Dear Editor

The trichodysplasia-spinulosa polyomavirus-associated (TSPyV) was first described in a solid organ transplant recipient with a rare skin disease. Initially mistaken as a side effect of cyclosporine treatment, 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.

Occasionally, the lack of eyelashes and hair shafts can occur. Despite the strong association between TS and TSPyV, the mechanisms of pathogenesis and virus transmission are still unknown. Seroprevalence studies 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.

The first complete TSPyV genome was obtained in 2010 through Sanger sequencing. Later, Siebrasse et al. 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 using Next Generation Sequencing (NGS). Lastly, Tsuzuki et al. have sequenced a complete TSPyV genome from cardiac tissues also using NGS method.

A detailed analysis on 13 TSPyV genomes isolated in USA, Australia and a sequence from 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). Interestingly, all the three lineages include viruses sampled in US, suggesting no geographic clustering of this virus. Nevertheless, more comprehensive genetic characterization of this virus needs to be done since only 13 samples were used to classify these lineages.

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 350 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). Cycling conditions for short PCRs were tested with an initial denaturation of 94 °C for 5 minutes followed by 45 cycles of 94 °C for 1 minute. A final extension step of 72 °C for 7 minutes was added after the fortieth cycle. The optimal annealing temperature for all ten primers was 55 °C.

In order to evaluate the effectiveness of the assays, DNA samples were extracted from spicules of a positive TSPyV sample 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 MgCl₂, 1U of Platinum Taq DNA polymerase (Invitrogen®, Inc., EUA), and 10 μL of template, reaching a final volume of 50 μL.

Cycling conditions used in short PCRs were tested with an initial denaturation of 94 °C for 5 minutes followed by 40 cycles of 94 °C for 30 seconds, annealing temperatures ranging from 50 °C to 60 °C for 30 seconds and extension times of 72 °C for 1 minute. A final extension step of 72 °C for 7 minutes was added after the fortieth cycle. The optimal annealing temperature for all ten primers was 55 °C, 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 °C for 5 minutes followed by 45 cycles of 94 °C

### Table 1

| Primer   | Sequences | Coordinates* | Fragment Size |
|----------|-----------|--------------|--------------|
| TSP_PA1_F | TTCAATTAACACAGGCTT | 3,666 | 670 |
| TSP_PA1_R | AGATATACCTCCCTATGGGC | | |
| TSP_PA2_F | CCTGCATGTTGTCAAAAACAG | 4,269 | 625 |
| TSP_PA2_R | ACCATCCAGATAAACAGGAGGA | | |
| TSP_PA3_F | CCTCAGCTCTCTGTTGTTAATA | 2 | 544 |
| TSP_PA3_R | CAGATAGGCTCTTCACTGTC | | |
| TSP_PA4_F | AAATGGGAGGATGAATACGAC | 405 | 855 |
| TSP_PA4_R | CCAACATCTCCAGTACCCAG | | |
| TSP_PA5_F | TGAGGCTGCCACAGATGAAT | | |
| TSP_PA5_R | ACCATCGCAAACATATGGA | | |
| TSP_PA6_F | TTTGAGAATCTTCTGTTGC | 1,149 | 641 |
| TSP_PA6_R | CCATCCAGATAAACAGGAGGA | | |
| TSP_PA7_F | CCCCCTATGTTGTTGCTTCTC | 4,811 | 620 |
| TSP_PA7_R | CCCCCCTGTTGTTGCTTCTC | | |
| TSP_PA8_F | CCCCCTATGTTGTTGCTTCTC | 3,914 | 534 |
| TSP_PA8_R | TTAAGCTACTGGAAGGAAAGC | | |
| TSP_PA9_F | AAATGGGAGGATGAATACGAC | 2,732 | 605 |
| TSP_PA9_R | AATTTGGACAGTACCAAACCA | | |
| TSP_PA10_F | CCCCCTATGTTGTTGCTTCTC | 2,020 | 805 |
| TSP_PA10_R | CCCCCTATGTTGTTGCTTCTC | | |
| TSP_PA11_F | CCCCCTATGTTGTTGCTTCTC | 1,608 | 575 |
| TSP_PA11_R | CCCCCTATGTTGTTGCTTCTC | | |

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

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 MgCl₂, 1U of Platinum Taq DNA polymerase (Invitrogen®, Inc., EUA), and 10 μL of template, reaching a final volume of 50 μL.
for 30 seconds, annealing temperatures ranging from 51 °C to 55 °C for 50 seconds and an extension time of 72 °C for 4 minutes. As for the short PCR, a final extension step of 72 °C for 7 minutes was also included. The annealing was optimal at 51 °C 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 that had been previously submitted to TSPyV DNA detection through real time PCR. 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)¹, 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.

Paulo Roberto URBANO
Ana Carolina Soares de OLIVEIRA
Camila Malta ROMANO

Universidade de São Paulo, Instituto de Medicina Tropical de São Paulo, Laboratório de Virologia, LIMHC-52, São Paulo, SP, Brazil.

Correspondence to: Paulo Roberto Urbano, Laboratório de Virologia, Instituto de Medicina Tropical de São Paulo, Av. Dr. Enéas de Carvalho Aguiar 470, 05403-000 São Paulo, SP, Brasil. Tel.: +55 11 30617020 ext. 112, Fax.: +55 11 30618680. E-mails: purbano@usp.br; cmromano@usp.br; anacarolina.soares@usp.br

FUNDING
This paper was supported by FAPESP, project # 2012/15381-7 and CNPq #446851/2014-0. Paulo Urbano holds a CAPES scholarship and Ana Carolina Oliveira holds a FAPESP scholarship #2012/15469-1.

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.

REFERENCES
1. van der Meijden E, Janssens RW, Lauber C, Bouwes Bavinck JN, Gorbalenya AE, Feltkamp MC. Discovery of a new human polyomavirus associated with trichodysplasia spinulosa in an immunocompromized patient. PLoS Pathog. 2010b;6:e1001024.
2. Izakovic J, Bückner SA, Düggelin M, Guggenheim R, Itin PH. Hair-like hyperkeratoses in patients with kidney transplants. A new cyclosporine side-effect. Hautarzt. 1995;46:841-6.
3. Haycox CL, Kim S, Fleckman P, Smith LT, Piepkorn M, Sundberg JP, et al. Trichodysplasia spinulosa: a newly described folliculocentric viral infection in an immunocompromised host. J Investig Dermatol Symp Proc. 1999;4:268-71.

4. Chen T, Mattila PS, Jartti T, Ruuskanen O, Söderlund-Venermo M, Hedman K. Seroepidemiology of the newly found trichodysplasia spinulosa-associated polyomavirus. J Infect Dis. 2011;204:1523-6.

5. Van Ghelue M, Khan MT, Ehlers B, Moens U. Genome analysis of the new human polyomaviruses. Rev Med Virol. 2012;22:354-77.

6. Sadler GM, Halbert AR, Smith N, Rogers M. Trichodysplasia spinulosa associated with chemotherapy for acute lymphocytic leukaemia. Australas J Dermatol. 2007;48:110-4.

7. Lee JS, Frederiksen P, Kossard S. Progressive trichodysplasia spinulosa in a patient with chronic lymphocytic leukaemia in remission. Australas J Dermatol. 2008;49:57-60.

8. Schwieger-Briel A, Balma-Mena A, Nga N, Dipchand A, Pope E. Trichodysplasia spinulosa: a rare complication in immunosuppressed patients. Pediatr Dermatol. 2010;27:509-13.

9. Tan BH, Busam KJ. Virus-associated trichodysplasia spinulosa. Adv Anat Pathol. 2011;18:450-3.

10. Fukumoto H, Li TC, Kataoka M, Hasegawa H, Waki T, Sacki H, et al. Seroprevalence of trichodysplasia spinulosa-associated polyomavirus in Japan. J Clin Virol. 2015;65:76-82.

11. Siebrasse EA, Bauer I, Holtz LR, Le BM, Lassa-Claxton S, Canter C, et al. Human polyomaviruses in children undergoing transplantation, United States, 2008-2010. Emerg Infect Dis. 2012;18:1676-9.

12. Urbano PR, Pannuti CS, Pierrotti LC, David-Neto E, Romano CM. Rapid detection of trichodysplasia spinulosa-associated polyomavirus in skin biopsy specimen. Genome Announc. 2014;2 pii:e00694-14.

13. Tsuzuki S, Fukumoto H, Mine S, Sato N, Mochizuki M, Hasegawa H, et al. Detection of trichodysplasia spinulosa-associated polyomavirus in a fatal case of myocarditis in a seven-month-old girl. Int J Clin Exp Pathol. 2014;7:5308-12.

14. Kazem S, Lauber C, van der Mejden E, Kooijman S, Kravchenko AA, TrichSpin Network, et al. Limited variation during circulation of a polyomavirus in the human population involves the COCO-VA toggling site of Middle and Alternative T-antigen(s). Virology. 2015;487:129-40.

15. Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol. 2000;132:365-86.

16. Urbano PR, Nali LH, Bicalho CS, Pierrotti LC, David-Neto E, Pannuti CS, et al. New findings about trichodysplasia spinulosa-associated polyomavirus (TSPyV)-novel qPCR detects TSPyV-DNA in blood samples. Diagn Microbiol Infect Dis. 2016;84:123-4.

Received: 22 July 2016
Accepted: 26 October 2016