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Complete genotyping of mucosal human papillomavirus using a restriction fragment length polymorphism analysis and an original typing algorithm

Rui Jorge Nobre [a,b,*], Luís Pereira de Almeida [b,c], Teresa C. Martins [a,c]

[a] *Molecular Pathology Laboratory, Portuguese Institute for Oncology at Coimbra, EPE, Avenida Bissaya Barreto 98, 3000-075 Coimbra, Portugal*

[b] *Faculty of Pharmacy, University of Coimbra, 3000-295 Coimbra, Portugal*

[c] *Center for Neurosciences and Cell Biology, University of Coimbra, 3004-517 Coimbra, Portugal*

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Abstract

Background: Due to the differences in the oncogenic activity of human papillomaviruses (HPV), it is clinically important to accurately identify HPV types in a simple and time effective manner.

Objectives: We aimed at developing a straightforward and cost-effective assay to individually identify all mucosal HPVs, based on the amplification of L1 gene using MY09/11 primers, and subsequent restriction fragment length polymorphism (RFLP) analysis.

Study design: We made use of bioinformatic tools to analyze all published DNA sequences of 49 mucosal HPV types for PstI, HaeIII, DdeI and RsaI restriction sites. Based on the RFLP patterns, we have designed an original genotyping algorithm.

Results: Each HPV type presented a distinct RFLP pattern, which was visually distinguishable on polyacrylamide gels. A set of 27 pre-selected patient samples of known HPV types was confirmed positive for the same HPV type using this RFLP assay. Furthermore, in a random and blind HPV typing experiment performed in 30 untyped clinical samples, RFLP data consistently matched DNA sequencing results.

Conclusions: Our polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method, using 4 restriction enzymes (PstI, HaeIII, DdeI, RsaI) and an original genotyping algorithm, allows discrimination of all individual mucosal HPV types in single infections, and even detection of multiple infections. This assay gives complementary information to commercially available methods, and may also be financially advantageous, particularly when financial resources are scarce.

Keywords: HPV; Genotyping; Polymerase chain reaction (PCR); RFLP; Cervical cancer

1. Introduction

Cervical cancer is the second most common cancer in women worldwide, and the most common cancer in most developing countries (Moore, 2006). Epidemiologic studies have established that certain types of human papillomavirus (HPV) are the main cause of cervical cancer (Muñoz et al., 1992; Walboomers et al., 1999; Bosch et al., 2002). HPVs are a heterogeneous group of double stranded, non-enveloped DNA viruses of the *Papillomaviridae* family (de Villiers et al., 2004). At present, more than 100 HPV types have been identified. However, only HPVs of the *Alpha-papillomavirus* genus, which includes mucosal and cutaneous types, infect the anogenital epithelium (de Villiers et al., 2004). The mucosal HPV types of this genus have been divided into four groups based on their oncogenic activity: high-risk types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59),

* Corresponding author at: Molecular Pathology Laboratory, Portuguese Institute for Oncology at Coimbra, EPE, Avenida Bissaya Barreto 98, 3000-075 Coimbra, Portugal. Tel.: +351 239400200x2272; fax: +351 239484317.

E-mail addresses: rui.jorge.nobre@gmail.com (R.J. Nobre), luispa@ci.uc.pt (L.P. de Almeida), tmartins@ipocoimbra.min-saude.pt (T.C. Martins).

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probable high-risk types (26, 53, 66, 68, 73, 82), low-risk types (6, 11, 13, 40, 42, 43, 44, 54, 61, 70, 72, 81 and 89) and types of undetermined-risk (30, 32, 34, 62, 67, 69, 71, 74, 83, 84, 85, 86, 87, 90, 91) (Muñoz et al., 2006). Due to differences in oncogenic activity, it is clinically important to detect HPV infection and to accurately identify HPV types in a simple and time effective manner.

In cervical screening programs, HPV-DNA is usually detected by a non-radioactive signal amplification method, the Hybrid Capture System II (Digene Diagnostics) (Lorincz, 1996; Bozzetti et al., 2000). This methodology allows the detection of 13 probable/high-risk and 5 low-risk types using two different cocktail RNA probes, in two separate reactions. Following approval by Food and Drug Administration (FDA), Hybrid Capture System II became the standard procedure for HPV DNA detection. However, Hybrid Capture System II has some important limitations, as it does not provide information about the specific type of HPV that is detected, giving a positive result when at least one of the 18 types is present. Furthermore, it is less sensitive than PCR (Smits et al., 1995; Cope et al., 1997) and the cross-reactivity of the two cocktail probes reduces the clinical relevance of a positive result (Castle et al., 2002; Poljak et al., 2002). Nucleic acid amplification has been developed to increase the sensitivity and the specificity of HPV-DNA detection. The majority of protocols use GP5+/6+ consensus primers (Van den Brule et al., 1990; de Roda Husman et al., 1997) or MY09/11 degenerate primers (Manos et al., 1989). Both sets of primers are directed to a highly conserved region of the HPV L1 gene and are potentially capable of detecting a large number of mucosal HPV types in a single PCR reaction (Hildesheim et al., 1994). After HPV detection using consensus primers, PCR amplimers can be analyzed by several HPV typing assays: restriction fragment length polymorphism (RFLP), reverse hybridization analysis, microarray platforms or sequencing. PCR using consensus primers with subsequent restriction fragment length polymorphism analysis (PCR-RFLP) has been demonstrated to be a sensitive and a financially advantageous methodology to detect and characterize HPV-DNA in clinical specimens (Lungu et al., 1992). However, the PCR-RFLP techniques previously described have some limitations: (a) they do not allow the genotyping of all high-risk HPV types (Pizzighella et al., 1995; Patti et al., 2002; Naqvi et al., 2004); (b) they often make use of a large number of restriction enzymes (Bernard et al., 1994; Astori et al., 1997); (c) they are ineffective or time consuming with regards to RFLP-pattern analysis on polyacrylamide gels and to identification of given HPV types (Bernard et al., 1994; Astori et al., 1997).

In this work we have developed an easy, rapid and cost-effective PCR-RFLP assay to identify all known mucosal HPVs, based on the amplification of a fragment of the L1 gene using MY09/11 primers and subsequent RFLP analysis with four restriction enzymes: PstI, HaeIII, DdeI and RsaI.

Table 1
Mucosal HPV types considered for typing (Family *Papillomaviridae*, genus *Alpha-papillomavirus*)

Species	HPV types
1	32, 42
3	61, 62, 72, 81, 83, 84, 86, 87, 89, 102
5	26, 51, 69, 82
6	30, 53, 56, 66
7	18, 39, 45, 59, 68, 70, 85, 97
8	40, 43, 91
9	16, 31, 33, 35, 52, 58, 67
10	6, 11, 13, 44, 74
11	34, 73
13	54
14	90
15	71, 106

2. Materials and methods

2.1. HPV DNA sequences and restriction site "in silico" analysis

All published DNA sequences of mucosal HPV types (Table 1) were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/). These sequences were subsequently aligned using the "Alignment tool" of CLC Free Workbench program, version 3.2 (CLC bio, Denmark) and DNA segments of the L1 gene that are bracketed by the MY09/11 primers (MY09/11 amplicons) were selected and compiled in a file. A second file was created, which included the selected restriction enzymes (PstI, HaeIII, DdeI, RsaI). Finally, using the "Restriction site analysis tool" of CLC Free Workbench program, the information from both files was crossed in order to predict the product sizes of each MY-amplicon restriction reaction.

2.2. HPV detection in clinical samples

A set of pre-selected clinical samples, in which 27 different mucosal HPV types had been previously identified by PCR and DNA sequencing (HPV 6, 11, 16, 18, 31, 33, 34, 35, 39, 44, 45, 52, 53, 56, 58, 59, 61, 62, 66, 67, 70, 71, 73, 81, 83, 84, and 102) and a representative collection of 30 untyped clinical samples (10 normal samples, 10 low-grade squamous intraepithelial lesions, 5 high-grade squamous intraepithelial lesions and 5 invasive squamous cervical carcinoma) were obtained from the ongoing cervical cancer screening program of the Portuguese Institute for Oncology at Coimbra (IPOCFG, EPE). Cervical samples were collected in PreservCyt (Cytyc Corporation, Boxborough, MA, USA), a liquid cytology medium, and centrifuged at 4500 rpm for 15 min. The pelleted cells were lysed in a Tris-buffered solution (0.3 M NaCl, 10 mM EDTA, 10 mM Tris pH 7.5), containing 0.5% SDS and 0.5 μg/μl proteinase K, at 56 °C. The DNA was purified by standard phenol/chloroform extraction and ethanol precipitation. HPV

DNA was detected by PCR using the degenerate consensus primers MY09 (5′-CGTCCMARRGGAWACTGATC-3′) and MY11 (5′-CMCAGGGWCATAAYAATGG-3′), which amplify a region of 449–458 nucleotides (depending on HPV type) of the highly conserved L1 ORF (Manos et al., 1989). Each PCR amplification was carried out with 50–100 ng of template DNA in 1× PCR Buffer (Biotools, Madrid, Spain), containing 1.5 mM $MgCl_2$, 12.5 pmol of each primer, 200 μM of each deoxynucleotide triphosphate and 1 unit of *Taq* DNA polymerase (Biotools, Madrid, Spain). Thermal cycling was performed in a Perkin-Elmer 9600 (PerkinElmer, MA, USA) as follows: denaturation of DNA template at 95 °C for 4 min, followed by 35 cycles of 94 °C for 30 sec, 56 °C for 30 sec, and 72 °C for 30 sec, and a final extension step at 72 °C for 8 min. To check the integrity of the DNA extracted from the specimens, a region of 501 base pairs of the cellular β-globin gene was amplified using primers Beta1 (5′-TCAACCCTACAGTCACCCAT-3′) and Beta2 (5′-CTAACAATTACGAACAGCAATGAG-3′). The PCR conditions were the same as described for HPV detection. All PCRs were carried out under conditions that minimize sample cross-contamination. DNA from HeLa and CaSki cell lines was used as positive control. A negative control containing all PCR reagents, except DNA, was added to monitor contamination. In order to verify the amplification efficiency and the absence of unspecific products, the amplified DNA was analyzed in a horizontal 2% agarose gel.

2.3. HPV typing by RFLP (PstI, HaeIII, RsaI, DdeI)

HPV-positive cases were typed by RFLP analysis. Each restriction reaction was performed separately in a final volume of 20 μl, using 1 μg of MY09/11 PCR product, 2 μl of 10× recommended restriction buffer, and 10 units of the following restriction endonucleases: PstI (Qbiogene, CA, USA), HaeIII (Fermentas Inc., Canada), DdeI (Qbiogene, CA, USA) and RsaI (Fermentas Inc., Canada), according to the manufacturers' instructions. Reactions took place at 37 °C for 1 h. Digested products were electrophoretically separated on 10% polyacrylamide gels, in the presence of 25 bp and 50 bp DNA molecular weight markers (Invitrogen, Carlsbad, CA, USA). Gels were stained with Gelstar (Cambrex Bio Science Rockland, Inc., ME, USA) for 15 min and patterns were visualized using a Versa Doc Imaging System (Bio-Rad, Hercules, CA, USA).

2.4. Validation of RFLP restriction reaction

To test the efficacy of each enzyme and to avoid the misidentification of types due to partial digestion, an additional restriction reaction was performed, in which MY09/11 PCR products and a control DNA (PCR product of β-globin fragment amplification, obtained in 2.2) were digested together. Each restriction reaction was performed in a final volume of 20 μl, using 1 μg of MY09/11 PCR product, 1 μg of Beta1/2 amplicon, 2 μl of 10× recommended restriction buffer, and 20 units of each restriction endonuclease, according to the conditions described in 2.3.

2.5. Confirmation of RFLP data using sequencing

All MY-PCR products obtained in Section 2.2 were sequenced to confirm the RFLP results. The PCR products were purified from agarose gel using a PCR clean-up Gel extraction Kit (NucleoSpin® Extract II, Macherey-Nagel, Germany) and cloned. At least five fragments of each amplicon were sequenced. Forward and reverse sequencing reactions were carried out using 20 ng of template DNA from PCR amplimers and 20 μM of the MY09 and MY11 primers, respectively. Automated DNA sequencing was performed by MWG DNA Biotech Company (Germany). Sequences obtained were compared with all sequences available in GenBank database. HPV type was identified based on >98% sequence homology over 449–458 nucleotides.

3. Results

3.1. HPV DNA sequences and restriction site "in silico" analysis

A total of 296 different DNA sequences of the L1 gene (HPV variants), corresponding to 49 mucosal HPV types, were obtained from the GenBank database. The *in silico* analysis of their MY09/11 amplicons for PstI, HaeIII, DdeI and RsaI restriction sites defined 74 different sets of RFLP patterns: 49 corresponded to reference HPV types, 2 corresponded to HPV subtypes (HPV 55 and HPV 64), and 23 corresponded to HPV variants, which had an altered RFLP-pattern with regards to their respective reference type (Table 2). Each type presented specific sets of RFLP patterns, which were not shared with any other HPV type. All HPV types were clearly identifiable by visual discrimination on 10 % polyacrylamide gels, except for one particular situation (case of HPV 85 and b variant of HPV 82), in which the size of the restriction fragments differed only by 9 bp. In this specific case, the problem can be solved using a more concentrated polyacrylamide gel, using type-specific PCR or by sequencing this MY09/11 amplicon.

3.2. The algorithm for mucosal HPV typing

To analyze the RFLP-patterns, we developed a novel HPV genotyping algorithm (Fig. 1). The algorithm design was based in: a) the 74 sets of RFLP-patterns obtained for each mucosal HPV type, subtype and variant, as described in Table 2; and b) the fact that each restriction enzyme does not cut some of MY09/11 amplicons, as described in Table 3.

Table 2
RFLP fragment sizes and GenBank accession numbers of sequences used as representative of the 49 mucosal HPV types

HPV type	GenBank sequence	MY PCR amplicon (bp)	RFLP fragment sizes (bp)			
			PstI	HaeIII	DdeI	RsaI
6	S73503	449	**449**	**217, 124, 108**	**382, 67**	**161, 149, 72, 67**
11	M14119	449	**242, 207**	**217, 124, 108**	**447,** 2	**216, 135, 72,** 26
13	X62843	455	**242, 213**	**204, 127, 124**	**326, 67, 62**	**175, 135, 73, 72**
16	U89348	452	**216, 210,** 26	**444,** 8	**452**	**310, 72, 70**
18	X05015	455	**242, 213**	**455**	**432,** 23	**135, 125, 85, 72,** 38
18[a]	U45891	455	**242, 213**	**455**	**432,** 23	**260, 85, 72,** 38
26	X74472	455	**353, 102**	**455**	**455**	**365, 72,** 18
30	X74474	449	**242, 207**	**232, 217**	**291, 158**	**449**
31	AJ831566	452	**216, 210,** 26	**328, 124**	**283, 167,** 2	**380, 72**
31[a]	DQ241373	452	**216, 210,** 26	**328, 124**	**283, 90, 77,** 2	**380, 72**
32	X74475	449	**449**	**317, 124,** 8	**320, 108,** 21	**216, 161, 72**
33	U45896	449	**242, 207**	**449**	**320, 77, 52**	**236, 102, 72,** 39
34	X74476	458	**253, 179,** 26	**334, 124**	**211, 151, 88,** 8	**186, 161, 96,** 15
35	U45898	452	**426,** 26	**261, 180,** 8, 3	**294, 135,** 23	**177, 161, 72, 42**
39	M62849	455	**330, 125**	**455**	**324, 131**	**260, 123, 72**
40	X74478	455	**455**	**447,** 8	**297, 158**	**365, 90**
42	M73236	449	**449**	**449**	**341, 108**	**242, 135, 72**
43	AJ620205	449	**273, 176**	**331, 118**	**447,** 2	**332, 72, 45**
44	U12493	455	**455**	**215, 124, 108,** 8	**297, 112, 46**	**222, 161, 72**
45	X74479	455	**242, 213**	**447,** 8	**324, 131**	**338, 72, 45**
45[a]	U45914	455	**242, 213**	**447,** 8	**324, 131**	**237, 101, 72, 45**
45[b]	U45907	455	**242, 213**	**447,** 8	**324, 131**	**203, 180, 72**
45[c]	U45911	455	**242, 213**	**447,** 8	**324, 131**	**180, 158, 72, 45**
51	U45917	452	**452**	**379, 73**	**362, 90**	**380, 72**
52	U45920	449	**423,** 26	**258, 183,** 8	**357, 92**	**449**
52[a]	U45919	449	**423,** 26	**258, 183,** 8	**246, 107, 96**	**449**
52[b]	U45918	449	**423,** 26	**258, 183,** 8	**449**	**449**
53	X74482	449	**449**	**232, 217**	**206, 158, 85**	**449**
53[a]	DQ241374	449	**449**	**232, 217**	**243, 206**	**449**
54	U12501	452	**452**	**217, 127, 108**	**452**	**138, 125, 117, 72**
54[a]	AF436129	452	**245, 207**	**217, 127, 108**	**452**	**138, 125, 117, 72**
55[*]	U12494	455	**455**	**215, 124, 108,** 8	**112, 111, 101, 85, 46**	**165, 161, 72, 57**
56	X74483	449	**242, 207**	**275, 166,** 8	**307, 142**	**310, 72, 49,** 18
58	U45927	449	**216, 207,** 26	**449**	**348, 101**	**306, 111,** 32
58[a]	U45929	449	**216, 207,** 26	**449**	**449**	**306, 143**
58[b]	DQ241375	449	**216, 210,** 23	**449**	**449**	**304, 111,** 34
59	X77858	455	**429,** 26	**399, 56**	**455**	**455**
59[a]	U45933	455	**429,** 26	**455**	**455**	**455**
61	U12500	455	**455**	**212, 211,** 24, 8	**455**	**185, 180, 72,** 18
62	U12499	449	**341, 108**	**232, 217**	**449**	**359, 72,** 18
64[*]	U12495	458	**432,** 26	**334, 124**	**211, 151, 87,** 9	**186, 161, 72,** 39
66	U12498	449	**207, 150, 66,** 26	**449**	**291, 158**	**449**
66[a]	DQ344527	449	**207, 150, 66,** 26	**441,** 8	**291, 128,** 30	**449**
66[b]	DQ486474	449	**207, 150, 66,** 26	**449**	**291, 158**	**377, 72**
67	U12492	449	**423,** 26	**266, 183**	**307, 92, 50**	**310, 72, 67**
68	U45934	455	**455**	**455**	**455**	**260, 85, 72,** 38
68[a]	AJ831567	455	**455**	**386, 69**	**455**	**260, 85, 72,** 38
69	U12497	455	**455**	**223, 183, 49**	**455**	**365, 72,** 18
70	U12476	455	**242, 213**	**232, 117, 106**	**455**	**231, 123, 72,** 29
70[a]	U22461	455	**242, 213**	**232, 117, 106**	**297, 158**	**231, 123, 72,** 29
70[b]	AF538717	455	**242, 213**	**232, 223**	**455**	**231, 123, 72,** 29
70[c]	U21941	455	**455**	**232, 117, 106**	**455**	**231, 123, 72,** 29
71	AY330622	452	**360, 92**	**217, 127, 108**	**320, 132**	**380, 72**
72	U12477	455	**455**	**220, 211,** 24	**300, 155**	**365, 72,** 18
73	U12491	458	**432,** 26	**458**	**243, 215**	**201, 161, 96**
74	AF436130	455	**455**	**218, 180, 49,** 8	**215, 126, 114**	**165, 158, 72, 60**
74[a]	Y12221	455	**455**	**455**	**455**	**222, 161, 72**
74[b]	U40822	455	**455**	**232, 215,** 8	**243, 212**	**222, 161, 72**
81	AJ620209	452	**341, 111**	**127, 121, 108, 96**	**452**	**452**
82	AB027021	455	**455**	**447,** 8	**455**	**310, 73, 72**
82[a]	AF293961	455	**455**	**447,** 8	**243, 212**	**383, 72**

Table 2 (*Continued*)

HPV type	GenBank sequence	MY PCR amplicon (bp)	RFLP fragment sizes (bp)			
			PstI	HaeIII	DdeI	RsaI
82[b]	U12483	455	**455**	**455**	**288, 167**	**383, 72**
83	U12489	452	**317, 135**	**383, 69**	**452**	**380, 72**
83[a]	LVX82	452	**452**	**383, 69**	**452**	**380, 72**
84	U12490	452	**452**	**346, 106**	**220, 142, 90**	**310, 142**
85	AF131950	455	**455**	**455**	**297, 158**	**383, 72**
86	AF349909	452	**317, 135**	**343, 106,** 3	**310, 125,** 17	**380, 72**
87	AF400628	452	**351, 101**	**232, 209,** 8, 3	**362, 90**	**380, 72**
89	U12478	452	**452**	**325, 127**	**246, 152, 54**	**380, 72**
90	AF042837	449	**449**	**232, 209,** 8	**449**	**310, 139**
91	AF070938	455	**455**	**455**	**357, 98**	**455**
97	DQ080080	455	**242, 213**	**447,** 8	**362, 93**	**135, 125, 123, 72**
102	DQ080083	452	**359, 93**	**166, 121, 96, 69**	**452**	**452**
106	DQ080082	452	**452**	**209, 127, 108,** 8	**452**	**452**

Restriction fragments larger than 40 bp are in bold. Fragments under this size are not considered for discrimination of HPV types, because they are not visualized with good resolution in polyacrylamide or agarose gels. HPV 55[*] is a subtype of HPV 44, and HPV 64[*] is a subtype of HPV 34, as reported by de Villiers et al. (2004). The superscript letters (a–c) show HPV variants that have an altered RFLP-pattern with regards to their respective reference HPV type.

Table 3
MY09/11 amplicons that are not cut by PstI, HaeIII, DdeI and RsaI restriction enzymes

Restriction enzyme	Restriction site not present in MY09/11 amplicons of HPVs
PstI	HPV 6, 32, 40, 42, 44, 51, 53, 53[a], 54, 55, 61, 68, 68[a], 69, 70[c], 72, 74, 74[a], 74[b], 82, 82[a], 82[b], 83[a], 84, 85, 89, 90, 91, 106
HaeIII	HPV 18, 18[a], 26, 33, 39, 42, 58, 58[a], 58[b], 59[a], 66, 66[b], 68, 73, 74[a], 82[b], 85, 91
DdeI	HPV 16, 26, 52[b], 54, 54[a], 58[a], 58[b], 59, 59[a], 61, 62, 68, 68[a], 69, 70, 70[b], 70[c], 74[a], 81, 82, 83, 83[a], 90, 102, 106
RsaI	HPV 30, 52, 52[a], 52[b], 53, 53[a], 59, 59[a], 66, 66[a], 81, 91, 102, 106

The superscript letters (a–c) show HPV variants that have an altered RFLP pattern with regards to their respective reference type.

3.3. Evaluation of RFLP restriction efficacy

Since digestion can be partial or fail due to several factors, we have designed a strategy, which was done in parallel to the HPV genotyping, to avoid misidentification of types, based in the co-digestion of MY09/11 amplicon with a control DNA. For that purpose, we have designed a pair of primers that target a region of β-globin gene (Beta1/2 amplicon), which is cleaved by the whole spectrum of selected restriction enzymes when optimal conditions are achieved, originating the following restriction fragments: PstI: 322 bp + 179 bp; HaeIII: 279 bp + 222 bp; DdeI: 271 bp + 198 bp + 32 bp; RsaI: 251 bp + 210 bp + 40 (Fig. 2). The size of these fragments does not coincide with any of the fragments listed in Table 2.

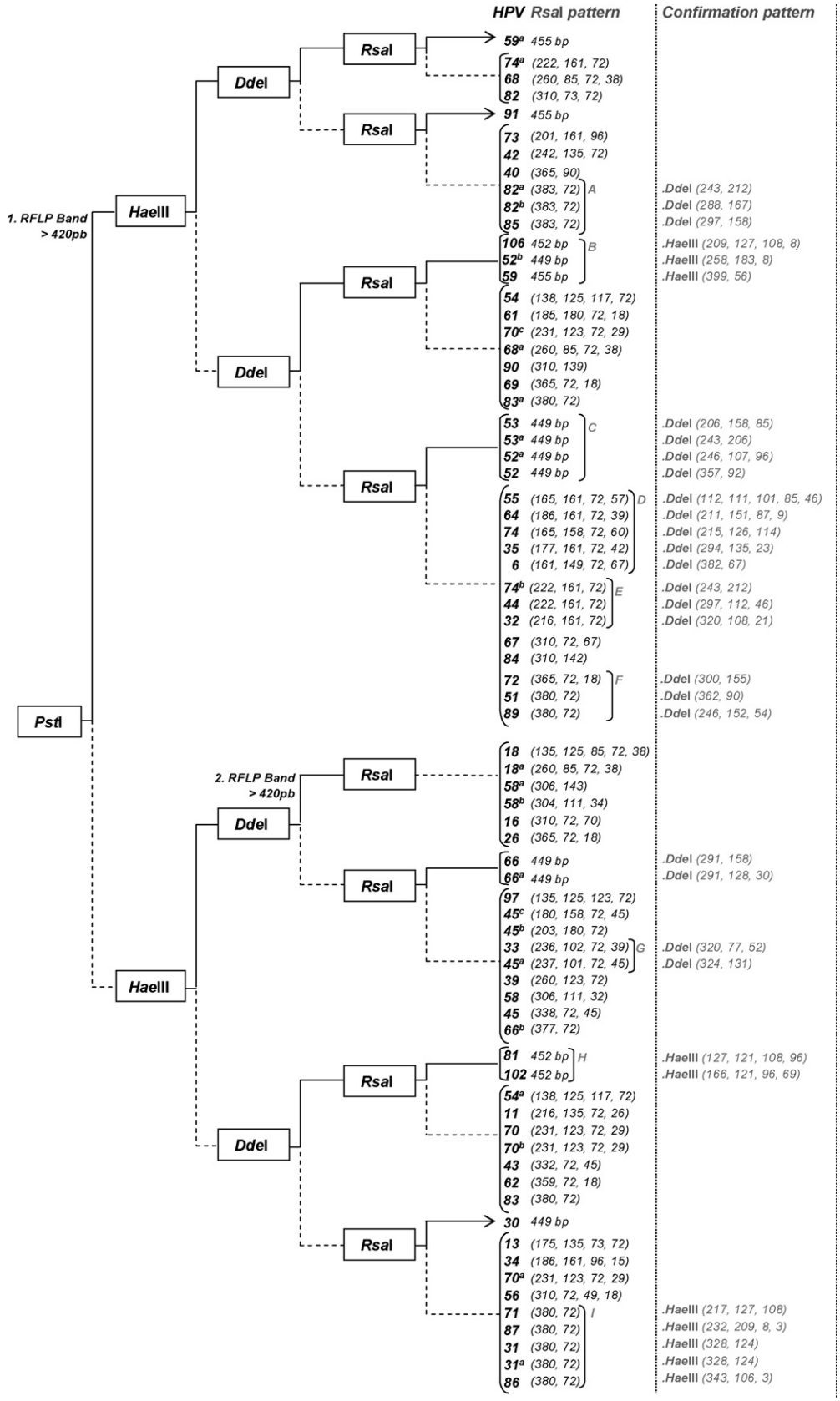
3.4. Evaluation of HPV typing approach in clinical samples

To confirm the reliability of this PCR-RFLP based methodology and the applicability of the HPV typing algorithm, two different experiments were performed. The first study was carried out, in a blind fashion, on pre-selected HPV-positive clinical samples, in which 27 different mucosal HPV types had been previously identified by PCR with MY09/11 primers and DNA sequencing (HPV 6, 11, 16, 18, 31, 33, 34, 35, 39, 44, 45, 52, 53, 56, 58, 59, 61, 62, 66, 67, 70, 71, 73, 81, 83, 84, and 102). In all samples, PCR-RFLP data consistently matched the DNA sequencing results. Fig. 3 shows the RFLP patterns of some HPV types (HPV 16, 58 and 102).

The second study, which has consisted in a random and blind experiment, was performed on a representative collection of clinical samples obtained from women attending the cervical screening program of IPOCFG, EPE. Of the 30 cervical samples analyzed, HPV DNA was detected in 16 cases (3/10 normal samples, 3/10 low grade-lesions, 5/5 high grade-lesions and 5/5 carcinoma samples). Corresponding MY09/11 PCR products were subsequently genotyped using both RFLP analysis and sequencing. Table 4 shows the results obtained using each method. RFLP data consistently matched the DNA sequencing results, with the exception of one single case, sample 22, in which it was impossible to discriminate the triple HPV infection using RFLP analysis, due to the complexity of the pattern obtained. Nevertheless, in all other HPV-positive samples, even in the case of multiple infections (e.g. samples 16 and 23), sequencing results confirmed RFLP genotyping. All the enzymes have shown a high cut efficiency and no partial digestions were visualized under the RFLP conditions previously described.

4. Discussion

Due to differences in the oncogenic activity of HPVs, it is important to detect HPV infection and to accurately identify HPV types in a simple and quick manner. This work aimed at developing an easy, rapid and cost-effective PCR-RFLP assay to identify all known and unknown mucosal HPV types,


HPV RsaI pattern
Confirmation pattern
PstI
1. RFLP Band > 420pb
HaeIII
DdeI
RsaI
59a 455 bp
74a (222, 161, 72)
68 (260, 85, 72, 38)
82 (310, 73, 72)
91 455 bp
73 (201, 161, 96)
42 (242, 135, 72)
40 (365, 90)
82a (383, 72) A
82b (383, 72)
85 (383, 72)
.DdeI (243, 212)
.DdeI (288, 167)
.DdeI (297, 158)
106 452 bp B
52b 449 bp
59 455 bp
.HaeIII (209, 127, 108, 8)
.HaeIII (258, 183, 8)
.HaeIII (399, 56)
54 (138, 125, 117, 72)
61 (185, 180, 72, 18)
70c (231, 123, 72, 29)
68a (260, 85, 72, 38)
90 (310, 139)
69 (365, 72, 18)
83a (380, 72)
53 449 bp C
53a 449 bp
52a 449 bp
52 449 bp
.DdeI (206, 158, 85)
.DdeI (243, 206)
.DdeI (246, 107, 96)
.DdeI (357, 92)
55 (165, 161, 72, 57) D
64 (186, 161, 72, 39)
74 (165, 158, 72, 60)
35 (177, 161, 72, 42)
6 (161, 149, 72, 67)
.DdeI (112, 111, 101, 85, 46)
.DdeI (211, 151, 87, 9)
.DdeI (215, 126, 114)
.DdeI (294, 135, 23)
.DdeI (382, 67)
74b (222, 161, 72) E
44 (222, 161, 72)
32 (216, 161, 72)
.DdeI (243, 212)
.DdeI (297, 112, 46)
.DdeI (320, 108, 21)
67 (310, 72, 67)
84 (310, 142)
72 (365, 72, 18) F
51 (380, 72)
89 (380, 72)
.DdeI (300, 155)
.DdeI (362, 90)
.DdeI (246, 152, 54)
2. RFLP Band > 420pb
18 (135, 125, 85, 72, 38)
18a (260, 85, 72, 38)
58a (306, 143)
58b (304, 111, 34)
16 (310, 72, 70)
26 (365, 72, 18)
66 449 bp
66a 449 bp
.DdeI (291, 158)
.DdeI (291, 128, 30)
97 (135, 125, 123, 72)
45c (180, 158, 72, 45)
45b (203, 180, 72)
33 (236, 102, 72, 39) G
45a (237, 101, 72, 45)
.DdeI (320, 77, 52)
.DdeI (324, 131)
39 (260, 123, 72)
58 (306, 111, 32)
45 (338, 72, 45)
66b (377, 72)
81 452 bp H
102 452 bp
.HaeIII (127, 121, 108, 96)
.HaeIII (166, 121, 96, 69)
54a (138, 125, 117, 72)
11 (216, 135, 72, 26)
70 (231, 123, 72, 29)
70b (231, 123, 72, 29)
43 (332, 72, 45)
62 (359, 72, 18)
83 (380, 72)
30 449 bp
13 (175, 135, 73, 72)
34 (186, 161, 96, 15)
70a (231, 123, 72, 29)
56 (310, 72, 49, 18)
71 (380, 72) I
87 (380, 72)
31 (380, 72)
31a (380, 72)
86 (380, 72)
.HaeIII (217, 127, 108)
.HaeIII (232, 209, 8, 3)
.HaeIII (328, 124)
.HaeIII (328, 124)
.HaeIII (343, 106, 3)

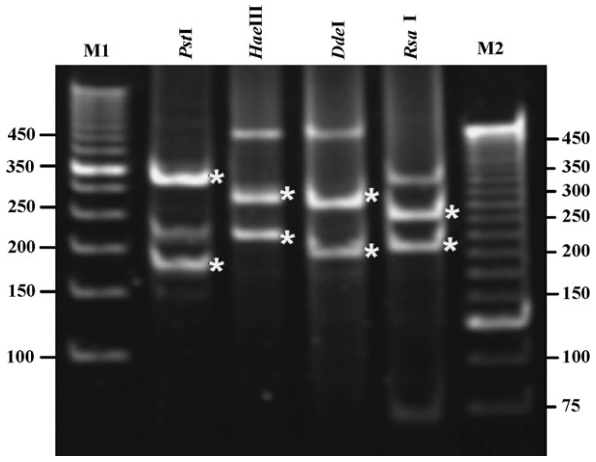


Fig. 2. Validation of RFLP restriction reactions on 10% polyacrylamide gel (RFLP patterns from co-digestion of MY09/11PCR products of HPV 16 and Beta1/2 amplicon). Each restriction reaction was performed in a final volume of 20 μl, using 1 μg of each PCR product, 20 units of each restriction endonuclease at 37 °C for 1 h. Asterisks indicate fragments from Beta1/2 amplicon restriction. Lane M1, 50 bp DNA molecular weight marker; Lane M2, 25 bp DNA molecular weight marker.

based on the amplification of a fragment of the L1 gene using MY09/11 primers and subsequent RFLP analysis.

In the impossibility of evaluating our method on the more than 290 published HPV variants, we made use of bioinformatic tools to evaluate if a PCR-RFLP based methodology, using PstI, HaeIII, DdeI and RsaI restriction enzymes, allowed discrimination of all individual HPV types. For that purpose, an extensive *in silico* analysis of all different DNA sequences of the HPV L1 gene, corresponding to 49 mucosal HPV types, was performed for PstI, HaeIII, DdeI and RsaI restriction sites. Of 296 HPV variants analyzed, only 23 showed a different set of RFLP patterns when compared with their reference type. Moreover, these 23 variants presented sets of RFLP patterns that were distinct from all the other types, thus not affecting the individual identification of mucosal HPV types. The bioinformatic results have confirmed that each HPV type had a specific set of PstI, HaeIII, DdeI and RsaI-RFLP patterns, which were not shared by any other HPV type and could be clearly identified by visual discrimination on 10% polyacrylamide gels.

In summary, the herein described PCR-RFLP based method permits discrimination of all known mucosal HPV types (49 HPV types and 2 subtypes). This range of HPV identification largely exceeds the number of types characterized by commercial assays currently available or by any other genotyping methodology based on RFLP analysis (Bernard et al., 1994; Pizzighella et al., 1995; Patti et al., 2002; Naqvi et al., 2004; Santiago et al., 2006).

A RFLP assay was recently reported that uses a single restriction enzyme (*Hpy*CH4V) to type 39 mucosal HPVs (Santiago et al., 2006). Although an effective discrimination between high- and low-risk types was achieved with this enzyme, it did not allow an individualized identification of all HPV types, as some HPV types show the same *Hpy*CH4V RFLP pattern (HPV 11 and 30; and HPV 44 and 55, for instance). Moreover, using *Hpy*CH4V, several HPV types (HPV 18 and 68, HPV 18 and 70, HPV 18 and 42, HPV 31 and 58, HPV 33 and 13, HPV 51 and 83, HPV 56 and 11, HPV 58 and 62, HPV 82 and 53, HPV 6 and 64, HPV 61 and 84) were indistinguishable on common, non-expensive agarose or polyacrylamide gels, because the size of their *Hpy*CH4V restriction fragments differed in less than 9 bp. Compared to this method, our PCR-RFLP assay requires substantially less sophisticated equipment in the analysis of RFLP patterns and may thus be more adequate in settings of poor economical resources.

In order to minimize errors and avoid time consuming procedures associated with the complexity of visual discrimination, in polyacrylamide gels, of RFLP patterns of the assays previously reported (Bernard et al., 1994; Santiago et al., 2006), we have developed an original genotyping algorithm to rapidly identify HPV types (Fig. 1). This algorithm allows ready identification of HPV types in two easy steps: (1) sequential analysis of the occurrence/non-occurrence of PstI, HaeIII, DdeI and RsaI restriction reactions; and (2) subsequent comparison of the restriction fragments obtained in RsaI digestion with the expected sizes defined for each HPV type. Finally, when a HPV type is identified, it is possible to confirm it, comparing all the RFLP-patterns obtained with data described in Table 2.

In addition to bioinformatics studies, the reliability of our method and the applicability of this new HPV genotyping algorithm were successfully confirmed in two sets of experiments, which were carried out on already pre-selected HPV-positive clinical samples and on a representative collection of unselected clinical samples. One limitation has come out of these studies. Our RFLP methodology is less efficient when multiple HPV genotypes are present in the same sample. In the case of multiple infections, RFLP patterns are often very complex and the resolution of fragment sizes may not allow the use of the HPV algorithm in the

Fig. 1. Algorithm for mucosal HPV typing after RFLP analysis of MY09/11 amplicons using PstI, HaeIII, DdeI, and RsaI restriction enzymes. In the algorithm, each box represents a restriction reaction using a specific enzyme. Draw lines (—) represent the possible results when digestion does not occur, whereas dashed lines (- - -) represent the possible results when digestion occurs. In two particular situations (designated by 1 and 2), draw lines also represent the possible results when digestion originates an RFLP-fragment larger than 420 bp. The HPV type is identified in two easy steps: (1) sequential analysis of occurrence/non-occurrence of PstI, HaeIII, DdeI and RsaI restriction reactions and (2) subsequent comparison of the restriction fragments obtained by RsaI digestion with the expected sizes (in brackets) defined for each HPV type by *in silico* analysis. In cases in which RsaI digestion does not occur (groups B, C and H), or when two or more HPVs present similar RsaI-RFLP patterns (groups A, D, E, F, G, I), HPV type can be identified by comparison of the restriction fragment sizes obtained by DdeI or HaeIII restriction digestion ("confirmation pattern").

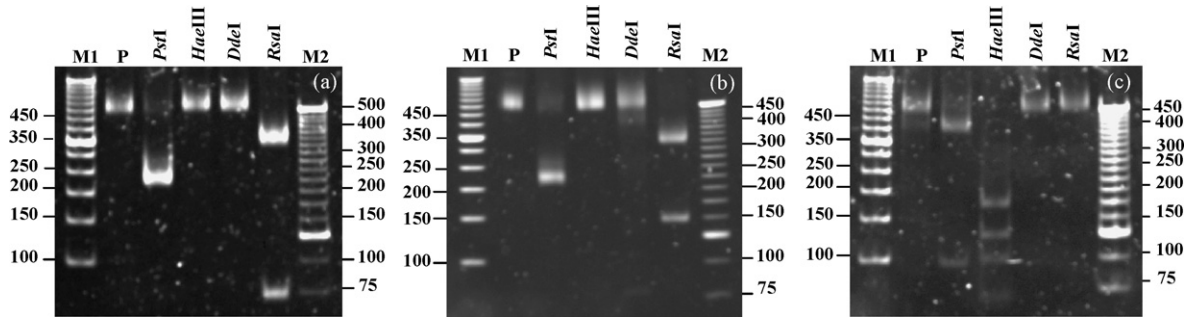


Fig. 3. RFLP patterns of MY09/11 PCR products of HPV 16, 58 and 102. 1 μg of MY09/11 PCR products were mixed in a total volume of 20 μl with 10 units of the PstI, HaeIII, DdeI, and RsaI at 37 °C for 1 h. The digested and undigested PCR products and DNA molecular weight markers were electrophoretically separated on 10% polyacrylamide gels, stained with Gelstar. RFLP patterns of: (a) HPV 16 (U89348), (b) a variant of HPV 58 (U45929), (c) HPV 102 (DQ080083). Lane M1, 50 bp DNA molecular weight marker; Lane P, undigested MY09/11 PCR amplicon (≈450 bp); Lane M2, 25 bp DNA molecular weight marker.

identification of genotypes involved. Thus, the efficacy of our method may be improved through its incorporation in a multi-methodology strategy. Taking this into account, the herein described assay can be used as a first-line genotyping method to determine the HPV genotype in single infections (or even to discriminate between single and multiple infections), while reverse hybridization analysis may be used as complementary method, when unknown or complex RFLP patterns are visualized.

The reproducibility and sensitivity of the MY09/11 amplification system were recently improved by the development of the new primer set PGMY09/11 (Gravitt et al., 2000). This system consists in a set of 5 upstream oligonucleotides (PGMY11 primer pool) and a set of 13 downstream oligonucleotides (PGMY09 primer pool), which bind to the same region of MY09 and MY11 primer, respectively. This new primer system increases the efficiency of the amplification of some HPV types and appears to be more sensitive than MY09/11 primers (Coutlée et al., 2002; Giovanelli et al., 2004). Bioinformatic analysis suggests that our HPV typing methodology can also incorporate the PGMY system, as it is based on the same primer binding regions used by MY primers. Although 2 of 18 PGMY primers are two bases shorter than MY primers, this difference is not significant and does not alter the herein described RFLP patterns.

In conclusion, our approach offers significant advantages, not only over the PCR-RFLP techniques previously described, but also over other currently available HPV typing assays (like hybridization with type-specific probes, type-specific PCR, microarray methodologies, or any of the commercial platforms): (a) it detects a higher number of mucosal HPV types (49 HPV types and 2 HPV subtypes); (b) it allows detection of unknown HPV types; and (c) it is financially advantageous, and therefore particularly suited for routine HPV detection and identification in settings of poor financial resources, like cervical screening programs in developing countries.

Table 4
Comparative genotyping results of HPV-positive clinical samples using RFLP analysis and DNA sequencing/cloning analysis of MY09/11 PCR products

Sample	Diagnosis	HPV typing	
		RFLP analysis results	DNA sequencing and cloning results
3	Normal	S–HPV 16	S–HPV 16 (U89348)
7	Normal	S–HPV 6	S–HPV 6 (S73503)
8	Normal	S–HPV 67	S–HPV 67 (U12492)
12	Low-grade lesion	S–HPV 62	S–HPV 62 (U12499)
13	Low-grade lesion	S–HPV 6	S–HPV 6 (S73503)
16	Low-grade lesion	M–HPV 53 + 84	M–HPV 53 (X74482) + 84 (U12490)
21	High-grade lesion	S–HPV 58 (a variant)	S–HPV 58 (U45929)
22	High-grade lesion	M–*	M–HPV 16 (U89348) + 33 (U45896) + 6 (S73503)
23	High-grade lesion	M–HPV 30 + 56	M–HPV 30 (X74474) + 56 (X74483)
24	High-grade lesion	S–HPV 31	S–HPV 31 (AJ831566)
25	High-grade lesion	S–HPV 31	S–HPV 31 (AJ831566)
26	Carcinoma	S–HPV 16	S–HPV 16 (U89348)
27	Carcinoma	S–HPV 16	S–HPV 16 (U89348)
28	Carcinoma	S–HPV 33	S–HPV 33 (U45896)
29	Carcinoma	S–HPV 18	S–HPV 18 (X05015)
30	Carcinoma	S–HPV 58 (a variant)	S–HPV 58 (U45929)

Accession numbers of DNA sequences analyzed are given in brackets. Letters S and M indicate single and multiple infections, respectively.
* Types could not be differentiated.

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