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Human Papillomavirus (HPV) genotype 18 variants in patients with clinical manifestations of HPV related infections in Bilbao, Spain

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Abstract

Background: Human papillomavirus (HPV) variants differ in their biological and chemical properties, and therefore, may present differences in pathogenicity. Most authors classified variants based on the phylogenetic analysis of L1 region. Nevertheless, recombination in HPV samples is becoming a usual finding and thus, characterizing genetic variability in other regions should be essential.

Objectives: We aimed to characterize the genetic variability of HPV 18 in 5 genomic regions: E6, E7, E4, L1 and the Upstream Regulatory Region (URR), working with both single infection and multiple HPV infection samples. Furthermore, we aimed to assess the prevalence of HPV 18 variants in our region and look for possible existence of recombination as well as analyze the relationship between these variants and the type of lesion.

Methods: From 2007 to 2010, Clinical Microbiology and Infection Control Department analyzed 44 samples which were positive for HPV 18. Genetic variability was determined in PCR products and variants were assigned to European, Asian-amerindian or African lineage. Recombination and association of variants with different types of lesion was studied.

Results: Genetic analysis of the regions revealed a total of 56 nucleotide variations. European, African and Asian-amerindian variants were found in 25/44 (56.8%), 10/44 (22.7%) and 5/44 (11.4%) samples, respectively. We detected the presence of recombinant variants in 2/44 (4.5%) cases. Samples taken from high-grade squamous intraepithelial lesions (H-SIL) only presented variants with specific-african substitutions.

Conclusions: Multiple HPV infection, non-european HPV variants prevalence and existence of recombination are considered risk factors for HPV persistence and progression of intraepithelial abnormalities, and therefore, should be taken into consideration in order to help to design and optimize diagnostics protocols as well as improve epidemiologic studies.

Our study is one of the few studies in Spain which analyses the genetic variability of HPV18 and we showed the importance of characterizing more than one genomic region in order to detect recombination and classify HPV variants properly.

Keywords: Human papillomavirus infection, Genotype 18, Variants, Recombination, Multiple infection

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Background

Based on the epidemiologic classification in terms of their risk to induce cervical cancer, human papillomaviruses (HPV) can be divided into 3 groups: “high-risk” genotypes associated with a greater risk of developing cancer, “low-risk” genotypes associated with low grade cell changes or benign epithelium proliferations in the genital area, but not with cancer, and “probable high-risk” genotypes from which there is not enough data about their relationship with cervical cancer to classify them [1].

About 15 genotypes are classified as high-risk types, and two of them (16 and 18) cause over 70% of all cervical cancer cases [2,3]. Nucleotide variability of these genotypes has been largely studied and different molecular variants were described [4,5]. These variants differ in their biological and chemical properties [6–8], and therefore, may become an important risk factor in cervical cancer due to possible differences in pathogenicity.

Most authors classified variants based on the phylogenetic analysis of one genomic region nucleotide variations [9]. Nevertheless, some publications have confirmed the presence of recombination in HPV samples [10,11]. This event may occur due to a homologous recombination or to a repeated infection of the same HPV genotype but different variant and it is more often found since coinfections with more than one HPV type are becoming a usual finding [12–14]. Therefore, it should be essential to determine HPV variants analyzing different genomic regions and multiple infections.

There are very few epidemiological national studies in Spain and all of them refer to HPV 16 which is the most investigated HPV type worldwide. However, there is no previous national work related to HPV 18 nucleotide variability, which is the second most prevalent HPV genotype found in cervical cancer.

The aim of the present study was: i) to characterize the genetic variability of HPV 18 in 5 genomic regions: E6, E7, E4, L1 and the Upstream Regulatory Region (URR), working with both single infection and multiple HPV infection samples, ii) assess the prevalence of HPV 18 variants in our region and look for existence of recombination, and iii) analyze the relationship between variants and types of lesion.

Results

Samples collected

Since 2007 to 2010, a total of 1085 positive samples for HPV were received and analyzed. HPV 18 was detected in 65 samples (6%). Forty-four patients consented to have their samples analyzed and studied, so this study was based on their samples: 10 single HPV infections (22.7%) and 34 multiple HPV infections (77.3%).

We were able to amplify HPV DNA in 43/44 samples for E6 region, 41/44 for E7, 35/44 for E4, 43/44 for L1 region and 44/44 for URR region. All PCR products were sequenced and sequences from each region were submitted to GenBank.

Nucleotide variations

Variant distribution was determined through E6, E7, E4, L1 and URR sequences. Genetic analysis of the regions revealed a total of 56 nucleotide variations (Figure 1).

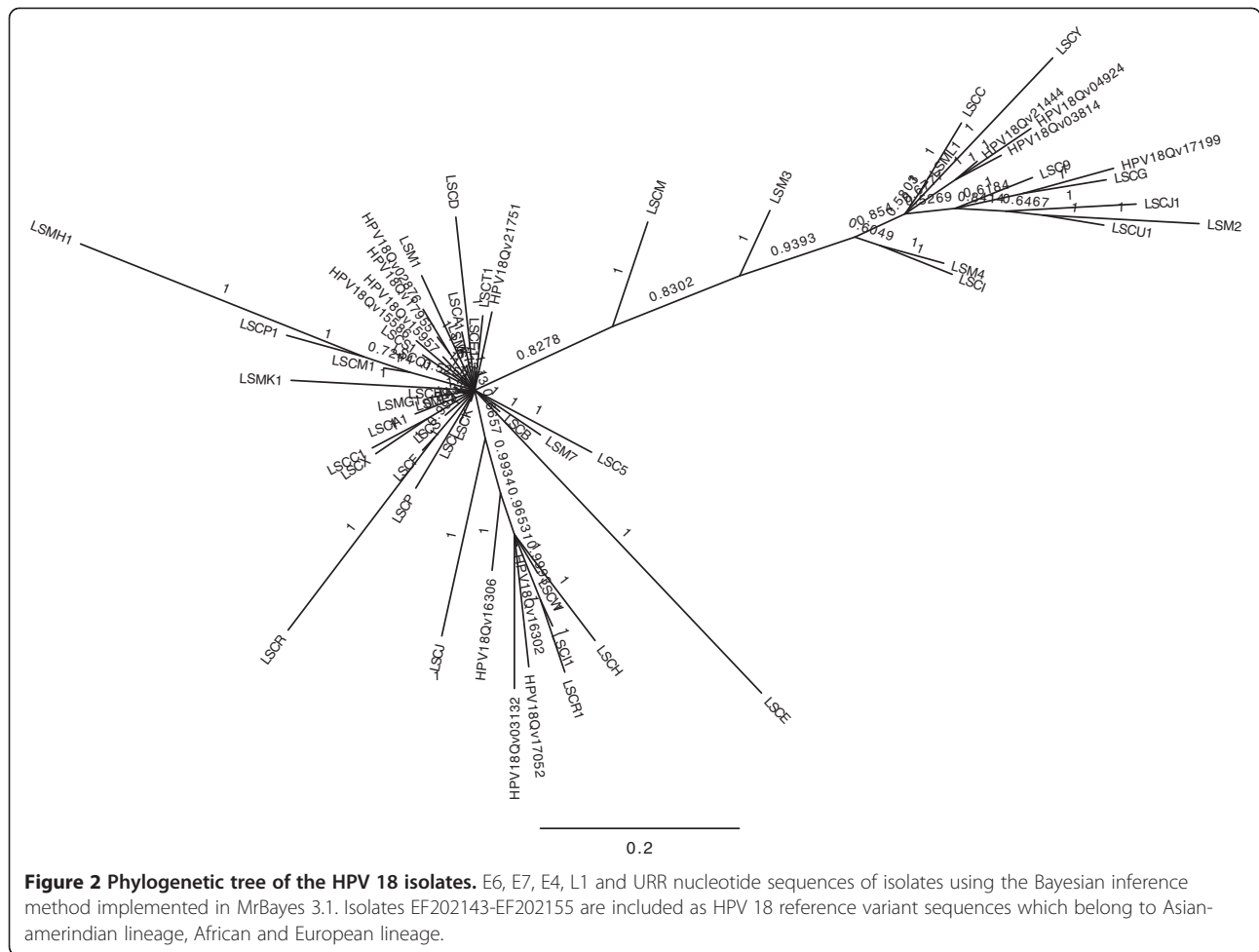
In the *E6* gene nine nucleotide variations were detected. Six of them were specific to the African lineage: T317C (6/10 African variants), T251C (9/10), A548G, G266A and G374A (present in all African isolates) and C342T (5/10 African variants) which lead to a non-synonymous amino acid alteration His/Tyr. A non-synonymous substitution T318C (Tyr/His) was found to be specific to the European lineage (2/25 European isolates), while the synonymous substitution C549A was detected among the three different branches (35/43 sequenced *E6* amplimers). In our study, C287G was observed in all HPV 18 isolates.

E7 gene genetic variability analysis revealed five nucleotide substitutions. Three nucleotide variations were specific for the African lineage: C665T (3/10 African variants), C593T (His/Tyr), C640C and T864G (Asn/Ser). All of them but C665T and T864G were present in all African variants. One synonymous substitution (C751T) was detected in both European and Asian-amerindian isolates (26/34 non-African variants).

E4 gene analysis presented most nucleotide variations (17 substitutions) and almost half of them (8/17) lead to amino acid changes. All African variants showed 4 non-synonymous substitutions (C3558A His/Gln, C3578T Ser/Leu, A3586C Ser/Arg and T3593G Ile/Ser), one synonymous variation (T3534C) and a deletion of 6 amino acids (3627–3632). European variants showed 4 specific non synonymous substitutions G3482A (Ser/Asn, 3/25 isolates), T3563A (Leu/Gln, 4/25), C3617T (Ser/Leu, 4/25) and C3630G (His/Gln, all European isolates). Two non-synonymous substitutions were also detected in two European isolates: T3492A and C3615T.

L1 gene and URR sequence analysis demonstrated the presence of substitutions C6842G and T7592C in all our isolates.

Most nucleotide variations found in our study have been already described in literature except for T318C, C665T, C3615T, C3617T, G6897A, G6993A, A7000T/C, T7001C, T7007G and T7765G. Only substitutions in positions 318 and 3617 lead to amino acid changes (Tir/His and Ser/Leu, respectively). T318C substitution was present in 2 European isolates whereas C3617T nucleotide change was not specific to any lineage and was detected in 5 samples.



as L-SIL while presence of H-SIL was detected in 3 specimens.

High grade lesions only presented African variants (2/3 isolates, 66.7%) and variants that presented both African and European substitutions (1/3 isolates, 33.3%) whereas

most European and Asian-amerindian variants were detected in negative cytologies (Table 2).

Presence of lesions associated with non-European variants was found to be statistically significant ($p = 0.01053$). Nevertheless, there was not a statistically significant

Table 1 Evidence for recombinant samples

Sample \ Gene	E6	E7	E4	L1	URR	Program inference
LSM3	Af	Af	X	X	E	No recombination detected.
LSCY	Af	Af	E	E	E	Maxchi detected recombination.(LSCM is one of the donors)
LSMK1	AsA	E	Af	E	Af	Maxchi detected recombination.(LSCI and LSCR1 are the donors)
LSCR	E	E	E	E	E	No recombination detected.
LSMH1	E	E	E	E	E	No recombination detected.
LSC5	E	E	E	E	E	No recombination detected.
LSM7	E	E	E	E	E	No recombination detected.
LSCB	AsA	E	E	E	E	No recombination detected.
LSCM	Af	E	Af	Af	E	No recombination detected.

Isolates analyzed for possible recombination. Phylogenetic trees were constructed for each sequenced region and isolates were classified as Asian-amerindian (AsA), European (E) or African (Af) variants. RDP, Maxchi and Chimaera were used for the detection of recombination. URR: Upstream regulatory region, X: absence of data.

Table 2 Human papillomavirus 18 variants vs type of lesion

Variants \ Type of Lesion	Neg	L-SIL	H-SIL
European	71.0%	3.3%	0%
Asian-amerindian	12.9%	1.1%	0%
African	12.9%	4.4%	100%
Recombinant	3.2%	1.1%	0%

Neg: no lesion, L-SIL: low-grade squamous intraepithelial lesion, H-SIL: high-grade squamous intraepithelial lesion.

association between type of infections (single vs multiple) and presence of lesions ($p = 0.18078$).

Discussion

There are almost no epidemiologic studies about HPV 18 variants carried out in Spain and even though national HPV prevalence is low, it cannot be forgotten that this genotype together with genotype 16 cause 70% of cervical cancer cases.

Many authors confirm that distribution of HPV variants is related to geographic or race distribution [6,17] and therefore, Spain should expect predominance of European variant, followed by African and Asian-amerindian variants. Our results show concordance with this stating: 25 European variants (56.8%), 10 African (22.7%) and 5 Asian-amerindian variants (11.4%).

HPV 18 has been associated with both recurrent lesions with very bad clinical prognosis [18] and benign lesions [19]. This fact may reflect the oncogenic potential difference among variants. Hecht et al [20] identified a HPV 18 variant with lower oncogenic potential due to its absence in cervical cancer but presence in 40% of intraepithelial lesions. Villa et al [21] suggested that non-European HPV 18 variants persisted more frequently and were more

associated with pre-invasive lesions. Since then, most studies confirm that different variants of the same genotype differ in their pathogenic characteristics and therefore, nucleotide substitutions may play an important role. Our study results show concordance with these statements as African variants and variants where most of specific-african substitutions were detected were the only type of variants detected in H-SIL.

Most nucleotide changes reported in our study have been previously described and some of them are of particular importance. In URR, the mutation A41G is located in the Sp-1 binding site and isolates with this nucleotide variation have shown to have an increased transcriptional activity [22]. Variations in positions 41 and 104 modulate Sp1 and YY1 activities and are associated to a higher activity of the E6/E7 promoter. Patients with T104C substitution are less likely to present tumour recurrence [23]. Other nucleotide changes like T7651C, A7658C and C7726T also lay within transcription factor binding sites.

Substitutions C287G, C6842G and T7592C were found in all our isolates. Variation C6842G has been previously reported as error in the original sequence [24], and H. Arias-Pulido et al. sequenced the original reference HPV 18 plasmid (provided by E-M de Villiers, Deutsches Krebsforschungszentrum, Germany) and observed the substitution T7592C [25], so it is considered as a sequencing error in the original HPV 18 reference sequence report.

Furthermore, ten "new" nucleotide variations have been detected and two of them were non-synonymous and lead to amino acid changes (T318C and C3617T, Tir/His and Ser/Leu, respectively).

Knowledge on HPV variants and their nucleotide variability is essential for three main reasons: i) nucleotide variations may interfere with the viral oncogenic potential, ii) host cellular immune response can be different when

Table 3 Polymerase chain reaction characteristics for Human papillomavirus 18

	Primer sequence (5' - 3')	Annealing Temp/Cycles	Nucleotides amplified*	Amplicon size
E6 F	AGTAACCGAAAACGGTCGGGA	55°C/40 cycles	38-491	454 pb
E6 R	GTTGTGAAATCGTCGTTTTCA			
E7 F	TGAAAAACGACGATTCACAAC	55°C/40 cycles	470-931	462 pb
E7 R	ACCTTCTGGATCAGCCATTG			
E4 F	GTAAGGAAGGGTACAACACG	57°C/35 cycles	3309-3792	484 pb
E4 R	CTGTCCAATGCCAGGTGGA			
LCR 1 F	TCGGTTGCCTTTGGCTTAT	55°C/40 cycles	7465-7775	311 pb
LCR 1 R	AAGGGTAGACAGAATGTTGGACA	55°C/40 cycles	7718-163	303 pb
LCR 2 F	GCTAATTGCATACTTGGCTTG			
LCR 2 R	TCCGTGCACAGATCAGGTAG			
MY11	GCACAGGGTCATAACAATGG	55°C/40 cycles	6558-7012	455 pb
MY09	CGTCCAAGGGATATTGATC			
L1 seq	ACAGTCTCTGTACCTGGG			

*Position numbering refers to the first nucleotide of the HPV 18 reference genome (accession number NC001357). F: forward primer, R: reverse primer.

Table 4 GeneBank accession numbers for the sequenced isolates

Isolate	E6	E7	E4	L1	URR
LSM1	JN416211	JN416162	X	JN416262	JN416313
LSM2	JN416212	JN416163	JN416121	JN416263	JN416314
LSM3	JN416213	JN416164	X	X	JN416315
LSM4	JN416214	JN416165	JN416122	JN416264	JN416316
LSC5	JN416215	JN416166	JN416123	JN416265	JN416317
LSM6	JN416216	X	X	JN416266	JN416318
LSM7	JN416217	JN416167	JN416124	JN416267	JN416319
LSC9	JN416219	JN416169	JN416125	JN416269	JN416321
LSCA	JN416220	JN416170	X	JN416270	JN416322
LSCB	JN416221	JN416171	JN416126	JN416271	JN416323
LSCC	JN416222	JN416172	JN416127	JN416272	JN416324
LSCD	JN416223	JN416173	X	JN416273	JN416325
LSC E	JN416224	JN416174	JN416128	JN416274	JN416326
LSCF	X	X	X	JN416275	JN416327
LSCG	JN416225	JN416175	X	JN416276	JN416328
LSCH	JN416226	JN416176	JN416129	JN416277	JN416329
LSCI	JN416227	JN416177	JN416130	JN416278	JN416330
LSCJ	JN416228	JN416178	JN416131	JN416279	JN416331
LSCK	JN416229	JN416179	JN416132	JN416280	JN416332
LSCL	JN416230	JN416180	JN416133	JN416281	JN416333
LSCM	JN416231	JN416181	JN416134	JN416282	JN416334
LSCP	JN416232	JN416182	JN416135	JN416283	JN416335
LSCR	JN416234	JN416184	JN416137	JN416285	JN416337
LSCS	JN416235	X	JN416138	JN416286	JN416338
LSCW	JN416238	JN416187	JN416141	JN416289	JN416341
LSCX	JN416239	JN416188	JN416142	JN416290	JN416342
LSCY	JN416240	JN416189	JN416143	JN416291	JN416343
LSCA1	JN416241	JN416190	JN416144	JN416292	JN416344
LSCC1	JN416243	JN416192	JN416146	JN416294	JN416346
LSCE1	JN416245	JN416194	X	JN416296	JN416348
LSMF1	JN416246	JN416195	JN416147	JN416297	JN416349
LSMG1	JN416247	JN416196	JN416148	JN416298	JN416350
LSCH1	JN416248	JN416197	JN416149	JN416299	JN416351
LSCI1	JN416249	JN416198	JN416150	JN416300	JN416352
LSCJ1	JN416250	JN416199	JN416151	JN416301	JN416353
LSMK1	JN416251	JN416200	JN416152	JN416302	JN416354
LSML1	JN416252	JN416201	JN416153	JN416303	JN416355
LSCM1	JN416253	JN416202	JN416154	JN416304	JN416356
LSCP1	JN416256	JN416205	JN416157	JN416307	JN416359
LSCQ1	JN416257	JN416206	JN416158	JN416308	JN416360
LSCR1	JN416258	JN416207	JN416159	JN416309	JN416361
LSCS1	JN416259	JN416208	JN416160	JN416310	JN416362

Table 4 GeneBank accession numbers for the sequenced isolates (Continued)

LSCT1	JN416260	JN416209	X	JN416311	JN416363
LSCU1	JN416261	JN416210	JN416161	JN416312	JN416364

there are substitutions in the amino acids on the viral capsid which may be relevant for the vaccination, iii) HPV infections with a variant may not give immunological protection against a subsequent infection with other variant of the same genotype.

Nowadays, HPV variants recombination has already been described and it is more often found since coinfection with more than one HPV type prevalence is not a unusual finding [10].

In our study we detected 2 recombinant variants (4.5%) which might have been missed or wrong classified if only amplifying one genomic region. Furthermore, non-recombinant samples as LSCM or LSM3 showed specific-african substitutions in some regions (for example E6) whereas they would be classified as european variants when only analyzing nucleotide variation in URR. Therefore, characterizing more than one genomic region may be essential in order to detect recombination and classify HPV variants properly.

We amplified URR and *E6*, *E7* and *L1* genes from at least 93% of samples. However, when characterizing *E4* region, we were only able to amplify 35 samples. *E4* gene is generally disrupted during DNA integration into the host genome and this disruption may explain the inability to amplify *E4* gene in some of our samples.

In conclusion, data and knowledge on geographic HPV intratypic variants distribution might help to establish a data base about the diversity and pathogenicity of different HPV variants, which may help to design and optimize diagnostics protocols in order to reduce the disease.

Methods

Recruitment of participants

Clinical Microbiology and Infection Control Department at Basurto University Hospital (Basque Country, North of Spain) analyzed samples which were remitted from different Hospital Services, especially the Consultation of Sexually Transmitted Diseases and the Department of Obstetrics and Gynecology, from 2007 to 2010.

All samples were collected from patients with clinical manifestations of HPV related infections. Lesions were classified by pathologists into three categories: negative (no lesion was found), low-grade squamous intraepithelial lesion (L-SIL) or high-grade squamous intraepithelial lesion (H-SIL).

Molecular genotyping was carried out using "Linear Array HPV Genotyping Test" kit (Roche Molecular

Diagnostics). In our study, we analyzed positive samples for HPV genotype 18 (both single infections and multiple HPV infections) from patients who had given written, informed consent.

Genomic DNA extraction

DNA extraction was performed by QIAamp DNA mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Extracted DNA was eluted with 200 µl AE buffer and stored at -20°C until amplification.

PCR amplification and sequencing

Amplification of HPV *E6*, *E7*, *E4* genes and the URR was performed using type-specific primers designed according to HPV 18 genome prototype sequence (GenBank accession number NC001357). The URR was amplified using 2 primer sets. In order to amplify L1 region, consensus HPV primers were used (Table 3).

PCR was performed in 30 µl of reaction mixture containing 10 × PCR buffer, 25 mmol/L MgCl₂, 25 mmol/L of each deoxynucleoside, 100 pmol/L of sense and anti-sense primer, 5 µl of template DNA and 2,5 U of Taq DNA polymerase (Qiagen).

The thermal program started with a pre-heat of 95°C for 15 min, followed by 35–40 cycles of suitable annealing temperature which depended on the primers and finished with a final extension at 72°C for 10 min (Table 3).

PCR products were confirmed based on specific bands of amplified DNA presence in agarose gel (2%). Afterwards, amplicons were automatically sequenced using the "Big Dye Terminator Cycle Sequencing kit" (Applied Biosystems) according to the manufacturer's instructions.

For *E6*, *E7*, *E4* and URR amplicons the same forward specific primers as those used in amplification were chosen as sequencing primers. In the L1 region, a specific primer was used in order to sequence HPV 18 and not other HPV types present in cases of multiple infection (Table 3).

Nucleotide variations, phylogenetic analysis: variants and recombination

HPV sequences were aligned and compared to the HPV 18 prototype sequence which belongs to the Asian-amerindian lineage (accession number NC001357), using BioEdit Sequence Alignment Editor v7.0.4.1 and Clustal W (<http://www.genome.jp/tools/clustalw/>).

Amplification and sequencing of the samples were repeated to confirm nucleotide variations which were present in less than three isolates.

Sequences were assigned to a lineage on the basis of their similarity to HPV 18 known variant sequences [26] which belong to Asian-amerindian lineage (GenBank accession numbers: EF202143 - EF202146), African (EF202152 - EF202155) and European lineage (EF202147- EF202149,

EF202151). Phylogenetic trees were built using the Bayesian inference method implemented in MrBayes 3.1 [27] and three methods (all implemented in RDP3 [15]) were used for the detection of recombination (RDP [15], Maxchi [16] and Chimaera) to analyze isolates which did not adjust to the clusters.

Lesions

Association of lesions and variants or infection type (single HPV vs multiple HPV infection) was analyzed. Fisher exact test was used for statistically significant association.

GenBank accession numbers

The following are the GenBank accession numbers for all the sequences used in this analysis. X indicates absence of nucleotide sequence data Table 4.

Ethical approval

All procedures followed were approved by the appropriate Ethics Committee related to our institutions (Basurto University Hospital and University of Basque Country) and complied with the guidelines and ethical standards for experimental investigation with human subjects of Helsinki Declaration of 1975, as revised in 2000. All study participants provided written, informed consent.

Additional file

Additional file 1: E4, E6, E7, L1, LCR_bay. Phylogenetic trees were constructed for each region individually.

Abbreviations

HPV: Human papillomavirus; H-SIL: High-grade squamous intraepithelial lesion; L-SIL: Low-grade squamous intraepithelial lesion; SIL: Squamous intraepithelial lesion; URR: Upstream regulatory region.

Competing interests

All authors declare no potential conflicts of interest.

Authors' contributions

LSA carried out the molecular studies, participated in the sequence alignment and drafted the manuscript. MB and EA participated in the design of the study and its coordination and have been involved in drafting the manuscript and revising it critically for important intellectual content. SH, DA and VE have made substantial contributions to acquisition of samples and data and have revised the manuscript critically. KGE and BMJ performed phylogenetic and statistical analysis. RC conceived the study, participated in its design and coordination and has given final approval of the version to be published. All authors read and approved the final manuscript.

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