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Detection and differentiation of opportunistic viral infections potentially contributing to renal graft rejection by tetraplex-nested PCR

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Abstract. The need for an intensive care protocol, sometimes weekly or biweekly, has led to a significant increase in laboratory costs for kidney recipients. In the present study, an in-house tetraplex nested PCR assay was developed and validated for the specific detection of BKV, JCV, HCMV and EBV in clinical samples. We determined the Limit of Detection (LOD) and analytical specificity. To demonstrate the diagnostic performance of the assay, a total of 102 archival plasma samples were tested and compared with a commercial uniplex real-time PCR kits. The analytical sensitivity of the in-house tetraplex nested PCR assay was 173 copies/ml, when all four viruses were present in the specimens. These values were 79.2, 58.7, 87.6 and 96.1 copies/ml when only, BKV, JCV, HCMV and EBV respectively, were present. The cross-reactivity assays were shown no detectable signal in the tetraplex PCR results. The estimated diagnostic sensitivities were 92.6% for BKV, 92.3% for JCV and 100% for both HCMV and EBV as compared with commercial kits. Regarding the sensitivity and specificity, it seems that the developed Multiplex Nested PCR assay could be used as a reliable virus-associated renal rejection (VRR) panel in post renal transplant surveillance.

INTRODUCTION

Solid organ transplantation is a therapeutic method for many human diseases and becomes an effective therapeutic option for end-stage renal diseases. Kidney transplant rejection can be reduced through the use of immunosuppressive agents (Chinen & Buckley 2010). Although, deliberate immunosuppression has been administered during last decades to reduce the chance of graft rejection, it has also augmented the sequels of opportunistic infections post-renal transplantation (Comoli & Ginevri 2012). Inappropriate monitoring and management of viral infections occasionally could hamper the graft survival due to direct viral-associated nephropathy or indirect prophylactic interventions such as reducing the dose of immunosuppressant to prevent extra-renal complications (Zaza *et al.*, 2014). Many opportunistic viral infections after renal transplantation result from the reactivation of latent viruses (Weikert & Blumberg 2008). Although more viral infections may have clinical significance in kidney transplant recipients, only some of them can be considered as the etiologic agents of graft rejection. In general, human Cytomegalovirus (HCMV) and BK virus have a lot of clinical significances due to potential ability in developing nephropathy and graft rejection post kidney transplantation, but some studies, though controversial, point to the role of JC

virus and Epstein-Barr virus (EBV) in creating similar consequences (Cukuranovic *et al.*, 2012; Phillips *et al.*, 2004; Purighalla *et al.*, 1997; Shenagari *et al.*, 2010; Kantarci *et al.*, 2011; Shenagari *et al.*, 2017). Due to the role of these viruses in the development of renal or extra-renal complications, many nephrologists, especially in the first year after transplantation, continuously monitor them to prevent probable serious complications (Comoli & Ginevri 2012). The need for an intensive care protocol, sometimes weekly or biweekly, has led to a significant increase in laboratory costs for these patients. Developing a cheap and reliable test for these viral infections can greatly reduce the cost of screening and encourages clinicians to use NATs for screening of viral infections associated with kidney transplant rejection in developing countries. The aim of this study was to develop a reliable, cost-effective and simultaneous tetraplex nested PCR method to detect four clinically important viruses potentially related to renal graft rejection.

MATERIAL AND METHODS

Samples and viruses

A total of 102 archived plasma samples from renal transplant recipients referring to Molecular Diagnostic Center were included in this study. These samples had been stored at -80°C until the performing tests. Some positive-control samples for optimization and validation test were prepared. For assay development and validation, control viruses, including HTLV-1, HIV-1, HSV-1, HSV-2, VZV, HSV-6, HSV-7, HSV-8, SV40, HCV, HBV, TTV, B19 and human genome were used for assessment of clinical specificity. The abovementioned control agents were clinically isolated from Iranian patients.

Primers design

The extent of homology between the genomes of BK and JC viruses which was estimated about 75% persuaded us to get all genomic sequences (215 sequences) related to these viruses available from NCBI GenBank database and run a large degree of multiple alignments to identify conserved region among various isolates and distinct areas between two viruses (Frisque *et al.*, 1984). Analysis of the BKV genome reveals a great deal of sequence variation. For EBV and HCMV conserved and species specific genes were collected to ensure coverage of all strains and analytical specificity. The nucleotide sequences were aligned using Clustal W and pairwise sequence comparisons, and phylogenetic analysis was performed with MEGA version 7. Altogether 8 pair primers were designed to run a tetraplex nested PCR for the viruses in separate two steps. Designing were done using AlleleID, version 7.0 and PrimerPlex version 2.0 softwares (Premier Biosoft International, Palo Alto, CA, USA). Primers were designed based on conserved regions of genome for each virus so they were able to recognize all the genotypes of each virus. For the first round the primers designed to amplify a 741 bp, a 736 bp, a 552 bp and 732 bp from of BK Large T antigen, JC Large T antigen, HCMV UL55 and EBV DNA polymerase genes, respectively. The nested primers were designed based on interior region of related first round amplicon as follows: BK virus 261 bp, JC virus 165 bp, CMV 367 bp and EBV 476 bp. The sequences and characteristics of designed primers are shown in Table 1.

Uniplex nested PCR and Real-time PCR

Viral DNA was extracted from 200 µl of plasma samples using QIAamp® DNA mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In-house Uniplex nested PCR was carried out simultaneously with 2 µM of specific external primer sets designed for the first round using Accupower Hotstart PCR premix (catalog no. K-5051; Bioneer, Daejeon, Korea). Amplification was carried out in a programmable thermocycler (ProFlex™ 96-well PCR System, Applied Biosystems™, USA). The amplification profile consisted of a single cycle of enzyme activation at 95°C for 5 min, followed by 30 amplification cycles of denaturation at 94°C for 30 s, annealing at 58°C for 15 s and extension at 72°C for 90 s, and a final extension at 72°C for 10 min. 5 µl

Table 1. The sequences and characteristics of designed primers were as follows:

Primer name	Primer sequence (First Round)	Primer length	Amplicon length
BK ForEx	TTGTCAGCAAGCAGTAGATACA	22	741 bp
BK RevEx	CCTAAACCAAATTAGCAGTAGC	25	
JC ForEx	TGATGATGAAAACACAGGATC	21	736 bp
JC RevEx	TCAACCCTTTGTTTGGCTGC	21	
HCMV ForEx	TCTGCGTTAACTTGTGTATCGTC	23	552 bp
HCMV RevEx	CTATAACGCGGCTGTAGGAAC	22	
EBV ForEx	CCACCAGAACCGGGGAGTTG	20	732 bp
EVB RevEx	TGGGCACCTGCGAAGACAT	19	

Primer name	Primer sequence (Second Round)	Primer length	Amplicon length
BK ForIn	GCCTTAAATGTAAACCTACCC	21	261 bp
BK RevIn	GGACAGGATACTCATTCATTGTA	24	
JC ForIn	GACAGCCATATGCAGTAGTG	20	165 bp
JC RevIn	GTCTAAGTACATGCCCATAAGC	23	
HCMV ForIn	AGTCACCATTCCTCTCATAC	20	367 bp
HCMV RevIn	TGTGGATGTAAGCGTAGC	18	
EBV ForIn	AATCTCTGCCACCTCCAC	18	476 bp
EBV RevIn	TGCTCTACGCCTTCTTCC	18	

of 1/10 diluted first-round product was then transferred into a second PCR solution mixture. The second-round reaction mix contained the same constituents as the first, but 2 µM of each internal primers and same premix. The second-round PCR amplification was performed as follows: 5 min initial denaturation at 95°C then thirty-five cycles of 94°C for 30 secs, 57°C for 15 secs, 72°C for 45 secs for, and a final extension for 5 minutes. Final reaction volume was always 20 µl for both rounds. The uniplex PCRs were included by blank and negative control reactions to check cross-contaminations. After PCR, 10 µl of the product was electrophoresed in a 1.5% agarose gel in 0.5× TBE buffer for 30 min at 3 V/cm and visualized by ethidium bromide staining. A 100-bp DNA Ladder (Thermo Scientific™ GeneRuler™ California) was used for molecular weight markers. Sequencing was done automatically using dyelabeled dideoxy nucleotides and DNA polymerase in an Applied Biosystems 3730XL DNA sequencer (Applied Biosystems, Foster City, CA, USA). In addition, real-time PCR was performed in a StepOne Plus™ instrument (Applied Biosystems, Foster City, CA, USA) using the GeneProof™ real-time PCR kits (Vídeňská, Czech Republic) simultaneously for BKV, JCV, CMV and EBV according to the manufacturer's instruction. An internal control was included in the reaction mix of Real-time PCR kits, controlling the possible inhibition of the PCR or excluded, controlling also the DNA extraction process quality.

Tetraplex nested PCR assay

Multiplex PCR was performed in 25 µl PCR reaction using Multiplex PCR plus kit (Qiagen, Germany). 12.5 µl of 2× Multiplex PCR Master Mix (Qiagen, Germany), 2.5 µl of Q-solution, 2.5 µl of CoralLoad (except first round), 2 µM of each 8 specific primers for BKV, JCV, HCMV and EBV (Totally 16 µM) and 5 µl extracted DNA were added to the master mix according to manufacturer's instruction. Here too, as in the case of uniplex reaction,

5 µl of 1/10 diluted first-round products were then transferred into a second PCR solution mixture. The nested multiplex thermocycling program adjusted according to manufacturer's instructions as follows: initial denaturation at 95°C for 5 s followed by 30 amplification cycles of denaturation at 95°C for 30 s, annealing at 58°C for 90 s and extension at 72°C for 90 s, and a final extension at 68°C for 10 min. The mentioned program was same for both round except cycling that were 30 and 35 for first and second rounds respectively. The tetraplex PCRs were included by blank and negative control reactions to check cross-contamination. The multiplex PCR reactions were performed using ProFlex™ 96-well PCR System and finally the PCR products were run on 1.5% agarose gel.

Analytical sensitivity and specificity

In order to assess the analytical sensitivity of designed uniplex and tetraplex PCR, the purified amplicons of BKV, JCV, HCMV and EBV were inserted into the TA cloning vector (Fermentas T/A cloning kit, PTZ 57R/T) and transformed into competent *DH5α* bacteria according to manufacturer's instructions. Plasmids were extracted from bacteria using Qiaprep® Spin Miniprep Kit (Qiagen, Germany). The concentrations of plasmids were determined using NanoDrop™ 2000/2000c Spectrophotometers (Thermo Fisher Scientific, USA), and standards were made based on copy number/ml. Serial dilutions of constructed plasmids related to BKV, JCV, HCMV and EBV were prepared as follows: 10, 50, 100, 200, 500 and 1000 copies/ml. Plasmid copy number was calculated as the DNA concentration in grams per µl times 6×10^{23} copies per mol/molecular weight of cloned plasmid in gram per mol. uniplex and tetraplex-PCR were performed on each standard in a quadruplicate mode and repeated five times with 5 days intervals to inspect the limit of detection (LOD). The sensitivity was assessed based on the minimum plasmid copy number at which amplification occurred. Therefore, the LOD for these particular uniplex and multiplex assays were estimated using probit analysis to match these probabilities for comparison purposes. Probit analysis (SPSS, vision 19; IBM) was used to determine the 95% LOD and the two-sided fiducial confidence intervals from the combined data of all replicates tested for each virus. Final LODs were expressed as a concentration, copies/ml. Since the analytical sensitivity of multiplex PCR could be affected by high concentration of each target sequence, a serial dilution of each viral standard were tested against a high copy number/ml (10^6) standard of the other one. To determine the analytical specificity and cross-reactivity of developed multiplex PCR, evaluation of 16 designed primers was performed by assessing potential homologies to all sequences deposited in NCBI using the BLASTn algorithm (http://www.ncbi.nlm.nih.gov). As well as, control samples that consisting HTLV-1, HIV-1, HSV-1, HSV-2, VZV, HSV-6, HSV-7, HSV-8, SV40, HCV, HBV, TTV, B19 and human genome were used as template in separate reactions.

Clinical sensitivity and specificity

Clinical sensitivity and specificity were determined by comparison between results of in-house uniplex and multiplex PCRs and the results of concurrently accompanying commercial Real-time PCR for simultaneous detection and quantitation of BKV, JCV, HCMV and EBV in archival plasma samples of 102 renal transplant recipients. Due to lack of sample with four co-infected viruses, two manually spiked samples, prepared from mixing plasma of patients with one agent and other plasmas including 3 agents were prepared.

RESULTS

Optimization of the tetraplex nested PCR

Gradient PCR was utilized to identify optimum annealing temperatures to all designed primers. The optimal temperature determined as 58°C for the first round and 57°C for the second round. The accuracy of amplicons produced in uniplex PCR reactions was confirmed by DNA sequencing using specific primers. Subsequently, the in-house multiplex PCR was optimized. As

shown in Figure 1, specific amplicons produced in Multiplex PCR format were readily detected and discriminated on a 2% agarose gel. Optimization of PCR using positive DNA samples with individual Multiplex PCR primers resulted in the amplification of the 261 bp for BKV DNA, the 165 bp for JCV DNA, the 367 bp for HCMV DNA, and the 476 bp of EBV DNA at an optimized annealing temperature of 57°C.

Specific detection of BKV, JCV, HCMV and EBV in the tetraplex nested PCR assay

The results indicated that designed specific primers for each virus did not cross-react with others. Especially BK specific primers amplified only this virus and did not amplify the genetically relevant JCV and vice versa. Similarly, HCMV and EBV species specific primers did not amplify genomes of each other's.

Analytical sensitivity and specificity of the single PCR and tetraplex nested PCR assays

Standard curve analysis used to calculate efficiency of Real-time PCR methods (Figure 2). Lower limit of 95% detection (LLOD) was measured for each uniplex and tetraplex PCR assays and expressed as the

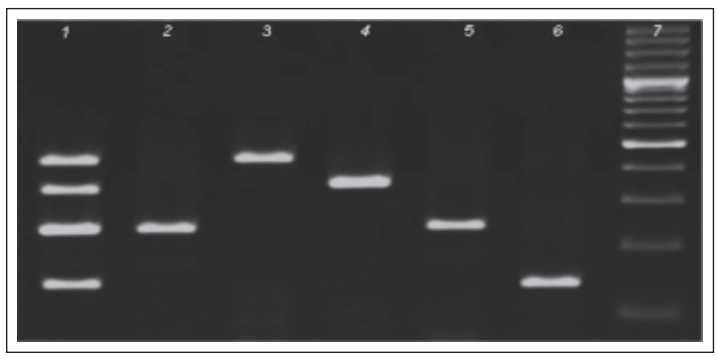

Figure 1. Agarose gel electrophoresis related to uniplex and tetraplex PCR in which specific targets have been amplified and separated based on their size. Lane 1: Four separated amplicons of JCV, BKV, CMV and EBV (tetraplex-PCR), lane 2 & 5: BKV amplicon (261 bp), lane 3: EBV amplicon (476 bp), lane 4: CMV amplicon (367 bp), lane 6: JCV amplicon (165 bp), lane 7: DNA size marker (100 bp). PCR products were resolved by electrophoresis in 1.5% agarose in TBE buffer and the gel was stained with ethidium bromide and photographed.

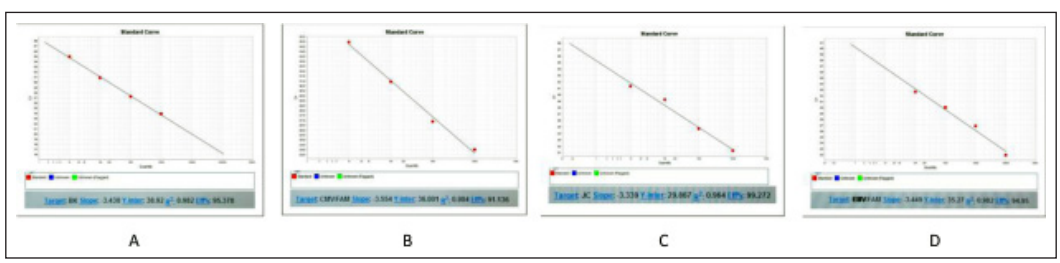

Figure 2. Standard curve used to calculate efficiency of Real-time PCR methods. A) Graph shows standard curve used for calculation of BKV Real-time PCR efficiency and were 95.378%. B) Graph shows standard curve used for calculation of CMV Real-time PCR efficiency and were 91.136%. C) Graph shows standard curve used for calculation of JCV Real-time PCR efficiency and were 99.272%. D) Graph shows standard curve used for calculation of EBV Real-time PCR efficiency and were 94.95%. The Real-time PCR assays were performed in StepOne Plus™ instrument and related standard curves analysis were generated using StepOnePlus™ Software v2.3 (Applied Biosystems, Foster City, CA, USA).

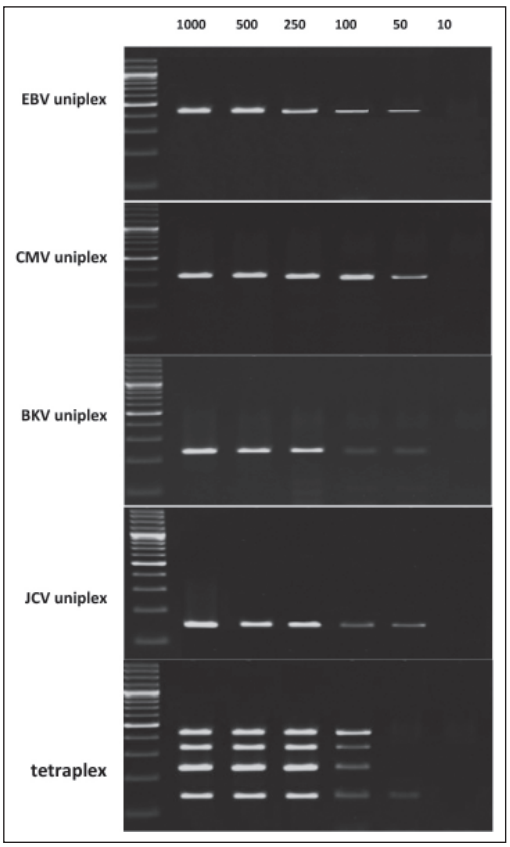

Figure 3. Agarose gel electrophoresis showing Limit of detection (LOD) of the uniplex and tetraplex PCR assays employed over mentioned serial dilutions of the four target gene-plasmids of EBV, CMV, BKV and JCV. The concentrations of each plasmid DNA were indicated above each lane. PCR products were resolved by electrophoresis in 1.5% agarose in TBE buffer and the gel was stained with ethidium bromide and photographed.

lowest copy number detected 95–100% of the time, depending on the assay. LLOD was determined for each virus in the multiplex assay using plasmid dilutions. In Figure 3 agarose gel electrophoresis showing Limit of detection (LOD) of the uniplex and tetraplex PCR assays employed over mentioned serial dilutions of the four target gene-plasmids of EBV, CMV, BKV and JCV. In addition, according to Probit regression analysis, the LLOD was calculated to be 173 copies/ml for lowest pooled equal concentration of BKV, JCV, HCMV and EBV plasmids. The lowest concentrations detected by the uniplex PCR assay were 79.2 copies/ml for BKV, 58.7copies/ml for JCV, 87.6 copies/ml for CMV and 96.1 copies/ml for EBV. In addition, our results showed high viral load of one target sequence does not influence the analytical sensitivity of the other virus with low concentration. The primers were tested *in silico* by querying the NCBI nucleotide database for related sequences using BLASTn. Full coverage and 100% identity for the primer sequences showed to correlated viruses included various strains and genotypes of four viruses. No organism represented the best match for both the forward and reverse primers for any of our primer pairs except related viruses. As a further test of specificity, Genomic DNA or cDNA from ten different samples including human genome and some blood transmitted viruses, including HTLV-1, HIV-1, HSV-1, HSV-2, VZV, HSV-6, HSV-7, HSV-8, SV40, HCV, HBV, TTV, B19 were assayed. No detectable signal in the Multiplex PCR was seen.

Clinical sensitivity and specificity of in-house tetraplex nested PCR

Totally 102 archived plasma samples that were referred to Molecular Diagnostics Center in Guilan province were tested using both the multiplex PCR and the commercial Real-time PCR Kits. In order to assess the clinical reliability and applicability of in-house Multiplex PCR, the results were compared with Real-time PCR results. As summarized in Table 2 based on Real-time PCR results, 26(25.4%) samples were JCV positive, 54(52.9%) samples were BKV positive, 16(15.6%) samples were HCMV positive and 10(9.8%) samples were EBV positive. The results of uniplex nested PCR were the same as Real-time PCR. The estimated sensitivity of tetraplex nested PCR for detection of four agents was 92.6% for BKV, 92.3% for JCV and 100% for both HCMV and EBV as compared with commercial uniplex Real-time PCR kits. On the other hand, 4(3.9%) samples (for BKV) and 2(1.9%) samples (for JCV) were positive only by the commercial uniplex Real-time PCR kits. Moreover, clinical specificities of in-house tetraplex nested PCR and uniplex nested PCR were assessed separately by using negative samples which were proved to be negative

Table 2. Samples that assessed for the presence of four viruses by real-time PCR, uniplex PCR and tetraplex PCR were as follows:

	Uniplex Real-time PCR				In-house Uniplex PCR				In-house Multiplex PCR			
	BKV	JCV	HCMV	EBV	BKV	JCV	HCMV	EBV	BKV	JCV	HCMV	EBV
Positive (n)	54	26	16	10	54	26	16	10	50	24	16	10
Negative (n)	48	76	86	92	48	76	86	92	52	78	86	92
Co-infected with 1 agent	27	20	7	4	27	20	7	4	25	18	7	4
Co-infected with 2 agent	9	6	3	2	9	6	3	2	6	3	3	2
Total samples	102	102	102	102	102	102	102	102	102	102	102	102
Sensitivity (%)					100	100	100	100	92.6	92.3	100	100
Specificity (%)					100	100	100	100	100	100	100	100

by commercial uniplex Real-time PCR kits previously mentioned. No positive results were observed, thus the clinical specificity of the assay considered 100% for four agents.

DISCUSSION

The probability of survival of the renal transplant has increased with the advent of new immunosuppressive drugs, nonetheless this also increases the likelihood of multiplying opportunistic viruses with the ability to boost the rejection rates among these patients. For this reason, many of the transplantation centers developed regular screening and monitoring system for accurate diagnosis and timely treatment in their post-transplant care plan (Chakera *et al.*, 2011). Both urine cytology and molecular-based methods can be used to detect polyomavirus infection, and cell cultures or antigenemia determination is effective for detecting CMV infection in RT recipients (Knipe *et al.*, 2007). However, PCR technology, which is simple, rapid, and sensitive and can distinguish virus subtypes, is gradually replacing urine cytology for polyomavirus infection detection and cell culture or antigenemia determination for CMV detection (Randhawa *et al.*, 2005; Kwon *et al.*, 2015) Additionally, PCR technology is increasingly considered to be the "gold standard" for virus infection detection. PCR based methods for detection of opportunistic viral infections are now considered as an integral part of the post-transplant monitoring and management (Cukuranovic *et al.*, 2012). Various Real-time PCR assays have been developed and used in the care and management of renal transplant recipients (Khansarinejad *et al.*, 2012; Funahashi *et al.*, 2010). For clinical purposes, it is desirable to have a standard, absolute viral load for the diagnosis and monitoring of these viruses; therefore, there is a need to compare the differences among various assays. Real-time PCR is the principal technology used for viral load measurement in these patients. Various testing protocols can have significant differences in the limit of quantitation and dynamic ranges, leading to different conclusions regarding the cutoffs and predictive values of viruses for nephropathy (Hoffman *et al.*, 2008). Also, financial constraints in developing countries can be considered as a negative factor in choosing simultaneous Real-time PCRs in detection and monitoring of important viral infections (Ahmed *et al.*, 2015). Up to our knowledge there is no available commercial kit based on Multiplex PCR or Multiplex Real-time PCR for monitoring of BKV, JCV, HCMV and EBV. As well as, there is no standardized and US Food and Drug Administration approved commercial assay for detection and quantification of these agents and substantial inter-laboratory variability and cost-ineffectiveness points to the need to supply standard and applicable method. Despite

this, many study have been designed to develop in-house Uniplex qualitative PCR or quantitative Real-time. Also, there are a few reports of in-house Multiplex-PCR assays for simultaneous diagnosis of opportunistic viral infections post renal transplantation (Funahashi *et al.*, 2010; Bergallo *et al.*, 2007; Wada *et al.*, 2007; Gunson *et al.*, 2009; Whiley *et al.*, 2001). The present study is the first attempt to design a PCR based method as virus associated renal rejection (VRR) panel to detect all recommended tests in monitoring of renal recipients. Multiplex PCR applications benefit diagnostics in a clinical laboratory due to their ability to detect and rule-out many related pathogens in a single reaction, reducing tech-time by more than 3 hours for a panel of many viruses (Pierce *et al.*, 2012). Despite some limitations of qualitative PCR assay in management of viral infections among renal transplant recipients such as inability in quantification and determination of severity of infection or monitoring antiviral treatment responses, it seems it could be suitable and cost-effective for primary screening of replication of viral infections in RT. Analytic sensitivity, or the lowest possible concentration necessary to produce a reliable result, is an important parameter to consider when replacing singleplex Real-time PCR assays with Multiplex PCR platforms evolving from newer, more expensive technologies. The analytical sensitivity of the in-house tetraplex nested PCR assay was 173 copies/ml, when all four viruses were present in the specimens. These values were 79.2, 58.7, 87.6 and 96.1 copies/ml when only, BKV, JCV, HCMV and EBV respectively, were present. To demonstrate the diagnostic performance of the assay, a total of 102 plasma samples were tested and compared with a commercial Real-time PCR kit. The results indicate that the established method is sensitive, specific and cost-effective, and can be used particularly in situations where the high cost of commercial kits prevents the use of molecular methods for the diagnosis of opportunistic viral infections among renal transplant recipients. Further characterization of viruses in clinical specimens may be of greater clinical importance, especially when particular subtypes are known to be more virulent in the population as is the case with BKV in particular populations. It should be noted that due to clinical importance of the HCMV, JCV and EBV and in some cases BKV in other immunocompromisation episode, the presented test can be utilized successfully in these patients. Finally, considerable sensitivity and specificity of developed qualitative tetraplex nested PCR make it a reliable virus associated renal rejection (VRR) panel, at least as a point of care screening of opportunistic viral infections in renal transplant patients.

Conflict of interest statement

The authors have no conflict of interest.

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