiprep gradient (see 2.2. Production and isolation of VLPs). In all preparations, we confirmed the presence of VP1 protein by SDS PAGE (results not shown) and visualized compact capsids (Fig. 2).

3.2. Characterization of serum samples by ELISA

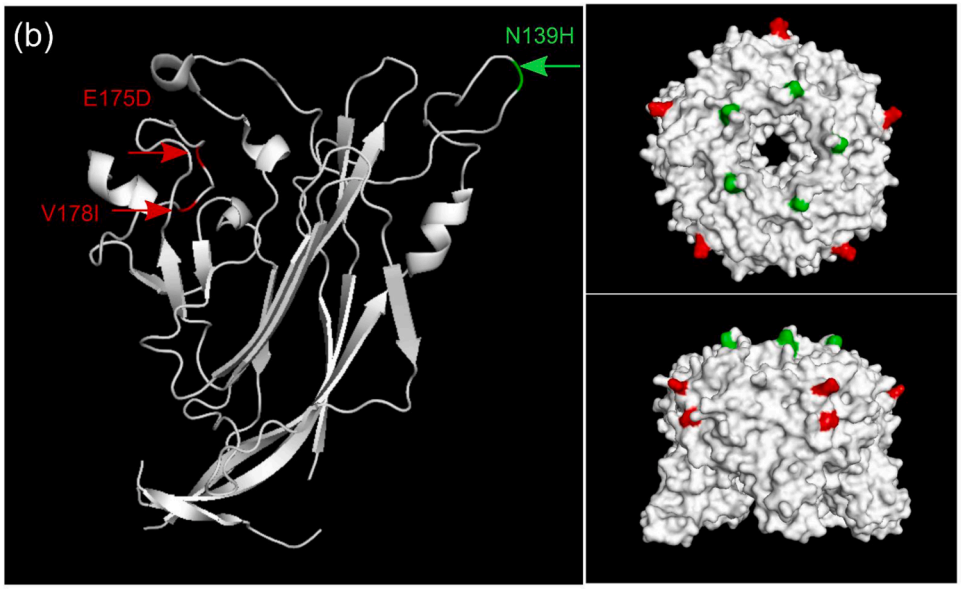
First, we assessed the serological characteristics of the serum samples (Fig. 3) from KTx patients that were pre-characterized for viremia and viruria previously (see Table 1) and divided them into three groups. Group 1 contained samples that were preferentially reactive with BKPyV-IV antigen (T01-T04). Samples T01, T02, and T03, which were determined as BKPyV-IV positive because of the subtype detected in urine (and blood in the case of sample T01), showed high reactivity with the BKPyV-IV antigen. Sample T04 reacted almost exclusively with BKPyV-IV antigen, but conclusive confirmation of the active BKPyV-IV infection was lacking. Group 2 (T05-T08) contained samples that displayed a very high reactivity with both antigens, yet the T07 and T08 were from patients with confirmed BKPyV-I viruria only. Group 3 (T09-T15) contained samples that reacted preferentially with BKPyV-I antigen even though their reactivity with BKPyV-IV antigen was quite high (the sample T14 was the only exception that contained subtype I specific antibodies). Cytotect CP (T15) was included in this category as well. Samples T09, T12, and T13 were from patients with confirmed BKPyV-I viruria, and the BKPyV infection status of other samples was either unclear or negative (see Table 1).

3.3. Antigen competition assay using mutant VLPs

After the initial screening, we further tested all samples with the antigen competition assay: when reactivity with both BKPyV-I and BKPyV-IV antigens coated on ELISA plates is completely eliminated after

(a)

	< DE loop >	...	< EF loop ...
BKPyV-I	NLHAGSQKVHEHGGGKPIQGSNF	...	GDPLEMQGVLMNYRTKYPDGTITP
BKPyV-IV	NLHAGSQKVHENGGGKPIQGSNF	...	GDPLEMQGVLMNYRTKYPEGTVTP
BKPyV-IV-DEmut	NLHAGSQKVHEHGGGKPIQGSNF	...	GDPLEMQGVLMNYRTKYPEGTVTP
BKPyV-IV-EFmut	NLHAGSQKVHENGGGKPIQGSNF	...	GDPLEMQGVLMNYRTKYPDGTITP



(c)

DE loop	N139H	polar neutral	→	polar charged (+)
EF loop	E175D	polar charged (-)	→	polar charged (-)
	V178I	non-polar neutral	→	non-polar neutral

Fig. 1. The design of two mutant BKPyV variants. A) Changes in sequences of VP1 DE loop (green) and EF loop (red); B) The structure of BKPyV-IV VP1 monomer (left) and top view (top right) and side view (bottom right) of VP1 pentamer with mutations introduced into the DE loop (green) or EF loop (red), please note that EF loop is partly buried between neighboring pentamers in assembled capsids. Created in PyMOL, based on BKPyV structure by (Neu et al., 2013), PDB: 4MJ1; C) Characteristics of the introduced changes of amino acid residues.

pre-incubation with competing soluble antigen, e.g., BKPyV-I VLPs, then no specific antibodies against BKPyV-IV are present, and vice versa. The BKPyV-IV-DEmut and BKPyV-IV-EFmut are identical to their parent BKPyV-IV VLPs except for the DE or EF loop sequence, respectively, which is identical to the BKPyV-I. Presumably, in case the antibodies that depend on the DE or EF loop are present in the sample, the competing capacity of the mutants should differ from BKPyV-IV. In case the context of the BKPyV-I sequence of the BC loop is essential for these antibodies, the competing capacity of the mutants should also differ from BKPyV-I.

First, we demonstrate the interesting phenomenon we observed in the antigen competition assay of Group 1 serum samples. When we performed the antigen competition assay (Fig. 4), the pre-incubation of sample T01 with BKPyV-I VLPs did not lower the reactivity of serum with BKPyV-I antigen, but pre-incubation with BKPyV-IV and both mutant VLPs did. A similar trend was observed for sample T03. On the contrary, in sample T02, only pre-incubation with BKPyV-I VLPs was able to lower the reactivity of sera on BKPyV-I antigen to cut-off values.

All three serum samples exhibited a similar pattern when tested on BKPyV-IV antigen: pre-incubation with BKPyV-I antigen did not diminish the reactivity with BKPyV-IV antigen, and pre-incubation with both mutant variants showed slightly lower efficiency in capturing BKPyV-IV antibodies than unmutated BKV-IV antigen. This implies that in sample T01, the reactivity with BKPyV-I antigen possibly stems from a very high level of anti-VP1 BKPyV-IV antibodies that might interact with immobilized BKPyV-I antigen „non-specifically“ due to, e.g., macromolecular crowding (Chapanian et al., 2014; Frutiger et al., 2021). On the contrary, pre-incubation of sample T01 with BKPyV-IV VLPs and both mutants lowered the concentration of these antibodies, thus resulting in negligible reactivity with coated BKPyV-I antigen.

On the other hand, serum sample T02 and T03 antibodies presumably target epitopes that might be common for both subtypes, and the binding of antibodies is sensitive to the changes in the DE and EF loop of BKPyV-IV. Consequently, BKPyV-IV-DEmut and BKPyV-IV-EFmut antigens did not capture BKPyV-I reacting antibodies as efficiently as BKPyV-IV. These findings suggest that samples T02 and T03 might contain poly- or pan-reactive antibodies that are frequently induced upon novel virus exposure (Guthmiller et al., 2020). Even with an ongoing BKPyV-IV infection (confirmed viruria), past infection with BKPyV-I cannot be excluded in serum samples T02 and T03. It was documented recently that serum samples of BKPyV-infected healthy donors contain cross-neutralizing monoclonal antibodies that are pan-reactive against all BKPyV subtypes, and this pan-reactivity is accomplished by tight binding to conserved regions of complex viral epitopes, including the EF loop (Lindner et al., 2019). The last sample from Group 1, T04, apparently contained subtype IV specific antibodies that were efficiently captured only by BKPyV-IV derived antigens.

Next, we compared four serum samples from Group 2 (Fig 5.). When the serum samples T05, T06, and T07 were pre-incubated with competing antigens and tested on BKPyV-I antigen, the reactivity was substantially decreased by pre-incubation with BKPyV-I VLPs. However, when samples were pre-incubated with the BKPyV-IV-derived VLPs, reactivity with BKPyV-I remained almost the same except T05, where BKPyV-IV-DEmut VLPs reduced the reactivity of serum by 40%. In addition, pre-incubation with BKPyV-I VLPs dramatically reduced the reactivity of either sample with BKPyV-IV coated antigen, which points to a high content of antibodies against BKPyV-I that potentially cross-react with BKPyV-IV. Interestingly, while the pre-incubation of

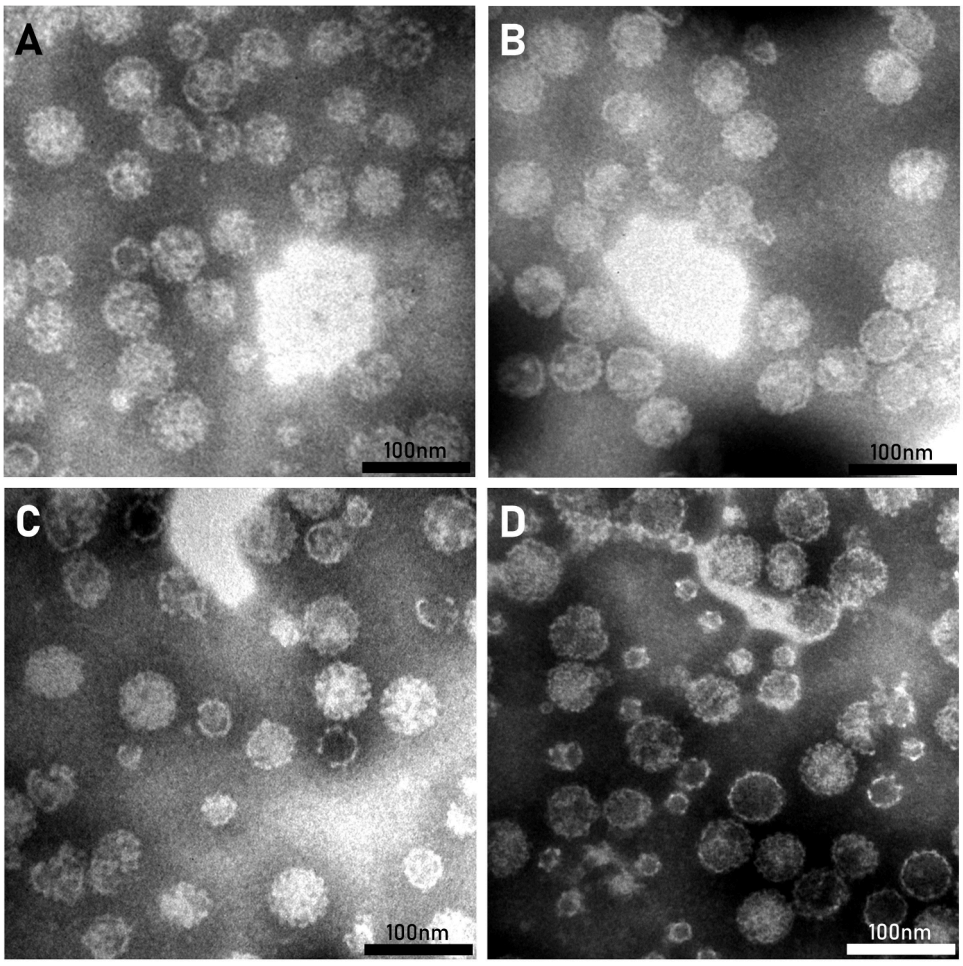


Fig. 2. All four types of purified VLPs were visualized by TEM; A - BKPyV-I, B - BKPyV-IV, C - BKPyV-IV-DEmut, D - BKPyV-IV-EFmut.

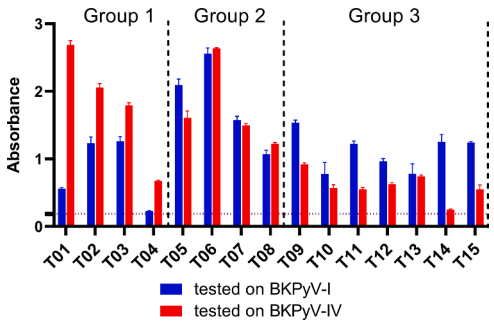


Fig. 3. Reactivity of serum samples on ELISA with BKPyV-I (blue) and BKPyV-IV (red) as coated antigens shown as mean absorbance of each sample. Error bars represent SDs; the experiment was carried out in quadruplicate (see Supplementary Table S1 for details). Cut-off values are shown as dotted lines of the corresponding color.

samples T05 and T06 with BKPyV-I VLPs or BKPyV-IV-DEmut reduces the reactivity with coated BKPyV-IV antigen dramatically, pre-incubation with BKPyV-IV VLPs was not as efficient (Fig. 5). In both samples, BKPyV-IV-EFmut did not substantially decrease the reactivity with either BKPyV-I or BKPyV-IV antigens. Sample T07 showed a different pattern when tested on coated BKPyV-IV antigen. Pre-incubation with BKPyV-IV or BKPyV-IV-DEmut VLPs reduced the reactivity less than pre-incubation with BKPyV-I VLPs or BKPyV-IV-EFmut. In fact, T07 was the only serum sample from our sera collection that efficiently recognized BKPyV-IV-EFmut VLPs during competition assay. We conclude that all three samples (T05, T06, T07) are indeed positive for anti-VP1 BKPyV-I antibodies (pre-incubation with BKPyV-I VLPs eliminates reactivity with both BKPyV-I and BKPyV-IV antigens) but differ in the content and quality of antibodies cross-reacting with BKPyV-IV antigen. In samples T05 and T06, anti-VP1 BKPyV-I antibodies cross-reactivity with BKPyV-IV VP1 could be primarily affected by DE loop composition, and when the DE loop sequence is changed to BKPyV-I, their affinity towards the mutant is greater than towards wild-type BKPyV-IV. The high titers of antibodies in T06 make it difficult for complete reactivity reduction with a standard amount of competing antigen, but T05 and T06 also showed a similar pattern with respect to pre-incubation with BKPyV-IV-EFmut. On the contrary, T07 probably contains antibodies cross-reacting with BKPyV-IV VP1 that do not recognize the immunodominant DE loop region but cross-react with EF loop epitope with BKPyV-I composition. The pre-incubation of sample T08 with all types of BKPyV VLPs was not informative. The reactivity with either BKPyV-I or BKPyV-IV antigens remained unchanged or even slightly increased. We suggest that in this particular case, the reactivity with BKPyV antigens might be caused by cross-reacting antibodies originally directed to other BKPyV subtypes (II, III) (as demonstrated by immunization experiments in mice (Pastrana et al., 2013)) or other polyomaviruses (e.g., JCPyV, SV40). Although the later cases are not common, they are well documented in the literature (Carter et al., 2003; Hejtmánková et al., 2019; Randhawa et al., 2009). Of note, serum T08 was later found to contain high titers of antibodies against polyomaviruses JCPyV, MCPyV, and SV40 (data not shown) indeed.

All serum samples from Group 3 (T09-T15) demonstrated a uniform trend (Fig. 6). The reactivity with coated BKPyV-I antigen could only be

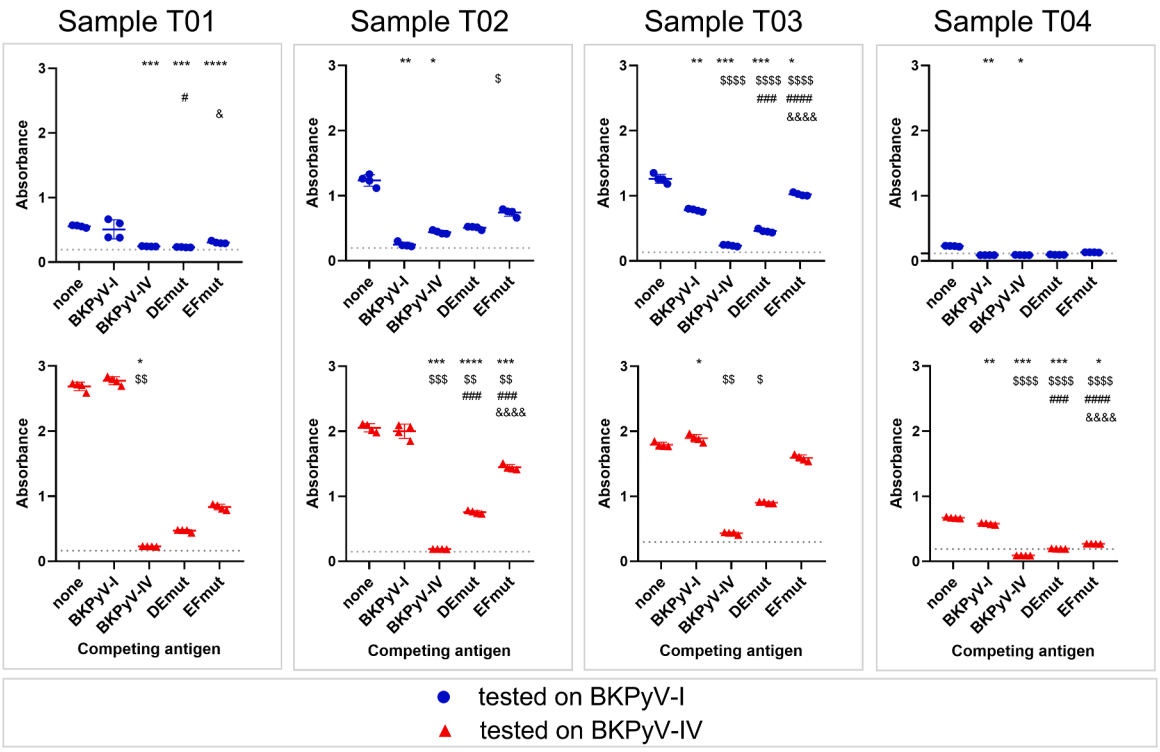


Fig. 4. Pre-incubation of serum samples from Group 1 with BKPyV-I, BKPyV-IV, BKPyV-IV-DEmut (DEmut) or BKPyV-IV-EFmut (EFmut) VLPs and their subsequent testing on BKPyV-I (blue) or BKPyV-IV (red) antigen on ELISA. Experiments were carried out in quadruplicate. gray dotted lines represent the mean cut-off value. Asterisks, dollar signs, hashes, or ampersands indicate statistical significance ($^{*/\$/\#/\&}$ $p<0.05$; $^{**/\$\$/\#\#/\&\&}$ $p<0.01$; $^{***/\$\$\$/\#\#\#/\&\&\&}p<0.001$; $^{****/\$\$\$\$/\#\#\#\#/\&\&\&\&}$ $p<0.0001$) in multiple comparisons between no antigen (none) and competing antigens (BKPyV-I, BKPyV-IV, DEmut, EFmut), between BKPyV-I antigen and BKPyV-IV-derived competing antigens (BKPyV-IV, DEmut, EFmut), between competing BKPyV-IV antigen and BKPyV-IV-mutant antigens (DEmut, EFmut), or, between competing BKPyV-IV-DEmut and BKPyV-IV-EFmut, respectively. Comparisons with p-value > 0.05 (non-significant) are not indicated. A complete statistical analysis can be found in Supplementary Table S2.

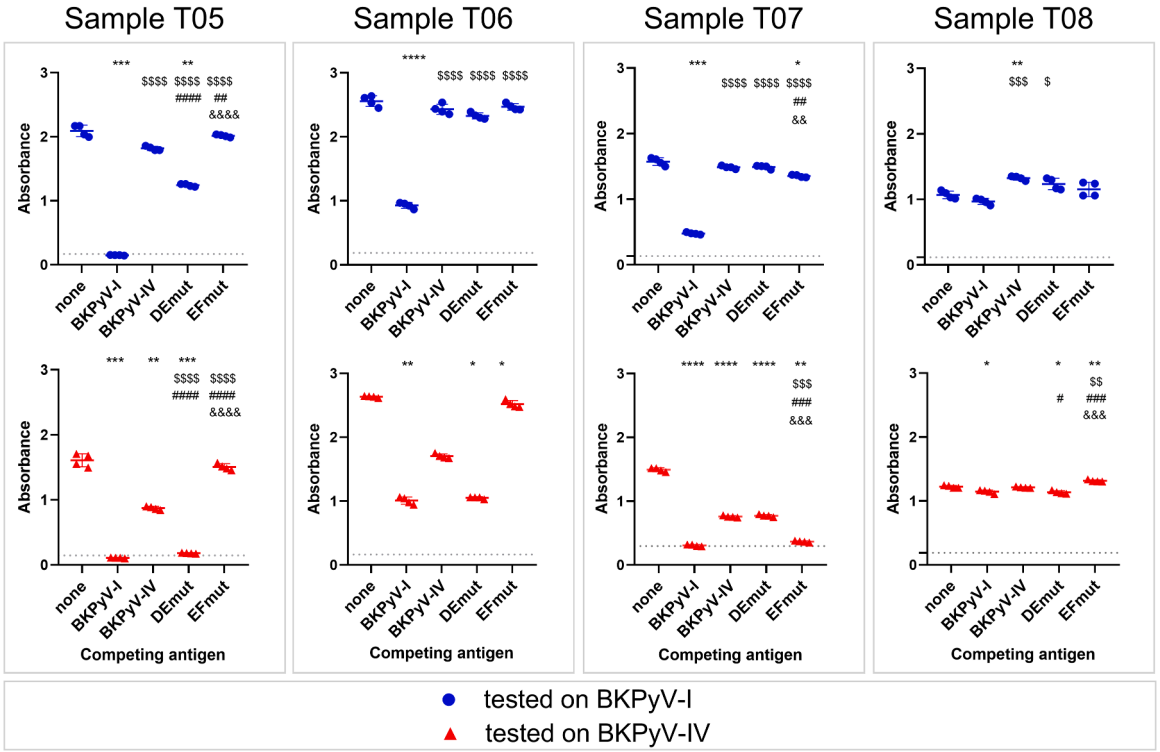


Fig. 5. Pre-incubation of serum samples from Group 2 with BKPyV-I, BKPyV-IV, BKPyV-IV-DEmut (DEmut) or BKPyV-IV-EFmut (EFmut) VLPs and their subsequent testing on BKPyV-I (blue) or BKPyV-IV (red) antigen on ELISA. Experiments were carried out in quadruplicate. For further description, see Legend in Fig. 4.

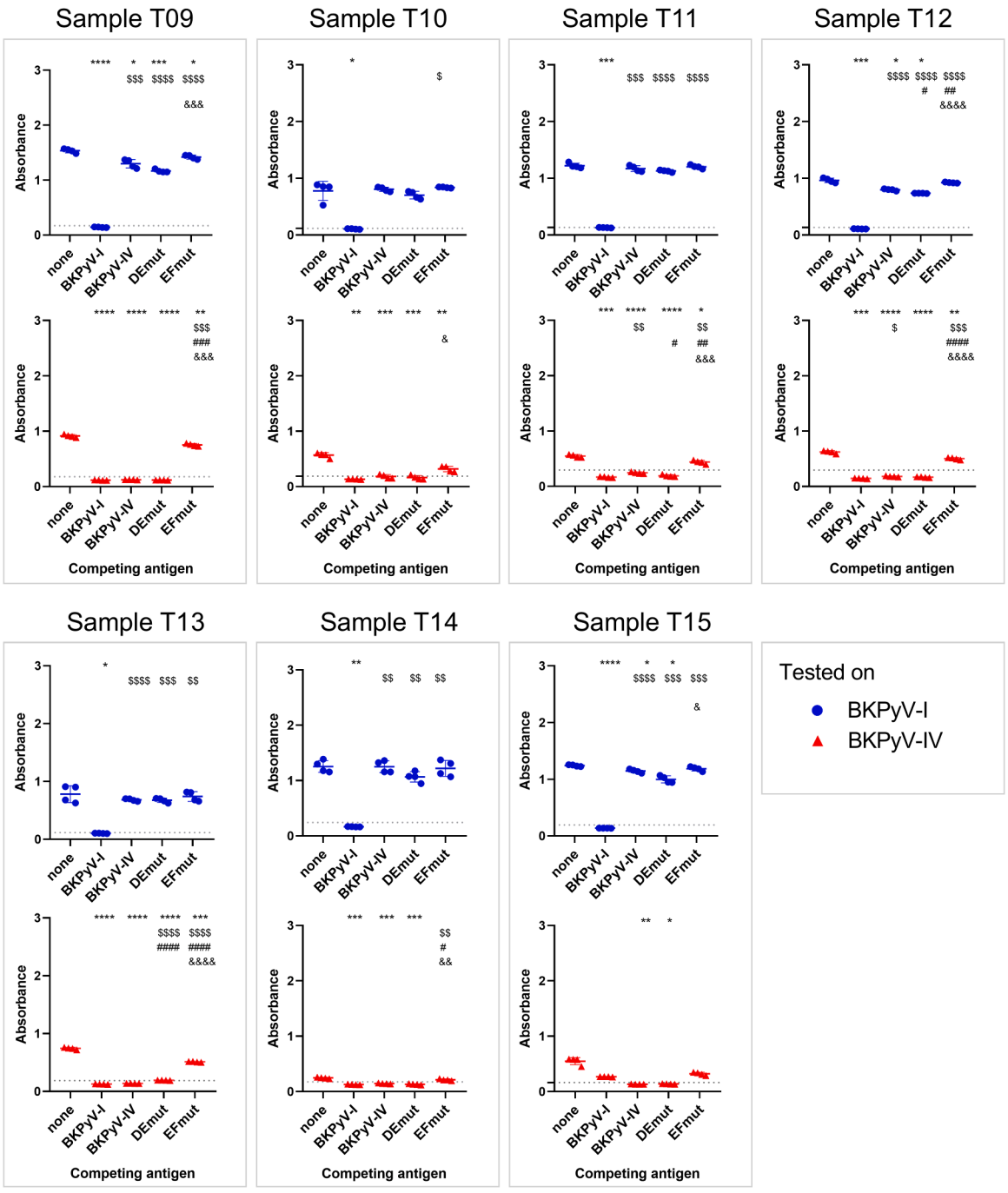


Fig. 6. Pre-incubation of serum samples from Group 3 with BKPyV-I, BKPyV-IV, BKPyV-IV-DEmut (DEmut) or BKPyV-IV-EFmut (EFmut) VLPs and their subsequent testing on BKPyV-I (blue) or BKPyV-IV (red) antigen on ELISA. Experiments were carried out in quadruplicate. For further description, see Legend in Fig. 4.

eliminated by pre-incubation with BKPyV-I VLPs, and the pre-incubations with BKPyV-IV VLPs variants had a negligible effect. The reactivity with coated BKPyV-IV antigen was almost completely eliminated by pre-incubation with BKPyV-I, BKPyV-IV, and BKPyV-IV-DEmut VLPs. At the same time, BKPyV-IV-EFmut reduced reactivity less efficiently, especially in cases where the original reactivity with BKPyV-IV antigen was rather high (e.g., by less than 25% in sample T09). T14 was not informative for analysis of the interaction of antibodies with BKPyV-IV derived VLPs antigens since it contains virtually no cross-reacting antibodies.

Taken together, changes in the EF loop region substantially restricted recognition of BKPyV-IV-EFmut by antibodies in almost all double-reactive serum samples. The effect was paradoxically lower in some samples from patients undergoing confirmed BKPyV-IV infection (samples T01 and T02); thus, changes in the EF loop did not restrict exclusively binding of BKPyV-IV specific antibodies, but the subtle alternation in the EF loop of BKPyV-IV antigen led to the change in the antibody recognition in general. However, there might be exceptions, as demonstrated by the competition assay results with serum T07. We suggest that this serum might represent a case when prevailing cross-reacting antibodies target linear epitopes in the EF loop region of VP1 rather than quaternary viral capsid epitopes. It has been demonstrated that such antibodies can be generated experimentally and usually recognize linear epitopes common to other polyomaviruses (Randhawa et al., 2009). Interestingly, the amino acid composition of the EF region in JCPyV VP1 is more similar to BKPyV-I and BKPyV-IV-EFmut than to

BKPyV-IV. In agreement, sample T07 was found to be highly reactive with the JCPyV antigen (data not shown). Consequently, T08 might represent an extreme example of sera that contains a high level of antibodies targeted to (linear) epitopes common to several polyomaviruses.

Furthermore, a recent publication revealed that the clonally complex anti-VP1 BKPyV antibody repertoire includes a high frequency of monoclonal antibodies that broadly neutralize BK polyomavirus subtypes and the related JC polyomavirus (JCPyV) via binding to a conserved viral epitope at the junction between capsid pentamers that includes EF loop (Lindner et al., 2019). This corresponds to our finding that the EF loop plays a fundamental role in the recognition of BKPyV antigen by antibodies in most of our test samples. Since 90% of antibodies raised against the same antigen in polyclonal sera are believed to recognize conformational epitopes (Van Regenmortel, 2009), we suggest that the presence of intact EF loop region of VP1 is critical for reliable detection of anti-BKPyV VP1 antibodies during diagnostics. This explains our previous setbacks in constructing a serotype-specific diagnostic kit by using synthetic peptides (mimotopes) that only covered hypervariable regions of the BC loop of VP1. Furthermore, amino acid alterations in the EF loop of VP1 were previously observed in neutralization escape mutants experimentally generated in SV40 (Murata et al., 2008) and MCPyV (Fleury et al., 2015), thus suggesting the general importance of this region in the antibody recognition in the polyomavirus family.

To explain our results, we suggest that antigen conformation could be altered specifically by introduced mutations in the EF loop of VP1. It is generally assumed that the maturation of antibodies depends on the "lock and key" mechanism. The dynamic nature of antibodies enables the selection of the most binding competent conformation. This is accompanied by a rigidification of the antigen-binding site and influenced by the probability of the conformation adopted by the antigen (Fernández-Quintero et al., 2021). In agreement with this notion, Führer et al. (2021) observed that the more flexible isoforms have the lowest binding potential when investigating allergic reactions to hazelnuts. In analogy, we propose that converting the VP1 EF loop of BKPyV-IV capsid to the VP1 BKPyV-I EF loop arrangement can presumably increase the surface flexibility of antigen and consequently decrease the reactivity of affinity matured antibodies in our assays.

The phenomenon of the development of pan-specific or poly-specific antibodies in natural infection by several virus variants has been recently intensively investigated (Greaney et al., 2022, 2021; Griffith and McCoy, 2021; Guthmiller et al., 2020; McCarthy et al., 2018; Pantaleo et al., 2022). BKPyV infection is a prototype of chronic infection (Doerries, 2006) and co-infection with several subtypes or related polyomaviruses is not exceptional (Rani et al., 2016). Therefore, the immunity of infected persons is possibly shaped by changes in "immunodominance hierarchies" (Greaney et al., 2022) over time, as it can also reflect different dynamics in the affinity enhancement of antibodies (Eisen, 2014) during exposure histories in individuals rather than the presence of "non-specific" antibodies against subtypes. Collectively, these notions suggest that distinguishing BKPyV subtype-specific antibodies in some human sera might not be possible. However, in the case of KTx patients, the presence of antibodies cross-reacting between subtypes could be beneficial for controlling the infection.

4. Conclusions

The original goal of our current study was to identify the subtype-specific epitopes recognized by anti-VP1 BKPyV antibodies. We focused on DE and EF loop regions of VP1 and prepared variants of BKPyV-IV VLPs that contained one or two "typical" BKPyV-I amino acids in the BKPyV-IV context of DE or EF loop regions of VP1, respectively. The arrangement of VLPs likely attracts antibodies that preferentially recognize conformational epitopes normally exposed on the virus surface. In the antigen competition assay, we noticed that changes in EF loop regions substantially restricted recognition of BKPyV-IV-EFmut by antibodies in the majority of serum samples. Thus, the EF loop of VP1 seems crucial for the recognition of these anti-VP1 BKPyV antibodies in general. Therefore, the presence of an intact EF loop region of VP1 is critical for the reliable detection of anti-BKPyV VP1 antibodies during diagnostics.

The antigen competition assay with BKPyV-IV-DEmut VLPs competing antigen showed that anti-BKPyV-I antibodies cross-reacting with BKPyV-IV antigen could be eliminated by BKPyV-IV-DEmut VLPs as efficiently as by BKPyV-I VLPs from two serum samples that displayed substantially higher reactivity with BKPyV-I than with BKPyV-IV antigen. Thus, changing the epitope in the DE loop by introducing histidine instead of asparagine in the context of BKPyV-IV attracts the binding of antibodies presumably originally targeted to BKPyV-I antigen. Therefore, the importance of the DE loop of the VP1 composition is worth further study in distinguishing the subtype-specific antibodies by simple ELISA.

We and others (Hejtmánková et al., 2019; Wunderink et al., 2019) previously showed that pre-incubation by heterologous competing antigen presents an easy way to eliminate cross-reactivity between the BKPyV subtypes, especially in ELISA setting. However, our careful examination of sera from several KTx patients shows that the actual composition of BKPyV antigens (e.g., mutations, genotypes, variants) can substantially influence the outcome, and interpretation of results might be challenging.

CRediT authorship contribution statement

Alžběta Hejtmánková: Funding acquisition, Formal analysis, Visualization, Writing – original draft. **Helena Caisová:** Investigation, Data curation, Writing – review & editing. **Tereza Tomanová:** Investigation, Data curation. **Hana Španielová:** Funding acquisition, Conceptualization, Supervision, Methodology, Validation, Resources, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2022.199031.

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