

Presence of Urinary Haufen Accurately Predicts Polyomavirus Nephropathy

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ABSTRACT

There are no accurate, noninvasive tests to diagnose BK polyomavirus nephropathy, a common infectious complication after renal transplantation. This study evaluated whether the qualitative detection of cast-like, three-dimensional polyomavirus aggregates (“Haufen”) in the urine accurately predicts BK polyomavirus nephropathy. Using negative-staining electron microscopy, we sought Haufen in 194 urine samples from 139 control patients and in 143 samples from 21 patients with BK polyomavirus nephropathy. Haufen detection was correlated with pathology in concomitant renal biopsies and BK viremia (decoy cell shedding and viral load assessments by PCR) and BK viremia (viral load assessments by PCR). Haufen originated from renal tubules containing virally lysed cells, and the detection of Haufen in the urine correlated tightly with biopsy confirmed BK polyomavirus nephropathy (concordance rate 99%). A total of 77 of 143 urine samples from 21 of 21 patients with BK polyomavirus nephropathy (disease stages A–C) contained Haufen, and during follow-up (3 to 120 wk), their presence or absence closely mirrored the course of renal disease. All controls were Haufen-negative, however, high viremia or viruria were detected in 8% and 41% of control samples, respectively. κ statistics showed fair to good agreement of viruria and viremia with BK polyomavirus nephropathy or with Haufen shedding and demonstrated an excellent agreement between Haufen and polyomavirus nephropathy (κ 0.98). Positive and negative predictive values of Haufen for BK polyomavirus nephropathy were 97% and 100%, respectively. This study shows that shedding of urinary Haufen and not BK viremia and viruria accurately mark BK polyomavirus nephropathy. It suggests that the detection of Haufen may serve as a noninvasive means to diagnose BK polyomavirus nephropathy in the urine.

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BK polyomavirus nephropathy (BKN) affects 1 to 9% of renal allografts.^{1–5} No specific and potent anti-polyomavirus therapy is available. Therapeutic attempts include reduction in overall immunosuppression often combined with some antiviral drugs (e.g., leflunomide, cidofovir).^{6–10} Outcome of BKN largely depends on the histologic stage at time of diagnosis. Whereas BKN early stage A fares favorably, advanced disease stage C generally results in chronic allograft failure/loss, making an early diagnosis of BKN imperative for long-term graft survival.^{2,7,11–15}

The definitive diagnosis of BKN requires a renal biopsy. For facilitation of diagnostic workup, patient screening strategies are used for the clinical risk assess-

ment. They are based on signs of BK virus (BKV) replication in urine/viruria and plasma/viremia: Urine cytology for polyomavirus inclusion-bearing “decoy cell” quantification and PCR assays for quantitative

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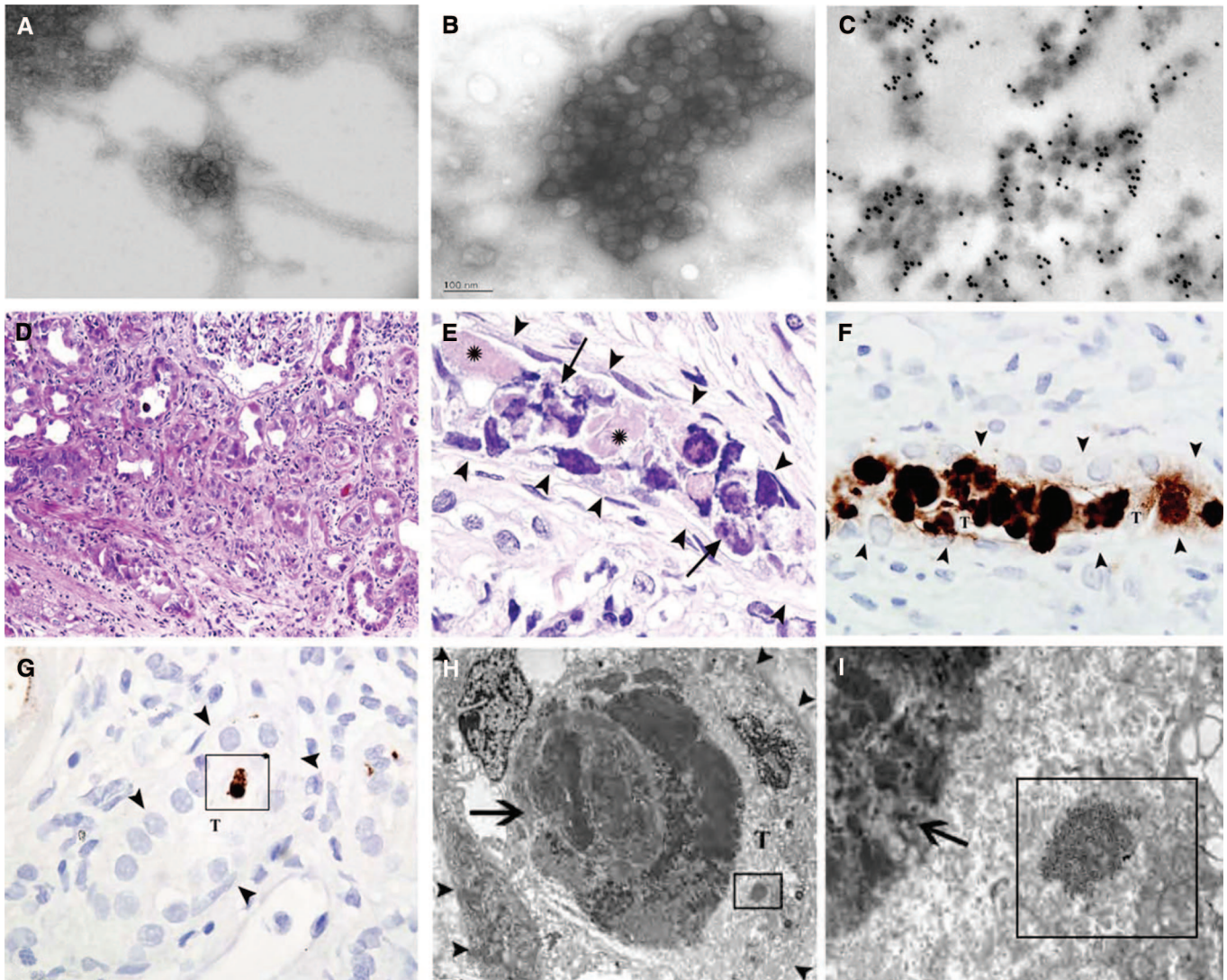


Figure 1. (A through C) Three-dimensional cast-like polyomavirus aggregates, termed Haufen, in voided urine samples. (A) A small Haufen (nine virions). (B) A large Haufen (>100 virions). (C) Haufen are rich in Tamm-Horsfall protein (marked by the black dots representing gold particles). (A and B, negative staining EM, uranyl acetate counterstaining; C, immunogold labeling EM with a mouse monoclonal anti-human Tamm-Horsfall antibody, 5-nm gold particles). (D and E) BKN shows diffuse, virally induced acute tubular injury and a predominantly mononuclear inflammatory cell infiltrate in the interstitium. (E) A high-power view of a tubular cross-section with severe, virally induced epithelial cell injury and host cell lysis (long arrows); the arrowheads mark the tubular basement membrane. *Intratubular proteinaceous material rich in Tamm-Horsfall protein. (D and E: hematoxylin- and eosin-stained, formalin-fixed, and paraffin-embedded tissue sections). (F through I) Intratubular release of mature daughter virions subsequent to BKV replication in epithelial cells, a hallmark of BKN. (F and G) Immunohistochemical incubations to detect polyomavirus capsid proteins demonstrate abundant virions in tubular lumens (T). The viruses form small and large aggregates, especially in areas of host cell lysis; the arrowheads mark the tubular basement membranes. (G) A small intratubular (T) intensely staining Haufen (boxed area). (F and G, immunohistochemical incubations with a monoclonal mouse anti-polyomavirus antibody directed against the VP1 capsid protein). (H and I) EM illustrates a tubular cross-section with a sloughed epithelial cell undergoing lysis subsequent to the replication of polyomaviruses (long arrow; compare with E and F). Daughter virions are released into the tubular lumen (T) and are located as aggregates (boxed areas) in a microenvironment rich in Tamm-Horsfall protein. The arrowheads in H mark the tubular basement membrane. (H and I, uranyl acetate staining). Magnifications: $\times 125,000$ in A through C; $\times 25$ in D; $\times 150$ in E; $\times 120$ in F and G; $\times 2500$ in H; $\times 15,000$ in I.

BKV load measurements.^{2,7,13,16–18} However, these screening techniques have only limited predictive value to diagnose accurately BKN; they are not “kidney disease specific” and cannot reliably distinguish between clinically insignificant polyomavirus replication and manifest intrarenal disease (BKN).^{7,19–24}

Given the dependence on renal biopsies for accurately diagnosing BKN and the limitations of currently available screening tests, we propose a new diagnostic method. In voided urine samples from patients with biopsy-proven BKN, we observed three-dimensional cast-like polyomavirus aggregates, hereaf-

Table 1. Patient groups and study samples

Group	Patients	Urine Samples	Plasma Samples	Renal Biopsies
Control group patients with no evidence of BKN (n = 139)				
renal transplant patients ^a	100	119	17	8 (negative for BKN)
bone marrow transplant patients ^b	15	45	45	0
native kidney patients	24	30	0	0
Study group patients with biopsy-proven BKN (n = 21)				
pre-BKN period: urine and plasma samples included up to 2 wk before initial histologic BKN diagnosis	10	13	13	2 (negative for BKN)
initial BKN histologic diagnosis: urine and plasma samples included ± 2 wk of biopsy diagnosis	21	24	24	21
follow-up: 4 to 96 wk after initial BKN diagnosis with biopsy				Stage A: n = 8 Stage B: n = 11 Stage C: n = 2
follow-up biopsy positive for BKN (persistent disease)	6	8	8	8
				Stage A: n = 2 Stage B: n = 4 Stage C: n = 2
follow-up biopsy negative for BKN (resolved disease)	2	2	2	2 (negative for BKN)
follow-up: 8 to 120 wk after initial BKN diagnosis without biopsy in patients with varying levels of viremia and viruria	12	96	96	0

^aSix patients underwent eight renal biopsies with no histologic evidence of BKN.

^bSeven patients with clinically documented hemorrhagic cystitis; confirmed by bladder biopsy in one patient.

ter termed “Haufen” (after the German word for “cluster or stack”) by electron microscopy (EM). Such densely arranged viral aggregates have not been described before. We hypothesized that Haufen were morphologic biomarkers of a productive intrarenal BKV infection. The data presented here support our hypothesis and show that the detection of Haufen can be clinically used to diagnose BKN accurately and noninvasively.

RESULTS

Haufen: Characteristic Features

In urine samples, Haufen were defined as discrete, tightly clustered, cast-like aggregates of a minimum of six polyomaviruses with an unequivocal three-dimensional architecture (Figure 1, A and B). Ultrastructurally, polyomaviruses were easily identified by their characteristic regular capsid structure and a size of approximately 40 to 45 nm in diameter. By definition, Haufen did not contain cell membrane fragments or debris as major core components. Single polyomaviruses and two-dimensional flat groups of virions, sometimes arranged in small sheets, were not classified as Haufen and were not further analyzed. Urine samples categorized as “Haufen-positive” generally contained multiple small and large polyomavirus aggregates that occasionally reached sizes > 100 virions. Haufen were typically identified within the first 10 min of grid examination, along with varying numbers of free, nonaggregated polyomaviruses in the

background. Three of three Haufen-positive urine samples that were studied by immunogold labeling demonstrated a significant Tamm-Horsfall protein content in the polyomavirus aggregates (Figure 1C).

Renal biopsies with BKN showed dense polyomavirus aggregates in injured renal tubular segments (Figure 1, D through I). These intratubular polyomavirus aggregates seemed to be washed out of the affected nephrons as “Haufen” (Figure 1G).

Interobserver Reproducibility/Agreement Rate of Haufen Detection

Thirty-eight of 40 representative urine samples (20 Haufen-positive and 20 Haufen-negative) re-analyzed by a second investigator (V.M.) in a so-called “blinded manner” were accurately reclassified ($\kappa = 0.90$; concordance rate 94.6%).

Effects of Long-Term Storage on Haufen

Eighteen Haufen-positive urine samples fixed in 2% paraformaldehyde were reexamined after 3 to 15 mo of storage at 4°C. All 18 samples demonstrated well-preserved polyomaviruses and typical Haufen without apparent ultrastructural changes.

Haufen in Urine Samples and Concomitant Signs of BKV Replication

Control Group.

All 194 urine samples from all control patients ($n = 139$) were Haufen-negative. Plasma PCR: a total of 66% (41 of 62) of

Table 2. Haufen shedding and signs of BKV activation^a

Patient Groups	Urine Samples (N)	Patients (N)	Viral Haufen-Positive in Urine		Decoy Cells in Urine (total number per ThinPrep slide)					
					0 (No Decoy Cells)		1 to 9 (Below Threshold)		≥10 (Above Threshold)	
			Samples	Patients	Samples	Patients	Samples	Patients	Samples	Patients
Control group										
renal transplant	119	100	0% (0/119)	0% (0/100)	30% (36/119)	36% (36/100)	26% (31/119)	27% (27/100)	44% (52/119)	34% (34/100)
bone marrow ^b	45	15	0% (0/45)	0% (0/15)	36% (16/45)	60% (9/15)	13% (6/45)	40% (6/15)	51% (23/45)	53% (8/15)
nontransplant	30	24	0% (0/30)	0% (0/24)	57% (17/30)	71% (17/24)	27% (8/30)	25% (6/24)	17% (5/30)	12% (3/24)
BKN group										
pre-BKN	13	10	8% (1/13)	10% (1/10)	8% (1/13)	10% (1/10)	15% (2/13)	20% (2/10)	77% (10/13)	90% (9/10)
BKN: initial histologic diagnosis	24 ^c	18 ^c	100% (24/24)	100% (18/18)	0% (0/24)	0% (0/18)	0% (0/24)	0% (0/18)	100% (24/24)	100% (18/18)
follow-up, biopsy confirmed										
persistent BKN: biopsy positive for BKN	8	6	100% (8/8)	100% (6/6)	0% (0/8)	0% (0/6)	0% (0/8)	0% (0/6)	100% (8/8)	100% (6/6)
resolved BKN: biopsy negative for BKN	2	2	0% (0/2)	0% (0/2)	100% (2/2)	100% (2/2)	0% (0/2)	0% (0/2)	0% (0/2)	0% (0/2)
follow-up: not biopsy confirmed										
high viremia: plasma PCR > 1 × 10 ⁴ viral copies/ml	25	8	88% (22/25)	12% (3/25)	8% (2/25)	12% (1/8)	0% (0/25)	0% (0/8%)	92% (23/25)	88% (7/8)
intermediate viremia: plasma PCR between 1 × 10 ³ and 1 × 10 ⁴ viral copies/ml	12	8	75% (9/12)	88% (7/8)	17% (2/12)	25% (2/8)	8% (1/12)	12% (1/8)	75% (9/12)	63% (5/8)
low viremia: plasma PCR <1 × 10 ³ viral copies/ml	36	12	36% (13/36)	50% (6/12)	42% (15/36)	33% (4/12)	8% (3/36)	17% (2/12)	50% (18/36)	50% (6/12)
no viremia: plasma PCR undetectable	23	5	0% (0/23)	0% (0/5)	96% (22/23)	80% (4/5)	4% (1/23)	20% (1/5)	0% (0/23)	0% (0/5)

samples were positive, 8% (five of 62) above threshold; 18% (three of 17) were from renal transplant patients, and 4% (two of 45) were from bone marrow transplant patients. Urine PCR: a total of 50% (98 of 194) of samples were positive, 33% (65 of 194) above threshold; 34% (40 of 119) were from renal transplant patients, 42% (19 of 45) were from bone marrow transplant patients, and 20% (six of 30) were from nontransplant patients. Decoy cells: a total of 64% (125 of 194) of samples were positive, 41% (80 of 194) above threshold; 44% (52 of 119) were from renal transplant patients, 51% (23 of 45) were from bone marrow transplant patients, and 17% (five of 30) were from nontransplant patients. Biopsies: eight renal biopsies from six patients were negative for BKN (Tables 1 and 2).

Study Group.

The time of initial histologic BKN diagnosis served as the reference point to define three major subgroups: “Pre-BKN,” “initial BKN diagnosis,” and “follow-up period.” The follow-up period was further subdivided on the basis of the results of repeat renal biopsies and BKV plasma load levels.

Pre-BKN (from Time of Transplantation until 2 Wk before Initial Histologic Diagnosis).

A total of 92% (12 of 13) of urine samples from 10 patients were Haufen-negative. Only 8% (one of 13; patient 3; Figure 2) were Haufen-positive 8 wk before initial diagnosis of BKN. Plasma PCR: a total of 85% (11 of 13) of samples were positive, 15% (two of 13) above threshold (patients 3 and 14; Figure 2). Urine PCR: all samples were positive, 92% (12 of 13) above threshold. Decoy cells: all samples were positive, 77% (10 of 13) above threshold. Biopsies: two of two renal biopsies (patients 10 and 14; Figure 2) were negative for BKN. They were triggered by viremia (750 and 2.7 × 10⁴ BKV copies/ml), viruria (1.7 × 10⁹ and 4.29 × 10⁷ BKV copies/ml), and decoy cell shedding above threshold (>50 decoy cells per ThinPrep slide; Tables 1 and 2).

Initial BKN Histologic Diagnosis (±2 Wk of Biopsy).

All 24 urine samples available from 18 of 21 patients were Haufen-positive. Plasma PCR: a total of 100% (24 of 24) of samples were positive, 71% (17 of 24) above threshold. In two patients, BKV loads were low (<250 and 590 BKV copies/ml;

Table 2. continued

Urine PCR (BKV copies/ml)						Plasma PCR (BKV copies/ml)						Renal Biopsy	
0 (Undetectable)		<1 × 10 ⁷ (Below Threshold)		>1 × 10 ⁷ (Above Threshold)		0 (Undetectable)		<1 × 10 ⁴ (Below Threshold)		>1 × 10 ⁴ (Above Threshold)			
Samples	Patients	Samples	Patients	Samples	Patients	Samples	Patients	Samples	Patients	Samples	Patients		
52% (61/119)	49% (49/100)	15% (18/119)	18% (18/100)	34% (40/119)	25% (25/100)	23% (4/17)	23% (4/17)	53% (9/17)	53% (9/17)	18% (3/17)	18% (3/17)	0% (0/8)	100% (8/8)
31% (14/45)	67% (10/15)	27% (12/45)	40% (6/15)	42% (19/45)	60% (9/15)	36% (16/45)	67% (10/15)	60% (27/45)	47% (7/15)	4% (2/45)	13% (2/15)	NA	NA
70% (21/30)	88% (21/24)	10% (3/30)	12% (3/24)	20% (6/30)	12% (3/24)	NA	NA	NA	NA	NA	NA	NA	NA
0% (0/13)	0% (0/10)	8% (1/13)	10% (1/10)	92% (12/13)	90% (9/10)	15% (2/13)	20% (2/10)	70% (9/13)	60% (6/10)	15% (2/13)	20% (2/10)	0% (0/2)	100% (2/2)
0% (0/24)	0% (0/18)	4% (1/24)	6% (1/18)	96% (23/24)	94% (17/18)	0% (0/24)	0% (0/18)	29% (7/24)	39% (7/18)	71% (17/24)	61% (11/18)	100% (18/18)	0% (0/18)
0% (0/8)	0% (0/6)	12% (1/8)	17% (1/6)	88% (7/8)	83% (5/6)	0% (0/8)	0% (0/6)	25% (2/8)	33% (2/6)	75% (6/8)	67% (4/6)	100% (8/8)	0% (0/8)
50% (1/2)	50% (1/2)	50% (1/2)	50% (1/2)	0% (0/2)	0% (0/2)	50% (1/2)	50% (1/2)	50% (1/2)	50% (1/2)	0% (0/2)	0% (0/2)	0% (0/2)	100% (2/2)
0% (0/25)	0% (0/8%)	0% (0/25)	0% (0/8%)	100% (25/25)	100% (8/8)	0% (0/25)	0% (0/8%)	0% (0/25)	0% (0/8%)	Per definition: 100%	Per definition: 100%	NA	NA
8% (1/12)	12% (1/8)	17% (2/12)	12% (1/8)	75% (9/12)	76% (6/8)	0% (0/12)	0% (0/8)	Per definition: 100%	Per definition: 100%	0% (0/12)	0% (0/8)	NA	NA
8% (3/36)	17% (2/12)	56% (20/36)	75% (9/12)	36% (13/36)	58% (7/12)	0% (0/36)	0% (0/12)	Per definition: 100%	Per definition: 100%	0% (0/36)	0% (0/12)	NA	NA
39% (9/23)	60% (3/5)	52% (12/23)	60% (3/5)	9% (2/23)	40% (2/5)	Per definition: 100%	Per definition: 100%	0% (0/23)	0% (0/5)	0% (0/23)	0% (0/5)	NA	NA

^aNA, not available.

^bThirty-one urine samples from seven patients with a clinical diagnosis of hemorrhagic cystitis.

^cThree patients with biopsy-proven BKN but with no urine samples available at the time of initial histologic diagnosis were not included.

patients 6 and 11; Figure 2). Urine PCR: a total of 100% (24 of 24) of samples were positive, 96% (23 of 24) above threshold. Decoy cells: a total of 100% (24 of 24) of samples contained decoy cells above threshold. Biopsies: renal biopsies from all 21 patients showed BKN; however, at time of initial diagnosis, urine and plasma samples were collected only from 18 of 21 patients with histologic disease stages A (six of 18), B (10 of 18), and C (two of 18; Tables 1 and 2).

Follow-up Period: Biopsy Confirmed

Persistent BKN (4 to 36 Wk after Diagnosis).

A total of 100% (eight of eight) of samples were Haufen-positive with concomitant renal biopsies positive for BKN. Plasma PCR: all eight samples were positive, 75% (six of eight) above threshold. Urine PCR: all eight samples were positive, 88% (seven of eight) above threshold. Decoy cells: all samples were positive above threshold (Tables 1 and 2).

Resolved BKN (20 to 32 Wk after Diagnosis).

A total of 100% (two of two) of samples were Haufen-negative with concomitant renal biopsies negative for BKN. Plasma PCR: one (50%) of two samples was positive below threshold. Urine PCR: one (50%) of two samples was positive below threshold. Decoy cells: all samples were negative (Tables 1 and 2).

Follow-up Period: Not Biopsy Confirmed

High Viremia ($\geq 1 \times 10^4$ BKV copies/ml; 3 to 120 Wk after Diagnosis).

A total of 88% (22 of 25) of samples were Haufen-positive. Urine PCR: a total of 100% (25 of 25) of samples were positive above threshold. Decoy cells: a total of 92% (23 of 25) of samples contained decoy cells above threshold (Tables 1 and 2).

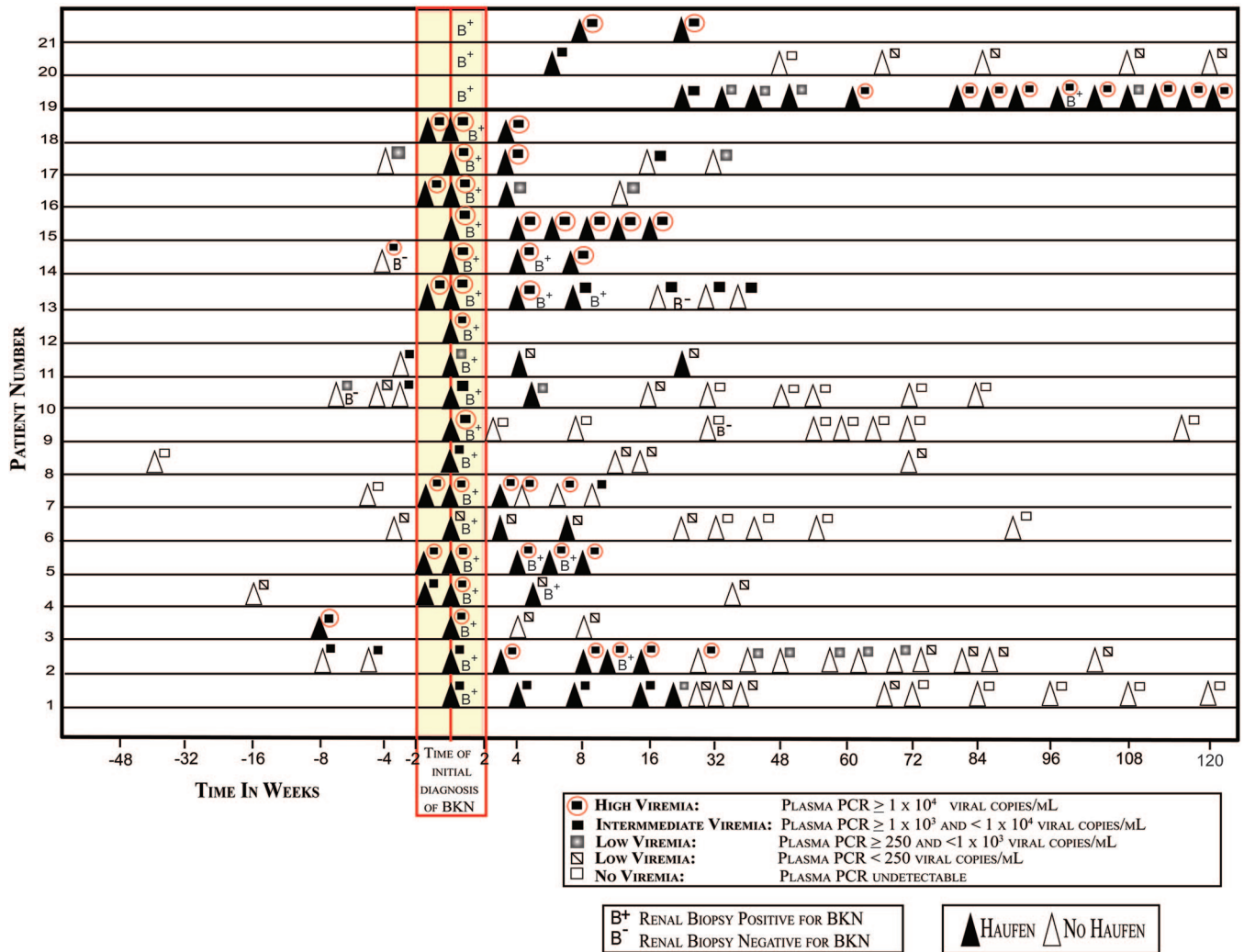


Figure 2. The dynamics of Haufen shedding over time in voided urine samples from patients with BKN. Illustrated are 21 study group patients with biopsy-proven BKN; in 18 patients, plasma and urine samples were available at time of the initial diagnostic biopsy. ▲, Haufen-positive urine samples; △, Haufen-negative urine samples; B⁺, renal biopsies with BKN; B⁻, renal biopsies without BKN. Plasma BKV load levels are marked by rectangles and grouped into five categories: solid black rectangle with red circle = $\geq 1 \times 10^4$ BKV copies/ml; solid black rectangle $\geq 1 \times 10^3$ BKV copies/ml and $< 1 \times 10^4$ BKV copies/ml; gray rectangle = 250 to 999 BKV copies/ml; open rectangle with diagonal line = < 250 BKV copies/ml; open rectangle = undetectable BKV copies/ml.

Intermediate Viremia (BKV loads $\geq 1 \times 10^3$ and $< 1 \times 10^4$ copies/ml; 4 to 38 Wk after Diagnosis).

A total of 75% (nine of 12) of samples were Haufen-positive. Urine PCR: a total of 92% (11 of 12) of samples were positive, 75% (nine of 12) above threshold. Decoy cells: a total of 83% (10 of 12) of samples were positive, 75% (nine of 12) above threshold (Tables 1 and 2).

Low Viremia ($< 1 \times 10^3$ BKV copies/ml; 3 to 120 Wk after Diagnosis).

A total of 36% (13 of 36) of samples were Haufen-positive. Urine PCR: a total of 92% (33 of 36) of samples were positive, 36% (13 of 36) above threshold. Decoy cells: a total of 58% (21 of 36) of samples were positive, 50% (18 of 36) were above threshold (Tables 1 and 2).

No Viremia (Plasma PCR Undetectable; 3 to 120 Wk after Diagnosis).

A total of 100% (23 of 23) of samples were Haufen-negative. Urine PCR: a total of 61% (14 of 23) of samples were positive, 9% (two of 23) above threshold. Decoy cells: a total of 4% (one of 23) of samples were positive below threshold (Tables 1 and 2).

Dynamics of Haufen Shedding

During the pre-BKN time period, samples from 10 patients were studied (Figure 2). Only one of 13 urine samples from one of 10 patients (patient 3) was Haufen-positive, accompanied by above threshold viremia (9.4×10^4 viral copies/ml), viruria (6.4×10^8 viral copies/ml), and decoy cell shedding (> 100 decoy cells per ThinPrep slide). A biopsy, however, could not be performed until 8 wk later, when BKN was ultimately diag-

nosed. Twelve of 13 urine samples from nine of 10 patients collected during the pre-BKN period were Haufen-negative, including three consecutive negative samples from patient 10 with varying concomitant viremia. In five of 10 patients, Haufen-negative samples were obtained 3 to 4 wk before initial histologic diagnosis of BKN. At the time of BKN diagnosis, all 18 patients from whom urine samples were available for analysis were Haufen-positive (Figure 2).

During the follow-up period (3 to 120 wk after BKN diagnosis), samples from 20 of 21 patients were studied. Six of six patients with biopsy-proven persistent BKN and ten additional patients had one to 14 sequential Haufen-positive urine samples. For seven patients, Haufen-positive samples were collected in the setting of BKV plasma loads of $<1 \times 10^3$ copies/ml 3 to 48 wk after the initial BKN diagnosis. After one to five consecutive Haufen-positive samples, 13 of 20 patients, including two patients (9 and 13) with biopsy-proven BKN resolution, turned Haufen-negative. Three patients (3, 7, and 9) converted within 3 to 5 wk after initial diagnosis. In patients 2 and 7, conversion occurred in the setting of high viremia ($>1 \times 10^4$ BKV copies/ml) and viruria ($>1 \times 10^7$ BKV copies/ml) and in patients 1, 2, and 6, in association with decoy cell shedding above threshold. Once converted, all patients remained Haufen-negative during further follow-up (up to 105 wk) when signs of BKV replication steadily decreased, although never became undetectable in nine of 13 patients. All 139 control patients were Haufen-negative despite marked signs of BKV replication in some individuals (Figure 3).

Haufen Shedding, BKV Replication, and BKN

The shedding of Haufen was not merely linked to the degree of viruria or viremia (e.g., decoy cell excretion, BKV loads in urine and plasma) (Table 3). Significant differences were found between the proportions of samples with high numbers of decoy cells, BKV gene copies in the urine, and plasma on the one hand and Haufen-positivity on the other hand (all differences $P < 0.001$; κ between 0.39 and 0.56; fair to modest agreement).

The shedding of Haufen was very tightly linked to intrarenal disease (BKN) (see Table 4 and Concise Methods section for definitions of BKN positivity and negativity; no significant difference between Haufen-positive/negative and BKN-positive/negative groups $P = 0.317$, $\kappa = 0.98$; excellent agreement; concordance rate 99%). The detection of Haufen had a sensitivity of 100%, a specificity of 99%, a positive predictive value (PPV) for BKN of 97%, and a negative predictive value (NPV) of 100%.

In contrast, intrarenal disease (BKN) was not tightly associated with viruria. The distribution of BKN-positive/negative groups and decoy cell-positive/negative groups or samples with high/low urine BKV gene copy numbers differed significantly (see Table 4 for definitions; all differences highly significant $P < 0.001$, $\kappa = 0.25$ and 0.34, respectively; fair agreement). A marginal difference was observed between the distribution of BKN-positive/negative groups and samples with high/low plasma BKV gene copy numbers (see

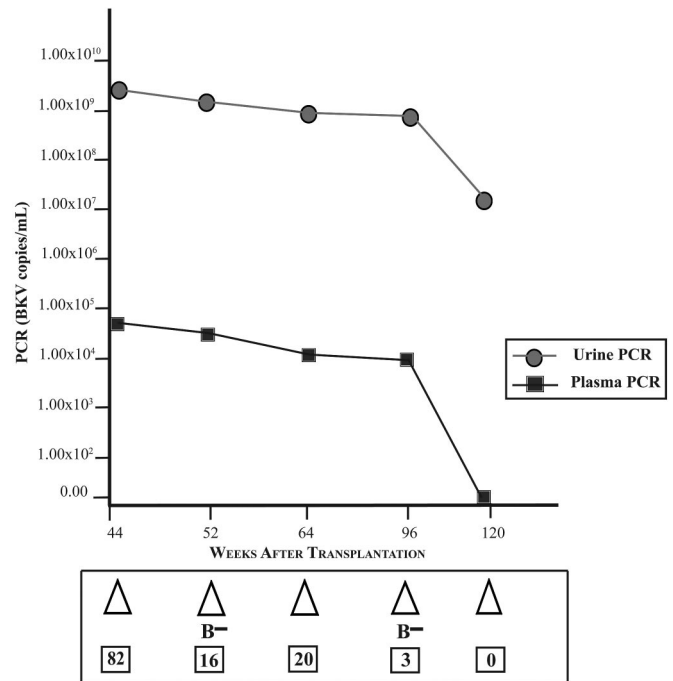


Figure 3. One renal transplant control group patient with marked polyomavirus replication and no BKN. Over months (weeks after transplantation plotted on x axis), this renal allograft recipient presented with very high viremia (■), viruria (●), and decoy cell counts (total decoy cell numbers per ThinPrep slide given in boxed areas). Two allograft biopsies performed 52 and 96 wk after grafting showed no evidence of BKN (B⁻). Five urine samples were consistently Haufen negative (△).

Table 4 for definitions; $P = 0.058$, $\kappa = 0.62$; good agreement). The PPVs for BKN ranged between 40% (high decoy cell shedding) and 74% (high plasma BKV load levels), and NPVs ranged between 88% (plasma BKV load levels) and 100% (decoy cell shedding and urine BKV load levels; Table 4).

A subgroup analysis limited to samples obtained from renal allograft recipients for whom all tests were performed in parallel gave similar results (BKN-positive time points $n = 30$; BKN-negative time points $n = 32$; thresholds as listed in Table 4; Haufen: sensitivity 100%, specificity 97%, PPV 97%, NPV 100%; high decoy cell shedding: sensitivity 100%, specificity 19%, PPV 54%, NPV 100%; high plasma BKV load levels: sensitivity 70%, specificity 81%, PPV 78%, NPV 74%; high urine BKV load levels: sensitivity 100%, specificity 22%, PPV 55%, NPV 100%).

DISCUSSION

In patients with biopsy-proven BKN, tight, three-dimensional polyomavirus clusters, termed Haufen, were easily detected in voided urine samples by negative staining EM with high interobserver agreement/reproducibility. The detection of Haufen is a new observation that differs from previous studies focusing on polyomavirus infections and the urinary shedding of free, nonclustered viruses.^{24–26} Not free polyomaviruses but rather

Table 3. Comparison of Haufen shedding with markers of viral replication/activation

Parameter	Haufen		Total	P (McNemar Test)	κ
	Positive	Negative			
Decoy cells				<0.001	0.43
positive (≥ 10 per ThinPrep slide)	77	97	174		
negative (<10 per ThinPrep slide)	0	163	163		
total	77	260	337		
Urine PCR				<0.001	0.39
positive ($>1 \times 10^7$ viral copies/ml)	67	88	155		
negative ($<1 \times 10^7$ viral copies/ml)	10	172	182		
total	77	260	337		
Plasma PCR				<0.001	0.56
positive ($>1 \times 10^4$ viral copies/ml)	46	9	55		
negative ($<1 \times 10^4$ viral copies/ml)	31	119	150		
total	77	128	205		

Table 4. Comparison of screening tests to detect BKN^a

Test	BKN		P (McNemar Test)	κ	Sensitivity (%)	Specificity (%)	PPV for BKN (%)	NPV for BKN (%)
	Positive ^b	Negative ^c						
Haufen			0.317	0.98	100	99	97	100
positive	100.0% (32/32)	1.3% (1/77)						
negative	0.0% (0/32)	98.7% (76/77)						
Decoy cells			<0.001	0.25	100	36	40	100
≥ 10	100.0% (32/32)	63.6% (49/77)						
<10	0.0% (0/32)	36.4% (28/77)						
Plasma BKV load			0.058	0.62	72	88	74	88
$\geq 10^4$ viral copies/ml	72.0% (23/32)	10.0% (8/77)						
<10 ⁴ viral copies/ml	28.0% (9/32)	90.0% (69/77)						
Urine BKV load			<0.001	0.34	100	47	44	100
$\geq 10^7$ viral copies/ml	100.0% (32/32)	53.2% (41/77)						
<10 ⁷ viral copies/ml	0.0% (0/32)	46.8% (36/77)						

^aAnalysis limited to a subset of samples in which all four tests were performed in parallel ($n = 109$ samples)

^bDefined as all samples obtained at time of biopsy-proven BKN (initial time of diagnosis $n = 24$ and follow-up biopsies $n = 8$; compare with Figure 2).

^cDefined as all samples from control patients with no clinical or biopsy evidence of BKN ($n = 62$) and all samples from patients with BKN during the pre-BKN period ($n = 13$) and at time of biopsy-proven BKN resolution ($n = 2$).

three-dimensional, polyomavirus aggregates (*i.e.*, Haufen) are described here for the first time as reliable markers of intrarenal polyomavirus-induced disease (BKN). Haufen were found in voided urine samples from all 21 patients with BKN in early and late disease stages (A through C) but not in any of the 139 control patients. BKN and Haufen showed an excellent level of agreement with a κ of 0.98 and a nearly perfect concordance rate of 99%. The qualitative detection of Haufen was 100% sensitive and 99% specific for identifying biopsy-proven BKN. It closely reflected the clinical disease course with PPV and NPV of 97 and 100%, respectively. The PPV may even be greater because only one of 209 urine samples from a patient with concurrent high viremia and viruria, who could not undergo biopsy until 8 wk later when BKN was diagnosed, was classified as having a false-positive test result. Conceivably, this sample may also be a true-positive Haufen test marking BKN.

Haufen shedding was not a mere reflection of marked BKV replication. The Haufen-positive group significantly differed from groups with high viruria and viremia. It is likely that specific factors exceeding viral replication are needed to promote Haufen genesis. We believe that Haufen form in the kid-

neys and not in extrarenal sites, such as the bladder. In our interpretation, intratubular polyomavirus replication with the release of mature daughter virions into injured nephrons rich in Tamm-Horsfall protein subsequent to the lysis of epithelial host cells creates the proper microenvironment for the formation of Haufen. Tamm-Horsfall protein presumably facilitates intratubular polyomavirus aggregation as previously shown *in vitro* for influenza, mumps, and Newcastle disease viruses.²⁷ Once formed in the diseased kidney, cast-like polyomavirus aggregates are flushed into the bladder and can be detected as Haufen in voided urine samples. The demonstration of dense, intratubular polyomavirus aggregates in biopsies with BKN and the high Tamm-Horsfall protein content of urinary Haufen shown in this study support our hypothesis. Thus, we believe that the intratubular aggregation of polyomaviruses in patients with BKN and the shedding of Haufen in voided urine samples follow the same general principle known from the formation and shedding of red or white blood cell casts in patients with kidney disease. We propose Haufen as powerful and accurate new urinary biomarkers that can be used clinically to diagnose BKN noninvasively.

A new diagnostic strategy to identify patients with BKN effectively is important because the prevalence of disease remains high (1 to 9% of all renal allograft recipients; 6.5% in Chapel Hill), and chronic allograft failure/loss is common as a result of the lack of potent antipolyomavirus drugs.^{1–3,5,9,11} Although preemptive therapeutic strategies to avoid BKN have been proposed²⁸ and will likely result in a decreased disease incidence in the future, the ultimate goal of disease prevention cannot always be achieved, leaving physicians with the constant challenge to diagnose intrarenal disease (BKN) early, accurately, and, best, noninvasively. Diagnostic tests that can easily and frequently be repeated are ideal. In this respect (the accurate, noninvasive diagnosis of BKN), the detection of Haufen in urine samples offers an attractive new method that can significantly improve diagnostic strategies.¹³

A renal biopsy is currently required for a definitive BKN diagnosis, but small/suboptimal single biopsy cores can give false-negative histologic diagnoses in 25 to 37% of patients with disease.^{12,13,15} Moreover, if virally induced cytopathic changes are absent, then early-stage BKN can be morphologically overlooked.^{12–14} Thus, even the “gold standard” renal biopsy can, in some cases, fail to provide an accurate diagnosis.

Because deterioration of allograft function does not necessarily accompany early viral nephropathy and multiple repeat biopsies are impractical for surveillance, general signs of BKV replication have been used for BKN risk assessment. This common clinical strategy is based on the fact that all patients with BKN have signs of viral replication in the urine and plasma.^{1,2,7,11,16–18,22,29} Screening assays include urine cytology for the detection of decoy cells and quantitative PCR assays in the plasma and urine. However, patient screening using signs of BKV replication have limitations because viral replication is common and not kidney disease-specific. Only a small subgroup of “viral replicators” ultimately develop manifest BKN.^{2,7,11,14,16,22,29} Similar phenomena are widely known from other viruses, such as cytomegalovirus or Epstein-Barr virus, that also establish latency in humans. Therefore, in an attempt to improve the predictive diagnostic value of BKV replication-based assays, clinically relevant quantitative cutoff levels have been suggested.^{2,16,18} Unfortunately, however, even the consideration of “thresholds” in the clinical risk assessment has diagnostic limitations: (1) Threshold levels should be exceeded on repeat (*i.e.*, for >4 wk) before a patient can be categorized as having “presumptive” BKN² thereby protracting the clinical decision-making process; (2) the PPVs of individual test results with readings exceeding the “threshold levels” to mark BKN are <80%, some even <50%; (3) threshold levels have not been validated in multicenter studies; (4) although usually high, BKV loads, especially plasma loads, can be low at the time of initial diagnosis of BKN (occasionally <1000 BKV copies/ml plasma as shown by us and others^{30,31}); (5) PCR assays are not standardized, with an interlaboratory variability of test results of up to 2 log 10, rendering general recommendations of clinically relevant virus load levels problematic³²; and (6)

threshold levels are valid only at time of initial BKN diagnosis and not suitable to monitor for BKN resolution.

As illustrated in this study, by using Haufen determination, a single, easily obtainable urine sample can provide specific diagnostic evidence of BKN. We believe that on the basis of the disappearance of Haufen from the urine during patient follow-up, BKN resolution occurs earlier than the cessation of BKV replication, as is currently assumed.

When do we advocate negative staining EM on voided urine samples? The search for Haufen is not suited as a mass screening tool but rather should amend other clinical tests, including renal biopsies. We recommend it as a targeted diagnostic tool for (1) all patients with signs of BK virus replication on the basis of currently recommended screening protocols to separate patients with BKN from those with asymptomatic BKV replication, (2) patients undergoing renal biopsy with BKN in the differential diagnosis, (3) patients with a negative biopsy result but with signs of persistent BKV replication that might precede the manifestation of disease for months,^{7,16} and (4) patients with BKN to monitor for the earliest signs of disease resolution. The detection of Haufen can shed additional light on the development, progression, resolution, and possible recurrence of BKN.

Although unfamiliar to most transplant physicians, negative staining EM of urine samples is a well-established, robust, and easily performed method that has been in routine use in diagnostic virology for more than four decades. It is not a research tool.³³ There are approximately 250 diagnostic EM laboratories in the United States that are geared toward the evaluation of clinical samples by a variety of methods, including negative staining EM. Major institutions, such as the Centers for Disease Control and Prevention (Atlanta, GA), heavily depend on this technique for the diagnosis of a variety of infectious diseases from clinical samples. In this study we followed standard sample preparation techniques,^{24,34,35} enabling any electron microscopist or pathologist involved in EM examination of other tissue samples to prepare and examine negatively stained urine samples for Haufen after brief adaptation. The technique itself is not “magic.” It does not involve costly and time-consuming fixation/embedding procedures used in standard EM evaluations of solid tissues. Rather, it is a whole-mount technique of fluid with a turnaround time of 2 to 3 h and costs of approximately \$300 at our institution that favorably compare with other laboratory assays, such as quantitative PCR tests. Because Haufen do not lose their characteristic ultrastructural features during extended storage at 4°C, fixed urine samples could potentially be sent to referral EM laboratories for evaluation, a practice frequently used for a variety of other clinical tests. Thus, we think that the search for Haufen is a practical and advantageous new clinical strategy.

In conclusion, we contend that Haufen in voided urine samples are specific and accurate structural biomarkers that can be used to diagnose BKN noninvasively and to improve patient treatment. The use of negative staining EM for the detection of Haufen requires no novel infrastructure but rather

“thinking outside the box.” Transplant physicians will have to simply incorporate a new application of an old, well-established diagnostic technique into their clinical decision-making process. Future prospective, multicenter studies are needed to refine and further validate our current single-center, retrospective findings.

CONCISE METHODS

Urine, blood, and renal biopsy samples were collected for diagnostic purposes following standard-of-care guidelines. The study protocol was approved by the institutional review board of the University of North Carolina at Chapel Hill.

A total of 337 urine samples from 160 patients (99 men, 61 women; 92 white, 56 black, 8 Hispanic, 4 Asian; median age 51 yr [range 6 to 82]) were analyzed by conventional cytology for decoy cells, negative staining EM for the detection of Haufen, and quantitative PCR assays for BKV DNA loads. PCR and negative staining EM analyses were conducted on leftover urine samples subsequent to cytology processing. For EM studies, urine was fixed in 2% paraformaldehyde and stored at 4°C until use as described previously.²⁴ In a subgroup of patients, we also performed quantitative PCR assays for BKV DNA plasma loads and renal biopsies (Table 1).

The study group consisted of 21 consecutive patients with biopsy-proven BKN (19 renal allograft recipients, one bone marrow transplant, and one nontransplant patient with chronic lymphocytic leukemia; 143 urine and plasma samples). They were seen at The University of North Carolina from 2005 to 2007 with a post-BKN observation period ranging from 0 to 120 wk. The control group consisted of 139 patients who were treated at The University of North Carolina and had no clinical or biopsy evidence of BKN (100 renal and 15 bone marrow allograft recipients and 24 patients without transplants; 194 urine and 62 plasma samples). Seven control group bone marrow transplant recipients had clinical evidence of hemorrhagic cystitis (confirmed by a bladder biopsy in one patient). Control group samples from bone marrow transplant patients were consecutively collected from 2005 to 2007. Samples from renal transplant and nontransplant patients were initially collected consecutively from 2005 to 2006; thereafter, from 2006 to 2007, samples were selectively collected from patients with signs of viruria (≥ 10 decoy cells per ThinPrep cytology slide) because, on the basis of current patient risk assessment, these individuals are at increased risk for BKN.

BKN Definition

According to *Heptinstall's Pathology of the Kidney*,¹² BKN was defined by morphologic changes in renal biopsies (*i.e.*, histologic evidence of polyomavirus replication in the renal cortex *and/or* medulla with intranuclear viral inclusion bodies *and/or* positive immunohistochemical *and/or in situ* hybridization signals accompanied by varying degrees of tubular injury, inflammation, and fibrosis; disease stages A, B, or C). Typically, BKN follows a protracted disease course and is accompanied by general signs of polyomavirus replication (*i.e.*, decoy cell shedding in the urine) and elevated BK polyomavirus load levels in the urine and plasma. General signs of polyomavirus replication,

however, are not pathognomonic for BKN, because they can frequently also be found in patients without disease as asymptomatic and clinically insignificant events.^{7,12,13,16,24,29}

Haufen Definition

“Haufen” (after the German word for “cluster or stack”) were defined as three-dimensional, cast-like, dense polyomavirus aggregates in urine samples analyzed by EM.

Cytologic and Histologic Studies

All 337 urine samples were processed for cytology using standard liquid-based procedures (Cytec/Hologic Corp., Marlborough, MA) and stained by the Papanicolaou method. Decoy cells were quantified as reported previously.^{7,11,16,17,22,29} Thirty-eight renal biopsies from 32 patients were processed for routine histology, including standard transmission EM studies in selected cases. Morphologic workup, diagnosis, and staging of BKN were conducted according to previous reports and the definitions given already.^{7,11,12}

All biopsy samples fulfilled the Banff '97 criteria for tissue adequacy.³⁶ Immunohistochemical detection of polyomavirus-associated antigens was performed on all biopsies with a monoclonal mouse antibody directed against the SV40 large T antigen (Clone 416; Calbiochem, San Diego, CA) and a monoclonal mouse anti-polyomavirus antibody against the VP1 capsid protein (clone 597) following previous reports.^{11,37} Transmission EM studies on renal biopsy samples were performed according to standard protocols.

Negative Staining Urine EM for the Detection of Haufen and Immunogold Labeling

Negative staining EM on voided urine samples was conducted according to previously published procedures.^{25,34} Important parts of the protocol include an initial urine clarification step followed by a second viral concentration step as described previously.^{24,25} Briefly, 10 ml of fixed urine was centrifuged at 12,500 rpm for 30 min (clarification step) to remove large cell fragments; the supernatant was subsequently centrifuged at 36,000 rpm for 1 h for viral concentration. After the last centrifugation round, the supernatant was mostly discarded, and the pellet including a minimal amount (400 to 500 μ l) of covering supernatant (containing Haufen) was saved for further analysis. Negative staining was performed using a previously described standard two-step method (adsorption followed by negative staining using 1% uranyl acetate).^{33,34} One grid was prepared for each sample. A detailed technical procedure manual will be published separately.

Polyomaviruses were identified by their characteristic ultrastructural morphology. Specimens were qualitatively categorized as either Haufen-positive (*i.e.*, containing one or more tight, three-dimensional polyomavirus clusters) or Haufen-negative. In positive cases, multiple Haufen were generally identified within 5 to 10 min of screening at $\times 50,000$ magnification. No attempt was made to quantify Haufen shedding. All Haufen-negative samples were examined for 30 min before the sample was categorized as negative. The analysis was performed by one investigator (H.K.S.), who was blinded to the clinical and histologic information. A subset of 40 representative urine samples were reevaluated by a second investigator (V.M.) in a

blinded manner to test the interobserver reproducibility of Haufen detection.

Three Haufen-positive urine samples were processed and stained by immunogold labeling with a Tamm-Horsfall antibody using standard protocols (monoclonal mouse anti-human Tamm-Horsfall antibody [cat. no. CL1032A; Cedarlane Laboratories, Hornby, ON, Canada] and goat anti-mouse IgGFab'2 5-nm gold beads [cat. no. EMGFAF5; British Biocell Int., distributed in the United States by Ted Pella, Inc., Redding, CA]).

Eighteen Haufen-positive, fixed urine samples were stored for 3 to 15 mo at 4°C. The specimens were reevaluated by negative staining EM to study effects of long-term storage on the structural integrity of polyomaviruses and Haufen.

Quantitative PCR

Plasma and urine BKV loads were determined by a real-time quantitative PCR assay using the ABI PRISM 7900HT Sequence Detection System (Foster City, CA) with well-characterized probes and primers specific for BK virus.¹⁸ Real-time detection of PCR products was achieved with a fluorescence hydrolysis (TaqMan) probe. Primers and probes were purchased from TIB Molbiol LLC (Adelphia, NJ). The primer and probe sequences were as follows: BK virus forward 5'-GCAGCTCCCAAAAAGCCAAA-3'; BK virus reverse 5'-CTGGGTTTAGGAAGCATTCTA-3'; BK virus TaqMan probe 5'-6-FAM-ACCCGTGCAAGTGCCAAACTACTAATAAAAAGG-TAMRA-3'.

Quantitative linearity of the assay extended from 10 to 10⁹ measured copies of BKV equivalents correlating with a dynamic linear range of 250 to 2.5 × 10¹⁰ 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.

Statistical Analysis

Statistical analyses were performed with standard programs (Excel [Microsoft Corp., Redmond, WA] and SAS [SAS Institute, Cary, NC]). Using previously proposed cutoff levels for identifying patients at high risk for BKN (plasma PCR ≥ 1 × 10⁴ BK virus copies/ml; urine PCR ≥ 1 × 10⁷ BK virus copies/ml; urine cytology ≥ 10 decoy cells per ThinPrep slide), samples were categorized as either positive (above threshold level) or negative (below threshold level).^{2,17,18,22}

For statistical analysis, the term "BKN positive" was restricted to time points of biopsy-confirmed BKN (as defined already; both at time of initial BKN diagnosis and during follow-up/persistent disease). All urine and plasma samples collected at these BKN-positive time points were classified as either true positive (containing Haufen or showing BK virus loads/decoy cells above cutoff levels) or false negative (no Haufen or showing BK virus loads/decoy cells below cutoff levels).

The term "BKN negativity" was applied to time points of biopsy-proven absence of BKN (including all biopsies obtained during disease resolution), all control patients, and all time points of the pre-BKN period (span after transplantation up to 2 wk before the initial histologic diagnosis of BKN). All samples collected at these BKN-negative time points were classified as either true negative (containing no Haufen or showing BK virus loads/decoy cells below cutoff levels) or false positive (containing Haufen or showing BK virus loads/decoy

cells above cutoff levels). The agreement rates among decoy cell shedding, urine and plasma BKV load levels, Haufen, and BKN were assessed using (1) McNemar test (significant differences reported as $P < 0.05$), (2) κ statistics, and (3) percentage of concordance.

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DISCLOSURES

None.

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