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Polyomavirus Nephropathy: Quantitative Urinary Polyomavirus-Haufen Testing Accurately Predicts the Degree of Intrarenal Viral Disease

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Background. A *qualitative* highly predictive urinary test for polyomavirus nephropathy (PVN) is the PV-Haufen test. This article evaluates whether a *quantitative* PV-Haufen analysis, that is, the number of PV-Haufen shed per milliliter urine, predicts PVN disease grades and the severity of intrarenal PV replication. **Methods.** Polyomavirus-Haufen were counted in 40 urine samples from patients with biopsy-proven definitive PVN. The number of PV-Haufen was correlated with both histologic PVN disease grades 1 to 3 and the number of SV40-T–expressing cells as indicators of intrarenal PV replication in corresponding renal allograft biopsies (manual counts and automated morphometry). Findings from *quantitative* PV-Haufen analyses were compared to conventional laboratory test results, that is, BK viremia (quantitative polymerase chain reaction [PCR]) and BK viruria (quantitative PCR and decoy cell counts). **Results.** Polyomavirus-Haufen counts showed excellent correlation (α0.77–0.86) with the severity of intrarenal PV replication and disease grades. In particular, low PV-Haufen numbers strongly correlated with early PVN grade 1 and minimal intrarenal expression of SV40-T antigen ($P < 0.001$). In comparison, BK viremia and viruria levels by PCR showed only modest correlations with histologic SV40-T expression (α0.40–0.49) and no significant correlation with disease grades or minimal intrarenal PV replication. No correlations were seen with urinary decoy cell counts. In contrast to conventional quantitative PCR assays or decoy cell counts, *quantitative* urinary PV-Haufen testing accurately reflects the severity of PV replication, tissue injury, and PVN disease grades. **Conclusions.** *Quantitative* PV-Haufen testing is a novel noninvasive approach to patient management for the diagnosis and prediction of PVN disease grades and monitoring of disease course during therapy.

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Polyomavirus nephropathy (PVN) is the most important viral infection in renal allografts. The incidence of PVN in kidney transplant recipients ranges from 4% to 12%.[1-4] ABO-incompatible grafts seem to be at increased risk with a prevalence of 18%.[5] Graft failure because of PVN occurs in approximately 20% to 30% with a higher prevalence of 50% in cases of sclerosed PVN disease grade 3 (data collected by the Banff working group on polyomavirus nephropathy). Specific anti-PV therapy is not available, and outcome largely depends on an early diagnosis of PVN with limited renal injury that tends to respond favorably to therapeutic intervention (mainly based on the reduction of immunosuppression).

Polyomavirus nephropathy is defined morphologically by the histologic demonstration of polyomavirus (PV) replication in the renal parenchyma, and a renal biopsy is required to render a definitive diagnosis.[6-9] However, establishing a diagnosis of PVN in a kidney biopsy can be challenging. Deterioration of allograft function and a rise in serum creatinine levels does not always accompany early PVN, resulting in a possible delay of a diagnostic renal biopsy.[6,8]

Patient management after kidney transplantation includes risk assessment for potential PVN with quantitative polymerase chain reaction (PCR) assays for BK virus loads in plasma or urine or quantitation of urinary decoy cells in urine cytology specimens. These assays have been the mainstays for both clinical care and intervention.[2,10-19] They are based

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on the observation that all patients with PVN show systemic signs of PV replication with viremia or viruria, and the assumption that plasma PCR levels for BK virus reflect intrarenal PV replication. However, only a minority of patients with PV viremia or viruria ultimately develops PVN, underscoring a well-established fact: latency establishing DNA viruses, such as PV, can be reactivated without causing manifest disease.[8,18,20-24] Moreover, in immunocompromised patients, BK virus–associated disease with viremia can also be seen in the bladder (hemorrhagic cystitis) or in the salivary glands (human immunodeficiency virus–associated salivary gland sclerosis), typically lacking concurrent PVN.[25-28] Thus, positive PCR-based BK viremia or viruria test results or the presence of decoy cells in the urine indicate viral activation and an increased risk for PVN, but the tests cannot reliably predict the actual presence of intrarenal viral disease, that is, definitive PVN. In the setting of PVN, an ideal biomarker would not only indicate the presence or absence of intrarenal disease but also provide information on the severity of virally induced tissue injury.

We recently described a noninvasive biomarker to accurately predict PVN in fixed voided urine samples, that is, the PV-Haufen test. We showed that a *qualitative* analysis, that is, PV-Haufen present or absent, marked intrarenal disease with positive and negative predictive values exceeding 95%.[20,29] The PV-Haufen test is based on a novel concept, that is, the detection of dense, cast-like PV aggregates in fixed voided urine samples. Polyomavirus-Haufen form in virally injured renal tubules subsequent to PV release from infected tubular epithelial cells. They are then flushed out of the diseased kidneys and can be detected in the urine. In our previous publication, we reported that PV-Haufen were exclusively found in patients with biopsy-proven definitive PVN and were never seen in 139 control patients with varying degrees of BK viremia and viruria and absence of PVN. In patients with a definitive biopsy-proven diagnosis of PVN who were followed longitudinally, PV-Haufen shedding went from negative to positive at the time of initial biopsy diagnosis. Polyomavirus-Haufen testing subsequently remained positive during persistent viral nephropathy. At the time of disease resolution, PV-Haufen testing turned from positive to negative and remained negative thereafter during long-term follow-up. Thus, the *qualitative* detection of urinary PV-Haufen, that is, present or absent, is disease-specific and represents a novel clinical biomarker for definitive PVN.[20,30]

Currently, it is undetermined whether a *quantitative* urinary PV-Haufen analysis, that is, recording the number of PV-Haufen shed per milliliter urine, could provide additional clinical information on the severity of intrarenal disease. It is also undetermined how a quantitative urinary PV-Haufen assay might compare to conventional mainly PCR-based laboratory screening tests for PVN.

We address these questions in our current study that expands on our previous *qualitative* PV-Haufen test findings by now focusing on *quantitative* testing. Because PV-Haufen shedding is only found in patients with biopsy-proven definitive PVN, we hypothesize that the degree of urinary PV-Haufen shedding reflects the severity of virally induced kidney injury. The aim of our current study is to correlate traditional screening assays and *quantitative* PV-Haufen test results with the severity of intrarenal PV replication or PV burden and disease grades in patients with biopsy-proven PVN. Which test is most accurate?

RESULTS

Correlation of Quantitative Screening Test Results With the Severity of Intrarenal PV Replication

The severity of intrarenal PV replication was determined by using an immunohistochemical stain for the SV40-T antigen and manually counting the number of positive staining nuclei, the number of positive staining tubular cross-sections, or alternatively performing (automated) morphometry. Irrespective of manual or automated analysis, the number of urinary PV-Haufen most accurately reflected the severity of intrarenal PV replication–SV40-T expression with excellent correlation coefficients between 0.77 and 0.86 (Table 1 and Figure 1). Of note, correlations were tightest for comparisons based on the manual counts rather than the (automated) morphometric deconvolution analyses using the Aperio system. Plasma and urine PCR readings (BK viremia and viruria) showed a modest correlation with intrarenal PV replication (correlation coefficients between 0.40 and 0.49). No correlation was seen between the number of decoy cells in the urine and the SV40-T expression in the corresponding biopsy samples.

For correlation studies of a subgroup of PVN cases with minimal PV burden, six biopsies with 25 or less intrarenal SV40-T antigen–expressing cells were selected. Five of these six biopsies lacked intranuclear PV inclusion bodies by light microscopy further supporting an early and limited PVN disease stage in these cases. All six cases showed low urinary PV-Haufen numbers that tightly correlated with the low intrarenal PV burden ($\sigma = 0.91$; $P = 0.01$). Lowest PV-Haufen counts were found in one case with only two intrarenal SV40-T antigen-expressing cells (Table 2). The PV-Haufen counts increased in increments in the remaining five cases parallel to increased intrarenal PV load levels. In contrast, in this cohort, no significant correlations were found between all other screening assays and minimal intrarenal PV replication: plasma PCR $\sigma = 0.20$, $P = 0.7$; urine PCR $\sigma = -0.09$, $P = 0.9$; and decoy cell shedding $\sigma = 0.02$, $P = 1.0$.

Correlation of Screening Tests With PVN Disease Grades

Low numbers of urinary PV-Haufen were statistically significantly associated with early or mild PVN disease grade 1 in 12 of the 12 cases with a median PV-Haufen count of 282/mL urine and a maximum count of 677 PV-Haufen/mL urine seen in one patient. Polyomavirus nephropathy disease grade 2 showed median PV-Haufen counts significantly higher at 1693/mL (range, 112–9977/mL urine; $P =< 0.0001$). On an individual case basis, PV-Haufen counts showed only little overlap between PVN disease grades 1 and 2 (Table 3 and **Figure 1S, A, SDC,** http://links.lww.com/TP/B46). Polyomavirus nephropathy disease grade 3 demonstrated median PV-Haufen counts of 2822/mL, which differed significantly from PVN disease grade 1 (1 vs. 3 $P = 0.002$). In comparison, the degree of BK viremia and BK viruria by PCR testing and the degree of decoy cell shedding by urine cytology were not significantly associated with PVN disease grades 1–3 (Table 3).

TABLE 1.
Correlation of quantitative screening test results with the severity of intrarenal polyomavirus replication/ viral burden in cases of PVN

Biopsies with PVN (n = 40)		Concomitant urine samples (n = 40)						Concomitant plasma samples (n = 40)	
		PV-Haufen (#/mL urine)		Urine BKV PCR (viral copies/mL urine)		Decoy cells (number/ThinPrep slide urine cytology)		Plasma BKV PCR (viral copies/mL plasma)	
		σ	*P*	σ	*P*	σ	*P*	σ	*P*
Intrarenal PV replication: immunohistochemical staining for the SV40 Large T antigen	A) Manual count: (also Figure 1) number of SV40 large T antigen–positive cells in biopsy	0.86	<0.0001	0.48	0.002	0.0038	1.0	0.43	0.006
	B) Manual count: number of SV40 large T antigen–positive tubules in biopsy	0.83	<0.0001	0.43	0.006	–0.065	0.7	0.40	0.01
	C) Morphometry: % SV40 large T antigen positively stained area in biopsy	0.77	<0.0001	0.49	0.001	0.058	0.7	0.48	0.002

σ = Spearman correlation coefficient: 0.0 to 0.2 very weak to negligible correlation. 0.2 to 0.4—weak, low correlation. 0.4 to 0.7—moderate correlation. 0.7 to 0.9—strong, high correlation.
PVN, polyomavirus nephropathy; PCR, polymerase chain reaction; BKV, BK virus.

DISCUSSION

Urinary PV-Haufen are tight three-dimensional cast-like viral aggregates that are easily detected in fixed urine samples by negative staining electron microscopy (EM).[20,29] In a previous study, we showed that the *qualitative* detection of PV-Haufen, that is, present or absent, in a fixed urine specimen predicted PVN with positive and negative predictive values greater than 95%.[20]

FIGURE 1. Correlation of quantitative screening test results with intrarenal PV replication, that is, the number of SV40-T–expressing tubular epithelial cells in a biopsy sample with PVN. A, Urinary PV-Haufen; (B) Plasma PCR for BK virus; and (C) Urine PCR for BK virus. Scatterplots with regression lines are depicted. PVN, polyomavirus nephropathy; PCR, polymerase chain reaction.

TABLE 2.

Correlation of quantitative screening test results with intrarenal polyomavirus replication or viral burden

	Intranuclear PV inclusions visible by light microscopy	[a]Number of SV40-T antigen-positive tubular cells	[a]Number of SV40-T antigen-positive tubules	PV-Haufen (number/mL urine)	Urine BKV PCR (viral copies/mL)	Decoy cells (number/ ThinPrep slide)	Plasma BKV PCR (viral copies/mL)
Selected biopsies with PVN and minimal PV replication (n = 6)	Absent	2	2	169	3.18×10^6	100	4.64×10^3
	Absent	7	3	225	3.25×10^7	50	1.10×10^3
	Absent	10	4	225	1.53×10^9	100	1.63×10^4
	Present	12	6	282	1.48×10^7	20	5.93×10^4
	Absent	16	12	451	2.27×10^7	45	2.97×10^3
	Absent	25	17	451	8.24×10^7	100	2.00×10^4
				$\sigma = 0.91$	$\sigma = -0.09$	$\sigma = 0.02$	$\sigma = 0.20$
				$P = 0.01$	$P = 0.9$	$P = 1.0$	$P = 0.7$

[a] Immunohistochemical stain for the SV-40 Large T antigen.

In PVN: selected biopsies with minimal polyomavirus replication/SV40 positivity in 25 tubular epithelial cells or less.

σ = Pearson correlation coefficient: 0.0 to 0.2—very weak to negligible correlation. 0.2 to 0.4—weak, low correlation. 0.4 to 0.7—moderate correlation. 0.7 to 0.9—strong, high correlation.

PVN, polyomavirus nephropathy; PCR, polymerase chain reaction; BKV, BK virus.

In the current study, we extended our analysis to *quantitative* urinary PV-Haufen testing. We correlated the number of PV-Haufen in voided urine samples with the severity of PVN and compared the data to standard PV screening assays. This comprehensive comparative analysis showed that *quantitative* urinary PV-Haufen testing most accurately reflected the severity of intrarenal PV replication and virally induced renal injury. Few PV-Haufen in urine samples were associated with limited intrarenal PV replication, including one case with only two intrarenal SV40-T–expressing cells. Counts increased in increments parallel to the increase of intrarenal PV replication and high numbers of urinary PV-Haufen reflected marked virally induced tubular injury, a high intrarenal PV burden (correlation coefficient, 0.86).

Quantitative PV-Haufen testing also reflected PVN disease grades as proposed by the Banff Working Group on PVN.[31] Early mild PVN disease grade 1 was reflected by low numbers of urinary PV-Haufen that differed significantly from much higher counts in the florid PVN disease grade 2, and the late and sclerosed PVN disease grade 3.

How can the striking correlation between the severity of virally induced tissue injury in PVN and the numbers of urinary PV-Haufen shed into the urine be explained? The formation of PV-Haufen appears to be similar to the formation of other intratubular casts such as red or white blood cell casts. Polyomavirus-Haufen form within virally injured renal tubules containing high concentrations of uromodulin (Tamm-Horsfall protein) that has previously been shown to facilitate aggregation of influenza, mumps, and Newcastle viruses.[32] High concentrations of Tamm-Horsfall protein facilitate the aggregation of PV in vitro (personal observation; manuscript in preparation).[30] Because PV-Haufen are flushed with the urine out of tubules and into the bladder, they can serve as specific urinary biomarkers for intrarenal PVN.

In contrast, quantitative PCR assays to detect BK virus in plasma and urine only showed modest correlation with the severity of intrarenal PV replication or PV burden (correlation coefficients, 0.43 to 0.48). No significant correlations were found between quantitative PCR test results and PVN disease grades or limited PVN with only minimal intrarenal disease. No significant correlations were seen with urinary decoy cell counts. These results could be explained by potential PV activation in sites other than the kidneys, such as the urothelium or salivary glands.[25-28]

In daily practice, we do not recommend using *quantitative* urinary PV-Haufen testing as a mass screening tool. Rather, it should be used as a targeted test to separate patients

TABLE 3.

Quantitative screening test results by PVN disease grade

Screening tests for polyomavirus	PVN grade 1 (n = 12)	PVN grade 2 (n = 23)	PVN grade 3 (n = 5)	P[a]
PV-Haufen (#/mL urine)	median, 282 range, 169–677	median, 1,693 range, 112–9,977	median, 2,822 range, 1,128–8,014	0.0001 1 vs. 2 $\leq$ 0.0001 2 vs. 3 = 0.3[b] 1 vs. 3 = 0.001
Urine PCR (# copies/mL urine)	median, 3.80×10^7 range, 1.25×10^5 to 1.53×10^9	median, 1.10×10^8 range, 2.21×10^5 to 4.79×10^9	median, 3.20×10^8 range, 7.24×10^7 to 1.57×10^{10}	0.09[b]
Decoy cells (#/ThinPrep slide urine cytology)	median, 100 range, 18–100	median: 50 range: 10–100	median, 100 range, 50 × 100	0.3[b]
Plasma BKV PCR (# copies/mL plasma)	median, 1.41×10^4 range, 4.99×10^2 to 2.22×10^5	median, 2.57×10^4 range, 2.50×10^2 to 1.88×10^7	median, 1.52×10^5 range, 3.50×10^3 to 6.18×10^6	0.1[b]

[a] Wilcoxon rank-sum test for between group comparisons with Bonferroni correction (performed only if overall group comparison by Kruskal-Wallis testing significant).

[b] Not significant.

PVN, polyomavirus nephropathy; PCR, polymerase chain reaction; BKV, BK virus.

with asymptomatic transient BK viral activation from those with intrarenal disease, that is, definitive PVN. The test provides specific diagnostic evidence of intrarenal PVN and gives prognostic information on the severity of virally induced tissue injury. It is particularly suited for the diagnosis of PVN in early disease grade 1 with minimal intrarenal disease. *Quantitative* urinary PV-Haufen testing could be used to monitor PVN progression or regression during persistent disease.

Urinary PV-Haufen testing is performed using negative staining EM, a well-established, easily performed technique specifically developed for the identification and quantitation of viruses in various body fluids.[33] It has been in routine use in virology since the early 1960s and is the method of choice for viral studies at major virology centers, such as The Centers for Disease Control in Atlanta, GA. The incorporation of urinary PV-Haufen testing only requires "thinking outside the box." The turnaround time from receipt of a voided urine sample to reporting of results is approximately 3 hr with costs lower or comparable to PCR-based assays.

Although this single center study has limitations because of relatively few cases analyzed, we nevertheless think that *quantitative* urinary PV-Haufen testing will help to better define, diagnose, and manage PVN.

In conclusion, urinary PV-Haufen are accurate biomarkers of PVN and the number of PV-Haufen shed in fixed voided urine samples accurately reflects the severity of intrarenal disease. *Quantitative* PV-Haufen testing allows for an early diagnosis of PVN and monitoring of changing intrarenal viral load levels during therapeutic interventions such as in the setting of new clinical drug trials.

MATERIALS AND METHODS

Based on the availability of concomitant blood, urine, and renal biopsy samples, cases of definitive PVN were selected from the archives of The University of North Carolina at Chapel Hill (total n = 40 sample sets). The study protocol was approved by the institutional review board. Per definition, all diagnostic renal allograft biopsies showed evidence of PVN.[6-8] Histologic material was evaluated by two pathologists (V.N., H.K.S.) at a multiheaded scope and scoring results based on consensus (including the count of SV40-T–positive cells and tubules) were used for statistical analysis. Urine samples collected on the day of biopsy were analyzed by conventional urine cytology (ThinPrep; Hologic Incorporated, Bedford, MA) for decoy cells, negative staining EM for the detection and quantitation of PV-Haufen, and quantitative PCR for BKV gene sequences. For negative staining EM, urine was fixed in a 1:1 ratio of 4% paraformaldehyde with subsequent EM grid preparation using previously published methods. This protocol does not require any embedding procedures and thin sectioning is not needed (**SDC,** Materials and Methods, http://links.lww.com/TP/B46).[20,29,33] Plasma samples collected on the day of biopsy were analyzed by quantitative PCR for BKV gene sequences.

Biopsy Samples and Diagnosis of PVN

All renal allograft biopsies fulfilled the Banff '97 criteria for tissue adequacy and were evaluated according to standard guidelines.[34] The PVN classification into disease grades 1 to 3 followed proposals made by the 2013 BANFF working group on the classification of Polyomavirus Nephropathy.[31] Polyomavirus nephropathy grades (1 = early/mild; 2 = florid; 3 = late/sclerosed) were defined based on the severity of PV replication or degree of staining for the SV40-T antigen in combination with the Banff "ci" interstitial fibrosis score. Twelve of 40 cases were diagnosed as early or mild PVN grade 1 (**Figure 2S, A** and **D, SDC,** http://links.lww.com/TP/B46), 23/40 as florid PVN grade 2 (**Figure 2S, B** and **E, SDC,** http://links.lww.com/TP/B46) and 5 of 40 as late sclerosed PVN grade 3 (**Figure 2S, C** and **F, SDC,** http://links.lww.com/TP/B46).

Quantitative Analysis of the SV40-T Antigen Immunostain in Renal Biopsies

Intrarenal polyomavirus replication was assessed by immunohistochemistry with a monoclonal mouse antibody directed against the SV40-T antigen following previously published protocols (Clone 416, Calbiochem, San Diego, CA) (**Figure 2S, D, E,** and **F, SDC,** http://links.lww.com/TP/B46).[35] Expression levels of the SV40-T antigen, that is, the number of epithelial cell nuclei expressing the T antigen, were quantitatively analyzed by manual count and also by (automated) morphometry using the Aperio system (Aperio, Vista, CA).

Manual Counts

(a) The total number of tubular epithelial cell nuclei expressing the SV40-T antigen and (b) the total number of stained tubular cross-sections lined by one or more epithelial cells expressing the SV-40 large T antigen were recorded.

Morphometric Analysis

The percentage area of a renal biopsy demonstrating positive expression with the SV40-T antigen was accomplished as follows:

Aperio Color Deconvolution Software version 10.0 was applied to 40× virtual images in "svs" format scanned on the Aperio CS scanner (Aperio, Vista, CA). The total biopsy area was determined using the total stained area of all cellular components read using a channel optimized for blue nuclear counterstain. To evaluate the % of SV40-T–positive staining in the total biopsy area (TS_1), the total immunopositive area including both specific intranuclear staining and nonspecific background staining was determined using the DAB channel as specified by Aperio ($\%_{DAB}$). To calculate the specific intranuclear SV-40 large T antigen signals, the weak positive threshold (TS_{DAB}) was set to eliminate presumed nonspecific background staining as, for example, seen in the cytoplasm. The final % of SV-40–positive staining ($\%_{IMMUNOPOSITIVE}$) was calculated as: $\%_{IMMUNOPOSITIVE} = (\%_{DAB}) \times \left(\frac{TS_{DAB}}{TS_1}\right)$.

Negative Staining EM for the Detection and Quantitation of PV Haufen

PV-Haufen Definition

"Haufen" (after a German word for "cluster" or "stack") were defined as discrete three-dimensional, cast-like, dense polyomavirus aggregates in voided urine samples analyzed by negative staining EM (Figure 2). Polyomavirus casts containing six or more polyomavirus virions were classified as "PV-Haufen" following our previously published approach.[20,29]

Negative Staining EM and PV-Haufen Quantitation

Negative staining EM was performed using previously published standard procedures with a two-step methodology

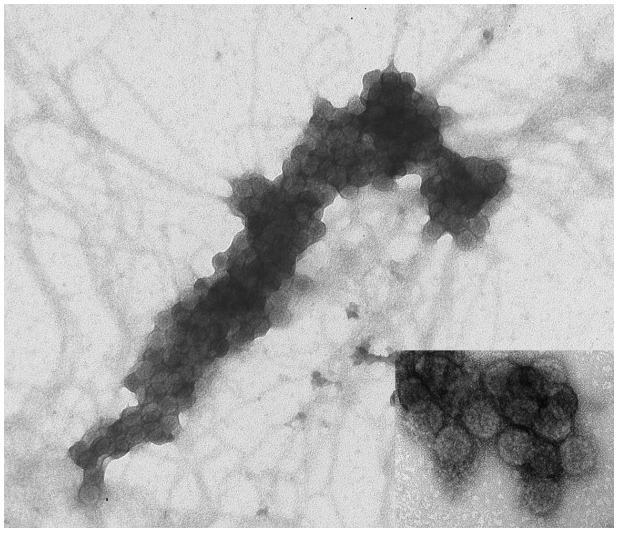

FIGURE 2. Negative staining electron microscopy depicting a characteristic PV-Haufen in a voided urine sample. Haufen are tight three dimensional viral casts composed of six or more individual PV virions. Note the large size of this PV-Haufen and the diagnostic viral capsid substructure and uniformity allowing easy identification by electron microscopy (*inset*) (uranyl acetate stain: ×100,000 magnification). PV, polyomavirus.

of urine clarification and urine concentration (**SDC,** Materials and Methods, http://links.lww.com/TP/B46 and **Table 1S, SDC,** http://links.lww.com/TP/B46).[20,29,33] One EM grid was prepared for each voided urine sample. Haufen quantitation was performed according to established previously published methodologies specifically designed for the quantitation of viruses in body fluids.[33,36] Briefly, PV-Haufen in the voided urine sample adhered to the EM grid by electrostatic binding properties (**Figure 3S, SDC,** http://links.lww.com/TP/B46). Electron microscopy grids were examined on a transmission electron microscope (LEO EM-910, LEO Electron Microscopy, Thornwood, NY, accelerating voltage, 80–100 kV). A total of 25 randomly selected grid fields were viewed at 50,000× magnification and PV-Haufen were counted in these 25 grid fields at 80,000–100,000× magnification. The examination time was, on average, 20 to 30 min per grid. The total number of PV-Haufen per mL urine was then calculated using the following formula:

$$\text{Haufen/25 grid fields} \times \left\{\frac{\text{whole grid area}}{\text{area of 25 grid squares}}\right\} \times \left\{\frac{\text{volume of total urine concentrate}}{\text{volume of applied concentrate}} \Big/ \text{total starting urine volume}\right\} = \text{number of Haufen/mL urine}$$

(methodology used by the Centers for Disease Control per Dr. Charles Humphrey, Chief, Infectious Disease Pathology).[33,36]

Quantitative PCR

Plasma and urine BK polyomavirus loads were determined by a real-time quantitative PCR assay using the ABI PRISM 7900HT Sequence Detection System with well characterized probes and primers specific for BK virus as previously published.[15,20] Real-time detection of PCR products was achieved with a fluorescent hydrolysis (Taqman) probe. Primers and probes were purchased from TIB Molbiol LLC, Adelphia, NJ. Quantitative linearity of the assay extended from 10 to 10^9 measured copies of BKV equivalents correlating with a dynamic linear range of 250 to 2.5×10^{10} BKV copies/mL urine or plasma. Detectable BKV DNA below the lower limit of linearity (<250 BKV copies/mL) was classified as a "low-positive" result.

Urine Cytology for Decoy Cell Quantitation

All urine samples were processed for cytology using standard liquid-based procedures (Hologic Incorporated, Bedford, MA) and stained by Papanicolaou method. Decoy cells were identified and quantified as reported previously.[11]

Statistical Analysis

Statistical analyses were performed with standard programs (Microsoft Excel and Stata 12.1; Stata Corp, College Station, TX). The number of urinary PV-Haufen, number of decoy cells, urine and plasma BKV DNA load levels were correlated with: (a) the severity of intrarenal PV replication/SV40-T staining using Pearson's correlation coefficient and Spearman's correlation coefficient and, (b) PVN disease grades 1 to 3 using Wilcoxon rank-sum testing, and Kruskal-Wallis testing with ties. Wilcoxon rank-sum testing for pairwise comparisons with Bonferroni correction was performed only if overall group comparison by Kruskal-Wallis testing was significant. All testing was performed with two-sided $\alpha = 0.05$.

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