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LETTER TO THE EDITOR

ALTERNATIVE METHODS FOR SEQUENCING FULL TSPyV GENOMES USING SANGER OR NGS

Sao Paulo, 22 July, 2016

Dear Editor

The trichodysplasia-spinulosa polyomavirus-associated (TSPyV) was first described in a solid organ transplant recipient with a rare skin disease[1]. Initially mistaken as a side effect of cyclosporine treatment[2], the disease trichodysplasia-spinulosa (TS) is characterized by the development of keratin spines (spicules), follicular papules, hair follicle dilation and keratotic plugging of the infundibulum, which usually manifests on the face of the patient[3,4,5].

Occasionally, the lack of eyelashes and hair shafts can occur[6,7,8]. Despite the strong association between TS and TSPyV, the mechanisms of pathogenesis and virus transmission are still unknown[7,9]. Seroepidemiological data report that TSPyV is ubiquitous and the latent form of infection is present in more than 70% of the healthy population. In general, the prevalence ranges around 5% in children and can reach 70% in the adult population[4,10].

The first complete TSPyV genome was obtained in 2010 through Sanger sequencing[1]. Later, Siebrasse *et al*.[11], used a set of four primer pairs to amplify and to sequence the complete viral genome, but the authors did not mention which sequencing platform was used. Our group has recently sequenced the whole TSPyV genome from biopsy fragments of spicules using Next Generation Sequencing (NGS) [12]. Lastly, Tsuzuki *et al*.[13], have sequenced a complete TSPyV genome from cardiac tissues also using NGS method -Illumina platform.

A detailed analysis on 13 TSPyV genomes isolated in USA, Australia and a sequence of another study carried out in the Netherlands revealed a sufficient degree of genetic diversity allowing this virus to be divided into three distinct lineages (TS-I, II, III)[14]. Interestingly, all the three lineages include viruses sampled in US, suggesting no geographic clustering of TSPyV. Nevertheless, more comprehensive genetic characterization of this virus needs to be done since only 13 samples were used to classify TSPyV in lineages, and viruses isolated in Brazil[12], the Netherlands[1] and Japan[14] were not included in this analysis.

Herein we present fast and cost effective methods to sequence the full-length genome of TSPyV. A set of 10 primer pairs, which amplify fragments from 530 to 850 nucleotides with overlaps between each other, and two overlapping primer pairs to perform long PCR assays that generate fragments of 3,497 and 2,024 nucleotides (Table 1 and Fig. 1) were designed using the Primer3 program[15]. Conserved regions to anchor the primer were chosen based on the consensus obtained by multiple alignment of TSPyV reference sequences available at GenBank (www.ncbi.nlm.nih.gov). Similarity analysis was done using the Blast tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to assure that the primers do not bind to other polyomaviruses.

In order to evaluate the effectiveness of the assays, DNA samples were extracted from spicules of a positive TSPyV sample[11] using the QIAamp DNA Mini Kit (Qiagen®, Germany) according to the manufacturer's instructions. Different primer concentrations, ranging from 0.2 to 1 μM were tested in solutions containing 1X PCR Buffer, 200 μM dNTPs, 1.5 mM $MgCl_2$, 1U of Platinum Taq DNA polymerase (Invitrogen®, Inc., EUA), and 10 μL of template, reaching a final volume of 50 μL.

Table 1
List of primers used to amplify all the TSPyV genome regions

Primer	Sequences	Coordinates*	Fragment Size
	Short PCR		
TSV_PA1_F	TTCCAATTAACCACAGGCTT	3,666	670
TSV_PA1_R	AGATATACCTCCCTATGGGC		
TSV_PA2_F	CCTGCATGGTGTCAAACAAG	4,269	625
TSV_PA2_R	ACCATCCAGATAAAGGAGGA		
TSV_PA3_F	CCTCAGCCTCCTGGTAATA	2	544
TSV_PA3_R	CAGATAGGCCTTCAACTGTC		
TSV_PA4_F	AAATGGGAGGAGTAATCAGC	405	855
TSV_PA4_R	CCATACAATCCCAGTACCAG		
TSV_PA5_F	TGAGGCTGCCACAGATGAAT	1,149	641
TSV_PA5_R	ACCCACTGCAAACATATGGA		
TSV_PA6_F	GTTGGAAACTCTTGTCTTGC	4,811	620
TSV_PA6_R	TCCTCAGGATAACGGTCTTA		
TSV_PA7_F	CCCACTATTGATTGGTCCTC	3,194	534
TSV_PA7_R	TTAAGCTACTGGAGGAAAGC		
TSV_PA8_F	AATTGGACAGTACCAAACCA	2,732	605
TSV_PA8_R	ATTCTTCAGTATATGGCGGG		
TSV_PA9_F	CACCTCCAGTTTTGCAATTT	2,020	805
TSV_PA9_R	ATCTTGCCTTCAGGTTAGTG		
TSV_PA10_F	CCCATGTTGAATGAGGACC	1,608	575
TSV_PA10_R	CCTAGGTAGGCCTCTGTATT		
	Long PCR		
TSV_T1F	TGAGGCTGCCACAGATGAAT	1,144	3,497
TSV_T1R	AAGCAAATGCACCAGAAGAAG		
TSV_T2F	CCAAAGGGAATCCAAGCAAT	4,498	2,024
TSV_T2R	CCCAGTACCAGAGGCAACAT		

*Coordinates correspond to the position of the primer forward and are based on Genbank reference KM007161.

Cycling conditions used in short PCRs were tested with an initial denaturation of 94 ºC for 5 minutes followed by 40 cycles of 94 ºC for 30 seconds, annealing temperatures ranging from 50 ºC to 60 ºC for 50 seconds and extension times of 72 ºC for 1 minute. A final extension step of 72 ºC for 7 minutes was added after the fortieth cycle. The optimal annealing temperature for all ten primers was 55 ºC, and 0.5 μM was the best primer concentration (data not shown).

Cycling conditions for long PCRs were also set up with an initial denaturation of 94 ºC for 5 minutes followed by 45 cycles of 94 ºC

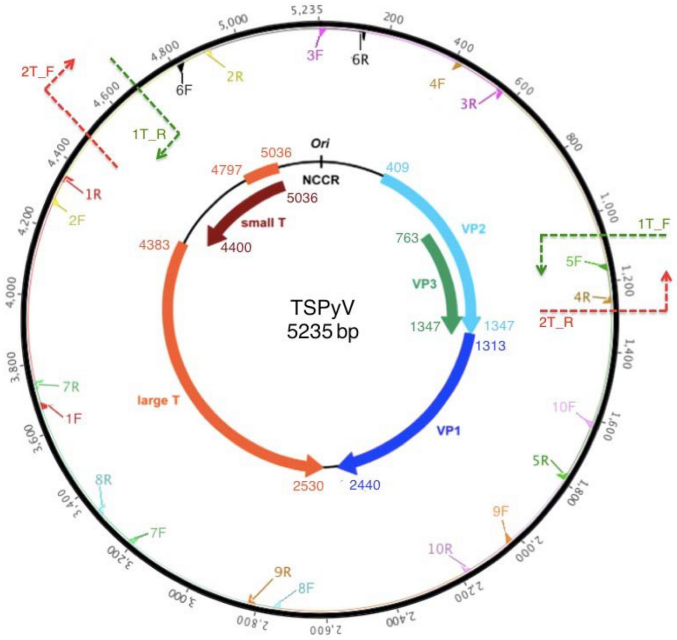

Fig. 1 - Schematic representation of the TSPyV circular genome and the primers used to amplify it. Dotted red and green lines in the outer circle denote primers of the long PCRs. Continuous colored lines represent the anchor sites of short PCR primers. The inner circle represents the TSPyV genome reference (ID KM007161), which is scaled to the primer coordinates.

for 30 seconds, annealing temperatures ranging from 51 ºC to 55 ºC for 50 seconds and an extension time of 72 ºC for 4 minutes. As for the short PCR, a final extension step of 72 ºC for 7 minutes was also included. The annealing was optimal at 51 ºC for both primer pairs and 0.5 µM of each primer has offered the best amplification performance. Amplification products were visualized in 1.5% agarose gel stained with 0.1 mL / 100 mL of SYBR Safe® (Invitrogen, Inc., USA). To validate the primers and protocols we used additional positive samples (blood and urine) from the same patient[11] that had been previously submitted to TSPyV DNA detection through real time PCR[16]. The products obtained from long PCRs were sequenced in NGS Ion Torrent PGM™ platform (Thermo Fisher®, USA) according to the manufacturer's instructions. Amplification products from short PCRs were sequenced by the method of Sanger in ABI Prism 3100 (Applied® Biosystems Incorporation, Foster City, CA, USA).

In both cases, the genome assemblies were obtained by means of CLC genomic workbench v6 (CLC Bio, Qiagen). In the case of sequences generated in Ion Torrent, reads were trimmed to remove short and low-quality reads. Reads obtained from Sanger were only trimmed to remove regions with low quality. Using the related TSPyV genome as reference (GenBank ID KM007161)[11], reads from Ion Torrent were assembled using map reads to reference tool. Sequences generated by the method of Sanger were assembled using regular tools available at CLC to assemble contigs and chromatograms. Complete genomes generated in this work are available at GenBank IDs KX249740 (Blood collected in April) and KX249741 (Blood collected in February) using NGS methodology; and KX249742 (Blood collected in April) and KX249743 (Urine collected in April) using the method of Sanger.

In this report we described two protocols that can be used to sequence the TSPyV complete genome through previous amplification products. Using the extended set of ten primer pairs, researchers can sequence either the whole genome or specific regions of interest, depending on the primers used (Fig. 1 for details).

In addition, the NGS approach described here makes larger epidemiological and phylogenetic studies possible. The amplicon method can be used in virtually any next generation platform, and barcoding allows several samples to be sequenced at once. In addition, the amplicon method is a good option for lower throughput platforms (i.e. Ion Torrent; Roche 454 Jr.).

Ultimately, the choice of the platform and sequencing approach will depend on the application, including the size of the research project and the number of genes/genomes to be sequenced. Therefore, the methods described here will ease the sequencing of the complete genome of TSPyV from all three already described lineages and significantly improve our ability to investigate the genetic diversity of this polyomavirus.

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COMPETING INTERESTS

None.

ETHICAL APPROVAL

The study was approved by the Ethics Committee of the *Faculdade de Medicina da Universidade de São Paulo*# 0234/10.

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