View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

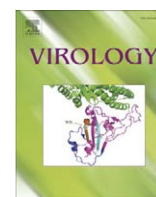
provided by Elsevier - Publisher Connector

Virology 391 (2009) 274–283

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro



Prevaccination genomic diversity of human papillomavirus genotype 6 (HPV 6)

Boštjan J. Kocjan [a], Mario Poljak [a], Mojca Cimerman [a], Nina Gale [b], Marko Potočnik [c], Željka Bogovac [a], Katja Seme [a,]*

[a] Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, Zaloška 4, 1105 Ljubljana, Slovenia
[b] Institute of Pathology, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia
[c] Department of Dermatovenereology, University Medical Centre Ljubljana, Ljubljana, Slovenia

ARTICLE INFO

Article history:
Received 20 April 2009
Returned to author for revision 2 June 2009
Accepted 17 June 2009
Available online 12 July 2009

Keywords:
Human papillomaviruses
HPV
HPV 6
Genomic diversity
Genomic variants
Genital warts
Laryngeal papillomas
Vaccine
Slovenia

ABSTRACT

Prevaccination genomic diversity of human papillomavirus genotype 6 (HPV 6) was established by sequencing 3798 bp of 77 clinically important HPV 6 isolates obtained from 45 and 32 patients with genital warts and laryngeal papillomas, respectively. By analyzing pooled L1, LCR, E6, E2, and E5 nucleotide data of an individual isolate, a total of 36 different genomic variants were identified, of which six (12 isolates), one (one isolate) and 29 (64 isolates) corresponded to HPV 6b, HPV 6a, and HPV 6vc genetic lineages, respectively. Several novel, potentially important mutations were identified. Non-prototypic HPV 6vc genomic variants were found in the majority of genital warts and laryngeal papillomas included in the study. The presence of serious HPV 6 genome sequence errors was confirmed and novel sequence errors were identified in sequence repositories.

© 2009 Elsevier Inc. All rights reserved.

Introduction

Human papillomavirus genotype 6 (HPV 6) is one of the most important HPV genotypes. Next to HPV 11, it is the major causative agent of genital warts and laryngeal papillomas, the most frequent benign tumours in the anogenital region and lower respiratory tract, respectively (Gissmann et al., 1983; Gale et al., 1994; Brown et al., 1999; Gale, 2005; Gale and Zidar, 2006; Potočnik et al., 2007). According to the latest papillomavirus classification criteria, HPV 6 is placed in the alpha genus — species 10, together with HPV 11, HPV 13, HPV 44, and HPV 74 (de Villiers et al., 2004). Based on its clinical association mainly with benign lesions, HPV 6 is regarded as a “low-risk” HPV genotype (reviewed in Grassmann et al. (1996)). HPV 6 is one of the four primary targets of the recently introduced quadrivalent HPV vaccine (Barr and Tamms, 2007; Garland et al., 2007).

HPV 6 was discovered by Southern blot hybridization in a tissue specimen of condyloma acuminata (Gissmann and zur Hausen, 1980). The complete genome, later designated HPV 6b, was cloned in 1981 (de Villiers et al., 1981) and fully sequenced and completely characterized two years later (Schwarz et al., 1983). Molecular studies performed in the years following the discovery of this virus demonstrated that HPV 6 is polymorphic and consists of several genomic variants. The focus of these studies has been on certain portions of the HPV 6 genome, particularly the long-control region (LCR), which contains regulatory elements for viral transcription and replication, and the coding regions for L1, L2, E6, E7, and E2 proteins (Rando et al., 1986; Kasher and Roman, 1988; Farr et al., 1991; Icenogle et al., 1991; Yaegashi et al., 1993; Kitasato et al., 1994; Heinzel et al., 1995; Roman and Brown, 1995; Grassmann et al., 1996; Suzuki et al., 1997; Caparros-Wanderley et al., 1999; Kovelman et al., 1999). In addition to HPV 6b, complete genomes of two closely related HPV 6 isolates, designated HPV 6a and HPV 6vc, have been cloned and fully characterized (Hofmann et al., 1995; Kovelman et al., 1999). On the basis of nucleotide sequence comparisons, HPV 6 isolates are usually grouped into prototype HPV 6b-related (prototypic) and HPV 6a/6vc-related (non-prototypic) genomic variants (Heinzel et al., 1995; Grassmann et al., 1996; Caparros-Wanderley et al., 1999; Kovelman et al., 1999). Non-prototypic HPV 6 genomic variants seem to predominate in genital warts (Gissmann et al., 1983; Rubben et al., 1992; Brown et al., 1993; Krige et al., 1997; Suzuki et al., 1997; Caparros-Wanderley et al., 1999).

Knowledge about the natural genetic diversity of HPV 6 is fairly limited, since the majority of HPV 6 genomic diversity studies performed so far have only investigated a limited number of isolates, which were almost exclusively from genital warts, and studies have focused on a single HPV 6 genomic region or even its short part only. Thus, in the present study we further investigated genomic diversity

* Corresponding author. Fax: +386 1 543 7418.
E-mail address: katja.seme@mf.uni-lj.si (K. Seme).

0042-6822/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2009.06.030

of HPV 6 in the prevaccination era, in order to provide important data for future epidemiological, functional and molecular assay development and vaccination studies. To achieve this, approximately half of the genome (3798 bp) of 77 clinically important HPV 6 isolates (e.g., those causing disease) was sequenced. The nucleotide sequence alignments were used to identify HPV 6 genomic variants and to investigate the linkage of these variants across the L1, LCR, E6, E2, E5a and E5b genomic regions. In addition, the coding regions were examined in detail to determine the prevalence, extent and distribution of amino acid changes that might have important biological properties. The LCR genomic region was used to determine the relationship between genomic variants described in our study and those described previously (Heinzel et al., 1995). This study, carried out on the largest number of HPV 6 isolates to date, is believed to be the first extensive work on the genetic diversity of the HPV 6 genotype.

Results

All 77 HPV 6 isolates included in the study were successfully amplified and sequenced across the L1 (nt 5700–7495), LCR-E6 (nt 7233–650) and E2–E5 (nt 3142–4538) genomic regions. Genomic sequences were established for 541 bp of E2 ORF (nt 3289–3829), for the entire LCR genomic region and for the entire L1, E6, E5a, and E5b ORFs. HPV 6 L1, E6, E2 and E5 genomic variants were identified using the prototype HPV 6b genome (GenBank accession no. X00203) as a standard for comparison and nucleotide position numbering. For the determination of HPV 6 LCR genomic variants, an LCR sequence amended by inclusion of a 94 bp segment at position 7350 in the prototype HPV 6b genome (Heinzel et al., 1995) was used.

HPV 6 L1 genomic variants

A total of fifteen L1 genomic variants were identified among 77 HPV 6 isolates (Fig. 1). Prototypic and non-prototypic HPV 6 genomic variants were determined in 12/77 (15.6%) and in 65/77 (84.4%) isolates, respectively. The prototype L1 sequence was identified in only 6/77 (7.8%) isolates. Sequence analysis of the entire *L1* gene revealed 25 nucleotide exchanges between the variants and the prototype HPV 6 sequence. The maximum distance between the variants and the prototype sequence was 11 nucleotides (0.7% of the entire L1 ORF). Nucleotide substitutions altered the L1 amino acid sequence of one prototypic (6-L1-3, represented by two isolates) and two non-prototypic (6-L1-13 and 6-L1-14, represented by ten and one isolate, respectively) HPV 6 variants; up to two amino acids (0.4% of the L1 protein) were exchanged (Fig. 1).

HPV 6 LCR genomic variants

A total of fifteen LCR genomic variants were identified among 77 HPV 6 isolates (Fig. 1). Prototypic and non-prototypic HPV 6 genomic variants were determined in 12/77 (15.6%) and in 65/77 (84.4%) isolates, respectively. None of the isolates corresponded to the prototype LCR sequence. Mutations were observed in 39 genomic positions: 30 single nucleotide exchanges, one 6 bp and one 1 bp deletion, and two 20 bp, one 14 bp and one 1 bp insertions. The maximum genomic distance between the variants and the prototype sequence was 20 mutations, thereby affecting 2.5% (20/806) of the entire LCR genomic region.

HPV 6 E6 genomic variants

A total of ten E6 genomic variants were identified among 77 HPV 6 isolates (Fig. 1). Prototypic and non-prototypic HPV 6 genomic variants were determined in 12/77 (15.6%) and in 65/77 (84.4%) isolates, respectively. None of the isolates corresponded to the reference E6 sequence. Molecular analysis of the entire E6 ORF revealed 13 point mutations observed in 12 genomic positions. The maximum distance between the variants and the prototype sequence was 7 nucleotides (1.5% of the entire *E6* gene). Nucleotide substitutions altered the E6 amino acid sequence of all non-prototypic HPV 6 variants (65 isolates); up to two amino acids (1.3% of the E6 protein) were exchanged (Fig. 1).

HPV 6 E2 genomic variants

A total of nine E2 genomic variants were identified among 77 HPV-6 isolates (Fig. 2). Prototypic and non-prototypic HPV 6 genomic variants were determined in 12/77 (15.6%) and in 65/77 (84.4%) isolates, respectively. The reference E2 sequence was identified in 9/77 (11.8%) isolates. Sequence analysis of the 541 bp segment of E2 ORF revealed 23 nucleotide exchanges between the variants and the prototype E2 sequence. The maximum variant divergence from the prototype sequence was 16 nucleotides, thereby affecting 2.9% of the evaluated E2 nucleotide sequence. Point mutations altered the E2 amino acid sequence of one prototypic (6-E2-2, represented by one isolate) and all non-prototypic (65 isolates) HPV 6 variants. As shown in Fig. 2, up to 10 amino acids (5.6% of the analyzed part of the E2 protein) were exchanged.

HPV 6 E5a and E5b genomic variants

A total of sixteen E5a genomic variants were identified among 77 HPV 6 isolates (Fig. 2). Prototypic and non-prototypic HPV 6 genomic variants were determined in 12/77 (15.6%) and in 65/77 (84.4%) isolates, respectively. The reference E5a sequence was identified in 12/77 (15.6%) isolates. There were 22 point mutations observed in 19 genomic positions. The maximum genomic distance between the variants and the prototype sequence was 14 nucleotides, amounting to a diversity of 5.1% of the entire E5a ORF. Nucleotide substitutions altered the E5a amino acid sequence of all non-prototypic HPV 6 variants (65 isolates); up to seven amino acids (7.7% of the E5a protein) were exchanged (Fig. 2).

A total of nine E5b genomic variants were identified among 77 HPV 6 isolates (Fig. 2). Prototypic and non-prototypic HPV 6 genomic variants were determined in 12/77 (15.6%) and in 65/77 (84.4%) isolates, respectively. The prototype E5b sequence was identified in 10/77 (13.0%) isolates. Sixteen point mutations were observed in 15 positions. The maximum distance between the variants and the prototype sequence was 11 nucleotides, thereby affecting 5.0% of the entire E5b ORF. Point mutations altered the E5b amino acid sequence of all non-prototypic HPV 6 variants (65 isolates) — up to seven amino acids (9.7% of the E5b protein) were exchanged (Fig. 2).

HPV 6 genomic variants

The nucleotide data of L1, LCR, E6, E2, and E5 genomic regions were combined in individual isolates and HPV 6 genomic variants were determined. Thus, among 77 HPV 6 analyzed isolates, 36 different genomic variants were identified (Fig. 3). As shown in Fig. 3, strong intergenomic co-variation between LCR, late (*L1*), and early (*E6*, *E2*, *E5a*, and *E5b*) viral genes was observed in all analyzed isolates. Twelve (15.6%) HPV 6 isolates represented genomic variants of the prototype HPV 6 isolate (six genomic variants) while 65 (84.4%) isolates represented closely related genomic variants of HPV 6a (one genomic variant, represented by one isolate) and HPV 6vc (29 genomic variants) (Fig. 3).

Distribution of HPV 6 genomic variants in genital warts vs. laryngeal papillomas

A total of 22 different HPV 6 genomic variants were identified among 45 HPV 6 isolates from genital warts. Prototypic HPV 6 genomic

A

	L1 genomic positions																											
HPV 6		5923	5992	6052	6073	6104	6166	6211	6217	6430	6433	6598	6623	6637	6661	6721	6967	7015	7079	7099	7121	7126	7162	7168	7219	7232	7282	f
	Ref-6b-O	A	G	C	A	A	T	T	A	A	G	A	C	G	G	A	T	A	G	G	A	A	T	A	A	C	T	6
6b	6-L1-1											T								A								3
	6-L1-2											T		A						A								1
	6-L1-3						G					T								A						T		2
6a	Ref-6a	T	A	T	G	C						T			A										C			0
	6-L1-4	T	A	T	G	C						T			A										C		C	1
6vc	Ref-6vc	T		T	G				T		T	T	A		A										C		C	0
	6-L1-5	T		T	G				T			T	A		A										C		C	45
	6-L1-6	T		T	G			G	T			T	A		A										C		C	1
	6-L1-7	T		T	G				T	C		T	A		A								G		C		C	1
	6-L1-8	T		T	G				T			T	A		A		A								C		C	1
	6-L1-9	T		T	G				T			T	A		A			G							C		C	2
	6-L1-10	T		T	G				T			T	A		A							G			C		C	1
	6-L1-11	T		T	G				T			T	A		A								G		C		C	1
	6-L1-12	T		T	G				T			T	A		A									G	C		C	1
	6-L1-13	T		T	G				T			T	A		A	G			C						C		C	10
	6-L1-14	T		T	G				T			T	A		A				C		G				C		C	1
	Codon	45	68	88	95	106	126	141	143	214	219	270	279	283	291	311	393	409	431	437	445	446	458	460	477	482	498	
	Prototype aa	G	R	F	L	R	V	V	V	S	D	T	R	G	P	K	V	L	E	K	N	L	P	G	T	P	T	
	Mutated aa	G	R	F	L	R	V	V	V	S	Y	T	R	G	P	K	V	L	Q	K	D	L	P	G	T	S	T	

B

	LCR genomic positions																																									
HPV 6		7349	7356	7366	7392	7400	7410	7452	7467	7513	7514	7515	7516	7517	7518	7519	7520	7594	7608	7612	7616	7617	7618	7623	7628	7661	7673	7679	7696	7700	7747	7748	7812	7838	7840	7884	7909	7911	7954	0020	0082	f
	Ref-6b-H	G	C	T	A	C	A	C	G	C	C	T	C	T	T	G	T	G	T	T	T	A	A	C	C	A	C	A	T	C	G	C	C	T	A	C	G	A	G	T	A	0
6b	6-LCR-1								T																																	6
	6-LCR-2					A			T																			C			C	A							A			2
	6-LCR-3					A			T																			C			C	A					C		A			4
6a	6-LCR-4*					A		A	T	A		Δ	Δ	Δ	Δ	Δ	Δ				A	C	Δ	A	T	G			G	G	C	A	I3		C	A			A	I4		1
6vc	Ref-6vc	T		D1		A		A	T	A	I2											C	C	A	T	G			G	G		A	I3						A	I4		0
	6-LCR-5	T				A		A	T	A	I2											C	C	A	T	G			G	G		A	I3						A	I4		41
	6-LCR-6	T	G			A		A	T	A	I2											C	C	A	T	G			G	G		A	I3						A	I4		5
	6-LCR-7	T			I1	A		A	T	A	I2											C	C	A	T	G			G	G		A	I3						A	I4		2
	6-LCR-8	T				A	C	A	T	A	I2											C	C	A	T	G			G	G		A	I3						A	I4		1
	6-LCR-9	T				A		A	T	A	I2							A				C	C	A	T	G			G	G		A	I3						A	I4		1
	6-LCR-10	T				A		A	T	A	I2									G		C	C	A	T	G			G	G		A	I3						A	I4		1
	6-LCR-11	T				A		A	T	A	I2											C	C	A	T	G			G	G		A	I3	G					A	I4		1
	6-LCR-12	T				A		A	T	A	I2											C	C	A	T	G			G	G		A	I3						A	I4	C	1
	6-LCR-13	T				A		A	T	A	I2											G	C	A	T	G	G		G	G		A	I3			A			A	I4		9
	6-LCR-14	T				A		A	T	A	I2								G			G	C	A	T	G	G		G	G		A	I3			A			A	I4		1
	6-LCR-15	T				A		A	T	A	I2											C	C	A	T	G			G	G		A	I3			A		G	A	I4		1

C

	E6 genomic positions												
HPV 6		156	221	251	269	299	323	350	365	392	473	479	506	f
	Ref-6b-O	A	A	C	A	C	A	T	A	C	G	C	T	0
6b	6-E6-1		T											1
	6-E6-2		T								A			11
6a	Ref-6a		T	G			C		T	T		T		0
	6-E6-3		T	G					T	T	A	T		1
6vc	6-E6-4*		T	G			T		T	T	A			48
	6-E6-5		T	G	C		T		T	T	A			1
	6-E6-6		T	G		T	T		T	T	A			1
	6-E6-7		T	G			T	C	T	T	A			1
	6-E6-8		T	G			T		T	T	A		C	2
	6-E6-9	T	T	G			C		T	T	A			10
	6-E6-10		T	G			C		T	T	A			1
	Codon	19	40	50	56	66	74	83	88	97	124	126	135	
	Prototype aa	T	T	H	R	C	I	A	T	I	A	F	G	
	Mutated aa	S	T	Q	R	C	I	A	T	I	A	F	G	

variants were detected in 4/45 (8.9%) and non-prototypic HPV 6 genomic variants in 41/45 (91.1%) isolates. Similarly, a total of 20 different HPV 6 genomic variants were identified among 32 HPV 6 isolates from laryngeal papillomas. Prototypic and non-prototypic HPV 6 genomic variants were determined in 8/32 (25.0%) and in 24/32 (75.0%) isolates, respectively. The difference in the prevalence of prototypic vs. non-prototypic variants in genital warts and laryngeal papillomas, calculated by the two-tailed Chi-square test (SPSS for Windows v15.0.0.0), did not reach statistical significance ($P = .1091$).

LCR sequences of HPV 6 isolates vs. LCR sequences of HPV 6b, HPV 6a, and HPV 6vc

All 77 HPV 6 isolates included in the study, classified as HPV 6b, HPV 6a or HPV 6vc genomic variants, contained a 94 bp segment (D1), which is still missing after genomic position 7350 in the prototype HPV 6b genome in available sequence repositories (GenBank acc. no. X00203) (Fig. 4). In addition, none of the HPV 6 isolates corresponded to the corrected version of this sequence (Heinzel et al., 1995). The LCR sequence of a single HPV 6a-related isolate identified in this study fully matched the LCR sequence of HPV 6a (GenBank acc. no. L41216). In contrast, none of the 65 HPV 6vc-related isolates was identical to the LCR sequence of HPV 6vc (GenBank acc. no. AF092932). As shown in Fig. 4, all isolates, including the HPV 6b- and HPV 6a-related isolates, contained a 19 bp segment (D2), which is missing after genomic position 7367 in the HPV 6vc genome sequence in available sequence repositories.

Phylogenetic analysis

Phylogenetic analysis of a 264–268 bp segment of the LCR genomic region of (i) 77 Slovenian HPV 6 isolates, (ii) the prototype HPV 6b sequence, (iii) the HPV 6a and HPV 6vc reference isolates and (iv) 31 isolates of HPV 6 originating from 7 different geographic regions (Brazil, Germany, India, Italy, Japan, USA-NY, and Senegal), revealed a phylogenetic tree with two dichotomically separated clusters (Fig. 5). As shown in Fig. 5, one cluster (P) contained the prototype HPV 6b and 24 related HPV 6 isolates, which represented six separated variant sub-clusters (P1–P6). The second cluster (N) contained HPV 6a and HPV 6vc, and 83 related HPV 6 isolates, which represented 13 distinct variant sub-clusters (N1–N13). The HPV 6 isolate SN6-1 (sub-cluster In), which is positioned between clusters P and N, can be considered to be an intermediate genome between the prototypic and non-prototypic HPV 6 genomic variants (Heinzel et al., 1995). Of the 12 Slovenian HPV 6 isolates that grouped to cluster P, six were identical to the HPV 6 isolate from Germany (sub-cluster P1), four to HPV 6 isolates from Brazil, India and USA (sub-cluster P5), and two isolates were identical to the HPV 6 isolate from USA (sub-cluster P4). Of the 65 Slovenian HPV 6 isolates from cluster N, 52 grouped together with HPV 6 isolates from Brazil, Germany, Italy, Japan and Senegal (sub-cluster N1), 10 with HPV 6 isolates from Germany and Senegal (sub-cluster N6), and one isolate clustered to HPV 6 isolates from Germany and USA (sub-cluster N12). In addition, one HPV 6 isolate was identical to isolate HPV 6ma (sub-cluster N7) and one Slovenian HPV 6 isolate formed a unique (Slovenian) sub-cluster N4.

Discussion

In this study, we describe HPV 6 prevaccination genomic diversity over a span of 3798 nucleotides (app. half of the viral genome), among the highest number of HPV 6 isolates to date. Our data set complements and significantly expands previous knowledge of HPV 6 genomic variants based on targeted analysis of L1, LCR, E6, and E2 genomic regions. In addition, this work is the first investigation of genetic variability of the HPV 6 E5 genomic region.

Sequence analysis of the six different genomic regions of 77 Slovenian HPV 6 isolates revealed a strong intergenomic co-variation between the LCR region and L1, E6, E2, E5a, and E5b ORFs. Nucleotide signatures specific for the prototypic and non-prototypic HPV 6 genomic variant lineages were clearly defined for all sequences. These regions showed, as a main feature, fixed nucleotide variations in more than one position. Similarly, all coding regions, except the *L1* gene, showed at least one fixed amino acid location that was characteristic for the non-prototypic HPV 6 variant group. In contrast to L1, LCR, E2, and E5b genomic regions, E6 and E5a ORFs failed to supply sufficient information to distinguish HPV 6a and HPV 6vc genetic lineages. By analyzing pooled L1, LCR, E6, E2, and E5 nucleotide data of an individual isolate, a total of 36 different genomic variants were identified among 77 HPV 6 isolates, of which 6 (12 isolates), 1 (one isolate), and 29 (64 isolates) corresponded to HPV 6b, HPV 6a, and HPV 6vc genetic lineages, respectively.

The L1 protein is the HPV major capsid protein and is the main target of neutralizing antibody responses during natural infection. Amino acid alterations of the L1 protein could potentially have important biological consequences. A total of 15 L1 genomic variants were identified in this study, of which three, represented by ten HPV 6 isolates from genital warts and three HPV 6 isolates from laryngeal papillomas, had specific amino acid substitutions in L1 ORF. One L1 genomic variant had an amino acid substitution at position 7079 (E431Q), one at positions 7079 (E431Q) and 7121 (N445D), and one variant had a specific amino acid substitution at position 7232 (P482S). The single amino acid change E431Q that was found in 10/77 (14.3%) HPV 6 isolates has been reported by Caparros-Wanderley et al. (1999) as the most frequent mutation encountered among HPV 6 isolates from genital warts. To our knowledge, the amino acid substitutions N445D and P482S that were characteristic features of one and two HPV 6 isolates, respectively, are described for the first time in this study. Similar to E431Q, both mutations clustered in the C-terminal part of the HPV 6 L1 protein, which ranks highly as a potential antibody-reactive site and might constitute a linear B-cell epitope on the surface of the HPV 6 capsid (Jenison et al., 1989; Caparros-Wanderley et al., 1999; Modis et al., 2002). However, the clinical significance of these mutations needs to be determined in further studies.

A series of previous reports have described HPV 6 genomic variants that were characterized by sequence rearrangements, such as insertions and deletions, in the LCR region. It was suggested that these sequence alternations may affect the HPV 6 pathogenic properties by influencing the expression of viral genes. Surprisingly, functional assays of the LCR region of different HPV 6 isolates from benign and malignant tumors, including HPV 6b, HPV 6a, and HPV 6vc, revealed no major difference in the activities of their early promoters that are responsible for the expression of E6 and E7 oncogenes (Heinzel et al., 1995; Grassmann et al., 1996). However, specific

Fig. 1. Sequence variations of the L1 ORF (A), long-control region-LCR (B), and E6 ORF (C) of 77 Slovenian HPV 6 isolates. Genomic positions at which mutations were observed are indicated across the top vertically. Positions for which no variation was detected compared to the reference HPV 6b sequences (Ref-6b-O; GenBank acc. no. X00203) are shaded. Nucleotide substitutions, deletions, and insertions are marked with letters, Δ, and I, respectively. The LCR sequence coded Ref-6b-H refers to the reference LCR sequence, which has been amended by inclusion of a 94 bp segment (Heinzel et al., 1995). Ref-6a, Ref-6vc, 6-LCR-4*, and 6-E6-4* indicate nucleotide sequences identical to the HPV 6a (GenBank acc. no. L41216) or HPV 6vc (GenBank acc. no. AF092932). The frequency (f) indicates the number of isolates identified for each HPV 6 genomic variant. The change in amino acid (aa) compared to the reference HPV 6b sequences is provided. Insertions (I) and deletions (D): I1 = TATGTGTATATGTGTGTATA; I2 = TACATTATTGTATA; I3 = ATATGTTTATTGCCACTGCA; I4 = T; D1 = ATGTGTGTTGTATATATGT.

A — E2 genomic positions

	HPV 6	3387	3404	3433	3460	3490	3507	3514	3518	3540	3551	3604	3642	3692	3734	3764	3774	3781	3787	3793	3805	3814	3815	f
6b	Ref-6b-O	T	A	G	C	T	G	C	C	A	C	C	G	T	G	G	A	C	C	C	A	A	C	9
	6-E2-1					C																		2
	6-E2-2																G							1
6a	6-E2-3*	C	C	A	A						A	T	A	C	C	A		A	T	G	G			1
6vc	6-E2-4*	C	C	A	A				G		A	T	A		C	A			T	A	G		A	49
	6-E2-5	C	C	A	A		C		G		A	T	A		C	A			T	A	G		A	1
	6-E2-6	C	C	A	A				G		A	T	A		C	A			T	A	G	C	A	1
	6-E2-7	C	C	A	A				G		A	T	A		C	A				A	G		A	3
	6-E2-8	C	C	A	A			G	G	C	A	T	A		C	A			T	A	G		A	10
	Codon	222	228	237	246	256	262	264	266	273	277	294	307	324	338	348	351	353	355	357	361	364	365	
	Prototype aa	L	K	R	S	I	G	H	L	Q	R	S	R	S	D	D	K	P	T	S	G	S	L	
	Mutated aa	P	Q	R	S	I	A	Q	V	P	R	S	K	P	H	N	R	P	T	R	G	S	M	

B — E5a genomic positions

	HPV 6	3892	3898	3912	3934	3979	4003	4048	4090	4099	4100	4123	4125	4126	4136	4139	4141	4147	4149	4158	f
6b	Ref-6b-O	A	G	C	C	C	G	T	C	T	C	A	T	G	T	T	T	T	C	A	12
6a	Ref-6a		A		A		C	C							C		C		A		0
	6-E5a-1		A		A		C	C	T				G	T	C		C		A		1
6vc	6-E5a-2*		A		A		C	C			T	T	G	T	C		C	G	A		32
	6-E5a-3		A		A	T	C	C			T	T	G	T	C		C	G	A		1
	6-E5a-4		A		A		C	C			T	T	G	T	C	C^{β}	C^{β}	G	A		1
	6-E5a-5		A		A		C	C		G	T	T	G	T	C		C	G	A		1
	6-E5a-6		A		A		C	C		G	T	T	G	T	C	C^{β}	C^{β}	G	A		2
	6-E5a-7	T	A		A		C	C			T	T	G	T	C		C	A	A		1
	6-E5a-8		A		A		C	C			T	T	G	T	C		C	A	A		1
	6-E5a-9		A		A		C	C			G^{α}	T	G	T	C		C	G	A		1
	6-E5a-10		A		A		C	C				T	G	T	C		C	G	A		11
	6-E5a-11		A		A		C	C				T	G	T	C		C	G			1
	6-E5a-12		A		A		C	C		C		T	G	T	C		C				1
	6-E5a-13		A		A		C	C				T	G	T	C		C		A		1
	6-E5a-14		A	G	A		C	C					G	T	C		C		A		9
	6-E5a-15		A	G	A		C	C					G	T	C		C		A	C	1
	Codon	2	4	9	16	31	39	54	68	71	72	79	80	80	84	85	85	87	88	91	
	Prototype aa	E	V	A	F	I	E	Y	F	T	L	A	L	L	Y	Y	Y	V	T	Q	
	Mutated aa	D	V	G	L	I	D	Y	F	T	V	A	C	C	H	H	H	V	N	P	

C — E5b genomic positions

	HPV 6	4194	4234	4251	4257	4267	4275	4296	4304	4308	4313	4322	4342	4345	4346	4352	f
6b	Ref-6b-O	C	A	G	C	G	G	G	A	A	A	A	T	A	C	A	10
	6-E5b-1									G							2
6a	6-E5b-2*	A	G	A			C	C			C	C^{α}	C	G	A	C	1
6vc	6-E5b-3*		G	A			C	C						G	A	C	50
	6-E5b-4		G	A	T		C	C						G	A	C	1
	6-E5b-5		G	A		A	C	C						G	A	C	1
	6-E5b-6		G	A			C	C	C					G	A	C	1
	6-E5b-7		G	A			C	C				C^{α}	C	G	A	C	1
	6-E5b-8		G	A			C	C				G^{β}		G	A	C	10
	Codon	12	26	31	33	37	39	46	49	50	52	55	62	63	63	65	
	Prototype aa	T	M	L	H	V	G	K	N	K	N	D	Y	T	T	D	
	Mutated aa	T	V	L	H	I	G	N	T	K	T	A/G	H	D	D	A	

Genetic lineage	Genomic variant	L1 variant	LCR variant	E6 variant	E2 variant	E5a variant	E5b variant	f
HPV 6b	6-G1	6-L1-R	6-LCR-1	6-E6-2	6-E2-R	6-E5a-R	6-E5b-R	5
	6-G2	6-L1-R	6-LCR-1	6-E6-1	6-E2-2	6-E5a-R	6-E5b-R	1
	6-G3	6-L1-1	6-LCR-3	6-E6-2	6-E6-R	6-E5a-R	6-E5b-R	2
	6-G4	6-L1-1	6-LCR-3	6-E6-2	6-E6-1	6-E5a-R	6-E5b-R	1
	6-G5	6-L1-2	6-LCR-3	6-E6-2	6-E6-1	6-E5a-R	6-E5b-R	1
	6-G6	6-L1-3	6-LCR-2	6-E6-2	6-E2-R	6-E5a-R	6-E5b-1	2
HPV 6a	6-G7	6-L1-4	6-LCR-4	6-E6-3	6-E2-3	6-E5a-1	6-E5b-2	1
HPV 6vc	6-G8	6-L1-5	6-LCR-5	6-E6-4	6-E2-4	6-E5a-2	6-E5b-3	17
	6-G9	6-L1-5	6-LCR-7	6-E6-4	6-E2-4	6-E5a-2	6-E5b-3	1
	6-G10	6-L1-5	6-LCR-9	6-E6-4	6-E2-4	6-E5a-2	6-E5b-3	1
	6-G11	6-L1-5	6-LCR-10	6-E6-4	6-E2-4	6-E5a-2	6-E5b-3	1
	6-G12	6-L1-5	6-LCR-12	6-E6-4	6-E2-4	6-E5a-2	6-E5b-3	1
	6-G13	6-L1-5	6-LCR-5	6-E6-6	6-E2-4	6-E5a-2	6-E5b-3	1
	6-G14	6-L1-5	6-LCR-5	6-E6-4	6-E2-6	6-E5a-2	6-E5b-3	1
	6-G15	6-L1-5	6-LCR-5	6-E6-7	6-E2-7	6-E5a-2	6-E5b-3	1
	6-G16	6-L1-5	6-LCR-5	6-E6-4	6-E2-4	6-E5a-4	6-E5b-3	1
	6-G17	6-L1-5	6-LCR-7	6-E6-4	6-E2-4	6-E5a-5	6-E5b-3	1
	6-G18	6-L1-5	6-LCR-5	6-E6-4	6-E2-4	6-E5a-6	6-E5b-3	2
	6-G19	6-L1-5	6-LCR-11	6-E6-10	6-E2-4	6-E5a-8	6-E5b-3	1
	6-G20	6-L1-5	6-LCR-6	6-E6-5	6-E2-4	6-E5a-9	6-E5b-3	1
	6-G21	6-L1-5	6-LCR-5	6-E6-4	6-E2-4	6-E5a-10	6-E5b-3	8
	6-G22	6-L1-5	6-LCR-6	6-E6-4	6-E2-4	6-E5a-10	6-E5b-3	3
	6-G23	6-L1-5	6-LCR-6	6-E6-4	6-E2-4	6-E5a-11	6-E5b-3	1
	6-G24	6-L1-5	6-LCR-5	6-E6-4	6-E2-4	6-E5a-2	6-E5b-4	1
	6-G25	6-L1-5	6-LCR-5	6-E6-4	6-E2-4	6-E5a-7	6-E5b-5	1
	6-G26	6-L1-5	6-LCR-5	6-E6-4	6-E2-4	6-E5a-2	6-E5b-6	1
	6-G27	6-L1-6	6-LCR-8	6-E6-4	6-E2-5	6-E5a-13	6-E5b-3	1
	6-G28	6-L1-7	6-LCR-5	6-E6-4	6-E2-7	6-E5a-3	6-E5b-3	1
	6-G29	6-L1-8	6-LCR-5	6-E6-4	6-E2-4	6-E5a-2	6-E5b-3	1
	6-G30	6-L1-9	6-LCR-5	6-E6-8	6-E2-4	6-E5a-2	6-E5b-3	2
	6-G31	6-L1-10	6-LCR-5	6-E6-4	6-E2-4	6-E5a-2	6-E5b-3	1
	6-G32	6-L1-11	6-LCR-5	6-E6-4	6-E2-7	6-E5a-2	6-E5b-3	1
	6-G33	6-L1-12	6-LCR-5	6-E6-4	6-E2-4	6-E5a-2	6-E5b-3	1
	6-G34	6-L1-13	6-LCR-13	6-E6-9	6-E2-8	6-E5a-14	6-E5b-8	9
	6-G35	6-L1-13	6-LCR-14	6-E6-9	6-E2-8	6-E5a-15	6-E5b-8	1
	6-G36	6-L1-14	6-LCR-15	6-E6-4	6-E2-4	6-E5a-12	6-E5b-7	1

Fig. 3. HPV 6 genomic variants found among 77 Slovenian HPV 6 isolates. The frequency (f) indicates the number of isolates identified for each HPV 6 genomic variant.

mutations in this region could affect the efficiency of viral replication by influencing the expression of HPV 6 E2 and E1 replicating proteins, as recently reported for HPV 16 (Hubert, 2005). In this study, a total of 15 HPV 6 LCR genomic variants were identified, all of which clearly catalogued to the HPV 6b, HPV 6a, and HPV 6vc genetic lineages. A single HPV 6a genomic variant was identical to the LCR sequence of HPV 6a, while other variants differed from the LCR of HPV 6b or HPV 6vc by a maximum of seven mutations (Fig. 1). We have thus demonstrated significant conservation of the LCR genomic region among all three HPV 6 genetic lineages.

The published genomic sequence of the prototype HPV 6b isolate (GenBank acc. no. X00203) has been a matter of confusion, since it misses a 94 bp segment in the LCR region that is present in the majority of HPV 6 isolates characterized so far. In 1995, Heinzel et al. recloned the LCR genomic region of the original HPV 6b isolate and showed that this sequence is an intrinsic part of the HPV 6b genome and was probably deleted during propagation of its clone in bacteria (Heinzel et al., 1995). In line with this observation, we detected this 94 bp "insert" in all analyzed HPV 6 isolates; in most cases, it differed from the original insert by one or two point mutations (Fig. 4). In addition, the results of our study indicate that a cloning artifact might occur also in the original HPV 6vc isolate (GenBank acc. no. AF092932). Namely, all of the investigated HPV 6 isolates contained a 19 bp sequence insertion that is missing after position 7367 in the published HPV 6vc genome sequence (Fig. 4). This assumption is further supported by the fact that this part of the HPV 6vc LCR coincides with the part of the HPV 6b LCR region in which a 94 bp deletion occurred. In addition, the LCR sequences of all HPV 6 isolates representing genomic variant 6-LCR-5 fully matched the LCR sequence of the HPV 6vc isolate, except for the missing part (Fig. 1).

Many important functions have been attributed to the high-risk HPV E6/E7 proteins, including binding and degradation of p53, interacting with retinoblastoma (Rb) proteins, facilitating the proliferation of infected epithelial cells and, consequently, inducing cellular transformation (Doorbar, 2006). In contrast, the physiological roles of the HPV 6 E6/E7 proteins remain unclear (Oh et al., 2004). Similarly, it is not clear whether genomic variants of HPV 6 *E6*/*E7* genes encode viral proteins with altered pathogenic properties. Among 10 E6 genomic variants characterized in this study,

Fig. 2. Sequence variations of a 3′ half of the E2 ORF (nt 3289–3829) (A), E5a ORF (B), and E5b ORF (C) of 77 Slovenian HPV 6 isolates. 6-E2-3*, 6-E2-4*, 6-E5a-2*, 6-E5b-2*, and 6-E5b-3* indicate nucleotide sequences identical to the HPV 6a or HPV 6vc. See legend to Fig. 1 for details. Note to E5a genomic variants: G^{α}: the aa change [leucine (L) into valine (V)] at codon 72 is for G (guanine); C^{β}: aa change [tyrosine (Y) into histidine (H)] at codon 85 is for C (cytosine) at nt 4139 plus C at nt 4141, only. Note to E5b genomic variants: C^{α}: the aa change, aspartate (D) into alanine (A) at codon 55 is for C; G^{β}: the aa change, aspartate (D) into glycine (G) at codon 55 is for G.

```
                 7340      7350      7360      7370      7380      7390      7400      7410      7420      7430      7440
HPV-6b-H         TATATGTGTGTATGTACTGTTATATGTATGTGTGTTGTATATATGTGTGTATATATGTGTCTGTGTGTATATGTATATGTATGTGTTGTGTATATATATGTGTGTGTGT
HPV-6b-X00203    .........D1----------------------------------------------------------------------------------------------....
LP22             .............................................................................................................
LP184            .............................................................................................................
CAC377           .............................................................................................................
CAC236           .............................................................................................................
LP130            ............................................................A................................................
LP143            ............................................................A................................................
CAC251c          ............................................................A................................................
CAC26            ............................................................A................................................
HPV-6a-L41216    ............................................................A................................................
CAC56            ............................................................A................................................
HPV-6vc-AF092932 .........T.............D2-----------------..................A................................................
LP137            .........T..................................................A................................................
LP100            .........T..................................................A................................................
LP145            .........T..................................................A................................................
CAC301           .........T..................................................A................................................
CAC1             .........T..................................................A................................................
CAC61            .........T..................................................A................................................
CAC96            .........T..................................................A.........C......................................
CAC231           .........T.....G............................................A................................................
LP129            .........T.....G............................................A................................................
```

Fig. 4. Multiple alignment of a 5′ part of the LCR genomic region (nt 7340–7448) of the prototype HPV 6b (GenBank acc. no. X00203), HPV 6a (GenBank acc. no. L41216), and HPV 6vc (GenBank acc. no. AF092932) and HPV 6 isolates characterized in this study; for simplicity, only 18 representative HPV 6 isolates (LP22-LP129) are shown. The sequence coded HPV-6b-H refers to the original HPV 6b LCR sequence, which has been amended by inclusion of a 94 bp segment (Heinzel et al., 1995). D1: a segment of 94 bp (ATGTACTGTTATATGTATGTGTGTTGTATATATGTGTGTATATATGTGTCTGTGTGTATATGTATATGTATGTGTTGTGTATATATATGTGTGT); D2: a segment of 19 bp (ATGTGTGTTGTATATATGT).

eight (all non-prototypic genomic variants), harbored specific amino acid substitutions in the HPV 6 *E6* gene; seven variants had a substitution at position 251 (H50Q) and one variant had a specific substitution at positions 156 (T19S) and 251 (H50Q). Since the amino acid substitution H50Q was also found in the majority of HPV 6 isolates previously characterized (Grassmann et al., 1996), we presume that it prevails in naturally occurring HPV 6 populations. However, this amino acid exchange clustered outside current functionally characterized domains of the HPV E6 protein (reviewed in Grassmann et al., 1996); its meaning is therefore at present unclear.

HPV E2 protein is a critical regulator of viral transcription and replication. In a study by Kovelman et al. (1999) it has been shown that E2 ORF is divergent between the three fully characterized HPV 6 genomes: HPV 6b, HPV 6a and HPV 6vc. In addition, this study revealed several amino acid substitutions in the E2 protein that were characteristic of HPV 6a and HPV 6vc genetic lineages. Using the HPV 6b and HPV 6vc E2 proteins, very similar activities in transcriptional activation and repression assays in primary human keratinocytes were observed, suggesting that natural variants of the HPV 6 E2 protein do not affect its regulatory function (Kovelman et al., 1999). In this study, we analyzed the genetic variability of a 3′ part of the *E2* gene that encodes for the E2 hinge and DNA-binding/dimerisation domains. Among nine E2 genomic variants identified, six (represented by 65 HPV 6 isolates) had amino acid sequences identical to the E2 protein of HPV 6b, HPV 6a, or HPV 6vc isolates. Additional novel amino acid substitutions were identified in one prototypic (K351R, nt 3774) and in two HPV 6vc-related genomic variants (G262A, nt 3507; H264Q, nt 3514 and Q273P, nt 3540). It would, however, be of great interest to investigate further whether identified natural variants of the HPV 6 E2 protein affect its key role in controlling HPV 6 replication in conjunction with the E1 protein and its variants.

The biological functions of the HPV E5 protein and its role in the HPV replication cycle are not well understood. Documented functions include transformation and stimulation of the proliferation of HPV-infected cells and induction of koilocytosis in conjunction with the HPV E6 proteins (Doorbar, 2006; Krawczyk et al., 2008). In contrast to other alpha-PVs, which have one E5 ORF, HPV 6 possesses two *E5* genes: *E5a* and *E5b* (Schwarz et al., 1983; Hofmann et al., 1995; Kovelman et al., 1999). It has been shown previously that both genes are actively transcribed in laryngeal papillomas (Ward and Mounts, 1989), indicating that they may take part in productive HPV 6 infection. We determined a total of 16 E5a genomic variants in this study, of which eight (represented by 60 isolates) had an amino acid sequence identical to the E5a protein of HPV 6b or HPV 6vc isolates. Novel amino acid substitutions detected at positions 3892 (E2D), 3912 (A9G), 3912 (A9G) and 4158 (Q91P), 4100 (L72V), and 4139/4141 (Y85H) in the E5a ORF constituted additional characteristic features of an additional six HPV 6vc-related genomic variants. Similarly, in the remaining two HPV 6vc genomic variants, a novel HPV 6 E5a protein variant was identified; this protein differed from the HPV 6vc E5a protein by a single amino acid substitution in codon 88 (Fig. 2, variants 6-E5a-11 and 6-E5a-12). In contrast to E5a, only nine E5b genomic variants were identified, five of which, represented by 65 isolates, had amino acid sequences identical to the E5b protein of HPV 6b, HPV 6a, or HPV 6vc isolates (Fig. 2). Novel amino acid exchanges identified at positions 4267 (V37I), 4304 (N49T), 4322 (D55A) and 4342 (Y62H), and 4322 (D55G) in the E5b ORF were additional characteristic features of the remaining four HPV 6vc-related genomic variants. Whether the reported amino acid variations in the HPV 6 E5a and E5b ORFs affect the biological functions of E5 proteins remains unknown.

The results of this study for the first time allowed phylogenetic comparison of HPV 6 genomic variants found worldwide (Heinzel et al., 1995) with a relatively large number of HPV 6 isolates from one geographically and ethnically closed cohort of patients (i.e., Slovenian patients). This LCR based phylogenetic comparison showed that 76/77 (98.7%) Slovenian HPV 6 isolates were identical to several phylogenetically diverse HPV 6 genomes identified previously in other parts of the world. Thus, in contrast to HPV 16 and HPV 18, the existence of specific and geographically restricted HPV 6 genomic variants seems highly unlikely.

In summary, the first extensive work on the genomic diversity of the HPV 6 genotype showed a substantial prevaccination genomic diversity of this HPV genotype. A total of 36 different HPV 6 genomic variants were identified in this study. Non-prototypic HPV 6 genomic variants belonging to the HPV 6vc genetic lineage were found in the majority of genital warts and laryngeal papillomas included in the study. The presence of serious sequence errors was confirmed and novel sequence errors were identified in sequence repositories. We believe that our study will be helpful in future epidemiological, functional, molecular assay development and vaccination studies.

Materials and methods

Clinical samples

A total of 77 HPV 6 isolates were included in the study. Forty-five isolates were obtained from the same number of male patients suffering from exophytic genital warts on the glans of the penis, the

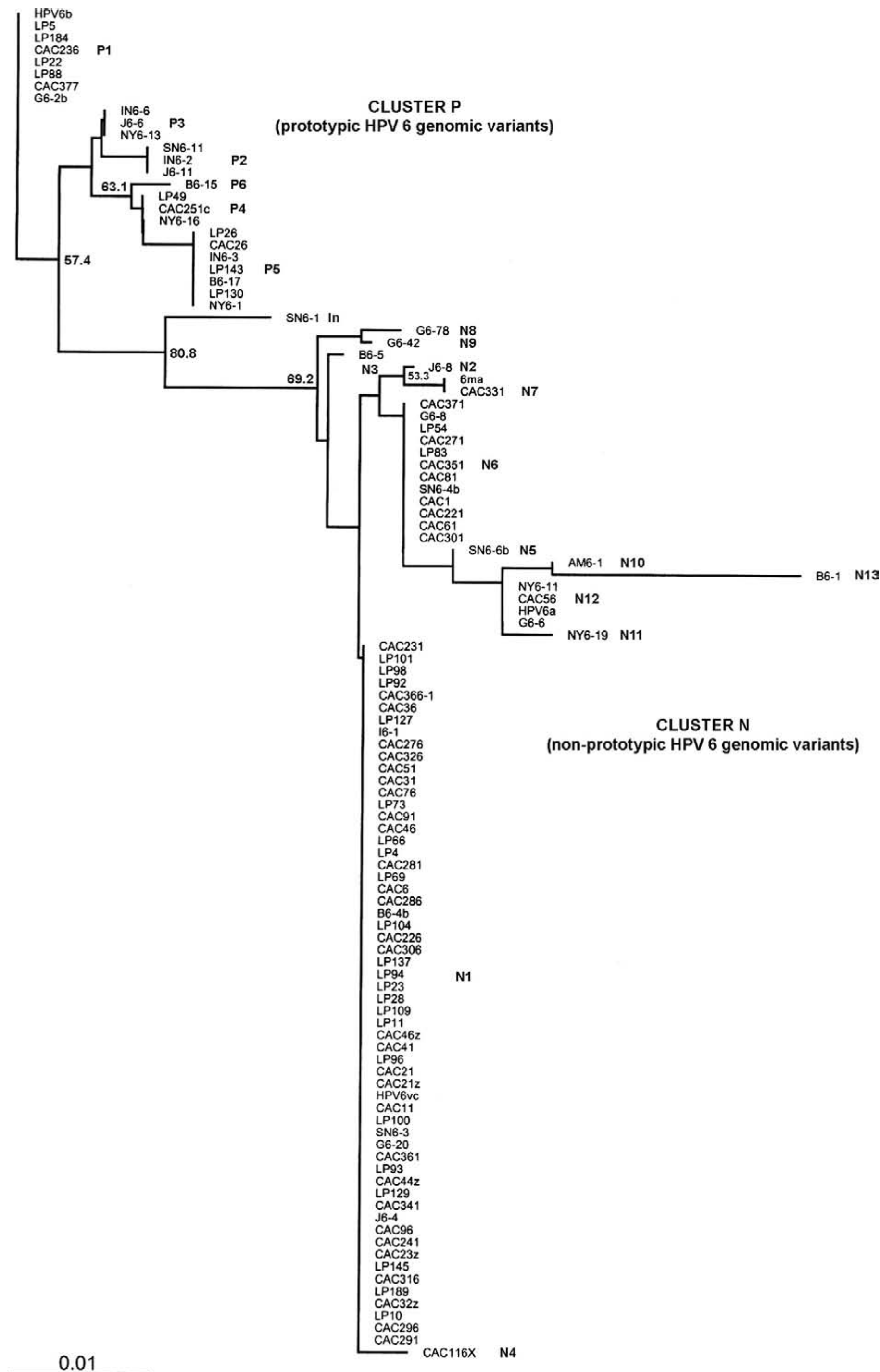


Fig. 5. Neighbor-joining algorithm based phylogenetic tree of HPV 6 LCR genomic variants from Slovenia (LP5-CAC116X), Brazil (AM6-1, B6-1, B6-4b, B6-5, B6-15, and B6-17), Germany (G6-2b, G6-6, G6-8, G6-20, G6-42, and G6-78), India (IN6-2, IN6-3, and IN6-6), Italy (I6-1), Japan (J6-4, J6-6, J6-8, and J6-11), USA-NY (NY6-1, NY6-11, NY6-13, NY6-16, and NY6-19), and Senegal (SN6-1, SN6-3, SN6-4b, SN6-6b, and SN6-11). Bootstrap values (%) above 50 are shown (1000 bootstrap replicates for each grouping of a tree).

coronal sulcus or the foreskin (Potočnik et al., 2007; Poljak et al., 2009) and 32 isolates were obtained from 17 male and 15 female patients suffering from laryngeal papillomas.

Amplification of HPV 6 L1, LCR-E6, and E2-E5 genomic regions

The genomic diversity of all HPV 6 isolates was investigated within the L1, LCR, E6, E2, E5a, and E5b regions of the HPV 6 genome. The 1796 bp fragment, which contained the complete L1 ORF (1503 bp) was amplified by PCR using the primer pair HPV6-L1F (5′-TGTTTTCAT-TACAGGTTCTGGA-3′, nt 5700–5721) and HPV6-L1R (5′-TAAACACA-CATACACATTACACAAA-3′, nt 7495–7471). The 1414–1430 bp fragment containing the complete LCR genomic region (806–822 bp) and complete E6 ORF (453 bp) was PCR amplified using the primer pair HPV6-LCRF (5′-CTGCTGTTTCCAAAGCCTCT-3′, nt 7233–7252) and HPV6-E6R (5′-CCACTTCGTCCACCTCATCT-3′, nt 650–631). The 1397 bp fragment encompassing the 3′ half of E2 ORF and the entire E5a (276 bp) and E5b (219 bp) ORFs was amplified by PCR using HPV6-E2F (5′-GGACAMTGACWCCTGGGTAAAG-3′, nt 3142–3163) and HPV6-E5R (5′-GTGTTGTGCTCCACCTTAG-3′, nt 4538–4520) primers. All primers

Table 1
List of primers used for sequencing the HPV 6 L1, LCR-E6, and E2-E5a-E5b PCR products.

Genomic region	Primer	Sequence (5′–3′)	Genomic position[a]
L1	HPV6-L1S	CGTAAACGTATTCCCTTATTTTTT (sense)	5761–5784
	HPV6-FS2[b]	CCCTGGACAGGATAAC (sense)	6190–6205
	HPV6-L1S1	TTCTACGGAAGGAACAAATG (sense)	6522–6541
	HPV6-L1S2	CAGACGTGCGATTTCCACTA (antisense)	6632–6613
	HPV6-FS5[b]	CCACACGCAGTACC (sense)	6783–6796
LCR-E6	HPV6-LCRS[c]	AATCCTATATATTTTGTGCCAGGT (sense)	7703–7726
	HPV6-LCRS1[c]	TTGGCAGGATATGATGCACT (antisense)	7774–7755
	HPV6-LCRS2	TGGTCTATGGTCGTTGCAGA (antisense)	145–126
	HPV6-E6S	ATAGGAGGGACCGAAAACG (sense)	26–44
E2–E5	HPV6-E2S	AATGTGCTGGTTGTTCCTGC (antisense)	3933–3914
	HPV6-E5S	GTGAGGAACAAAGGCAACAGT (sense)	3738–3758

[a] Nucleotide positions of primers were compared to the prototype HPV 6 genome (GenBank acc. no. X00203).
[b] Primers reported by Caparros-Wanderley et al. (1999).
[c] Nucleotide positions of these primers were compared to the corrected LCR sequence of the prototype HPV 6 (Heinzel et al., 1995).

used in the study were designed according to the genomic sequences of the prototype HPV 6b isolate (GenBank acc. no. X00203), and HPV 6a (GenBank acc. no. L41216) and HPV 6vc (GenBank acc. no. AF092932) isolates using Primer3 (http://frodo.wi.mit.edu/) and Netprimer (http://www.premierbiosoft.com) programs. The corrected LCR sequence of prototype HPV 6b (Heinzel et al., 1995) was used for designing and genomic position numbering of primers located in the HPV 6 LCR genomic region.

All PCR reactions were performed in 0.2 ml reaction tubes, each containing up to 200 ng of template DNA, 25 μl of High Fidelity PCR Master (Roche Diagnostics, Manheim, Germany), 300 nm of each of the "gene"-specific primers and water up to 50 μl. The cycling conditions used were 2 min at 94 °C, and 10 cycles of 10 s at 94 °C, 70 s at 55 °C and 1 min at 72 °C. This was followed by additional 25 cycles of 15 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C (the extension step increased by 5 s for each successive cycle). The final extension step was performed at 72 °C for 7 min and the reaction mixtures were cooled to 4 °C. All PCR amplifications were carried out on the GeneAmp® PCR instrument type 9700 (PE Applied Biosystems, Foster City, USA).

Sequencing and identification of HPV 6 genomic variants

The PCR products were analyzed on ready-to-use PCR CheckIT Wide Mini S-2x25 gels (Elchrom Scientific, Zurich, Switzerland) using a 500 bp DNA ladder (Roche Diagnostics) and purified with a QIAquick PCR purification kit (Qiagen, Hilden, Germany). Concentrations of purified amplicons were estimated on a 0.8% agarose gel (A9539-Agarose, Sigma-Aldrich, St. Louis, USA) using a High DNA Mass Ladder (Invitrogen, Carlsbad, USA) and set to 50 ng/μl. Sequencing of PCR products was performed at Macrogen, Ltd. (Seoul, Korea) with the same primers as those used for PCR. Additional sequencing primers that were also used are given in Table 1. HPV 6 L1, E6, E2, E5a, and E5b genomic variants were identified with the BioEdit Sequence Alignment Editor v7.0.5.3 (North Carolina State University, Raleigh, USA), using the genome of prototype HPV 6b isolate (GenBank acc. no. X00203) as a standard for comparisons and nucleotide position numbering. The corrected LCR sequence of prototype HPV 6b (Heinzel et al., 1995) was used for determination of HPV 6 LCR genomic variants.

Phylogenetic analyses of HPV 6 variants were based on multiple alignment of LCR sequences between HPV 6 genomic positions 7676 and 7939 (a segment of 264–268 bp) and performed using the Phylip program package (v3.65), as described previously (Heinzel et al., 1995); a neighbor-joining (NJ) method was used to construct a phylogenetic tree, which was visualized with the Treeview program (v1.6.6) of the University of Glasgow.

Nucleotide-sequence accession numbers

The HPV 6 nucleotide sequence data reported in this paper are deposited in the DDBJ, EMBL and GenBank databases under the following accession numbers: L1 sequences (FM876121–FM876165, FM897134–FM897165), LCR sequences (FM876166–FM876210, FM897166–FM897197), E6 sequences (FM875941–FM875985, FM897006–FM897037), E2 sequences (FM875986–FM876030, FM897038–FM897069), E5a sequences (FM876031–FM876075, FM897070–FM897101), and E5b sequences (FM876076–FM876120, FM897102–FM897133).

Acknowledgments

This study was partially supported by the Slovenian Research Agency — contract Z3-0220-0381-08.

References

Barr, E., Tamms, G., 2007. Quadrivalent human papillomavirus vaccine. Clin. Infect. Dis. 45, 609–617.

Brown, D.R., Bryan, J.T., Cramer, H., Fife, K.H., 1993. Analysis of human papillomavirus types in exophytic condylomata acuminata by hybrid capture and Southern blot techniques. J. Clin. Microbiol. 31, 2667–2673.

Brown, D.R., Schroeder, J.M., Bryan, J.T., Stoler, M.H., Fife, K.H., 1999. Detection of multiple human papillomavirus types in Condylomata acuminata lesions from otherwise healthy and immunosuppressed patients. J. Clin. Microbiol. 37, 3316–3322.

Caparros-Wanderley, W., Savage, N., Hill-Perkins, M., Layton, G., Weber, J., Davies, D.H., 1999. Intratype sequence variation among clinical isolates of the human papillomavirus type 6 L1 ORF: clustering of mutations and identification of a frequent amino acid sequence variant. J. Gen. Virol. 80, 1025–1033.

de Villiers, E.M., Gissmann, L., zur Hausen, H., 1981. Molecular cloning of viral DNA from human genital warts. J. Virol. 40, 932–935.

de Villiers, E.M., Fauquet, C., Broker, T.R., Bernard, H.U., zur Hausen, H., 2004. Classification of papillomaviruses. Virology 324, 17–27.

Doorbar, J., 2006. Molecular biology of human papillomavirus infection and cervical cancer. Clin. Sci. (Lond) 110, 525–541.

Farr, A., Wang, H., Kasher, M.S., Roman, A., 1991. Relative enhancer activity and transforming potential of authentic human papillomavirus type 6 genomes from benign and malignant lesions. J. Gen. Virol. 72, 519–526.

Gale, N., 2005. Papilloma/papillomatosis. In: Barnes, L., Eveson, J.W., Reichart, P., Sidransky, D. (Eds.), Pathology and Genetics of Head and Neck Tumours. IARC*Press*, Lyon, pp. 144–145.

Gale, N., Zidar, N., 2006. Benign and potentially malignant lesions of the squamous epithelium and squamous cell carcinoma. In: Cardesa, A., Slootweg, P.J. (Eds.), Pathology of the Head and Neck. Springer, Heidelberg, pp. 1–38.

Gale, N., Poljak, M., Kambič, V., Ferluga, D., Fischinger, J., 1994. Laryngeal papillomatosis: molecular, histopathologic, and clinical evaluation. Virchows Arch. 425, 291–295.

Garland, S.M., Hernandez-Avila, M., Wheeler, C.M., Perez, G., Harper, D.M., Leodolter, S., Tang, G.W., Ferris, D.G., Steben, M., Bryan, J., Taddeo, F.J., Railkar, R., Esser, M.T., Sings, H.L., Nelson, M., Boslego, J., Sattler, C., Barr, E., Koutsky, L.A., Females United to Unilaterally Reduce Endo/Ectocervical Disease (FUTURE) I Investigators, 2007. Quadrivalent vaccine against human papillomavirus to prevent anogenital diseases. N. Engl. J. Med. 356, 1928–1943.

Gissmann, L., zur Hausen, H., 1980. Partial characterization of viral DNA from human genital warts (Condylomata acuminata). Int. J. Cancer 25, 605–609.

Gissmann, L., Wolnik, L., Ikenberg, H., Koldovsky, U., Schnurch, H.G., zur Hausen, H., 1983. Human papillomavirus types 6 and 11 DNA sequences in genital and laryngeal papillomas and in some cervical cancers. Proc. Natl. Acad. Sci. U.S.A. 80, 560–563.

Grassmann, K., Wilczynski, S.P., Cook, N., Rapp, B., Iftner, T., 1996. HPV6 variants from malignant tumors with sequence alterations in the regulatory region do not reveal differences in the activities of the oncogene promoters but do contain amino acid exchanges in the E6 and E7 proteins. Virology 223, 185–197.

Heinzel, P.A., Chan, S.Y., Ho, L., O'Connor, M., Balaram, P., Campo, M.S., Fujinaga, K., Kiviat, N., Kuypers, J., Pfister, H., Steinberg, B.M., Tay, S.K., Villa, L.L., Bernard, H.U., 1995. Variation of human papillomavirus type 6 (HPV-6) and HPV-11 genomes sampled throughout the world. J. Clin. Microbiol. 33, 1746–1754.

Hofmann, K.J., Cook, J.C., Joyce, J.G., Brown, D.R., Schultz, L.D., George, H.A., Rosolowsky, M., Fife, K.H., Jansen, K.U., 1995. Sequence determination of human papillomavirus type 6a and assembly of virus-like particles in *Saccharomyces cerevisiae*. Virology 209, 506–518.

Hubert, W.G., 2005. Variant upstream regulatory region sequences differentially regulate human papillomavirus type 16 DNA replication throughout the viral life cycle. J. Virol. 79, 5914–5922.

Icenogle, J.P., Sathya, P., Miller, D.L., Tucker, R.A., Rawls, W.E., 1991. Nucleotide and amino acid sequence variation in the L1 and E7 open reading frames of human papillomavirus type 6 and type 16. Virology 184, 101–107.

Jenison, S.A., Yu, X.P., Valentine, J.M., Galloway, D.A., 1989. Human antibodies react with an epitope of the human papillomavirus type 6b L1 open reading frame which is distinct from the type-common epitope. J. Virol. 63, 809–818.

Kasher, M.S., Roman, A., 1988. Characterization of human papillomavirus type 6b DNA isolated from an invasive squamous carcinoma of the vulva. Virology 165, 225–233.
Kitasato, H., Delius, H., zur Hausen, H., Sorger, K., Rösl, F., de Villiers, E.M., 1994. Sequence rearrangements in the upstream regulatory region of human papillomavirus type 6: are these involved in malignant transition? J. Gen. Virol. 75, 1157–1162.
Kovelman, R., Bilter, G.K., Roman, A., Brown, D.R., Barbosa, M.S., 1999. Human papillomavirus type 6: classification of clinical isolates and functional analysis of E2 proteins. J. Gen. Virol. 80, 2445–2451.
Krawczyk, E., Suprynowicz, F.A., Liu, X., Dai, Y., Hartmann, D.P., Hanover, J., Schlegel, R., 2008. Koilocytosis: a cooperative interaction between the human papillomavirus E5 and E6 oncoproteins. Am. J. Pathol. 173, 682–688.
Krige, D., Mills, H.R., Berrie, E.L., Doherty, N.C., Jones, D.K., Ryan, C.A., Davies, H., Myint, S., McCance, D.J., Layton, G.T., French, T.J., 1997. Sequence variation in the early genes E1E4, E6 and E7 of human papilloma virus type 6. Virus Res. 49, 187–191.
Modis, Y., Trus, B.L., Harrison, S.C., 2002. Atomic model of the papillomavirus capsid. EMBO J. 21, 4754–4762.
Oh, S.T., Longworth, M.S., Laimins, L.A., 2004. Roles of the E6 and E7 proteins in the life cycle of low-risk human papillomavirus type 11. J. Virol. 78, 2620–2626.
Poljak, M., Kocjan, B.J., Potočnik, M., Seme, K., 2009. Anogenital hairs represent an important reservoir of alpha-papillomaviruses in patients with genital warts. J. Infect. Dis. 199, 1270–1274.
Potočnik, M., Kocjan, B.J., Seme, K., Poljak, M., 2007. Distribution of human papillomavirus (HPV) genotypes in genital warts from males in Slovenia. Acta Dermatovenerol. Alp. Panonica Adriat. 16, 91–98.
Rando, R.F., Groff, D.E., Chirikjian, J.G., Lancaster, W.D., 1986. Isolation and characterization of a novel human papillomavirus type 6 DNA from an invasive vulvar carcinoma. J. Virol. 57, 353–356.
Roman, A., Brown, D., 1995. Sequence variation in the extreme 5′ end of the human papillomavirus type 6a long control region. J. Infect. Dis. 171, 697–700.
Rubben, A., Beaudenon, S., Favre, M., Schmitz, W., Spelten, B., Grussendorf-Conen, E.I., 1992. Rearrangements of the upstream regulatory region of human papillomavirus type 6 can be found in both Buschke-Lowenstein tumours and in condylomata acuminata. J. Gen. Virol. 73, 3147–3153.
Schwarz, E., Dürst, M., Demankowski, C., Lattermann, O., Zech, R., Wolfsperger, E., Suhai, S., zur Hausen, H., 1983. DNA sequence and genome organization of genital human papillomavirus type 6b. EMBO J. 2, 2341–2348.
Suzuki, T., Tomita, Y., Nagata, H., Kono, A., Simizu, B., 1997. Nucleotide and amino acid sequence variations in the L1 open reading frame of human papillomavirus type 6. J. Med. Virol. 53, 19–24.
Ward, P., Mounts, P., 1989. Heterogeneity in mRNA of human papillomavirus type-6 subtypes in respiratory tract lesions. Virology 168, 1–12.
Yaegashi, N., Xi, L., Batra, M., Galloway, D.A., 1993. Sequence and antigenic diversity in two immunodominant regions of the L2 protein of human papillomavirus types 6 and 16. J. Infect. Dis. 168, 743–747.