NOVEL LIGHT-UPON-EXTENSION (LUX) REAL-TIME PCR PRIMER SYSTEM FOR RAPID DETECTION OF *POLYOMAVIRUS HOMINIS* 1 (BKV) IN CLINICAL SAMPLES

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ABSTRACT

Polyomavirus hominis 1 (BKV) has important clinical significance in the kidney transplant centers. It is the cause for the development of polyomavirus associated nephropathy (PVAN), which may be connected with graft dysfunction and loss. Yet there is no standard means of BKV diagnosis, but PVAN confirmation is made by examining graft biopsy. Nevertheless, it is risky and laborious procedure, moreover the results are difficult for interpretation. Thus a non-invasive and at the same time specific diagnostic method for demonstrating viral replication in the patient should be regarded. A novel molecular technique is the light-upon extension (LUX) real-time PCR using fluorogenic primers. A primer pair for BKV detection was developed and optimized for BKV real-time PCR. We confirm its high specificity but also the rapidity and non-invasiveness of the method for demonstration of ongoing viral replication.

Keywords: *Polyomavirus hominis* 1 (BKV), BK virus, Light-Upon Extension (LUX) real-time PCR

Introduction

Polyomavirus hominis 1 (BKV) is a pathogen with marked significance in the renal-transplant centers. Its primary infection is followed by latency in the kidneys but under different and yet not completely defined conditions it can reactivate and exert a direct cytopathic effect on the graft. This is connected with the development of severe disease entity – polyomavirus associated nephropathy (PVAN), which can result in graft dysfunction and loss. Clinical diagnosis of PVAN is a difficult task. It relies on examination of biopsy material, but the procedure is risky and results are difficult to be interpreted. Thus a non-invasive but at the same time clinically reliable assay must be used to define BK viral replication in patients at risk of PVAN development. Different real-time PCR techniques provide sensitive, rapid and highly specific tool for demonstration of ongoing viral replication. Development of novel techniques would be of clinical value for establishment of the diagnosis and management of PVAN (2, 8).

The novel real-time primer design called LUX (Light Upon Extension) has recently been introduced for viral gene detection. The system has been applied for the specific amplification of several human viral agents- Noroviruses (4, 7), quantitative measurements of HIV-1 (9) and in veterinary research as well-Newcastle disease virus and avian influenza viruses (1, 3). According the obtained results, the technique is found even more sensitive than TaqMan real-time PCR technology.

In LUX real time PCR detection the generation of a signal is based on a single fluorescent dye molecule that is attached to an oligonucleotide close to its 3'end (5). No quencher dye is required. A tail of 5-7 nucleotides is added to the 5'end of the primer to form a blunt end hairpin when it is not incorporated into the PCR product. This design provides a low initial fluorescence of the primers that increases upon formation of the PCR product. The hairpin oligonucleotides are as efficient as linear primers and provide additional specificity to the PCR by preventing primer-dimers and mis-priming. They could be designed by specialized software on a set of rules for optimum signal development and could be also used for gene quantitation. Self-quenched primers are an efficient and cost-effective alternative to fluorescence resonance energy transfer-labeled oligonucleotides (5, 6).

Our objective was to design a LUX real-time PCR primer system for BK virus for the improvement of its diagnosis among the renal-transplant recipients. The conditions and specificity were optimized in order for the reliable interpretation of the results and monitoring of the patients with kidney allograft.

Materials and Methods

Primer design software

D-LUX™ designer software was used to design BK virus fluorogenic primers (www.invitrogen.com/lux). The technique requires input of the BK viral genome sequence by its GenBank accession number (V0 1108) and uses default parameters in generating the labeled primers. Their suitability is marked with a number of asterisks as the best are usually first in the

displayed order. The chosen sequences for synthesis were named BKLUX-1 and BKLUX-2.

Evaluation of Specificity of the real-time PCR assay

BK virus DNA previously evaluated from urine and serum samples by a patient developed BK associated nephropathy was used as a positive BK control (2). For ensuring specific BK virus amplification related (Simian Virus 40 genome, JC virus DNA) and distant (Human Papilloma Viruses, Herpes Simplex Virus) viral genomes as well as normal human urothelial DNA were also included in the optimization protocol. The specificity of the LUX real-time PCR was determined by comparing the level of fluorescence above the threshold and melting curves obtained from BK virus DNA and the other viral (human) DNA.

LUX-Real-time PCR and quantifying the fluorescence

For this assay an in-house optimized mixture consisting of the following components was used: 5 µl 10X PCR buffer; 2.5 mM $MgCl_2$ (Abgene, USA); 1.25U of thermostable Taq DNA polymerase (Invitrogen, USA); 200 µM of each of the four nucleotides (USB, USA); FAM-labeled LUX primer and corresponding unlabeled primer (400 nM each, final concentration), 1 µl ROX reference dye (Invitrogen, USA) and 5 µl (~5 ng) DNA up to a final volume of 50 µl. MJ Research Opticon (Bio-Rad, USA) was programmed for the optimized in house-protocol: initial denaturation at 95°C for 10 min, followed by 45 cycles consisting of denatiration at 95°C (15s) and one-step of annealing/elongation at 60°C (1 min). Fluorescence spectra were recorded during the annealing/elongation phase of each PCR cycle. The cycle threshold was calculated as the cycle number at which the reaction became exponential. To identify the specific PCR products generated at the presence of LUX-primers, Tm analysis was performed by increasing the temperature from 60 to 95°C at a transition rate of 0.1°C/s. The software calculates the Tm, i.e. the rate of change of fluorescence (Tm= -dI/dT) is displayed as a function of temperature. To confirm additionally the obtained results of 25 µl aliquots of the PCR products were analyzed by electrophoresis on ethidium bromide stained 2% agarose gel.

Results and Discussion

Primer design and characteristics

LUX –fluorogenic primers were designed from the genome of BK virus strain Dunlop available in the GeneBank with accession number V01108. Of all fifteen displayed oligonucleotide sequences, these with best properties were from the specific genomic region of small t antigen. They were named BKLUX-1 and BKLUX-2 and used for further analysis. For additional insurance of their specificity a nucleotide blast for short sequences was performed (BLAST). A 100% similarity was found only with the BKV strain J3B-3. No similarities with other polyomaviruses/viruses were observed. They flanked a region of BKV genome shown on **Fig. 1**. (51 bp) and have characteristics given in **Table 1**.

Specificity of BK virus detection

For the evaluation of specific BKV amplification, in the optimization different viral genomes and DNAs were included. BK virus control from urine became positive at the 11 cycle considered as a threshold cycle (Ct=11) and BK virus DNA from sera at the 34 (Ct=34) (**Fig. 2**). No increase in fluorescence

4621 ataagttagt taccttaaag ctttagatct ctgaagggag tttctccaat tatttggacc
4681 caccattgca gagtttcttc agttagg**tct aagccaaacc actgtgtg**aa gcag**tcaatg**
4741 cagtagcaat ctatccaaac caagggctct tttcttaaaa attttctatt taaatgcctt
4801 aatctaagct gacatagcat gcaagggcag tgcacagaag gctttttgga acaaataggc
4861 cattccttgc agtacagggt atctgggcaa agaggaaaat cagcacaaac ctctgagcta
4921 ctccaggttc caaaatcagg ctgatgagct acctttacat cctgctccat ttttttatac
4981 aaagtattca ttctcttcat tttatcctcg tcgccccctt tgtcagggtg aaattcctta
5041 cacttcctta aataagcttt tctcattaag ggaagatttc cccaggcagc tctttcaagg
5101 cctaaaaggt ccatgagctc catggattct tccctgttaa gaactttatc cat

Fig. 1. Genomic region of the small t antigen of the BK virus genome (Strain Dunlop); note: **in bold** - primer sequences and the region they flank

TABLE 1

General characteristics of the designed LUX-primers

Name	5'→3' LUX sequences	Position	Ta[1]	Size[2]
BK virus (strain Dunlop); GenBank accession number V0 1108				
BKLUX-1	**cacaca**TCTAAGCCAAACCACTGTGTG-FAM[3]	4708-4728		
BKLUX-2	TTGGATAGATTGCTACTGCATTGA	4735-4759	60°C	51bp

Note: **in bold symbols:** six nucleotide tail quencher; [1]Optimized annealing temperature; [2]Size of the amplified PCR product; [3]Reporter fluorophore (6-carboxyfluorescein, acr onym "FAM")

spectra was observed in the other closely related and distant viral genomes as well as the human DNA. The melting curve analysis of PCR product of the BKV DNA revealed a maximum peak at ~78.2° C corresponding to the increase of the fluorescence of BKV DNA controls (urine, sera) above the threshold. No melting peaks were observed in the rest of the DNAs (**Fig. 3**). The size of the amplified product (51 bp) was verified also by gel-electrophoresis (data not not shown).

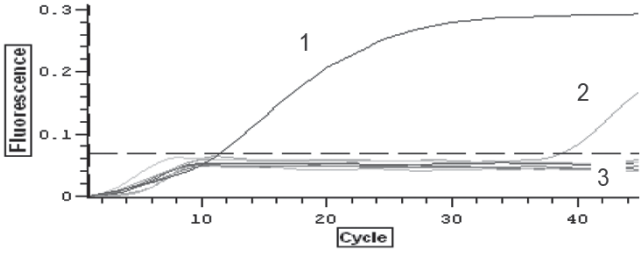

Fig. 2. Amplification plot of the BK virus DNA and the rest of the viral genomes (human DNA):

1. Positive amplification of BK virus DNA from urine (Ct=11);
2. Positive amplification of BK virus DNA from sera (Ct=34);
3. Negative amplification of the viral genomes (below the threshold)

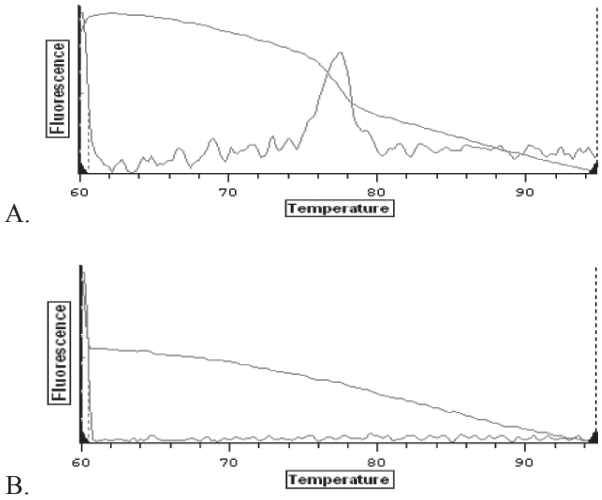

Fig. 3. Melting curve analysis of the PCR products of the positive control and a negative control genome (closely related JC virus). **A.** Melting curve of the BK virus amplification product with Tm ~ 78.2 (-dI/dT=78.2) **B.** No melting peak at the JC virus genome

It was demonstrated that the designed BK virus LUX primer pair is a specific and reliable tool for detecting on-going BK virus replication. This was confirmed not only by the increase of fluorescence emission of the positive BKV control DNA from serum and urine but also by the performed melting curve analysis and the demonstration of a single peak at ~78.2°C. The gel-electrophoresis also served as a confirmation of the specificity. The reaction could be applied in the diagnosis and routine screening for BK virus replication in renal-allograft patients.

The fluorogenic primer method has several advantages over other methods including also ease of design of the primers. Their design is based on studies that demonstrate the effects of primary and secondary structure of oligonuclotides on the emission properties on a conjugated fluorophore. The primers are chemically synthesized oligonucleotides (22-29 nt in length) with a fluorophore attached to the C5 position of thymidine, which increases its fluorescence when incorporated into the double-stranded PCR product. This phenomenon results from several important facts: a) having the fluorophore close to the 3'-end of an oligonucleotide with G or C at the end, b) the existence of a G within a few bases of the label and also c) the ability of the oligonucleotide to form blunt ended hairpin at temperatures close to the annealing temperatures of the primer (6).

Conclusions

In conclusion the flouorogenic primer method (LUX) is rapid, uses few toxic chemicals and is useful for the specific and accurate detection of BK virus DNA among renal-allograft patients. However, further studies should be performed in order to establish a diagnostic agreement with the other available real-time used methods in the field of BKV detection for clinical purposes.

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