Challenging Issues in Diagnosis and Screening of BK Virus Nephropathy in Kidney Transplant Recipients, A Multicenter Experience in Iranian Population

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Introduction. BK virus nephropathy (BKVN) is an important complication of kidney transplantation and kidney biopsy remains the gold standard for its diagnosis. Urine/serum polymerase chain reaction (PCR) is a more sensitive diagnostic method, although it has some potential limitations.

Methods. This study enrolled all kidney transplant recipients who underwent kidney transplant biopsy, collected from three medical centers. Urine and serum PCR results of the patients were also collected from the molecular laboratories. The cut-off value for positive viral DNA load in serum and urine were > 10^4 and > 10^7 copies/mL, respectively. Sensitivity, specifity, positive and negative predictive values (PPV, NPV) and cut off values for PCR results were compared with pathologic diagnosis among laboratories.

Results. Among 369 biopsy samples, 33 (8.9%) had definite diagnosis of BKVN. PCR results were available for 138 cases. Three patients with definite BKVN had negative PCR results. In 22 patients, PCR was positive without evidence of BKVN. The overall sensitivity, specificity, PPV and NPV of PCR for detecting BKVN, based on a unique cut-off value, were 88, 81, 51, and 97%; respectively. The overall accuracy of PCR in all laboratories was high (82 to 86%), however significant inter-laboratory differences in sensitivity and specificity was found . A 2-log difference in threshold value for positive results was observed in one laboratory.

Conclusion. PCR may show a significant variability between different laboratories. Interpretation of PCR results using a single cut-off value for all laboratories, may decrease the sensitivity for the diagnosis and screening of BKVN.

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Original Paper

INTRODUCTION

BK virus nephropathy (BKVN) is an important cause of graft loss in patients with kidney transplantation.[1] BK virus is a non-enveloped double stranded DNA virus and belongs to the Polyomaviridae family.[2] After primary infection, the virus binds to GT1b receptors and enters the urothelial cells, and its DNA integrates into the host genome. The virus can survive in urinary bladder, ureters and renal pelvis epithelial cells for a long

time.[3] More than 90% of the adults are seropositive for the virus.[4] The virus can become activated and damage urothelial cells after transplantation and immunosuppression. Shedding of replicated virus in the urine (viruria) is the first step of BKV infection. Following virus proliferation in renal parenchyma, the virus DNA can be measured in serum. Tubulo-interstitial inflammation, tubular damage, fibrosis and nephropathy are the pathogenic causes of graft loss.[1,3, 5, 6]

In practice, several guidelines have recommended molecular polymerase chain reaction (PCR) screening and/or urine cytology for early diagnosis of graft rejection.[7,8] Persistent high plasma viral load (more than 10000 copy/mL) for 4 weeks is defined as "presumptive BKV nephropathy".[9] Early detection of the disease enables the clinician to alter the immunosuppression dose and reduce the risk of graft loss.[10]

Definitive diagnosis of BKVN is based on kidney biopsy and histopathological detection of viral cytopathic effect in tubular epithelial or parietal glomerular cells nuclei. It is usually associated with epithelial cell necrosis in early stages followed by tubular atrophy and interstitial fibrosis, which is seen in more advanced disease. Typical viral inclusion may not be detected in early stage of kidney infection. Immunohistochemistry (IHC) or in situ hybridization (ISH) assays can confirm the infection in these situations.[11,12] Typically, BKVN involves focal areas of kidney parenchyma and predominantly involves the medulla. Therefore, two biopsy cores, containing medullary tissue are needed for accurate diagnosis.[13] In this way, sampling error may occur in 10 to 30 percent of the patients and therefore a negative pathology result does not exclude BKVN.[12,14,15]

Quantitative plasma PCR has the highest positive predictive (PPV) value for the diagnosis of BKVN. A plasma viral load more than 10^4 copies/mL have 74% chance of predicting the disease. This cut-off point is not approved in multicenter investigations; however, inter-laboratory variations exist in this regard.[16,17]

In light of diagnostic challenges in screening and detection of BKVN, we conducted this study to compare the accuracy of PCR results in different laboratories with histopathological findings, as the gold standard technique.

MATERIALS AND METHODS

Patient Selection And Follow-up

In this retrospective cross-sectional study, kidney transplant recipients who underwent allograft biopsy and who were referred to three tertiary centers affiliated to Tehran and Mashhad universities of medical sciences between 2017 and 2019 were included. The study was approved by local ethics committee of our university (IR.TUMS.IKHC.REC.1397.043).

Histopathological Assays

Buffered formalin fixated biopsy samples were embedded in paraffin blocks. Serial 2-3μm thick sections were stained with hematoxylin & eosin (H&E), periodic acid Schiff (PAS), Masson's trichrome and Jones' methods. Presence of viral inclusion bodies and other histopathological changes, including interstitial fibrosis (ci), tubular atrophy (ct), tubulitis (t), and total inflammation (ti) were evaluated and categorized based on Banff classification, 2019.[18] IHC study for simian vacuolating virus 40 (SV40) antigen (Biocare medical company: USA, California) was performed based on the manufacturer protocol, to confirm the diagnosis in typical and suspicious cases. Nuclear staining with SV40 $\geq$ 1+ intensity in tubular epithelial cells with IHC was considered as definite BKVN.

Molecular Assay

Urine and serum samples were analyzed for BKV DNA load in three different molecular laboratories. In all laboratories, virus nucleic acid PCR was evaluated in whole blood and/or urine samples. Urine samples were centrifuged for 20 min at 1000 rpm. Whole blood samples were collected in plastic tubes, containing EDTA anti-coagulant, kept at room temperature, and transferred to the laboratories within 6 hours. Viral DNA load of more than 10^4 copies per mL for serum and 10^7 DNA virus copies per mL for urine were considered positive result.

In the first laboratory (#1), commercial kit (Roche Company, Germany) was used for virus DNA isolation. PCR for quantitative viral load evaluation was performed using Gene Proof kit (GeneProof a.s. Vídeňská 101/119 Dolní Heršpice 619 00 Brno Czech Republic) on Rotor-Gene 3000 (Qiagen Str. 1, 40724 Hilden, Germany) machine.

In the second laboratory (#2), viral DNA was

extracted by using Prepito magnetic based DNA/RNA kit (Perkin Elmer, USA) and Prepito-D instrument (Perkin Elmer, USA). The extracts were dissolved in 50 µL deionized water. Serum and urine BK viral loads were evaluated by real-time PCR method using the QIAGEN artus BK virus PCR kit on Rotor-Gene 3000 (Qiagen Str. 1, 40724 Hilden, Germany) device.

In the third laboratory (#3), the Magcore nucleic acid extraction magnetic beads automatic machine (RBC Bioscience, Taiwan) was used to extract viral DNA. The extracts were dissolved in 60 ML elution buffer. Plasma and urine PCR were evaluated by real time PCR method with GeneProof BK virus PCR kit (5 Plx-HRM, Germany) device.

Validated and FDA approved commercial extraction, and PCR kits were used in all laboratories. The extracted DNA were kept at -20 ºC before evaluation.

Statistical Analysis

Normal distribution of the results was assessed by Kolmogorov-Smirnov test using SPSS software version 23 (IBM Inc, Chicago, Illinois, USA). Continuous data were compared using either student's t-test or Mann-Whitney test. Qualitative data were compared, using Chi-square or Fisher's exact test. The level of statistical significance was considered $P < .05$ for all tests. Sensitivity, specificity, positive predictive and negative predictive values (PPV, NPV) for PCR results in different laboratories were compared with pathology findings, as gold standard, using 2×2 tables.

RESULTS

A total of 369 kidney biopsy samples, obtained from transplant patients, were referred to our centers for histopathological evaluation. To calculate the incidence of biopsy proven BKVN, all samples were included and analyzed. As BKVN primarily involves the medullary area, even biopsy samples from medulla with small number of glomeruli were not excluded.

Sixty-four percent of our patients were male. The mean age of the patients were 42.4 ± 14 years, ranging from 9 to 77 years old. Most of the patients presented with increased serum creatinine (more than 25% rise over previous creatinine) (72.3%) and pure significant proteinuria (more than 3000 mg/24 h) (7.3%). The remaining presented with malignant hypertension (blood pressure more than 180/120 mmHg), oliguria (urinary output less than 400 mL/d), persistent gross hematuria (presence of blood in urine) and elevated plasma glucose (Hb A1c more than 7%) (each one less than 2%). The most frequent causes of graft dysfunction were acute T-cell mediated rejection (ATCMR), chronic active T-cell mediated rejection, chronic active and inactive antibody mediated rejection (AMR), BKV nephropathy, calcineurin inhibitor (CNIs) toxicity, acute tubular necrosis (ATN) and mixed AMR and TCMR rejections.

In this study, of 369 biopsy samples, 33 (8.9%) were diagnosed with BKVN, out of which, 63.3% were male. There was no significant difference in sex distribution between patients with and without PVBKN ($P > .05$). The mean age of the patients with and without BKVN were 39.6 ± 17 and 42.8 ± 13 years, respectively ($P > .05$). All patients with definite diagnosis of BKVN, presented with elevated serum creatinine, as their only complain. Mean serum creatinine level at the time of admission was 3 ± 1.5 mg/dL. The basic transplantation data was available only for 245 patients, including 19 (7.8%) cases of BKVN. The mean duration of transplantation was 23.2 ± 17.3 (1 to 60) months in BKVN patients, which was significantly lower than the patients without BKVN (48.5 ± 60 (1 to 360) months). According to the Banff working group new classification, the prevalence of mild, moderate and severe interstitial fibrosis were 21, 33.3, and 0.9%; respectively.

There were only 138 (22 both serum and urine, 17 urine and 99 serum) PCR results, including 25 cases with biopsy proven BKVN. Although there was a strong relationship between the PCR and pathology results ($P < .001$), 22 patients exhibited positive PCR without pathologic evidence of BKVN. In individuals with confirmed BKVN, 3 tested negative with at least one PCR test (serum or urine). All of these 3 patients with false negative PCR results were re-evaluated in the second laboratory. Serum BKV viral loads were 500, 700, and 5000 copies/mL in these patients. Testing for urine PCR was not performed. However the first and third laboratories accounted for the vast majority of samples with PCR viral load above the cut-off values (21 out of 22 patients) (Table 1).

When compared with histopathology, the PCR

Sensitivity, Specifity, and Accuracy of PCR in Different Laboratories Using Universal Recommended Cut-off Values (10^4 DNA copies/mL in Serum and 10^7 DNA copies/mL in Urine)

Lab	PCR Result		Total	Sensitivity (%)	Specificity (%)	Accuracy (%)
	Negative	Positive				
#1						
Histopathology and IHC Result						
Negative	42	12	54			
Positive	0	13	13	100	77.8	82
Total	42	25	67			
#2						
Histopathology and IHC Result						
Negative	30	1	31			
Positive	3	2	5	40	96	86
Total	33	3	36			
#3						
Histopathology and IHC Result						
Negative	20	5	25			
Positive	0	9	9	100	80	86
Total	20	14	34			
Total						
Histopathology and IHC Result						
Negative	90	22	112			
Positive	3	23	26	88	81	85
Total	93	45	138			

results for detection of BKVN had a sensitivity of 88% (88% serum and 100% urine), specificity of 81% (87.5% serum and 61.3% urine), PPV of 51% (64.7% serum, 40% urine) and NPV of 97% (97% serum, 100% urine). While the overall accuracy was almost similar in all laboratories, the sensitivity was 100% and specificity was 77.8 and 80% in the first and third laboratories, respectively. In contrast, the second laboratory has a sensitivity of 40% for detection of BKV, while its specificity was much higher (96%) (Table 1).

DISCUSSION

Since the first isolation of BK virus from a kidney transplant recipient in 1970s, the definite diagnosis and effective therapy of BKVN remained a challenging issue.[19] In order to compare PCR results with pathology findings in this study, patients with either serum viral load of more than 10^4 copies/mL or urine viral load more than 10^7 copies/mL were considered to have a positive PCR test. Accordingly, PCR was negative in 12% (3/25) of definitive cases of BKVN, while it was positive in 16% (22/138) of patients, without confirmed pathological diagnosis of BKVN. Sensitivity, specificity, positive and negative predictive values of real-time PCR were 88, 81, 51, and 97%; respectively.

Hirsch *et al.* reported the sensitivity, specificity, and positive predictive value of PCR to be 100, 88, and 50%; respectively, while Singh *et al.* reported that serum PCR results were below 10,000 in 39% and below 1000 in 2 patients with confirmed pathologic evidence of BKVN. [20,21] Some of the earlier studies, including the study of Singh *et al*, emphasized that laboratory test alone, has a positive predictive value of less than 80% and even less than 50%.[21] While the cut-off values for the detection of BKVN, between different centers, are not validated, there was up to 2 log difference between the results of different laboratories.[21]

A study by Pinto *et al.* in Brazil reported that the cut-off values for serum PCR, using commercial and in-house kits were 10^4 and10^6, respectively.[22] In our study, PCR tests were carried out in three different referral laboratories, with different validation methods. It should be noted that the findings of these centers are frequently applied for follow-up and treatment of the patients. The accuracy of quantitative PCR for BK viral load was high and acceptable in all laboratories (82 to 86%), but using a single cut-off value for interpretation of positive test results, yielded different sensitivity and specificity values. In addition, three out of 25 patients with definitive pathologic diagnosis of

BKVN had negative serum PCR (viral load 10^2 in two and 10^3 in one patient), performed in lab#2. False positive test results in this laboratory were lower compared to the other two laboratories (higher specificity and lower sensitivity compared to other laboratories). As we use PCR as a screening test for BKV infection, it seems that the cut-off value from lab#2 should be considered 2 logs lower than the defined thresholds, to obtain the highest sensitivity. These findings were in line with the findings of the study of Pinto *et al*. which showed two logs variation in cutoff value for PCR test between two different methods. The observed differences in study results could be attributed to the potential differences in laboratory methods and different polymorphisms in the target gene regions. PCR is currently the "gold standard non-invasive method" for detecting polyomaviruses. To achieve the best performance in real-time PCR methods, target sequence should be carefully selected from conserved regions and evaluated on a regular basis against new sequences. The BKV genome has a level of sequence variation, which is also observed in different subtypes. Due to these variations, it is difficult to design the primer and probe for real-time PCR analyses to detect BKVs.[23]

In the line with prior research, we further emphasize on the importance of laboratories in validating their methods to establish the best cut-off values rather than relying on the suggested 'universal' thresholds (4 log copies/mL in serum and 7 log copies/mL in urine). Obviously, the clinicians should to be aware of the type of PCR test used in the laboratory.[17,24] To reduce the variability, testing in the same laboratory is also recommended.

This study had few limitations. The main limitation of most studies in this field, is the lack of a gold standard method for accurate determination of false negative and false positive results fin different methods. In a recent study by Singh *et al.*, a non-invasive biomarker was proposed for the evaluation of BKVN.[25,26] This assessment was based on the detection of three-dimensional polyomavirus aggregates by using negative staining electron microscopy in urine samples. The positive and negative predictive values for the presence of urinary cast-like structures, named "Haufen", were over 90%.[10, 21, 25, 26] But this method is too expensive and is not provided by most centers. Therefore, histopathologic diagnosis of renal parenchymal infection by the virus remains the gold standard method for definite diagnosis of BKVN. Tissue involvement in early stage of BKVN is focal, which means that in 10 to 30% of cases infected areas can be skipped during core biopsy.[27] Typical viral inclusions may also be undetectable in early stages of tubular epithelial cell infection. In this regard, simultaneous use of PCR results and routine IHC study in suspected cases will enhance the diagnostic accuracy.[11] Because of financial limitations, routine PCR screening and serial monitoring of BK viral load after few months, in patients with viral load less than the cut- off values was not performed, which is another limitation of our study. Additionally, due to lack of registry system in our centers, detailed clinical information regarding medications and the underlying comorbidities could not be reported. The last limitation of our study was the provision of PCR result in only 138 patients.

To the best of our knowledge, this study is the largest multicenter cohort on 369 biopsy samples, reported from Iran. We identified BKVN in 33 patients (8.9%). The prevalence and demographic characteristics of BKVN in this study were almost similar to our previous study.[15]

To obtain the best cut-off value, it is recommended that future prospective studies be designed to evaluate PCR in all patients who are candidate of graft biopsy and compare the different PCR test results.

CONCLUSION

There may be a large variation in PCR results between laboratories. Using a single cut-off value in all laboratories (4 log copies/mL in serum and 7 log copies/mL in urine) may reduce the sensitivity of PCR screening in BKVN diagnosis. PCR method should be validated and performed in certified laboratories for the diagnosis of BKVN in transplant cases. Clinicians should also be aware of the limitations of these diagnostic tests.

CONFLICT OF INTEREST

Nothing to be disclosed.

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