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NONINVASIVE DIAGNOSIS OF BK VIRUS NEPHRITIS BY MEASUREMENT OF MESSENGER RNA FOR BK VIRUS VP1 IN URINE[1]

RUCHUANG DING,[2] MARA MEDEIROS,[2] DARSHANA DADHANIA,[2] THANGAMANI MUTHUKUMAR,[2] DAVID KRACKER,[2] JIN M. KONG,[2] SUSANNA R. EPSTEIN,[2] VIJAY K. SHARMA,[2] SURYA V. SESHAN,[3] BAOGUI LI,[2] AND MANIKKAM SUTHANTHIRAN[2,4]

Background. **Polyoma virus type BK (BKV) nephritis has emerged as an important cause of renal allograft dysfunction and graft failure. Its diagnosis is contingent on the invasive procedure of allograft biopsy. A noninvasive diagnostic test for BKV nephritis could improve clinical outcome.**

Methods. **We obtained 25 urine specimens from 8 renal allograft recipients with biopsy-confirmed BKV nephritis, 31 samples from 28 recipients in whom BKV nephritis was excluded by allograft biopsy, and 74 specimens from 34 patients with stable allograft function. RNA was isolated from the urinary cells and reverse transcribed to complementary DNA. We designed gene-specific oligonucleotide primers and probes for the measurement of messenger RNA (mRNA) encoding BKV VP1 protein and a constitutively expressed 18S ribosomal RNA (rRNA) by real-time quantitative polymerase chain reaction. We explored the hypothesis that BKV VP1 mRNA levels predict BKV nephritis.**

Results. **The levels of BKV VP1 mRNA but not the levels of 18S rRNA predicted BKV nephritis. Analysis involving the receiver operating characteristic curve demonstrated that BKV nephritis can be predicted with a sensitivity of 93.8% and a specificity of 93.9% with the use of a cutoff value of 6.5×10^5 BKV VP1 mRNA copy number per nanogram of total RNA ($P<0.00001$). In the receiver operating characteristic curve analysis, the calculated area under the curve was 0.949 (95% confidence interval, 0.912 to 0.987, $P<0.00001$) for BKV VP1 mRNA levels and 0.562 (95% confidence interval, 0.417 to 0.708, $P>0.2$) for 18S rRNA.**

Conclusions. **Measurement of BKV VP1 mRNA in urinary cells offers a noninvasive and accurate means of diagnosing BKV nephritis.**

Infections, especially of viral origin, remain a major cause of morbidity and mortality after organ transplantation (*1*,*2*). Recently, BK virus (BKV), a polyoma virus with a special predilection for the urinary tract, has emerged as a significant posttransplantation complication (*3–6*). Human polyomavirus are members of the *Polyomaviridae* family and are approximately 40-nm icosahedrons with double-stranded, supercoiled DNA of about 5130 base pairs (*7*,*8*). BKV and JC virus (JCV) were both named after the patients in whom they were identified: BKV from the urine of a renal transplant recipient with ureteral stenosis (*9*) and JCV from the brain tissue of a patient with progressive multifocal leukoencephalopathy (*10*). The nucleotide sequences of BKV and JCV show 75% homology.

Primary BKV infection occurs during childhood, through the respiratory or gastrointestinal route. The primary infection is usually asymptomatic but occasionally is associated with upper respiratory or urinary tract disease. After the primary infection, the virus remains latent in blood cells and in the urinary tract and can be reactivated in the immunocompromised host. BKV infection results in hemorrhagic or nonhemorrhagic cystitis in bone marrow transplant recipients, whereas renal allograft recipients present with hematuria, ureteral stricture, and/or interstitial nephritis with graft dysfunction that can progress to graft loss (*3–6*,*11–13*).

The diagnosis of active human polyomavirus infection is based on the examination of urine, direct visualization using electron microscopy, or by cytopathologic methods; by virus isolation; and by techniques that detect viral antigens or nucleic acids (*3–6*,*14*). Detailed evaluation of renal allograft core biopsy tissue is the most accurate way of diagnosing BKV nephritis, and the histologic features include characteristic epithelial intranuclear inclusion bodies, cytopathic changes in tubular cells, and interstitial cellular infiltrate. Evaluation of the allograft tissue by electron microscopy (*15*) or by immunohistochemical staining or both using antibodies against SV40 (a simian polyoma virus that shares 70% homology with BKV and JCV) firmly establishes the diagnosis.

A noninvasive and accurate procedure to diagnose active BKV infection in renal allograft recipients would be of considerable value. Indeed, polymerase chain reaction (PCR)-based assays have been developed to identify BKV DNA in plasma, serum, and peripheral blood leukocytes and in urine samples (*16–19*). However, the ubiquitous nature of BKV is a frequent source of false-positive results in DNA-based tests (*20–23*).

The viral genome of BKV can be functionally divided into three regions: a noncoding regulatory region, an early region

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[2] Division of Nephrology, Departments of Medicine and Transplantation Medicine, Weill Medical College of Cornell University, New York-Presbyterian Hospital, New York, NY.

[3] Department of Pathology, Weill Medical College of Cornell University, New York-Presbyterian Hospital, New York, NY.

[4] Address correspondence to: M. Suthanthiran, M.D., 525 East 68th Street, Box 3, New York, NY 10021. E-mail: msuthan@med.cornell.edu.

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that codes for small and large T proteins that are transcribed before to DNA replication, and a late region that codes for the agnoprotein and the capsid proteins VP1, VP2, and VP3, which are transcribed after DNA replication (*7*,*8*). Because VP1 messenger RNA (mRNA) expression is contingent upon BKV DNA replication, we reasoned that productive infection could be readily assessed by measurement of mRNA encoding BKV VP1 protein. Thus, we designed and synthesized oligonucleotide primers and a probe for measurement of BKV VP1 mRNA by real-time quantitative PCR assay. We targeted a region in the VP1 region that does not undergo alternative splicing and one that is highly conserved among the major subtypes of BKV (*23*,*24*).

In the current investigation, we have explored the hypothesis that measurement of BKV VP1 mRNA in urine is an accurate and noninvasive means of diagnosing BKV nephritis.

METHODS

Collection of Urine Samples and Renal Biopsy Specimens

We measured the levels of BKV VP1 mRNA in 180 urine specimens obtained from 120 individuals (Table 1). Among the 120 subjects, 70 were renal allograft recipients; the remaining 50 individuals were normal subjects. Eight of the renal allograft recipients (age 47±11 years, mean ± SD; six females and two males; five cadaver donor grafts and three living donor grafts) had biopsy-confirmed BKV nephritis, and 25 urine specimens were obtained from this group at the time of diagnostic core needle biopsies. The diagnosis of BKV nephritis was established in this group by renal histologic findings and by positive immunostaining for BKV. Twenty-eight additional patients with allograft dysfunction (age 42±15 years, mean ± SD; 17 females and 11 males; 15 cadaver donor grafts and 13 living donor grafts) underwent 31 diagnostic core needle biopsies, and 31 urine specimens were obtained at the time of biopsies. The diagnosis of BKV nephritis was excluded in this group by renal histologic findings and by negative immunostaining for BKV. All kidney biopsy specimens were obtained under ultrasound guidance using a 14-gauge biopsy needle. The biopsy specimens were fixed in 10% neutral buffered formalin or 4% paraformaldehyde and processed. Paraffin-embedded sections (2-μm thick) were examined by light microscopy after staining with hematoxylin-eosin, periodic acid-Schiff, trichrome, and immunoperoxidase stain. The renal biopsy tissues were examined by a single pathologist who did not know the results of the molecular studies. BKV immunostaining was performed using monoclonal antibodies directed at the SV40 large T and small t antigens and cross-reactive with the large T antigen of BKV (Chemicon International, Temecula, CA). The biopsy specimens were classified with the use of the Banff '97 classification (*25*).

Seventy-four urine specimens were obtained from 34 renal allograft recipients with stable renal allograft function (age 49±16 years, mean ± SD; 13 females and 21 males; 17 cadaver donor grafts and 17 living donor grafts). In these patients, the serum creatinine levels had not changed by more than 0.2 mg per deciliter during the 7 days before and the 7 days after urine collection. We also obtained 50 urine specimens from 50 normal individuals (age 41±17 years, mean ± SD; 33 females and 17 males) for measurement of BKV VP1 mRNA levels in the urine specimens.

The institutional review board at the Weill Medical College of Cornell University approved the study. Each patient gave written (or oral, if only urine samples were involved) informed consent.

Isolation of RNA from Urine Specimens

Urine was centrifuged at 10,000×*g* for 30 min at 4°C. RNA was extracted from the pellet with use of a commercial kit (RNeasy minikit; Qiagen, Chatsworth, CA). For each sample, 1 μg of RNA was reverse transcribed to complementary DNA (cDNA) as previously described (*26*).

Design of Primers and Probes for the Quantification of BKV VP1 mRNA by Real-Time Polymerase Chain Reaction Assay

We designed and synthesized the primers and the probe for the measurement of levels of BKV VP1 mRNA by real-time quantitative PCR assay. The sequences and location of the oligonucleotide primers and the fluorogenic probe are illustrated in Figure 1. We targeted a region in the VP1 region that is highly conserved among the major subtypes of BKV (*23*,*24*) and does not undergo alternative splicing. The primer sequence and location also ensured a high degree of mismatch with the polyoma virus type JCV.

We also designed and synthesized the primers and the probe for the quantification of 18S ribosomal RNA (rRNA) by real-time PCR assay. 18S rRNAs (GenBank accession number K03432) were measured as a control for the quality of RNA and the real-time PCR assay. The sequences and location of the primers and the probe were as follows: sense primer: 5'- GCCCGAAGCGTTTACTTT GA-3' (929–948); antisense primer: 5'-TCCATTATTCCTAGCTGCGGTATC-3' (1009–985); probe: 5'-6-FAM-AAAGCAGGCCCGAGCCGCC- TAMRA-3' (965–984).

The probes for BKV VP1 mRNA and 18S rRNA were labeled with 6-carboxy-fluorescein (FAM) at the 5' end and with 6-carboxy-tetramethylrodamine (TAMRA) at the 3' end. FAM functioned as the reporter dye and TAMRA as the quencher dye.

mRNA levels of BKV VP1 and the levels 18S rRNA were measured using an ABI Prism 7700 sequence detection system (PE Biosystems, Foster City, CA). PCR amplification reactions were set up in a

TABLE 1. Study groups for measurement of BKV VP1 mRNA in urinary cells

Study group	Number of subjects (n = 120)	Number of allograft biopsy specimens (n = 56)	Number of urine specimens (n = 180)
BKV nephritis confirmed by renal allograft biopsy[a]	8	25	25
BKV nephritis excluded by renal allograft biopsy[b]	28	31	31
Stable allograft function[c]	34	ND	74
Normal subjects	50	ND	50

ND, not done.

[a] The diagnosis of BKV nephritis was confirmed by renal allograft biopsy showing characteristic histologic changes of BKV nephritis and by positive immunostaining for BKV.

[b] The diagnosis of BKV was excluded by absence of histologic findings characteristic of BKV nephritis and by negative immunostaining for BKV.

[c] The clinical diagnosis of stable graft function was based on the finding that serum creatinine had not changed by more than 0.2 mg per deciliter during the 7 days before and the 7 days after the urine samples were collected.

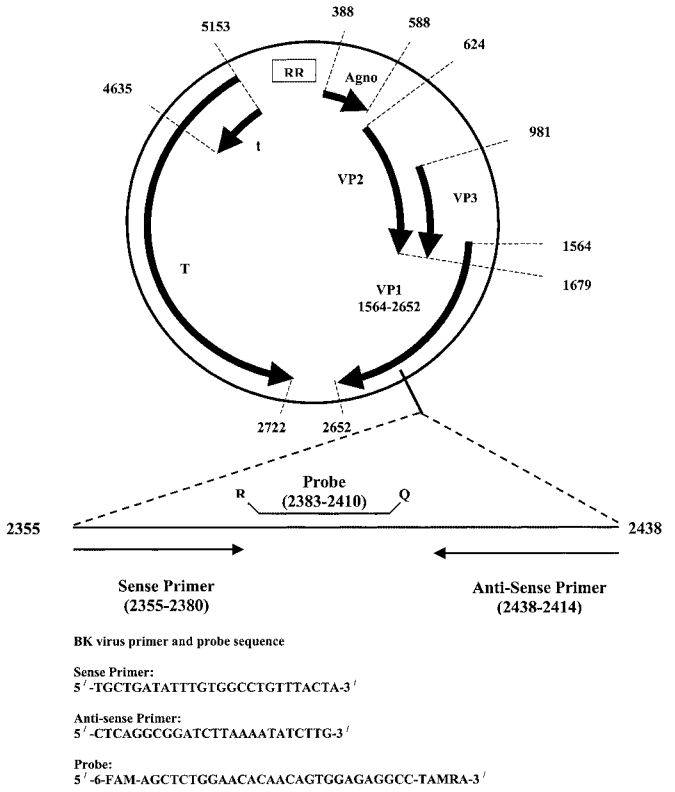

FIGURE 1. Design, sequence, and location of primers and probe for the measurement of BKV VP1 mRNA by real-time quantitative PCR. Schematic illustration of BKV genome illustrates the regulatory region (RR) and T and t proteins that are expressed early and before DNA replication and the Agno, VP1, VP2, and VP3 proteins that are expressed late and after DNA replication. The primers and the probe were designed to be complimentary to a region in VP1 that is highly conserved among the major subtypes of BKV (GenBank accession number J02038) and one that is mismatched with the JCV VP1 region. (*After* Major EO. Human polyomavirus. In: Knipe DM, Howley PM, eds. Fields' Virology, vol II. Philadelphia, Lippincott Williams & Wilkins 2001, p 2175.)

reaction volume of 25 μl using 12.5 μl of the TaqMan Universal PCR Master Mix, 2.5 μl of 1:1000 diluted template cDNA, 200 nM of primers, and 200 nM probe. The PCR amplification profiles included an initial incubation at 50°C for 2 min, denaturation at 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. The PCR amplicons for BKV cDNA and human 18S rRNA were prepared and quantified for developing the gene-specific standard curves. The standard curves were developed based on the principle that a plot of the log of the initial target copy number of a set of standard versus threshold cycle results in a straight line. The mRNA level in samples were expressed as number of copies per nanogram of total RNA.

The PCR amplification efficiency was 95.3 (95% confidence interval, 92.9–97.7). The correlation coefficient for the standard curve was 0.997 (95% confidence interval, 0.995–0.999). The interassay coefficient of variation was 3.10%.

Statistical Analysis

We used GraphPad Prism 3.02 statistical software for Windows (GraphPad Software, San Diego, CA) for data analysis. The levels of BK VP1 mRNA and 18S rRNA deviated from a normal distribution, which was substantially reduced by use of natural log (ln) transformation. The log transcript levels were used as the dependent variable in a one-way analysis of variance to compare levels among the different study groups. We then used Dunnett's test for multiple comparisons to control for the risk of a type I error while comparing the transcript levels in the BKV nephritis group with the three other groups shown in Table 1. Conventional receiver operating characteristic (ROC) curve analysis of transcript levels was used to determine cutpoints (thresholds) that maximized the sensitivity and specificity for distinguishing patients with BKV nephritis from those without. Area under the curve was calculated and the 95% confidence interval was determined. The association between the BKV nephritis status and the dichotomized BKV VP1 mRNA levels as determined by the ROC curve was analyzed using Fisher's exact test.

RESULTS

BKV VP1 mRNA Levels in Urinary Cells

Eight patients with renal allograft dysfunction who underwent diagnostic core needle biopsies qualified for the diagnosis of BKV nephritis on the basis of renal allograft histologic features and positive immunostaining of biopsy specimens for BKV. The histologic features included abnormal tubular cells containing large, irregular hyperchromatic nuclei with many demonstrating characteristic inclusions. In addition, there was patchy–diffuse distribution of varying degrees of interstitial inflammatory infiltrate consisting predominantly of lymphocytes, varying population of plasma cells, and scattered polymorphonuclear leukocytes and eosinophils. Immunoperoxidase staining with anti-SV40 antibodies revealed positive staining of the nuclei.

Table 2 shows that 15 of 16 urine specimens, obtained at the time the renal allograft biopsy specimens were classified as BKV nephritis, have high levels of BKV VP1 mRNA. Table 2 also shows that sequential urine specimens are informative and that the development of BKV nephritis and its resolution are reflected in the alterations in the levels of mRNA encoding BKV VP1 protein. In one patient (Table 2, MA), urine BKV VP1 mRNA levels continued to be high despite histologic resolution suggested by negative immunostaining of the allograft biopsy specimen obtained on posttransplantation day 147. However, the allograft that was removed in this patient on posttransplantation day 155 for persistent graft dysfunction was positive for BKV. Two other patients (Table 2, WC and ER) with persistent BKV VP1 mRNA in their urine specimens also lost their grafts within 2 to 6 months.

In striking contrast to the high levels of BKV VP1 mRNA in the urine of patients with BKV nephropathy, urine specimens from renal allograft recipients without BKV nephritis were seldom positive. Table 3 shows that only 2 of the 31 urine specimens obtained from the 28 renal allograft recipients without BKV nephritis (absence of histologic changes and lack of immunostaining for BKV) showed detectable levels of mRNA encoding BKV VP1 protein.

Figure 2 illustrates the mean (±SE) copy number of BKV VP1 mRNA in the patients with biopsy-confirmed BKV nephritis and in the patients in whom BKV nephritis was excluded by negative immunostaining for BKV in the renal allograft biopsy specimens. The mean (±SE) copy number was $3.93\times10^7 \pm 8.79\times10^6$ in urine samples from patients with BKV nephritis, whereas it was $2.23\times10^6 \pm 1.30\times10^6$ in the urine specimens from patients in whom BKV nephritis was excluded by allograft biopsy. Figure 2 also illustrates BKV VP1 mRNA levels in the 74 urine samples obtained from the patients with stable allograft function and in the 50

TABLE 2. BKV VP1 mRNA levels in urinary cells of patients with BKV nephritis

Patient	Posttransplantation day	Allograft histopathologic diagnosis	Allograft BKV immunostain	BKV VP1 mRNA (Copies per ng of total RNA)	18s rRNA (Copies per ng of total RNA)
WC	179	BKV nephritis, CAN gr5l	Positive	7.80E + 07	9.29E + 07
	203	BKV nephritis, CAN gr5l	Positive	1.06E + 08	9.89E + 07
JC	606	BKV nephritis, CAN gr5ll	Positive	4.96E + 07	3.46E + 06
	622	BKV nephritis, CAN gr5ll	Positive	6.54E + 07	1.90E + 07
ER	5	AR gr4lb	Negative	< 25	2.86E + 06
	132	BKV nephritis	Positive	3.10E + 07	5.07E + 06
	154	BKV nephritis	Positive	6.40E + 07	1.43E + 07
WL	141	Normal, gr1	Negative	4.42E + 07	2.37E + 06
	281	CAN gr5ll	Negative	6.90E + 06	2.26E + 06
	306	BKV nephritis	Positive	2.54E + 07	1.08E + 06
	327	BKV nephritis	Positive	5.00E + 07	1.88E + 07
SK	104	BKV nephritis	Positive	9.60E + 07	8.52E + 07
	125	AR grllb	Negative	< 25	4.04E + 08
JS	83	BKV nephritis	Positive	3.70E + 07	1.68E + 07
	106	AR gr4la	Negative	6.53E + 05	1.00E + 08
	139	AR gr4la & CAN gr5l	Negative	< 25	1.01E + 07
	350	CAN gr5ll	Negative	< 25	8.53E + 07
RA	58	BKV nephritis	Positive	2.08E + 07	1.62E + 07
	69	BKV nephritis	Positive	< 25	2.58E + 07
	80	AR gr4lb	Negative	8.84E + 04	1.49E + 07
MA	88	BKV nephritis	Positive	1.78E + 06	6.26E + 07
	91	BKV nephritis	Positive	2.22E + 06	6.50E + 07
	131	BKV nephritis	Positive	7.76E + 05	1.72E + 07
	137	BKV nephritis	Positive	1.45E + 06	3.58E + 07
	147	AR gr4lb	Negative	9.87E + 05	1.67E + 07

CAN, chronic allograft nephropathy; AR, acute rejection.

Urine specimens were obtained on indicated posttransplantation days, and levels of BKV VP1 mRNA and 18S rRNA were measured using gene-specific primers and probes in real-time quantitative PCR assay. All kidney biopsy specimens were obtained under ultrasound guidance using a 14-gauge biopsy needle. A single pathologist who did not know the results of the molecular studies examined the renal biopsy specimens. The biopsy specimens were classified with the use of Banff' 97 classification, and BKV immunostaining was performed using monoclonal antibodies directed at the SV-40 large T and small t antigens and cross-reactive with the large T antigen of BK virus.

urine specimens obtained from the normal subjects. Among the 74 urine specimens collected from the stable allograft function group, BKV VP1 mRNA was detected in only 6 urine samples, and the mean (±SE) copy number in stable allograft function group was $1.04\times10^5 \pm 8.5\times10^4$. BKV VP1 mRNA was not detected in 48 of 50 samples obtained from the normal subjects, and it was detected at a very low copy number (108 and 193) in two urine samples.

Statistical analysis of BKV VP1 mRNA levels in all four groups studied showed that the levels of BKV VP1 mRNA in urinary cells from patients with BKV nephritis are significantly higher than the levels in patients in whom BKV nephritis was excluded by renal allograft biopsy ($P<0.0001$), in patients with stable graft function ($P<0.0001$), and in normal subjects ($P<0.0001$). The levels of constitutively expressed 18S rRNA did not vary significantly among the study groups (BKV nephritis group vs. no BKV nephritis by allograft biopsy, $P=0.77$; BKV nephritis vs. stable function group, $P=0.17$; BKV nephritis vs. normal subjects, $P=0.38$).

Receiver Operating Characteristic Curve Analysis of mRNA Levels

The ROC curves (Fig. 3) show the fraction of true-positive results (sensitivity) and false-positive results (1-specificity) for various cutoff levels of BKV VP1 mRNA and 18S rRNA in urine specimens. The threshold that gave the maximal sensitivity and specificity for BKV VP1 mRNA was 6.54×10^5 copies per nanogram of total RNA. At this threshold, the sensitivity was 93.8% and the specificity was 93.9% ($P<0.00001$) (Table 4). Furthermore, at the cutoff value of 6.54×10^5 BKV VP1 mRNA level per nanogram total RNA, only 2 of 74 urine specimens from the stable allograft function group were positive (97% specificity) and none from the normal subjects were positive (100% specificity).

In the ROC curve analysis, the area under the curve represents the probability that a randomly chosen individual with the disease has a diagnostic test value greater than that for a randomly chosen individual without the disease. Thus a value of 1.0 reflects a perfect diagnostic test, whereas a value of 0.5 is no better than expected by chance. The calculated area under the curve (±SE) for BKV VP1 mRNA levels was 0.949±0.018 ($P<0.00001$), and the 95% confidence interval was 0.913 to 0.987 (Fig. 3A). In contrast to BKV VP1 mRNA levels, the levels of 18S rRNA were not useful in predicting BKV nephritis. The calculated area under the curve was 0.562±0.074 ($P>0.2$) and the 95% confidence interval was 0.417 to 0.708 (Fig. 3B).

The ROC curve analysis, shown in Figure 3(A) and (B), included all 130 urine specimens obtained from all 70 renal allograft recipients studied. Fifty-six of the 130 urine specimens were from 36 patients who had undergone renal allograft biopsy at the time of urine collection, and 74 urine specimens were from 34 patients who had stable graft function and did not undergo allograft biopsy at the time of urine collection. Although the presence or absence of BKV nephritis is known with a high degree of certainty in patients who

TABLE 3. BKV VP1 mRNA levels in urinary cells of patients without BKV nephritis

Patient	Posttransplantation day	Allograft histopathologic diagnosis	Allograft BKV immunostain	BKV VP1 mRNA (Copies per ng of total RNA)	18s rRNA (Copies per ng of total RNA)
EA	31	AR gr41a	Negative	<25	2.14E + 07
TC	17	AR gr4la-b	Negative	<25	2.40E + 08
JC	8	AR gr4la-b	Negative	<25	1.73E + 07
AH	6	AR gr4lb	Negative	<25	3.43E + 07
KF	6	AR gr3	Negative	<25	1.01E + 06
GG	90	AR gr3	Negative	2.70E + 07	1.22E + 08
PI	13	AR gr3	Negative	<25	1.13E + 08
TK	17	AR gr3	Negative	<25	2.17E + 08
LH	682	AR gr4la, CAN gr5l	Negative	<25	1.55E + 08
RM	16	AR gr4la	Negative	<25	2.05E + 08
MR	6	AR gr4la	Negative	9.31E + 06	2.17E + 07
IT	11	AR gr4la	Negative	<25	5.69E + 06
JT	157	AR gr4la-b, CAN gr5l	Negative	<25	2.24E + 06
AN	34	AR gr4lb	Negative	<25	1.03E + 08
	42	AR gr4lb	Negative	<25	8.92E + 07
NP	262	AR gr4lb	Negative	<25	6.06E + 06
DR	168	AR gr4lb	Negative	<25	4.93E + 06
KS	120	AR gr4lb	Negative	<25	1.97E + 08
PM	160	AR gr4lb	Negative	<25	3.13E + 06
PK	4	AR gr4ll	Negative	<25	8.19E + 06
ZS	4	AR gr4lla	Negative	<25	1.13E + 08
SG	9	AR gr4lb	Negative	<25	1.54E + 07
MM	102	AR gr4lla	Negative	<25	2.36E + 08
	341	AR gr4la, CAN gr5ll	Negative	<25	3.43E + 07
FB	107	AR gr4lb, CAN gr5ll	Negative	<25	1.38E + 06
	10	Other-ATN, TT	Negative	<25	5.26E + 07
WM	9	Other-ATN	Negative	<25	2.78E + 07
SM	8	Other-ATN	Negative	<25	3.29E + 06
JE	56	Other-TT	Negative	<25	1.74E + 07
CG	570	CAN gr5ll	Negative	<25	5.00E + 07
VJ	2142	CAN gr5ll	Negative	<25	1.47E + 07

ATN, acute tubular necrosis; TT, toxic tubulopathy.

Urine specimens were obtained on indicated posttransplantation days, and levels of BKV VP1 mRNA and 18S rRNA were measured using gene-specific primers and probes in real-time PCR assay. All kidney biopsy specimens were obtained under ultrasound guidance using a 14-gauge biopsy needle. A single pathologist who did not know the results of the molecular studies examined the renal biopsy specimens. The biopsy specimens were classified with the use of Banff' 97 classification, and BKV immunostaining was performed using monoclonal antibodies directed at the SV-40 large T and small t antigens and cross-reactive with the large T antigen of BK virus.

had undergone allograft biopsies, the diagnosis is less certain in the patients who did not undergo allograft biopsy at the time of urine collection. To eliminate this uncertainty, we repeated the ROC curve analysis using only the data from the renal allograft recipients who had undergone diagnostic allograft biopsies and in whom the presence or absence of BKV nephritis was established by renal allograft histologic findings and by immunostaining for BKV. This analysis showed that BKV nephritis can be predicted with a sensitivity of 93.8% and specificity of 87.5% using a BKV VP1 mRNA level of 6.54×10^5 copies per nanogram of total RNA ($P<0.00001$) (Table 4). The calculated area under the curve (±SE) for BKV VP1 mRNA levels was 0.927±0.033 ($P<0.00001$), and the 95% confidence interval was 0.861 to 0.994 (Fig. 3C). The levels of 18S rRNA, as expected, were not useful in predicting BKV nephritis; the calculated area under the curve was 0.521±0.085 ($P>0.2$) and the 95% confidence interval was 0.354 to 0.689 (Fig. 3D).

DISCUSSION

We found that BKV nephritis, a posttransplantation infectious complication that can result in allograft dysfunction and graft loss, can be diagnosed accurately and noninvasively by measurement of BKV VP1 mRNA in urinary cells.

Needle biopsy of the renal allograft is currently the most definitive test for the diagnosis of BKV nephritis (*3–6*). However, allograft biopsies can be complicated by hematuria, anuria, perirenal hematoma, bleeding and shock, arteriovenous fistula, and even graft loss. The complications have been reduced but not eliminated with recent refinements in the biopsy procedure, such as the use of ultrasonography. The importance of noninvasive diagnosis of BKV nephritis is emphasized also by the considerations that the renal allograft is often placed intra-abdominally in pediatric recipients thus making it difficult to biopsy and that repetitive assessment of intragraft status is often required to guide treatment decisions.

Infection with BKV is widespread in the normal population, rendering the current serologic tests uninformative with respect to identification of active infection (*27*,*28*). Urine cytology has yielded conflicting results. It has been reported that the decoy cells (renal tubular cells with intranuclear viral inclusion bodies) are absent in the urine of 72% of patients with BKV nephritis (*4*), and it has also been ob-

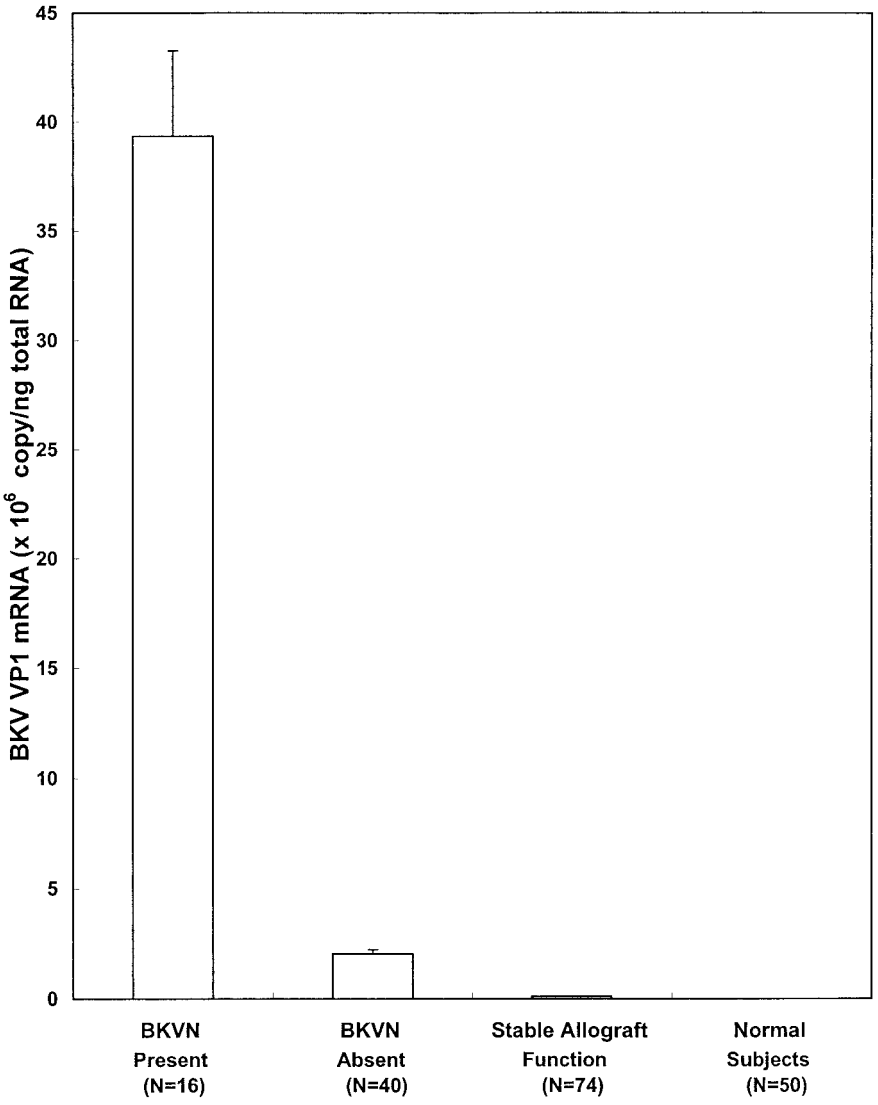

FIGURE 2. Levels of BKV VP1 mRNA in urinary cells. The mean (±SE) levels of BKV VP1 mRNA in urine samples from renal allograft recipients with biopsy-confirmed BKV nephritis (BKVN Present), renal allograft recipients in whom BKV nephritis was excluded by allograft biopsy (BKVN Absent), renal allograft recipients with stable allograft function (Stable Allograft Function), and Normal Subjects are shown. The clinical diagnosis of stable allograft function was based on the finding that serum creatinine levels had not changed by more than 0.2 mg per deciliter during the 7 days before and the 7 days after the urine samples were collected. The levels of BKV VP1 mRNA were significantly higher in the samples from patients with BKV nephritis than in the samples from the other three groups ($P<0.0001$, one-way analysis of variance). Dunnett's procedure was then used to compare the BKV VP1 mRNA levels in samples from patients with BKV nephritis with the mRNA levels in each of the three other groups of samples, and it showed that levels of BKV VP1 mRNA were significantly higher than those in specimens from patients without BKV nephritis ($P<0.001$), in specimens from patients with stable allograft function ($P<0.001$), or in normal subjects ($P<0.001$). Values in parentheses are the numbers of urine samples.

served that the decoy cells are present in the urine of 94% of patients without BKV nephritis (*16*). Moreover, a 97% accuracy rate in predicting the presence of renal histologic alterations caused by polyoma virus has also been reported after cytologic evaluation that included Papanicolaou staining of urine samples (*29*). Electron microscopic examination of negatively stained urine specimens is an accurate way of identifying polyoma virus but is nonquantitative and requires additional procedures to distinguish BKV from JCV (*5*,*15*). Nucleic acid–based assays have been reported to have a high degree of sensitivity. Nickeleit et al. (*16*) have observed that testing for BKV DNA in plasma is a specific and sensitive way of identifying renal allograft recipients with BKV nephritis. Limaye et al. (*17*) have quantified BKV load in sequential serum samples from four patients with biopsy-confirmed BKV nephritis and found that measurement of BKV DNA in serum identifies those at risk for the development of BKV nephritis. However, Tuzuner et al. (*30*) reported that testing for BKV DNA in blood specimens is not a sensitive predictor of BKV nephritis and that viral load in the urine specimens of renal allograft recipients is more informative.

A major limitation of DNA-based assays, especially qualitative ones, is that they cannot distinguish between latent and reactivated infection. In contrast to the 100% specificity found in our studies after measurement of BKV VP1 mRNA in urine of normal subjects, Arthur et al. (*21*) found that 10% of healthy adults have positive results. A high false-positive rate has also been found in additional populations after measurement of BKV DNA in urine specimens. Markowitz et al. (*22*) found that 15% of urine specimens from pregnant women yield positive results, and Jin et al. (*23*) found that 5% of urine samples from children and 29% of urine specimens from pregnant women are positive for BKV DNA.

In view of the limitations associated with DNA-based assays, we adapted real-time PCR assay for the measurement of BKV VP1 mRNA, and we tested the hypothesis that measurement of BKV VP1 mRNA in urine would be an accurate and noninvasive diagnostic test for BKV nephritis. VP1, a major capsid protein, is responsible for the characteristic icosahedron feature of the virus and enables its entry into cells (*7*,*8*). Although the transcription and translation of early T antigen precedes DNA replication, the transcription and translation of VP1 is contin-

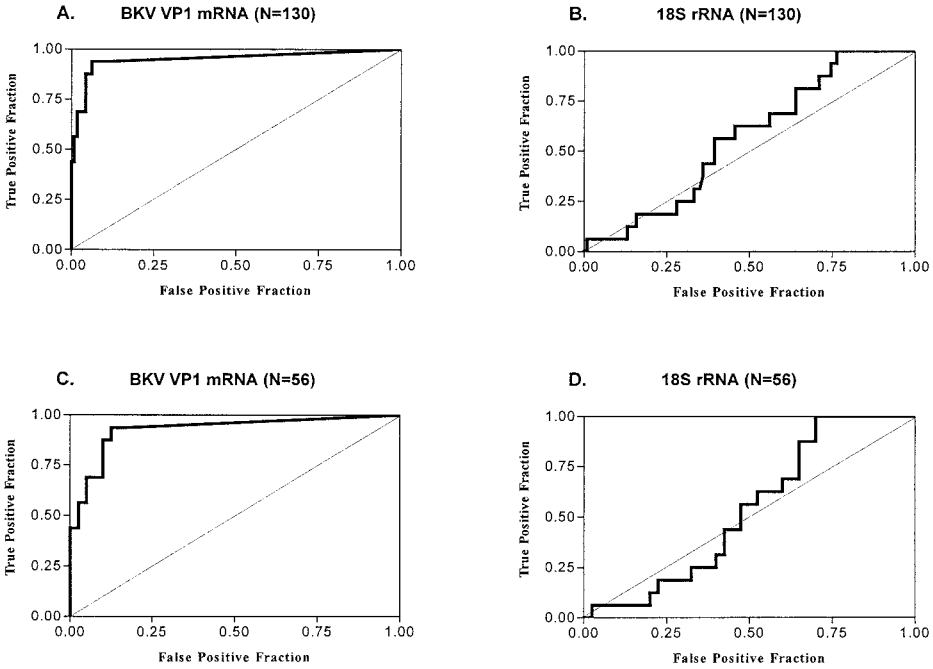

FIGURE 3. ROC curve of BKV VP1 mRNA levels and 18S rRNA levels. The fraction of true-positive results (sensitivity) and false-positive results (1-specificity) for BKV VP1 mRNA levels and 18S rRNA levels as markers of BKV nephritis are shown. The calculated area under the curve was 0.949 for BKV VP1 mRNA levels (A; $P<0.00001$) and 0.562 for 18S rRNA (B; $P>0.2$) when the ROC curve analysis included urine specimens from all renal allograft recipients studied. The calculated area under the curve was 0.927 for BKV VP1 mRNA levels (C; $P<0.00001$) and 0.521 for 18S rRNA (D; $P>0.2$) when the ROC curve analysis included urine specimens only from renal allograft recipients who had undergone renal allograft biopsy. A value of 1.0 reflects a perfect diagnostic test, whereas a value of 0.5 is no better than expected by chance.

TABLE 4. Levels of BKV VPI mRNA predict BKV nephritis

BKV VPI mRNA level[a]	BKV nephritis present	BKV nephritis absent	*P* value[b]
Urine specimens from all renal allograft recipients studied			
$\geq 6.54\times10^5$ copies/ng of total RNA	15	7	
$<6.54\times10^5$ copies/ng of total RNA	1	107	<0.00001
Urine specimens from the renal allograft recipients who had undergone renal allograft biopsy			
$\geq 6.54\times10^5$ copies/ng of total RNA	15	5	
$<6.54\times10^5$ copies/ng of total RNA	1	35	<0.00001

[a] An ROC curve was used to select the best cutoff points.
[b] *P* values are based on Fischer's exact test using dichotomized measures of BKV VP1 mRNA level.

gent upon viral DNA replication. (*7*,*8*). Viral early mRNAs such as mRNA encoding T antigen thus are detected in nonproductively infected cells, and late mRNAs such as VP1 mRNA are detected only in productively infected cells. Furthermore, the abundance of late mRNAs is several hundred times more than that of early mRNAs (*7*,*8*). The replicative cycle of BKV thus suggests that measurement of BKV VP1 mRNA in clinical specimens would be of value.

Nucleotide sequencing of the VP1 region has identified sequences that correspond to the major subtypes of BKV and regions that are highly conserved among the major subtypes (*23*,*24*). Our design of oligonucleotide primers for the measurement of BKV VP1 mRNA was informed by the VP1 sequence information among the BKV subtypes, and our primers and the probe were designed to target a highly conserved region of the BKV VP1 region. Moreover our primer design incorporated multiple sequence mismatches with the JCV VP1 region.

BKV nephritis was diagnosed accurately and noninvasively in this investigation using the primers and probe we designed for measuring BKV VP1 mRNA by real-time PCR. BKV, however, can infect any portion of the urinary tract, and it is likely that infection of the lower urinary tract would also yield a positive test result with our assay system. Whether quantitative differences in the viral load would help discriminate BKV nephritis from a lower urinary tract infection remains to be determined. We believe that the test system we have developed would be most helpful in the differential diagnosis of renal allograft dysfunction by excluding BKV nephritis as the basis of graft dysfunction.

To the best of our knowledge, this is the first study to evaluate the diagnostic accuracy of measuring BKV VP1

mRNA in urine specimens. The noninvasive and quantitative assessment of BKV productive infection, facilitated by measurement of BKV VP1 mRNA levels, might be of value for the characterization of the natural history of BKV infection and for the refinement of antiviral therapeutic strategies.

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