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Characterization of a new genotype of *Betapapillomavirus* HPV 17 through L1, E7, E7 and LCR sequences

L. OLIVEIRA, L. SANTOS, F. NEVES

Department of Microbiology and Parasitology, Fluminense Federal University, Rua Prof. Ernani Melo, 101 Niterói, RJ 24210-130, Brazil, 24210-130

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Summary. – Human papillomavirus (HPV) exhibits epithelial and mucosal tropism. HPV type 17 belongs to the *Betapapillomavirus* genus and molecular cloning experiments have identified two subtypes (17a and 17b) isolated from *epidermodysplasia verruciformis* (EV). HPV subtypes are characterized by dissimilarities from 2 to 10% at the nucleotide level from their referenced HPV. The aim of this study was to characterize the L1, E6, E7 and LCR sequences from an isolate, which was recovered from the oral mucosa of an asymptomatic 63 year-old woman. The whole late gene 1 (L1) was amplified using several sets of primers. The complete early genes 6 and 7 (E6, E7) and the long control region (LCR) were amplified using specific primers. Potential binding sites for transcriptional factors within the LCR were also investigated. Within these sets, the DNA sequence was altered at 91 positions (68 in L1, 13 in E6, 8 in E7, and 2 in LCR sequences). L1 analysis showed high dissimilarity compared with the HPV 17 prototype, reaching 4% of nucleotide substitutions and leading to a probable third 17 subtype. The E6 oncoprotein presented the highest modification among the sequences studied, with four amino acid changes in comparison with the prototype isolate. One amino acid was modified at a position 62 (S-T), a zinc-binding domain (CxxC(C)29 CxxC). Our findings provide data on genetic variations seen in this genotype, reaching to dichotomic branching and pointing to an evolutionary process.

Keywords: betapapillomavirus; HPV 17; subtype; oral cavity

Introduction

To date, 170 human papillomavirus (HPV) types have been wholly characterized, and approximately 60 of them exhibit epithelial and mucosal tropism (De Villiers *et al.*, 2013). They are among the most common sexually transmitted viral agents and are normally associated with both benign and malignant genital diseases. The virus can be observed in several body regions, such as the skin and the genital and oral mucosa, being quite well adapted to these ecological niches. Papillomavirus genotypes have been classified according to the entire L1 gene, a highly conserved open reading frame (ORF), which has 1,524 bp length in HPV 17. Similarity at least 90% characterizes the viral type and dissimilarity from 2–10% classifies a subtype. HPV 17 belongs to cluster *b2* of *Betapapillomavirus* (De Villiers *et al.*, 2013). This cluster is characterized by a short genome length (7,427 bp). Molecular cloning experiments have identified two subtypes (17a and 17b) isolated from patients with *epidermodysplasia verruciformis* (EV). HPV 17 normally infects the skin epithelium and was first isolated from benign maculas and subsequently from a squamous carcinoma (SCC) and malignant melanoma in one immunocompromised patient. It has also been found in keratoacantoma (benign skin tumor) and actinic keratosis (considered as precursor lesion for SCC) (Ekström *et al.*, 2011). The HPV types associated with EV are rare in typical skin lesions. A partial key to the host restriction of these viruses may be due to the unusual organization of their LCR region, which is shorter than other HPV genotypes (May *et al.*, 1991).

Since HPV type 17 exhibits tropism to skin cells, its detection in oral mucosa is very unusual, although the

*Corresponding author. E-mail: virologia.uff@gmail.com; phone: +55-2126292430.

Abbreviations: EV = *epidermodysplasia verruciformis*; HPV = human papillomavirus; SCC = squamous carcinoma

amplification of viral DNA with new primers has increased the spectrum of HPV 17 isolates in the oral cavity (Bottalico *et al.*, 2011). However, few of these genotypes have been sequenced to date, as can be seen in GenBank accessions. HPV 17 has been associated with the development of skin cancer in EV patients (Nindl *et al.*, 2007) and with non-genital seborrheic keratosis (Li *et al.*, 2004). However, the cutaneous HPV types are not classified as cancer risks. Betapapillomavirus species were previously considered almost exclusively skin types. They have been found in healthy people and individuals with skin tumors, including immunosuppressed and immunocompetent ones (Antonsson *et al.*, 2000; Pfister *et al.*, 2003). Regardless of being found in asymptomatic people or associated with benign and malignant skin lesions, their study has implication for understanding the oral colonization by HPV types previously discovered in other body sites. New variants isolated from oral mucosa could also differ in biological properties. We began this study by searching concordant viral genotypes in the oral and genital regions from 84 randomly selected asymptomatic women. After genotyping the positive HPV samples from both sites, we found HPV type 17 in the oral mucosa from one individual. In order to identify this one, we sequenced the whole viral genes L1, E6, E7 and the LCR region located between L1 and E6 genes.

Material and Methods

DNA extraction. HPV 17 DNA was extracted from an oral smear of a woman previously analyzed for HPV cervical infection, for which the result was negative. This woman was 63 years old, negative for HIV infection, a widow, with no schooling, of low income class, and a smoker of over 20 cigarettes a day. The Ethics Committee of the College of Medicine at the University approved the protocols for collection and informed consent (CAAE 0037.0.258.000-10). DNA was extracted from the oral and cervical samples using a commercial assay kit (Invisorb, Unisciencel, São Paulo, Brazil).

PCR reactions and sequencing. HPV detection was done by polymerase chain reaction (PCR). MY09/11 consensus primers were used to amplify a 450 bp L1 sequence, comprising one third of the major capsid protein. The primers were aligned to reveal the sequence from 6,761 to 7,090 bp, within the complete sequence 5,724–7,247 bp. Each cycle included a denaturation step at 94°C for 1 min, an annealing step at 55°C for 2 min and an elongation step at 72°C for 2 min using a DNA Thermal Cycler (Applied Biosystems, California, USA). The amplified PCR product was purified using a Illustra GFX PCR DNA and Gel Band Purification Kit (GE HealthCare, São Paulo, Brazil) and directly sequenced using a Big Dye Terminator v3.1 Cycle sequencing kit in an ABI DNA sequencer 3130 (Applied Biosystems, California, USA) with the same primers used for PCR amplification. The forward and reverse sequences were aligned and analyzed using Clustal W in the BioEdit Sequence Alignment Editor (North Carolina State University, Raleigh, NC) (Thompson *et al.*, 1994). The similarity analysis was performed by BLAST (Alignment Search Tool Analysis) (Altschul *et al.*, 1997).

After genotype identification, different sets of primers were used to investigate the HPV 17 genes, according to the reference sequence for HPV type 17 isolate (GenBank accession number X74469) (Delius and Hofman, 1994). To amplify the whole L1 sequence, the primers designed are listed in the Table 1. The annealing temperature of all primers was 58 °C. These primers were used to sequence partials fragments of the whole sequence. To amplify E6, E7 and LCR sequences, specific primers were designed: (i) for E6 gene (product length 567 bp): FWE6 (nt138–158) 5'GTG ACC GCC TTC GTT ACC TTA 3' and RVE6 (nt685–704) 5' TCG TAG CAA TGC AGG TCA GT 3'; (ii) for E7 gene (product length 497 bp): FWE7 (nt593–612) 5'GGC TTG TGC AGA CAT TGT GG 3'; RVE7 (nt1070–1089) 5'TCG CGG GAG TTT CCC TGT AT 3', and (iii) for LCR gene (product length 426 bp): Primers were used to cover the whole sequence (622–909 bp), flanking 604 to 1,040 bp; FWLCR (nt7250–7272) 5' GCT TTC GGT CTC TCA ATA AAC AA 3' and RVLCR (nt229–248) 5'ACA AGG TAT CAG CAA GCT CC 3'.

The PCR amplification for the whole E6 and E7 genes was performed using 50 pmol of different sets of primers. Amplifica-

Table 1. Primers designed for the whole L1 HPV 17 sequence

Gene	Primer name	Sequence 5' to 3'	Primers positions	Product size(bp)
17L1	BFL1	GCAGAAGCAGGAGGCCAGATT	5640-5659	451
	BRL1	AGGGATGTCCTGTAGTGCCT	6090-6071	
	CFL1	GCAGGCCTTGAGATAGGACG	6033-6052	464
	CRL1	GCATAGACTGCTCTCGTCT	6496-6477	
	DFL1	GGACAGGGCTCCTGTATGTG	6230-6249	586
	DRL1	TGTAACAGCCCCAGCTTCTG	6814-6795	
	EFL1	TGGCAGTTAGGATTTGTGCCT	6960-6980	452
	ERL1	CGGTGCTTCCACAAAAGCA	7411-7392	

tion was conducted in 50 μl of reaction mixture (1x PCR buffer, 200 mmol/l dNTPs, 1.5 mmol/l $MgCl_2$, 50 pmol of each primer, 0.25 U Taq polymerase (Invitrogen Brazil, São Paulo, SP, Brazil) and 5 μl of the extracted DNA sample with 30 amplification cycles. Each cycle included a denaturation step at 94°C for 30 s, an annealing step at 60°C for 1 min and a chain elongation step at 72°C for 1 min, on DNA Thermal Cycler (Verity, Applied Biosystems, California, USA). The LCR sequence was amplified at a 57°C annealing temperature using the same protocol. The amplified PCR product of each selected gene was sequenced in both strands with the same primers used for PCR amplification.

In silico analyses. The results were analyzed using the BioEdit Sequence Alignment Editor (North Carolina State University, Raleigh, NC) and aligned using uMuscle (Edgar, 2004). Nucleotides that differed from the prototype were recorded as genomic variations. Nucleotide distances were determined by p-distance. The GenBank database (Benson *et al*., 2008) was queried to retrieve all available sequences for HPV 17. Phylogenetic trees were created using the neighbor-joining method, and bootstrap analyses (1,000 replicates) were performed to test the tree confidence for the sequences (Mega package, v 6.0) (Tamura *et al*., 2013). Phylogenies on aligned amino acid sequences were constructed using the same program.

The MATCH™ Gene regulation software (Kel *et al*., 2003) and the TRANSFAC database (Matys *et al*., 2006) were used to search for transcriptional factors within the LCR sequence that had been previously described to bind this HPV 17 region: E2, API and the TATA box (Ensser and Pfister, 1990). Cut-off values and coincidence levels between consensus binding sites and LCR sequence of each type were adjusted in order to minimize both the number of negative and positive faults.

Results and Discussion

Using the traditional My09/My11 primers pair, a new HPV 17 variant far from the prototype was detected. Hazard *et al*. (2007) validated the FA fragment (about 480 nucleotides) as a robust tool to classify HPV types and subtypes, comparing L1 ORF, My and FA fragments. According to their results, My fragments are appropriated to taxonomic purposes. Very few subtypes have been described within the family *Papillomaviridae* in the past (De Villiers *et al*, 2004), but at the present, the more primer designs are appearing, more subtypes are being detected (Hazard *et al*., 2007), including those belonging to the Betapapillomavirus genus, uncommonly reported.

The L1, E6, E7 genes and the LCR sequence from an oral sample infected with the HPV 17 type were analyzed for possible nucleotide alterations and compared to the HPV 17 prototype. Within these regions, the DNA sequence

Table 2. Specific nucleotide changes within KY34886 isolate at nucleotide positions from 5724 to 7247 (L1)

Nucleotide position	5	5	5	5	5	5	5	5	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
	8	8	8	8	9	9	9	9	0	0	0	0	0	0	1	1	1	1	2	2	3	3	3	4	4	5	5	5	5	6	6	6	6	7
	4	5	8	9	0	3	5	6	0	1	5	7	8	9	2	2	5	6	3	9	0	0	1	7	9	1	3	4	6	0	0	1	1	1
	3	8	8	7	3	9	1	3	5	4	6	4	9	2	5	8	2	4	5	0	2	5	7	3	7	8	0	7	6	5	8	1	4	9
KY348861	C	A	A	T	G	G	T	C	A	G	G	T	T	A	C	T	C	T	A	A	T	A	C	T	C	C	C	A	T	T	A	A	A	A
X74469	T	T	T	A	A	A	C	T	T	A	A	C	C	G	T	C	T	G	G	G	C	T	T	C	T	A	T	G	C	C	T	G	T	T
																			1									2						
AA position																			7									7						
																			1									2						
KY348861																			K									D						
X74469																			R									G						

Nucleotide position	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
	7	7	7	7	7	7	7	8	8	8	8	8	9	9	9	9	0	0	0	0	1	1	1	1	1	1	1	1	2	2	2	2	2
	3	3	4	4	5	8	9	0	3	5	6	7	5	7	7	9	0	6	7	8	9	1	2	4	5	5	7	9	9	1	1	2	2	2
	1	7	6	9	2	8	4	0	9	7	3	5	9	1	7	3	7	1	6	5	1	9	7	5	2	4	8	5	6	7	8	0	8	9
KY348861	A	A	C	C	G	T	C	C	G	G	A	C	T	C	A	T	T	C	C	A	T	C	G	T	A	G	A	G	C	T	T	T	C	C
X74469	G	G	G	T	A	C	T	A	A	A	G	T	C	A	G	C	A	A	T	G	A	T	A	C	C	A	G	C	T	G	C	C	T	T
							3									4		4										4	4	4				5
AA position							5									2		4										9	9	9				0
							9									4		6										1	1	9				2
KY348861							D									S		N										S	T	S				I
X74469							E									P		K										S	T	P				T

Each column and the numbers in first four rows represents a specific nucleotide position in the L1 sequence. The fifth row shows the nucleotides changes for the HPV 17 prototype, Acc. No. X74469 in the NCBI BLAST (sixth row). The seventh row shows the position of non-conservative amino acid changes.

Table 3. Specific nucleotide changes within KP701189 (E6), KP70190 (E7) and KP701191 (LCR) isolates

E6														**E7**									**LCR**		
Nucleotide	0	0	0	0	0	0	0	0	0	0	0	0	0	Nucleotide	0	0	0	0	0	0	0	0	Nucleotide	0	0
position	2	2	3	3	3	3	3	4	4	4	4	5	5	position	7	7	7	7	7	8	8	8	position	0	0
	5	6	0	4	8	8	9	1	2	4	5	1	6		1	4	8	8	9	0	4	4		1	1
	6	5	7	6	4	5	4	9	2	2	1	9	5		1	7	0	8	5	7	3	4		2	3
KP701189	T	C	T	G	C	C	C	A	C	A	A	G	A	KP70190	G	G	T	A	A	C	A	C	KP701191	G	T
X74469	A	T	C	A	G	T	G	T	A	G	G	A	G	X74469	A	T	C	G	G	T	G	G	X74469	T	C
												1													
AA					6	6		7	7			0		AA				5				7	AA		
position					2	2		4	5			7		position				6				5	position	4	5
KP701189	P	D	Y	L	T	T	A	T	Q	V	R	R	Q	KP70190	A	D	C	D	K	I	E	E	KP701191		
X74469	P	D	Y	L	S	S	A	S	K	V	R	Q	Q	X74469	A	D	C	G	K	I	E	Q	X74469		

Specific nucleotide changes within E6 gene (200 to 625 bp), E7 (622 to 909 bp) and LCR (1 to 329 bp). Each column and the numbers in first four rows represents a specific nucleotide position in the sequence. The fifth row shows the nucleotides changes for the HPV 17 prototype, Acc. No. X74469 in the NCBI BLAST (sixth row). The seventh row shows the position of non-conservative amino acid changes.

was changed at 91 positions (68 in L1, 13 in E6, eight in E7, and two in LCR non coding region) (Tables 2 and 3). These sequences were submitted to GenBank and recorded under the accession numbers KY348861 (88L1WRJ), KP701189 (88E6RJ), KP70190 (88E7RJ) and KP701191 (88LCRRJ). No deletions or insertions were observed. The isolate 88L1WRJ shared 94.0% (1456/1524 bp) identity in the whole L1 sequence with HPV type 17. The complete E6, E7 and LCR gene sequences were analyzed, displaying 96.9% (413/426), 97.2% (280/288) and 99.4% (327/329) identity with the prototype sequence, respectively. These findings revealed a genome not very close to it. Although two subtypes ("a" and "b") have been described for HPV 17, the alignment of these regions with known sequences showed dissimilarity higher than 2%. Isolates differing between 2–10% identity in the L1 sequence are considered to be subtypes, but the final result is defined by the whole genome sequencing (De Villiers *et al.*, 2004). However, Ekstrom *et al.* (2011) not only classified but described new HPV types using small L1 DNA sequences. They also affirmed that shorter sequences within L1 ORF amplified with FA or My primers offer substantial results to identify new HPV types.

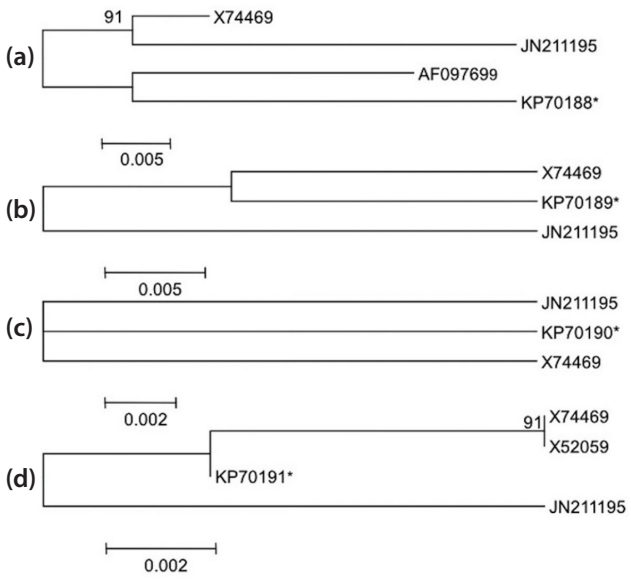

Fig. 1

Phylogenetic trees of L1 (a), E6 (b), E7 (c) and LCR (d) genetic sequences of HPV 17 (isolate compared with HPV 17 sequences available in the GenBank database)

Values of 1,000 bootstraps above 60% replicates are given at the branch of the L1 and LCR sequences. X74469, Prototype (Delius and Hofman, 1994); JN 21195, Isolate S 410, unpublished; Isolate 17b, L1 partial sequence (Berkout *et al.*, 2000), X52059.1, LCR (Enser and Pfistter, 1990). Asterisks indicate the isolates detected in this study.

The L1 DNA sequence analysis showed a high number of nucleotide variations, reaching over 2% of the partial region. Eight of them resulted in non-conservative amino acid changes, at positions 6235 (R-K), 6547 (G-D), 6794 (E-D), 6993 (P-S), 7061 (K-N), 7195/7196 (T-S), 7217 (P-S) and 7229 (T-I). The remaining variations led to silent substitutions (Table 2).

The E6 oncoprotein presented the highest degree of variation among the sequences studied, with four amino acid changes in comparison with the prototype. The amino acid sequence was modified at positions 62 (S-T), 74 (S-T), 75 (K-Q) and 107 (Q-R) (Table 3). The alteration at position 62 (S-T) is supposed to affect a zinc-binding domain (CxxC(C)29 CxxC).

The complete E7 nucleotide sequence showed two non-conservative alterations at positions 56 (G-D) and 75 (Q-E). Although not used as a classification criterion, the E6 and E7 oncogenic proteins revealed a significant distance from the

prototype (Table 3). As the function of this motif is undefined, the meaning of the substitution is also unknown. On the other hand, alterations in the E7 protein did not lead to mutations in the zinc-binding domains or the pRb binding core sequence (LxCxE).

The LCR region, considered the most variable sequence of the HPV genome, displayed only two point mutations, which did not promote any modifications in potential binding sites (Table 3). TRANSFAC database revealed 10 putative binding sites in this region: one AP-1, two TATA and TATAA signals, five early proteins E2, two transcription factors (one myeloblastosis viral oncogene, v-MyB, and one nuclear transcription factor, Y–NFY). In general, LCR has less conserved genome segments. This regulatory segment has low restriction of mutations.

Concerning the phylogenetic tree presented in the Fig. 1, we could note the further L1 genetic distance among 88L1WRJ variant and the prototype (subtype A) or AF097699 isolate (subtype B). The concatenated E6, E7, LCR and L1 sequences generated a phylogenetic tree that showed a well delimited split between the isolate of this study and the others available in the GenBank (Fig. 2). This dichotomical division was quite evident when the most common

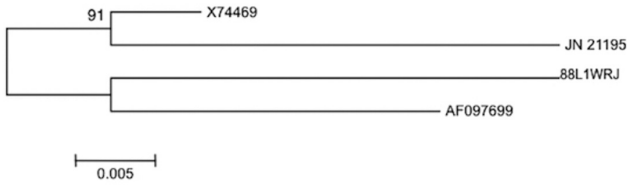

Fig. 2

Phylogenetic tree derived from a concatenated 88WRJ sequence aligment of L1, E6, E7 and LCR compared with HPV 17 sequences deposited in the GenBank database

X74469.1 (Delius and Hofman, 1994), JN 21195.1 (Isolate S 410, unpublished), AF097699.1 (Isolate 17b, L1 partial sequence, Berkout *et al*., 2000), X52059.1, LCR (Enser and Pfistter, 1990).

EV-associated HPV were clustered together in a partial L1 gene-based phylogenetic tree.

Phylogenetic comparisons were also performed by concatenating the sequences of the four genes analyzed in the present study. Due to the small number of sequences of HPV 17 type available in the GenBank database, the analysis of phylogenetic trees was somewhat impaired. The isolate was identified as a variant highly dissimilar from the other

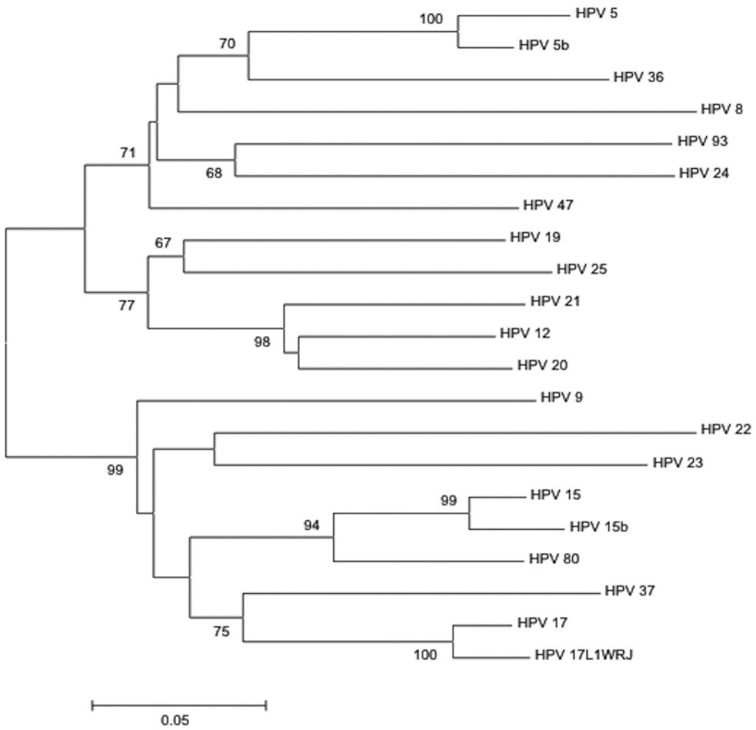

Fig. 3

Phylogenetic neighbor-joining tree constructed using L1 HPV 17 isolate (KY34886) and other sequences available in GenBank

M17463, HPV 5 (Zachow *et al*., 1987); D90252, HPV 5b (Yabe *et al*., 1991); X74471, HPV 8 (Fuchs *et al*.,1986); X74464, HPV 9 (Delius and Hofmann, 1994); X74466, HPV 12 (Delius and Hofmann, 1994); X74468, HPV 15 (Delius and Hofmann, 1994); AF091437, HPV 15b (Berkout *et al.* 2000); X74469, HPV 17 (Delius and Hofmann, 1994); X74470, HPV 19 (Delius and Hofmann, 1994); U31778, HPV 20 (Kremsdorf *et al*., 1984); U31779, HPV 21 (Kremsdorf *et a*l., 1984); U31780, HPV 22 (Kremsdorf *et al*., 1984); U31781, HPV 23 (Kremsdorf *et al*., 1984).

subtypes. Additionally and supported by the taxonomic tree constructed with the most common EV-associated types, our isolate belongs to HPV 17 type but it is clearly segregated from the prototype. The divergence was further characterized by a bootstrap value of 100%, similar to HPV 5 and 15 subtypes (Fig. 3). Then, despite the limitation of this study regarding the complete sequencing of the L1 gene, our results point to the description of a probable novel subtype of HPV 17.

HPV 17b was categorized as a subtype after PCR and sequence analysis from renal transplant recipients (AF097699) (Berkhout *et al.*, 2000). Our results revealed 95.1% (254/267 bp) identity with subtype b, which was partially sequenced. This finding reinforces the idea that we identified a possible new subtype. The phylogenetic tree showed that the isolate analyzed is closer to the prototype than the genome of subtype b, potentially making it a deep evolutionary variation from type 17. The commensal nature of cutaneous types (Antonsson *et al.*, 2000) favors infection at several sites and the mucosal tropism of the identified isolate was probably not random. The oral cavity has a large HPV spectrum and may be implicated in the evolution of this virus, allowing it to adapt to sites other than its original niche, may drive to produce adaptive variants of this genotype.

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