

The analytical performance of a real time BKV PCR assay

Real time BKV PCR testinin analitik performansı

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ABSTRACT

Objective: BKV is a virus usually undergone asymptotically in the childhood and can remain latent in the peripheral blood, brain and especially in kidneys. Reactivation of BKV under immunosuppression can cause diseases like interstitial nephritis, haemorrhagic or non- haemorrhagic cystitis, ureterostenosis and nephropathy. Especially in transplant recipients nephropathy frequency can reach 5% and can be the cause of premature loss 30-60% of transplanted organs and poor outcome. Quantification of BKV viral load in urine and serum with real time polymerase chain reaction (PCR) plays important role for early diagnosis and management of the therapy. Since the Real time PCR assay is more sensitive than classical PCR, can do quantification and have a less risk of contamination and short turn-around time. The aim of our study was to evaluate the analytical performance of a real time quantitative BKV PCR assay which was developed in our laboratory.

Method: Standards were prepared from BKV plasmid (ATCC 45025). BKV plasmid that contained 15×10^7 copies/ml to 3×10^1 copies/ml serial dilutions was measured by spectrophotometry. Primers for BKV VP1 gene and dual labelled probe at the 5' end with 6-carboxyfluoresceine (FAM) and the 3' end with 6-carboxytetramethylrhodamine (TAMRA) as described previously were used for the amplification reactions.

ÖZET

Amaç: BKV, çocukluk çağında genellikle asemptomatik olarak geçirilen ve özellikle böbreklerde olmak üzere periferik kan ve beyinde latent olarak kalabilen bir virustür. BVK'nın aktivasyonu immünsuprasyonu olan hastalarda hemorajik veya hemorajik olmayan sistit, ürostenoz ve nefropatiye neden olabilir. Özellikle transplant alıcılarında nefropati sıklığı %5'lere kadar ulaşabilmekte ve transplante edilen organın %30-60 erken kaybı ve kötü bir klinik seyirle sonuçlanabilmektedir. BKV viral yükünün serum ve idrar örneklerinde polimeraz zincir reaksiyonu (PZR) ile kantitasyonu, erken tanı ve tedavinin yönetiminde önemli rol oynamaktadır. Real time PZR; klasik PZR'ye göre daha duyarlı, kantitasyon yapabilen, kontaminasyon riski az ve sonuçlanma süresinin kısa olması nedeniyle son yıllarda yaygın olarak kullanılmaktadır. Bu çalışmamızda BKV tanısı için laboratuvarımızda tasarlanan kantitatif real time BKV PZR testinin analitik performansının değerlendirilmesi amaçlanmıştır.

Yöntem: BKV plazmidinden (ATCC 45025) standartlar hazırlanmıştır. 15×10^7 kopya/ml'den 3×10^1 kopya/ml'ye kadar seri dilüsyonlar içeren BKV plazmidinin spektrofotometrik ölçümü yapılmıştır. Amplifikasyon reaksiyonları için BKV VP1 gen bölgesine uygun, 5' ucu 6-karboksifloresceinle (FAM), 3' ucu 6-karboksitetrametilrodamin (TAMRA) ile işaretli primerler daha önce belirtildiği gibi kullanılmıştır.

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Results: To evaluate the analytical performance of the assay; analytical sensitivity, specificity, linearity, accuracy and precision was determined. The analytical sensitivity and the limit of detection of the assay were found 15×10^2 copies/ml and 5×10^2 copies/ml, respectively. Standard deviation (SD) of dilutions varied from 0.02 to 0.644 and CV varied from 0.79% to 11.47% between 15×10^7 to 15×10^1 copies/ml concentrations. Nineteen proficiency samples results of quality control program were in close agreement (100%). The assay demonstrated a linear range from 15×10^2 to 15×10^8 copies/ml. Specificity of the assay was found 100%. In addition proficiency samples results of the external quality control program were in close agreement.

Conclusion: According to our results the real time PCR protocol of BKV developed in our laboratory was found sensitive, specific, precise and reproducible with a broad dynamic range.

Key Words: Analytical performance, Real Time BKV PCR, validation experiments

Bulgular: Çalışmada testin analitik performansının değerlendirilmesi için analitik duyarlılık, özgüllük, doğrusalılık, doğruluk ve kesinlik parametreleri belirlenmiştir. Testin analitik sensitivitesi 15×10^2 kopya/ml ve saptama limiti 5×10^2 kopya/ml olarak belirlenmiştir. 15×10^7 kopya/ml ile 15×10^1 kopya/ml konsantrasyonlar arasında çalışılan dilüsyonların standard deviasyonu (SD) 0,02 ile 0,644 arasında, varyasyon katsayısı (CV) ise %0,79 ile %11,47 arasında değişmiştir. 19 dış kalite kontrol örneğinin sonuçları ilgili programla %100 uyumludur. Testimiz 15×10^2 kopya/ml ile 15×10^8 kopya/ml arasında lineer bir seyir göstermiştir. Özgüllüğü ise %100 olarak belirlenmiştir. Ayrıca dış kalite kontrol programı ile kalite kontrol örneklerinin sonuçları çok yakın uygunluktadır.

Sonuç: Elde edilen sonuçlarımız doğrultusunda laboratuvar tasarımı real time PCR protokolümüz; duyarlı, özgül, kesin ve geniş dinamik aralıklı tekraranabilirliği olan bir test olarak bulunmuştur.

Anahtar Kelimeler: Analitik performans, Real Time BKV PCR, Validasyon deneyleri

INTRODUCTION

BK virus (BKV) infection typically occurs in early childhood. Following primary infection, BKV establishes subclinical and persistent infection of which predominantly remains in kidneys, peripheral blood and brain (1, 2). Reactivation of BKV under immunosuppression is associated with diseases such as interstitial nephritis, haemorrhagic and non-haemorrhagic cystitis, ureterostenosis and nephropathy (1-3). Usually in transplant recipients BKV associated nephropathy (BKVAN), leading to graft loss in 50% of infected patients within 2-3 years of follow-up and subsequent return to haemodialysis (4, 5). Progression of BKVAN often occurs without clinical signs or symptoms except a decline in renal function (4, 6). Monitorization of BKV infection after transplantation can facilitate early diagnosis which provides an opportunity for pre-emptive treatment (6, 7).

For the diagnosis of BKV related conditions cytological analysis of urine samples, urine cell culture and electron microscopy is widely used but they are time-consuming and lack sensitivity (8, 9). Quantification of BKV viral load by a PCR based method has a prognostic value for diagnosis of BKV related diseases and monitoring (3, 4, 6). The aim of our study was to evaluate the analytical performance of a real time quantitative BKV PCR assay which was developed in our laboratory.

MATERIALS and METHODS

For the evaluation of analytical performance; analytical sensitivity, specificity, linearity, accuracy and precision were determined. Standard curves for the quantification of BKV were constructed using five, ten fold serial dilutions of a plasmid containing

the entire linearized genome of the BK virus Dunlop strain inserted into the Bam H1 restriction site of the pBR322 plasmid (pBKV, ATCC 45025). The plasmid was amplified using a transfection system. Plasmids were selected and isolated from competent bacteria and to determine concentrations a ultraviolet spectrofotometer was used. Serial dilutions of BKV plasmid that contained 15×10^7 copies/ml to 3×10^1 copies/ml were measured by spectrofotometry. Every dilutions were tested three times in the same run and different runs. Totaly standards prepared from BKV plasmid were tested 19 times in different runs. According to the assay results mean of measured BKV DNA levels, standard deviation and coefficient of variation values were calculated (Table 1). Primers for BKV VP1 gene and dual labelled probe at the 5' end with 6- carboxyfluoresceine (FAM) and the 3' end with 6-carboxytetramethylrhodamine (TAMRA) as described previously were used for the amplification reactions (10).

Analytical sensitivity: 15×10^7 copies/ml to 3×10^1 copies/ml serial dilutions were studied as three replicates in the same run and three different runs. Totally each dilutions were tested nine times for the analytical sensitivity.

Linearity: Three replicates of dilutions containing 15×10^7 copies/ml to 15×10^1 copies/ml were studied in 4 different runs. Totally each dilutions were tested 12 times.

Precision and accuracy: 15×10^7 copies/ml to 15×10^1 copies/ml dilutions were studied as three replicates in the same run and 19 times in different runs.

Specificity: The specificity of the assay was assessed by the testing of 66 BKV negative human plasma samples. We participated to the external quality control program of Quality Control for Molecular Diagnostics (QCMD) and QCMD 2007 JC virus and BK virus DNA External Quality Assessment (EQA) Panel samples were tested.

Table 1. Serial dilutions of the plasmid and their standard deviation and coefficient of variation values

Target Value (copies/ml)	Target value (\log_{10})	Number of determinations (n)	Mean of Measured BKV DNA (\log_{10})	Standard deviation	Coefficient of variation (%)
15×10^7	8.17	3	8.11	0.07	0.88
15×10^6	7.17	3	7.24	0.08	1.2
15×10^5	6.17	3	5.74	0.1	1.87
15×10^4	5.17	3	4.99	0.07	1.54
15×10^3	4.17	3	4.01	0.09	2.35
15×10^2	3.17	3	3.06	0.24	8.08
15×10^1	2.17	3	3.14	0.02	0.79
15×10^7	8.17 (standard 1)	19	8.13	0.06	0.84
15×10^6	7.17 (standard 2)	19	7.25	0.104	1.44
15×10^5	6.17 (standard 3)	19	5.61	0.644	11.47

Statistical analysis: Descriptive statistical analysis was performed with Microsoft Excell 2003 version, using the database of assay.

RESULTS

For absolute quantification; standards 1, 2, 3 containing known input gene copy numbers were analyzed in each run.

Analytical sensitivity of the assay was assessed by testing 15×10^7 copies/ml to 15×10^2 copies/ml dilutions 9 times in different runs. All replicates were found positive. Four of the 9 replicates were positive for 15×10^2 copies/ml to 1×10^3 copies/ml dilutions. No positivity were found for the dilutions of 3×10^2 copies/ml to 3×10^1 copies/ml. The analytical sensitivity of the assay was found 15×10^2 copies/ml.

Limit of detection was found 5×10^2 copies/ml greater than 95% positivity rate for all replicates with Microsoft Excell 2003 version. The results were expressed as target not detected below 5×10^2 copies/ml.

None of the 66 negative human plasma samples were positive, so the specificity of the assay was found 100%.

To determine the linearity; the correlation coefficient (r) and coefficient of variation (CV) were

checked according to the results of 15×10^7 to 15×10^1 copies/ml dilutions. As shown in Figure 1 (Linearity plot) a significant relationship was found between the average measured and expected BKV DNA levels ($r = 0.9986$ and $p < 0.005$). Precision and accuracy was determined by testing of serial dilutions. As shown in Table 1 standard deviation (SD) of dilutions varied from 0.02 to 0.644 and CV varied from 0.79% to 11.47% between 15×10^7 to 15×10^1 copies/ml concentrations. Nineteen proficiency samples results of quality control program were in close agreement (100%). The assay demonstrated a linear range from 15×10^2 to 15×10^8 copies/ml.

DISCUSSION

Infections by BKV are gaining important clinical problem in the management of renal transplant patients because it can be the cause of BKVAN and consequently loss of the transplanted organ. Patients with BKVAN show evidence of viral activation, but all of them do not have any clinical sign or specific symptom, except a breakdown in renal function (4, 7). The efficient early diagnosis of BKVAN, with close monitoring of renal function could be the best strategy for early clinical intervention and efficient treatment, with

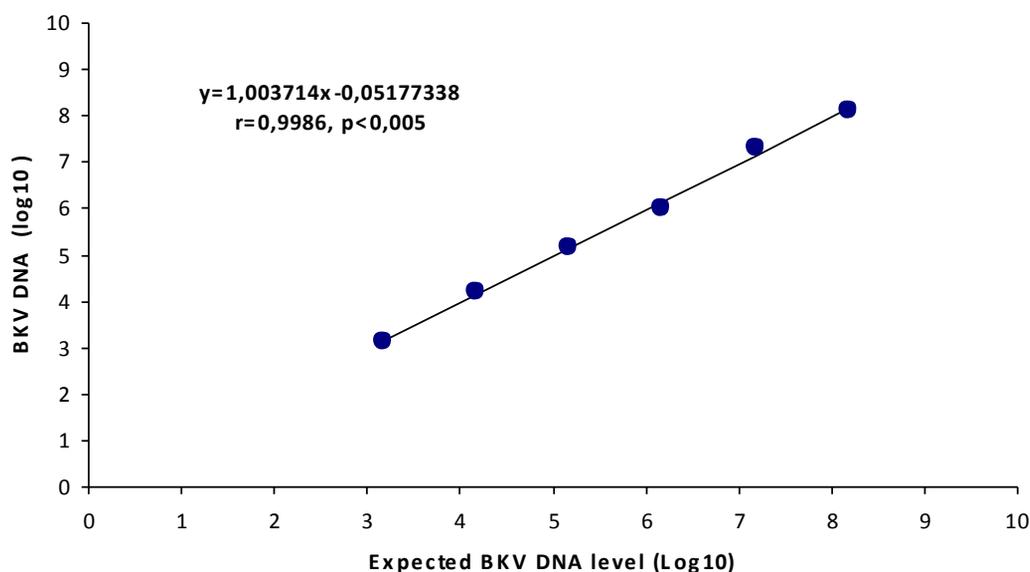


Figure 1. Scatter plot and regression equation

benefits regarding damage progression and successful outcome.

BKV quantitation by PCR is a key point on clinical management. The advantages of BKV quantitation is the early diagnosis with high predictive value, a window period of 6-12 weeks before viremia and nephropathy and being non invasive technic for patients comparison to renal biopsy (11). Recent studies have assessed the effectiveness of quantification of BKV and monitoring therapy to investigate the correlations between viral shedding and disease conditions (12-14). In fact, there is no effective antiviral agents for patients under immunosuppressive conditions (2, 15). Medical relevance of BKV infection is increasing among transplant patients due to immunosuppressive agents. For rapid diagnosis serial measurement of BKV viral load with real time PCR assay became a usefull diagnostic tool. Real time PCR assays have increased sensitivity and wide dynamic range compared to conventional PCR methods. Risk of contamination reduced during the analysis, early viral monitoring and the turn around time for resulting the reports within hours are the other advantages of real time PCR methods (3, 16-19). Quantification of viral load with laboratory developed tests must be ideally sensitive, specific, accurate, precise and have a broad dynamic range. When a new test is introduced to laboratory for routine diagnostic, validation experiments must be performed including the analytical sensitivity, specificity, precision, accuracy and linearity (20, 21). During the validation process stable control materials with known copies per mililiter are essential. Even so

there is no international standard material or method for quantitation of BKV viral load. Owing to this, laboratories must prepare their own internal standards for a measure of reproducibility and the analytical performance. In a study target sequences of BKV and CMV were diluted and used as external standard for the newly developed real time PCR assay detecting both CMV and BKV DNA simultaneously. After the evaluation process the sensitivity of the assay was found 5×10^3 copies/ml (22). In a similar study by Wu et al., a new quantitative PCR method for detection of BKV and CMV DNA simultaneously was established and the sensitivity of the assay was found 5×10^3 copies/ml (23). The analytical sensitivity of our assay was found 15×10^2 copies/ml. In several reports the sensitivity of BKV quantitation were found between 10^1 to 10^3 copies/ml (20, 21, 24, 25). For laboratory developed real time PCR assay the reportable range of viral load must be determined by measuring linear regression line and the correlation coefficient. Our PCR assay has a reportable range of 15×10^2 to 15×10^8 copies/ml. After validation experiments the laboratories must check the feasibility of the new test with an external quality control program. The intralaboratory variability can be tested by this quality control program material. In conclusion, this study shows that our real time PCR assay of BKV DNA is sensitive, specific, precise and reproducible and that it has a broad dynamic range of quantification. Analytical performance of the assay should be evaluated before the routine workflow for the standardization of laboratory developed real time PCR methods.

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