



Nucleotide and phylogenetic analysis of human papillomavirus types 6 and 11 isolated from recurrent respiratory papillomatosis in Brazil

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ABSTRACT

There are few studies about the distribution of natural molecular variants of low-risk HPVs. Our aim was to evaluate the E6 early gene variability among HPV-6 and HPV-11 isolates detected in recurrent respiratory papillomatosis (RRP) samples obtained in a cohort of Brazilian patients. We also performed a phylogenetic analysis in order to compare nucleotide sequences identified in our study with previously reported isolates from different anatomic sites (laryngeal papillomas, genital warts, cervical cancer and anal swabs) obtained from other parts of the world to determine the phylogenetic relationships of variants detected in Brazil. The complete coding region of the E6 gene of 25 samples was cloned and sequenced: 18 isolates of HPV-6 (72%) and 7 isolates of HPV-11 (28%). A total of four different HPV-6 genomic variants and two HPV-11 genomic variants was identified. It was not possible to correlate specific variants with disease severity. Phylogenetic trees for both HPV types were constructed enclosing both E6 sequences detected in our study and formerly published sequences. In both phylogenetic trees, the sequences from Brazil did not group together. We could not establish a geographical association between HPV-6 or HPV-11 variants, unlike HPV-16 and HPV-18.

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1. Introduction

Recurrent respiratory papillomatosis (RRP) is a benign disease of the upper respiratory tract (usually the larynx), which affects both pediatric and adult populations. RRP is characterized by solitary or multiple benign tumors in the respiratory tract, and is associated with infections with human papillomavirus (HPV) types 6 and 11 (Mounts and Kashima, 1984). Despite the benign nature of RRP, lesions tend to grow and extend throughout the entire respiratory tract, causing severe airway obstruction. Recurrence can also occur after surgical resection. For these reasons, RRP can result in considerable morbidity and mortality rates, which increase in rare cases in which malignant transformation occurs (Gallagher and Derkey, 2008).

The HPV genome consists of approximately 8,000 bp of double-stranded circular DNA encapsulated within an icosahedral protein

capsid that is 55 nm in diameter (Nebesio et al., 2001). HPVs infect cells from the basal layer of the stratified epithelium, and viral gene expression is closely linked to the differentiation program of the host cells (McMurray et al., 2001). The upstream regulatory region (URR) or long control region (LCR) consists of a non-coding segment of the genome that encloses cis-regulatory elements that are important to viral replication and gene expression control (Bernard, 2002; Chow et al., 2010). Functionally, the coding part of the viral genome is divided into early (E) and late (L) regions. The late region encodes the viral capsid proteins L1 and L2. In contrast, the early region contains genes designated E1–E7 that encode proteins involved in the regulation of viral DNA replication and transcription (Tyring, 2000). E6 and E7 have been found to be the most important pathogenic HPV proteins. The genes E6 and E7 from high-risk HPVs induce cellular immortalization by functionally interfering with proteins involved in cell cycle regulation (Longworth and Laimins, 2004). E6 and E7 have also been shown to be necessary for the maintenance of extrachromosomal forms of HPV in undifferentiated basal cells (Thomas et al., 1999).

More than 150 HPV types have been identified to date (de Villiers et al., 2004). HPVs can be categorized into low- and high-risk types based on the risk that the virus will cause squamous cell cervical carcinomas (IARC, 2007). The most often detected low-risk types are HPV-6 and HPV-11, which are causally associated with the development of benign lesions such as laryngeal papillomatosis and genital or perianal warts (Klozar et al., 2010). A distinct HPV type is established when the DNA sequence of the L1 gene differs from that of any other characterized type by at least 10%. In addition, isolates of the same HPV type are referred to as variants or subtypes when the nucleotide sequences differ by less than 10% (Bernard et al., 2006).

HPV-6 isolates are classified into HPV-6b-related (prototypic) and HPV-6a/6vc-related (non-prototypic) genomic variants (Heinzel et al., 1995). HPV-6b, the reference or prototype sequence, was originally isolated from a *condylomata acuminatum* (de Villiers et al., 1981; Gissmann and zur Hausen, 1980; Kovelman et al., 1999). The HPV-11 prototype isolate was detected in a laryngeal papilloma specimen in 1982 through Southern blot hybridization and in 1986, the complete nucleotide sequence of this clone was fully characterized (Dartmann et al., 1986; Gissmann et al., 1982).

Genetic variability analysis has proven essential to a better understanding of the natural and evolutionary history of papillomavirus. HPV nucleotide diversity has been investigated more extensively among high-risk HPV types. In contrast, few studies of this kind have been performed involving natural molecular variants of low-risk HPVs. Our aim was to evaluate E6 early gene variability among HPV-6 and HPV-11 detected in laryngeal papillomas samples obtained in a cohort of Brazilian patients. We also conducted phylogenetic analysis in order to compare nucleotide sequences identified in our study with isolates previously described from other parts of world.

2. Materials and methods

2.1. Clinical samples

Biopsy specimens were obtained from 25 patients between 3 and 52 years of age who had been treated at the Laryngology Clinic of the Department of Otorhinolaryngology of the School of Medicine of the University of São Paulo, in Ribeirão Preto, Brazil, and who had been diagnosed with RRP. Disease severity was determined at each surgical intervention using the Derkay staging system (Derkay et al., 1998).

2.2. HPV genotyping

Total DNA was extracted from biopsies of the lesions using the QIAmp DNA Micro Kit (Qiagen) according to the manufacturer's instructions. DNA quality assessment was performed using a PCR of endogenous gene *GSTP1*.

HPV positivity was accessed by a PCR protocol (Polymerase Chain Reaction) using the PGMY09/11 primer set which amplifies a 450 bp segment of the L1 gene from a broad range of mucosal HPV types (Gravitt et al., 2000). The amplification mix was comprised of 2.5 U of a proofreading polymerase (High Fidelity Enzyme Mix, Fermentas), 2.5 µl of 10X High Fidelity PCR Buffer, 4.0 mM MgCl₂, 0.2 mM dNTPs, 0.32 mM of each primer, 80 ng of extracted DNA to a final volume of 25 µl in nuclease free water. The reaction was submitted to an initial denaturation step at 95 °C for 13 min, 40 cycles at 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, and then to a final extension step at 72 °C for 5 min.

HPV positive samples were genotyped using RFLP (Restriction Fragment Length Polymorphism). In brief, samples were reamplified as described though in a final reaction volume of 100 µl. PCR products were independently digested by seven different restriction enzymes (*Bam*HI, *Dde*I, *Hae*III, *Hin*fI, *Pst*I, *Rsa*I and *Sau*3AI). Digestion profiles were compared to previously described restriction patterns (Bernard et al., 1994).

2.3. Molecular variants identification

For the nucleotide variability analysis, the complete E6 gene sequence of HPV-6 and HPV-11 positive samples was amplified using PCR with specific primers (Table 1). The amplification mix consisted of 6.0 U of a proofreading polymerase (High Fidelity Enzyme Mix, Fermentas), 5.0 µl of 10X High Fidelity PCR Buffer, 1.5 mM MgCl₂, 0.24 mM dNTPs, 0.4 mM of each primer, 500 ng of DNA, and nuclease free water, all of which added up to a final volume of 50.0 µl. Initial denaturation step at 95 °C for 5 min was conducted, followed by 35 cycles at 95 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, and a final extension at 72 °C for 8 min.

Amplified fragments were cloned using the TOPO XL Cloning™ Kit (Invitrogen). Five clones from each patient were purified using the GeneJET Plasmid Purification Kit (Fermentas). Sequencing reactions were performed using the BigDye Terminator kit (Applied Biosystems) and products were sequenced in an ABI 3130XL sequencer (Applied Biosystems). The sequencing reaction mixture consisted of 2.0 µl of 5X Sequencing Buffer (Applied Biosystems), 0.4 mM of specific E6 primers, 2.0 µl of Big ET Dye Terminator, and 5.0 µl of the sample. Sequencing reactions consisted of a hot start step of 10 min at 95 °C, followed by 30 cycles of 96 °C for 15 s, 50 °C for 15 s and 60 °C for 4 min. To control for nucleotide changes introduced by the polymerase, two different PCR products were cloned and sequenced.

The quality of the sequences obtained was evaluated using the Eletropherogram Quality Analysis, available online at <<http://www.biomol.unb.br/phph>>. Comparisons between the sequences acquired and those previously added to GenBank were conducted using BLAST (Basic Local Alignment Search Tool, available at <<http://www.ncbi.nlm.nih.gov/BLAST>>.) The alignment between

Table 1
Sequence of specific primers used for the amplification of the HPV E6 gene.

Primer	Sequence (5' → 3')
HPV6-E6F	GGGGGATCCGAATTCATGGAAAGTGCAAATGC
HPV6-E6R	GTGAAGCTTGGATCCTAGGGTAACATGTCTTCC
HPV11-E6F	AAAATTAGCAGACGAGGCATT
HPV11-E6R	CCACCTTGTCACCTCATCT

the prototype and all sequences obtained was performed using the CLUSTAL W software nested in the Bioedit 7.0.9.0 package (Hall, 1999; Thompson et al., 1997). All sequences were edited using Bio Edit (Hall, 1999) in order to remove vector fragments and analyze solely the complete sequence of the E6 gene. HPV-6 and HPV-11 nucleotide sequences generated in this study were submitted to GenBank (accession numbers: KC285838–KC285862).

2.4. In silico secondary structure analysis

The Predict Protein Server <<http://www.predictprotein.org>> (Rost et al., 2004) was used to compare the secondary structures of the E6 protein of HPV-6vc and the HPV-6vc variant with the amino acid change.

2.5. Phylogenetic analysis

Phylogenetic analysis was performed using the PhyML tool available online at South of France Bioinformatics Platform (<<http://www.atgc-montpellier.fr/phyml/>>) (Guindon et al., 2010; Guindon and Gascuel, 2003). Maximum-Likelihood trees were constructed using the TrN model for HPV-6 sequences and the HKY85 model for HPV-11 sequences. Models were determined using Modeltest 3.7 (Posada and Crandall, 1998) run in PAUP 4.0 beta10 (Swofford, 2002). Bootstrap was calculated based on 1,000 replicates, and values above 70% were considered significant.

The phylogenetic analysis included all 25 E6 sequences obtained in this study in addition to other full length E6 sequences reported previously: 77 and 12 HPV-6 samples isolated in Slovenia (Kocjan et al., 2009) and in South Africa (Combrinck et al., 2011), respectively, and 63 HPV-11 samples obtained in Slovenia (Maver et al., 2011). Sequences described by others were isolated from different anatomic sites: laryngeal papillomas, genital warts, cervical cancer and anal swabs.

3. Results

Our study used data from 25 patients diagnosed with RRP. Details from the patients, including clinical status and the number of surgical procedures performed, are presented in Table 2. The disease was classified as severe when more than three procedures per year were required with Derkey scores higher than 20. Only patients BR_LP19 and BR_LP 20 presented with severe disease.

All 25 biopsy specimens were positive for HPV DNA (100%). HPV-6 was detected in 18 (72%) cases, and HPV-11 was detected in 7 (28%) cases. All 25 isolates were successfully amplified and sequenced across the entire E6 genomic region (nt positions 102–554). HPV-6 E6 molecular variant classification was conducted through a comparison with the prototype HPV-6b (GenBank accession number X00203) and the non-prototypes HPV-6a (GenBank accession number L41216) and HPV-6vc sequences (GenBank accession no. AF092932). HPV-11 E6 variants were characterized by comparing obtained sequences with the HPV-11 prototype genome (GenBank accession number M14119).

3.1. HPV-6 and HPV-11 E6 molecular variants

Among the 18 HPV-6 samples analyzed, 17 isolates (94.4%) were found to possess a high identity to non-prototypic sequences (HPV-6a and HPV-6vc). A total of four E6 genomic variants were identified: BR_LP12, BR_LP8, Ref 6vc, and BR_LP14 (Fig. 1A). The Ref 6vc variant was the most prevalent – it was detected in 48% of the samples. Sequence analysis of the entire E6 gene revealed two synonymous point mutations compared to the prototype HPV-6b genome: A120T and G372A. Five isolates were identical to the non-prototype HPV-6a sequence with the exception of the G327A silent mutation detected in all samples. The nucleotide substitution A296G was detected in 2/12 HPV-6vc-related isolates (BR_LP14 and BR_LP16). This substitution resulted in an amino acid change from aspartate to glycine, a change which corresponded to an alteration rate of 0.66% within the entire E6 protein.

Table 2
Characterization of samples and clinical presentation of patients with recurrent respiratory papillomatosis.

HPV-Type	ID Sample	Gender	Age at diagnosis (years)	Age at last follow up (years)	Total procedures since diagnosis	Average procedures/year	Derkey score	Disease severity
HPV-6	BR_LP1	F	51	51	1	1	08	Mild
	BR_LP2	F	4	4	3	3	13	Mild
	BR_LP3	F	3	5	9	4.5	17	Mild
	BR_LP4	***	***	***	***	***	***	***
	BR_LP5	M	1	6	10	2	16	Mild
	BR_LP6	F	5	8	6	2	26	Mild
	BR_LP7	F	5	6	5	5	15	Mild
	BR_LP8	M	23	25	2	1	07	Mild
	BR_LP9	M	30	36	4	0.7	07	Mild
	BR_LP10	M	32	33	2	2	07	Mild
	BR_LP11	M	newborn	1	4	4	16	Mild
	BR_LP12	M	28	28	2	2	13	Mild
	BR_LP13	M	32	32	1	1	10	Mild
	BR_LP14	F	8	8	1	1	13	Mild
	BR_LP15	M	41	42	3	3	11	Mild
	BR_LP16	M	47	47	1	1	13	Mild
	BR_LP17	M	31	31	3	3	10	Mild
	BR_LP18	M	45	46	3	3	05	Mild
HPV-11	BR_LP19	M	7	13	22	3.7	22	Severe
	BR_LP20	F	2	3	6	6	21	Severe
	BR_LP21	F	2	5	9	3	15	Mild
	BR_LP22	M	2	5	1	0.3	06	Mild
	BR_LP23	M	3	3	2	2	20	Mild
	BR_LP24	M	29	31	5	2.5	31	Mild
	BR_LP25	M	33	33	4	1	04	Mild

***Data unavailable.

A		E6 nucleotide position					Number of samples	Disease Severity	
		1	2	2	2	3		Mild	Severe
		2	9	9	9	7			
HPV 6		0	5	6	7	2			
6b	Ref. 6b	A	G	A	C	G			
	BR_LP12	T				A	1	1	
6a	Ref. 6a	T							
	BR_LP8; BR_LP9; BR_LP10; BR_LP13; BR_LP915					A	5	5	
6vc	Ref. 6vc	T				A	10	10	
	BR_LP14; BR_LP16			G			2	2	
Codon				9					
				9					
Prototype aa				D					
Mutated aa				G					

B		E6 nucleotide position		Number of Samples	Disease Severity	
		0	2		Mild	Severe
		3	7			
HPV11		6	9			
Ref. HPV11		T	C	4	3	1
BR_LP19; BR_LP21; BR_LP24		C	T	3	2	1

Fig. 1. Summary of point substitutions identified in the E6 coding region. Sequence variation of the E6 gene of HPV-6 (A) and HPV-11 (B). At the top, nucleotide positions are indicated vertically. Nucleotide positions without mutations compared to the reference sequences are shaded in grey. At the bottom, codon positions and amino acid changes (aa) compared to reference sequence HPV-6vc are indicated. The column “number of samples” indicates the number of isolates identical to the variants specified in each lane. The last column correlates the number of each isolates with disease severity. The nucleotides at positions 295, 296 and 297 of HPV-6 represent the full codon of amino acid in the position 99.

The maximum genetic distance between the variants detected and the reference HPV-6b sequence was two nucleotide (0.44% of the entire gene). Two distinct HPV-11 molecular variants were detected: the prototype and the BR_LP19 (Fig. 1B), which is characterized by two synonymous nucleotide substitutions (T36C; C279T).

The 18 patients positive for HPV-6 and five patients for HPV-11 presented mild disease. Severe disease was observed only in two patients infected by HPV-11 (BR_LP19 and BR_LP_20). These patients presented different variants.

3.2. Secondary structure analysis

We analyzed the secondary structure of the HPV-6vc ref and the isolate harboring the A296G substitution (D99G). The physicochemical properties of aspartate and glycine are different because the former is a polar amino acid while glycine is apolar. The *in silico* analysis showed an alteration in the secondary structure composition of the HPV-6vc mutated protein over the reference. The percentage of β -sheet and of coil structures was

13.3% and 36.0%, respectively, and 14.67% and 34.67% for the mutated and the reference protein, respectively. There was no divergence in the percentage of α -helix structures among these variants.

3.3. Phylogenetic analysis of the HPV-6 E6 molecular variants

Phylogenetic analysis included all 18 HPV-6 E6 sequences obtained in this study, in addition to E6 sequences retrieved from Kocjan et al. (2009) ($n = 77$) and Combrinck et al. (2011) ($n = 12$).

The phylogenetic tree segregated into two major branches (Fig. 2). The HPV-6b prototype sequence and 13 HPV-6b related isolates clustered together in a monophyletic branch. The second major branch was subdivided into two sub-clusters: one that grouped together with HPV-6a related sequences and another that enclosed all HPV-6vc-related sequences. The 18 HPV-6 E6 sequences that we described did not group together. We observed that overall sequences did not cluster according to their geographical origin, nor did they cluster according to the anatomic site of infection.

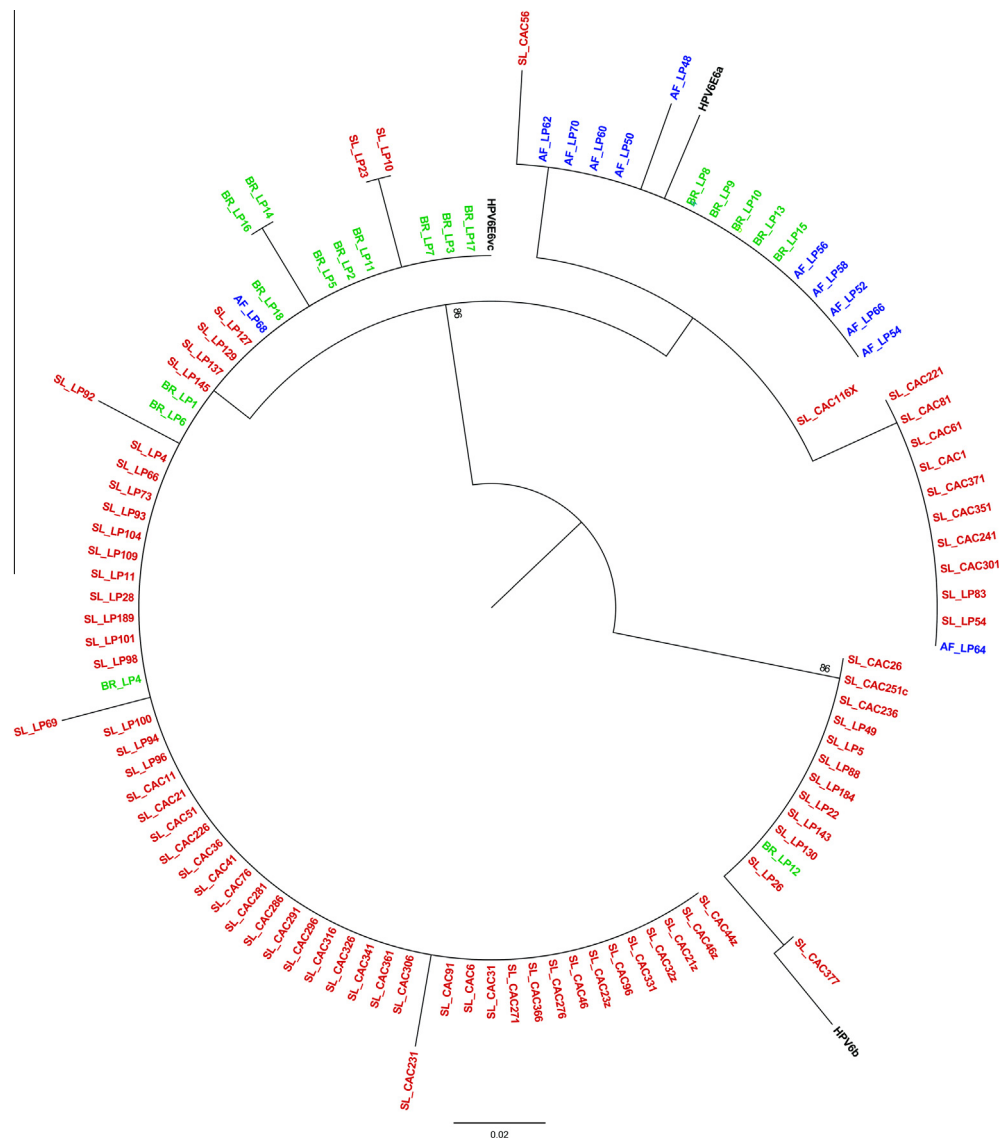


Fig. 2. Phylogenetic tree of HPV-6 variants. Unrooted Maximum Likelihood phylogenetic tree of HPV-6 E6 variants from Slovenia (red), Brazil (green), Africa (blue), and reference sequences (black), constructed with the TrN substitution model. Bootstrap was performed with 1,000 replicates. Values above 70% were considered significant. The prefix of each isolate represents the anatomic site of infection. (CAC: condyloma acuminata; LP: laryngeal papillomatosis). (For interpretation of color in this Figure, the reader is referred to the web version of this article).

3.4. Phylogenetic analysis of the HPV-11 E6 molecular variants

A phylogenetic tree was generated including all seven E6 isolates that we obtained. We also included the data from the 63 samples detected in Slovenia (Maver et al., 2011). As observed for the HPV-6 phylogenetic tree, HPV-11 sequences from Brazil did not cluster together (Fig. 3).

4. Discussion

HPV E6 has been described as an intrinsically disordered protein, which implies that small changes in the protein can strongly impact its tertiary structure and function (Donne et al., 2010; Uversky et al., 2006). In fact, the influence of E6 nucleotide variability upon the oncogenic potential was described more than two decades ago. It was observed that HPV-6 E6 was unable to replace HPV-16 E6 immortalizing activity in cooperation with HPV-16 E7 (Halbert et al., 1991). Differences in E6 transformation ability were even observed among high-risk HPV types (Villa and Schlegel,

1991), and at a more refined level among HPV-16 molecular variants (Richard et al., 2010; Sichero et al., 2012).

In this study, we describe the E6 early gene diversity of HPV-6 and HPV-11 isolated from 25 patients diagnosed with RRP. This work is the most extensive investigation concerning low-risk HPV nucleotide diversity in Brazil. Sequences obtained were compared to the prototypic HPV-6b and non-prototypic HPV-6a and HPV-6vc isolates. We identified four different E6 molecular variants, of which only one variant harbored an amino acid change. This alteration is described for the first time, and functional implications resulting from this variation need further analyses. It would be important to access the variant using bioinformatic tools if the tertiary structure of the new variant with the amino acid change D99G detected in this study could result in any important alteration in the protein's structure or function.

The E2 viral protein is the main regulator of viral gene transcription and affects viral expression by binding to specific elements within the LCR. Furthermore, E2 is involved in viral DNA replication and interacts with and recruits E1 to the origin. Thus,

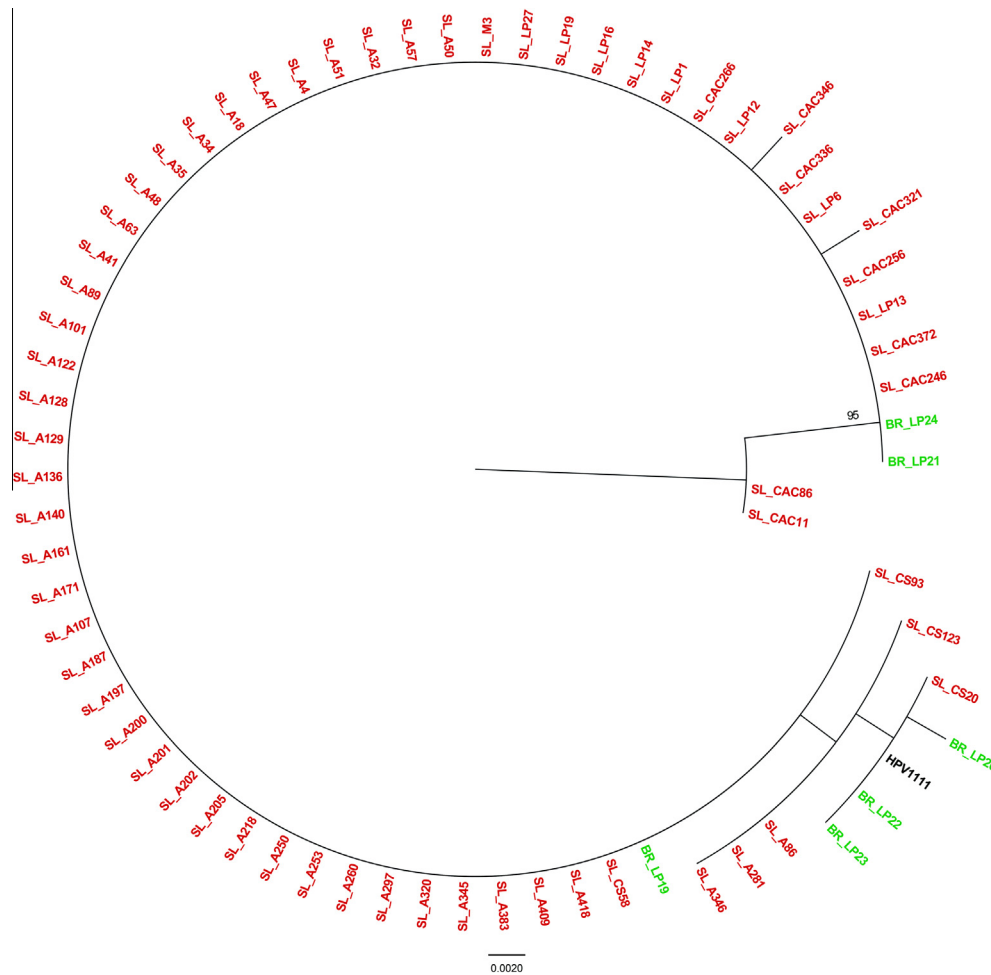


Fig. 3. Phylogenetic tree of HPV-6 variants. Unrooted Maximum Likelihood phylogenetic tree of HPV-11 E6 variants from Slovenia (red), Brazil (green), and reference sequences (black). Constructed with the HKY85 substitution model. Bootstrap was performed with 1,000 replicates. Values above 70% were considered significant. The prefix of each isolate represents the anatomic site of infection. (CAC: condyloma acuminata; LP: laryngeal papillomatosis; CS: Swab Cervical; A: Swab Anal). (For interpretation of color in this Figure, the reader is referred to the web version of this article).

it is reasonable to suppose that mutations in these regions may affect the clinical outcome of the infection. Among HPV-16 molecular variants, the influence of E2 variability upon the transcriptional transactivation potential has been described. We also observed that specific HPV-16 variants exhibit lower replication efficiency than others.

Although we have only sequenced the E6 gene of the HPV-6 and -11 variants analyzed in this study, the E2 and E1 viral nucleotide sequences of these isolates could be presumed to be a strong inter-gene sequence co-variation observed for HPV and the complete viral genome sequences of several variants of these viral types are available (Burk et al., 2011). However, no functional analyses of these proteins have been conducted, which, as of yet, prevents any further conclusions concerning the impact of the variability that we observed upon viral transcription and replication.

Among the individuals analyzed, only two were diagnosed with severe disease: BR_LP19 and BR_LP20. Both of these patients were positive for HPV-11, results which are in line with the opinion of several otolaryngologists who believe that HPV-11-associated disease is more aggressive than cases harboring HPV-6 (Wiatrak et al., 2004). However, the small number of samples analyzed prevents the correlation of disease severity with any particular E6 sequence.

In our results, we found four different nucleotide substitutions that did not result in any amino acid changes. Although synonymous changes are not reflected in the amino acid composition of

the protein, they represent an important aspect of papillomavirus (PV) evolution due to a strong codon usage bias (Zhao and Chen, 2011; Zhao et al., 2003, 1999). The codon preference in virus is driven by co-evolution with hosts by adapting to immune response and tissue tropism (Zhao and Chen, 2011). Interestingly, the preference of codon observed in PV is significantly different from the one seen in mammalian proteins, which allows the virus to use tRNAs that are being used less (Wada et al., 1990; Zhou et al., 1999). Though this strategy relies on using a less advantageous codon, it also avoids impairing host cell protein synthesis, which is important for the production of viral proteins (Aragones et al., 2010). Two synonymous mutations found in this study, one for HPV-6 and one for HPV-11, changed a codon preferred by humans to one chosen by papillomavirus (HPV-6 - A120T and HPV-11 - C279T). Conversely, a synonymous change found in HPV-11 altered a codon found in higher frequencies in PV to one used more often during human protein synthesis (HPV-11 - T36C). Studies converting rare codons to those more frequently used in mammals showed up to 100-fold increases in protein expression levels while keeping the levels of mRNA constant (Cid-Arregui et al., 2003; Disbrow et al., 2003; Mossadegh et al., 2004). Further studies showed that expression levels are related to tRNA abundance (Gu et al., 2004). To determine whether the synonymous mutations that we described have any impact in E6 protein expression, additional analysis is required.

Most of the HPV-6 isolates identified in this study have identical sequences to those previously described among laryngeal papillomatosis and *condyloma acuminata* specimens obtained in Slovenia and Africa (Combrinck et al., 2011; Kocjan et al., 2009). Comparative analysis revealed that 12.35% and 10.1% of the HPV-6 isolates described by Kocjan et al. (2009) and Combrinck et al. (2011) were identical to variants BR_LP 12 and BR_LP8, respectively. Additionally, among the 63 HPV-11 sequences characterized by Maver et al. (2011), 82.5% were identical to the BR_LP19 variant that we described. Maver et al. (2011) evaluated the nucleotide diversity of HPV-11 among 63 isolates and identified six E6 molecular variants, of which only one resulted in amino acid substitutions. We also consistently observed low diversity of the HPV-11 E6 protein. No geographical clustering was observed in the phylogenetic analysis of HPV-11 isolates. This same pattern was described by Kocjan et al. (2009), results which strongly suggest that HPV-6 and HPV-11 variants are detected with similar frequencies around the world. Furthermore, phylogenetic analysis of a 252 bp segment of the LCR of 62 isolates collected worldwide (Brazil, Germany, India, Italy, Japan, New York, Senegal and Singapore) revealed that HPV-6 and HPV-11 variant distribution show only minor correlations between geographic origin and ethnicity of the population studied (Heinzel et al., 1995).

Nucleotide analysis of worldwide collections of HPV-18 and HPV-16 isolates points toward co-evolution of these viruses with the three major ethnic groups: Africans, Caucasians, and East Asians. In contrast to the phylogenetic analysis of HPV-6 and HPV-11, HPV-16 and -18 LCR molecular variants robustly segregate into five major variant lineages: European (E), Asian (As), Asian-American (AA), and two African lineages (Af1 and Af2) (Ho et al., 1993; Ong et al., 1993). The distribution of HPV-16 variants worldwide varies significantly and correlates with the intrinsic admixture level of each population. In fact, the results of the analysis of HPV-16 and HPV-18 isolates detected in Brazil reflect the historic colonization of Brazil by European and Africans (Sichero et al., 2007).

Unfortunately, the small number of samples analyzed prevents the evaluation of the association between specific molecular variants and the anatomical site of infection. In contrast, an uneven distribution of HPV-16 variants among cervical and tonsillar cancer was observed in Stockholm (Du et al., 2012).

We observed a 25% amino acid variation rate (1/4 nt substitution resulted in amino acid substitutions) for HPV-6 samples, and no amino acid variation was found in the case of HPV-11 samples. However, Vrtacnik Bokal et al. (2010) and Shang et al. (2011) studied HPV-16 E6 found an amino acid variation rate of 62.5% (10/16) and 57.69%, (30/52) respectively. We do not know the reason for this divergence.

5. Conclusions

In this study, we demonstrated the low genomic diversity of HPV-6 and HPV-11 E6 sequences. A total of four different HPV-6 genomic variants and two HPV-11 genomic variants were identified. However, it was not possible to correlate disease severity with any particular variant. We could not establish an association between geographical location HPV-6 or HPV-11 variants. *In silico* analysis showed that the amino acid mutation detected in our study has an impact on the protein's secondary structure. Functional studies are required to evaluate the influence of this mutation on replication and protein expression as well as on the impact on the tertiary structure. Additionally, LCR variability may be linked to alterations in coding regions of the genome, which could, in turn, influence the risk of disease recurrence and may potentially indicate a more severe disease process in RRP.

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