

Polymerase chain reaction amplification and restriction enzyme typing as an accurate and simple way to detect and identify human papillomaviruses

S. PIZZIGHELLA*, M. RASSU, I. PIACENTINI*, B. MASCHERA and G. PALÚ†

*Institute of Microbiology, University of Padua Medical School, Padua and *Microbiology Department, OCM Verona, Italy*

Summary. A simple and economic method for the detection and identification of human papillomaviruses (HPV) is described. The method has been developed with cloned HPV DNA and DNA from clinical samples. Genomic fragments were obtained from several different HPV types, including the ones most frequently encountered in the genital tract by polymerase chain reaction (PCR) amplification directed by degenerate general primers. The amplification fragments were identified by a form of miniature fingerprinting, with a set of restriction enzymes that gave a unique digestion pattern for each HPV type. Different strategies are proposed, based on PCR and restriction analysis, and this approach to identification was compared with more classic methods such as Southern hybridisation.

Introduction

The oncogenic properties of papillomaviruses were first demonstrated in animal models and in 'in-vitro' experiments. Clinical and epidemiological studies established strong correlations between genital and anal cancers and papillomavirus (HPV) infection in man.¹ Epithelial neoplasia, other than anogenital cancer, has also been related to HPV infection.^{2–6} Various HPVs have been identified that usually infect the genital tract. They can be divided into two groups: those (HPV-6 and HPV-11) which are associated with non-malignant lesions, such as condylomata, and are thought not to play an aetiological role in neoplasia;^{7,8} and those which are found in neoplastic lesions. Among the latter, HPV-16 and HPV-18 are the most frequently isolated, but HPV-31,⁸ HPV-33,⁹ HPV-35,¹⁰ HPV-52b,¹¹ HPV-58¹² and possibly other types¹³ are also involved.

Although different chemicals and infectious agents have been ranked among the pathogenetic factors of cervical cancer—including byproducts of tobacco smoking,¹⁴ oral contraceptives,¹⁵ herpes simplex virus,¹⁶ cytomegalovirus and Epstein-Barr virus,¹⁷—HPV infection has been strongly linked to both cellular transformation¹⁸ and maintenance of the malignant phenotype. HPV DNA can be detected in virtually all patients with genital cancer,¹⁹ even if no data are available to assess how long the infection might have preceded development of neoplasia. Assuming that infection by HPV is a necessary event in the process of

neoplastic transformation, it could be expected that HPV would be found in cervical tissue well before any other cytopathological change or clinical finding could alert the clinician. The ability to find "oncogenic" HPVs in healthy patients could be very useful in identifying a subpopulation of women at risk of developing cancer, for whom more careful clinical surveillance is needed. Indeed, a significant percentage of women who carry "oncogenic" HPVs (10–20% in different reports),^{20,21} have a higher relative risk of developing cancer than the non-infected population.¹

It is clear that, for diagnostic purposes, it is necessary to devise a system that is sensitive enough to allow the detection of HPVs very early in the disease. In this respect, DNA amplification by polymerase chain reaction (PCR) appears to be the technique of choice to reveal minimal amounts of HPV DNA.^{22–24}

We have developed a simple and cheap method of detecting and typing HPVs in which a single pair of degenerate consensus primers enable the amplification of genomic sequences from at least six different HPVs. Typing is done by restriction enzyme digestion of the PCR product. The effectiveness of this approach has been assessed with cloned HPV DNA and DNA from clinical samples.

Materials and methods

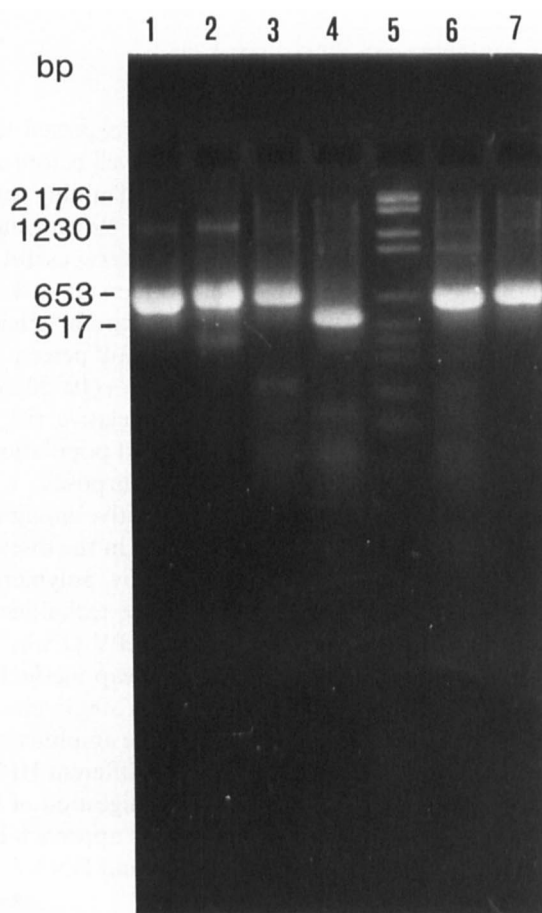
Plasmids containing complete HPV genomes were propagated in *Escherichia coli* strain HB 101, supE44 hsdS20 (r_B⁻ m_B⁻) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1, and purified by conventional techniques.²⁵ DNA was also extracted from paraffin-

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† Correspondence should be sent to Professor G. Palú.

Table. Expected size of fragments after digestion of HPV-specific PCR products by restriction endonucleases

Amplified DNA	Sizes of fragments (bp) expected in products from HPV					
	-6	-11	-16	-18	-31	-33
Total length	654	654	648	538	645	649
Digestion fragments						
<i>AccI</i>	643 11	654 —	386 146 116	359 179	282 112	405 244
<i>TaqI</i>	536 118	654 —	648 —	504 34	645 —	649 —
<i>HincII</i>	654 —	654 —	648 —	538 —	370 275	518 131
<i>SspI</i>	388 266	469 185	458 107	538 —	538 107	326 188
	—	—	83	—	—	135
<i>NdeI</i>	494 160	494 160	648 —	538 —	645 —	649 —
<i>HinfI</i>	419 181 54	419 235 —	538 110 —	538 — —	410 189 46	389 260 —

**Fig. 1.** PCR amplification of cloned HPV DNA by general primers. Lane 1, HPV-6; 2, HPV-11; 3, HPV-16; 4, HPV-18; 5, mol. wt markers; 6, HPV-31; 7, HPV-33.

embedded samples of codylomata obtained for histopathological examination. The extraction technique was essentially that described by Wright and Manos²⁶ with two octane and two ethanol extraction cycles and extensive proteinase K digestion.

Nucleotide sequence analysis

Genomic sequences of the different HPVs were obtained by EMBL data bank and analysed for the presence of homologies and restriction sites by the Microgenie (Beckman) program.

Primer synthesis

Primers were synthesised with a Beckman synthesiser (DNA SM model) and purified by high performance liquid chromatography (HPLC). Throughout the study, degenerate consensus primers designed by Evander and Wadell were used;²⁴ these could direct the amplification of DNA from several different HPVs, including the six types found most frequently in the genital tract—HPV-6, 11, 16, 18, 31 and 33. Computer analysis showed that these primers should be able to amplify sequences of HPV-35 too,²⁷ but this was not tested. The amplified fragment is localised in the E7–E1 genomic region of all viruses, encompassing nucleotides 607–1260 for HPV-6 and -11, nucleotides 636–1283 for HPV-16, nucleotides 805–1342 for HPV-18, nucleotides 634–1278 for HPV-31, and nucleotides 647–1295 for HPV-33. The size of fragments varied from 645 bp to 654 bp for all viruses tested except for HPV-18, in which a 538-bp product was obtained that represents a useful marker for typing.

PCR

For amplification, 10–100 pg of recombinant DNA or 0.1–1 µg of DNA extracted from clinical samples, were mixed with a solution of dATP, dTTP, dCTP and dGTP (final concentration 50 mM each) in PCR buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM MgCl₂; gelatin 100 µg/ml), containing 24 pmols of each primer and 2U of Taq polymerase, in a final volume of 100 µl. The mixture was overlaid with 100 µl of paraffin oil and subjected to 30–40 cycles of amplification. Each cycle included denaturation at 94°C, annealing at 50°C and elongation at 72°C, 1 min/step. At the end of these cycles there was an additional extension step of 10 min at 72°C. Negative controls with pBR322 were included in each run.

Detection and identification of PCR product

After amplification, a 20-µl sample was digested with several restriction enzymes—*AccI*, *TaqI*, *HincII*, *SspI*, *NdeI* and *HinfI*; the predicted sizes of the digestion fragments are shown in the table. Digested fragments were loaded on to a NuSieve agarose 3% gel (FMC Bioproducts), electrophoresed in TBE buffer (0.045 M Tris-borate, 0.001 M EDTA), stained with ethidium bromide and photographed under UV illumination with a Polaroid MP-4 Land Camera. Restriction enzyme analysis was also performed in the

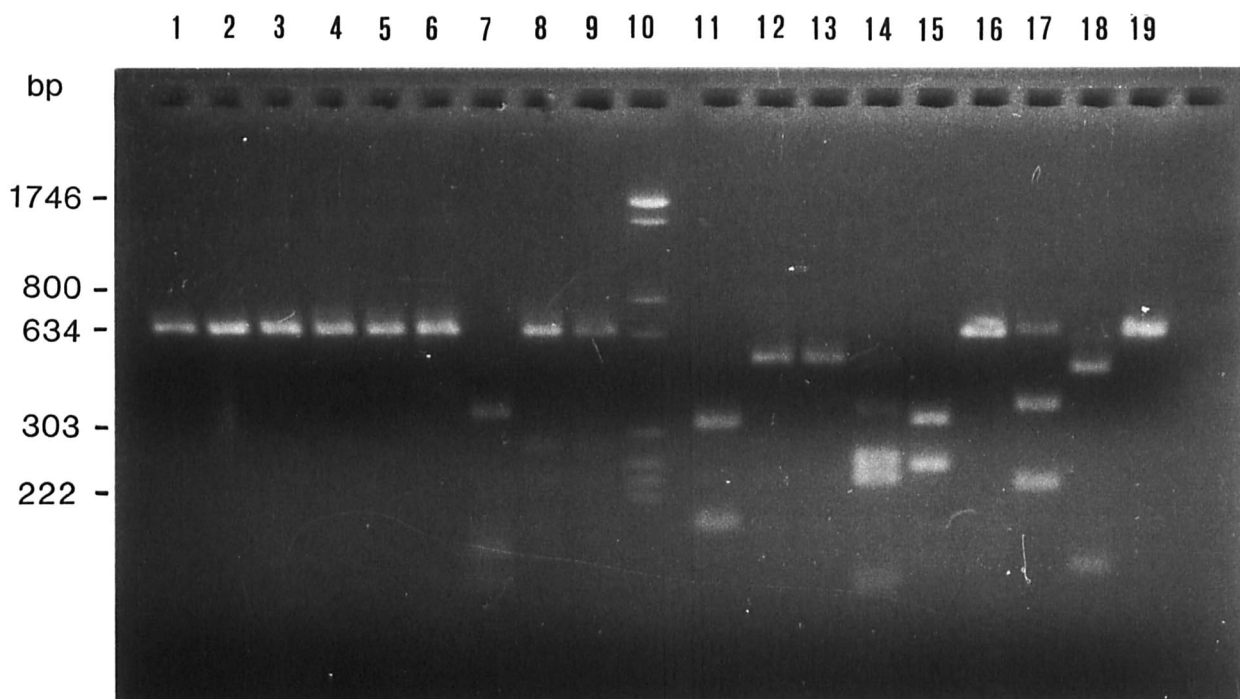


Fig. 2. Restriction endonuclease analysis of amplification fragments shown in fig. 1 with *AccI* and *HincII*. Lane 1, HPV-6, *AccI*; 2, HPV-6, *HincII*; 3, HPV-6 uncut control; 4, HPV-11, *AccI*; 5, HPV-11, *HincII*; 6, HPV-11 uncut control; 7, HPV-16, *AccI*; 8, HPV-16, *HincII*; 9, HPV-16 uncut control; 10, mol. wt markers; 11, HPV-18, *AccI*; 12, HPV-18, *HincII*; 13, HPV-18 uncut control; 14, HPV-31, *AccI*; 15, HPV-31, *HincII*; 16, HPV-31 uncut control; 17, HPV-33, *AccI*; 18, HPV-33, *HincII*; 19, HPV-33 uncut control.

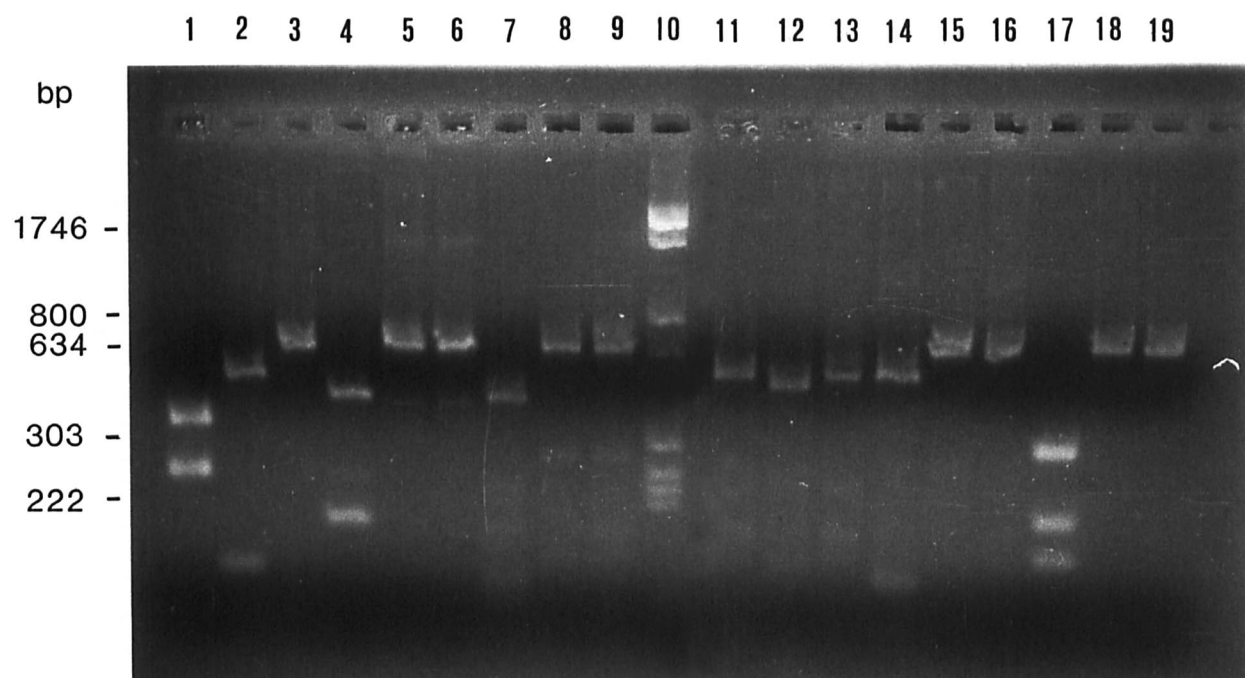


Fig. 3. Restriction endonuclease analysis of amplification fragments shown in fig. 1 with *SspI* and *TaqI*. Lane 1, HPV-6, *SspI*; 2, HPV-6, *TaqI*; 3, HPV-6 uncut control; 4, HPV-11, *SspI*; 5, HPV-11, *TaqI*; 6, HPV-11 uncut control; 7, HPV-16, *SspI*; 8, HPV-16, *TaqI*; 9, HPV-16 uncut control; 10, mol. wt markers; 11, HPV-18, *SspI*; 12, HPV-18, *TaqI*; 13, HPV-18 uncut control; 14, HPV-31, *SspI*; 15, HPV-31, *TaqI*; 16, HPV-31 uncut control; 17, HPV-33, *SspI*; 18, HPV-33, *TaqI*; 19, HPV-33 uncut control.

case of HPV-18, although the specific amplification fragment could be identified by its lower mol. wt.

Results

The restriction enzymes that were selected are reported in the table, together with the sizes of the fragments generated and the respective attribution to each HPV genotype. It is readily apparent that the use

of one or more enzymes can lead to an accurate identification of the PCR product.

The degenerate consensus primers used in this study were able to direct the amplification of DNA from all six HPCVs tested, giving a band of the expected size after gel electrophoresis (fig. 1). The assay sensitivity was of the order of 10 fg of template DNA for five of the six HPVs tested—HPV-6, 11, 16, 18 and 33. Amplification efficiency in the case of HPV-31 was

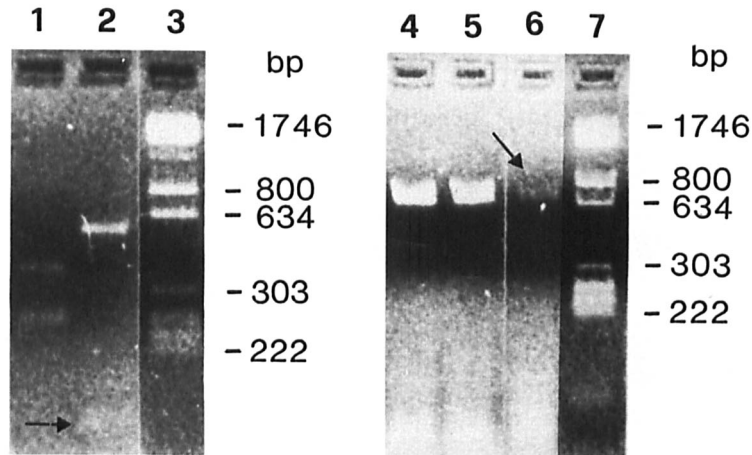


Fig. 4. PCR amplification and restriction enzyme typing of HPV from human condylomata. Lane 1, sample 1, *SspI*; 2, sample 2, *TaqI* (→: 118 bp); 3, mol. wt markers; 4, sample 1; 5, sample 2; 6, sample 3 (→): 7, mol. wt markers.

somewhat lower, a result which is consistent with the presence of three mismatches in the central region of the sense primer.²⁴ The specificity of the detected products was tested by restriction enzymes. Results are shown in figs. 2 and 3. Restriction enzymes gave fragments of the expected size, demonstrating that the amplified DNA was specific for the different HPVs. It is also noticeable (fig. 2) that *AccI* produced more bands than expected (lanes 11, 14 and 17), due to a partial digestion of the amplification product by this enzyme. As shown in lane 11, HPV-18 was cut at position 983, giving two bands of 359 and 179 bp, plus the original uncut segment of 538 bp. The same also holds true for HPV-33 (lane 17), which was only partially digested at position 1051. For HPV-31, the pattern is more complicated; the PCR fragment was cut at positions 745 and 1027, giving three bands of 112, 282 and 251 bp, which are shown clearly in lane 14. The two additional bands that are significant, one of 533 bp and the other of 394 bp, were generated by *AccI* digestion only at position 745 and 1027 respectively. In another set of experiments, when a new batch of *AccI* was used with the same substrate DNA, a complete pattern of digestion was observed (not shown). Further analysis with *NdeI* and *HinfI* gave fragments of the expected sizes. Spurious, very faint bands of low mol. wt can be seen only occasionally (lane 8, fig. 2; lanes 5 and 6, fig. 3). This result, which may be due to either contamination or mis-priming, did not affect detection and, consequently, the specificity of the assay.

A few clinical specimens were studied by the approach described above. In fig. 4, an analysis of three condylomata is shown. Clearly visible bands were obtained with samples 1 and 2, whereas only a low intensity band was observed with sample 3. Restriction digestion of the PCR products 1 and 2, with *SspI* and *TaqI* respectively, gave a pattern typical of HPV-6b. The band generated from sample 3 was too faint to be analysed by restriction endonuclease digestion.

Discussion

A method is described for the detection and typing of HPVs which could be very useful for clinical purposes, because it is simple and economic. Taking advantage of the sequence homology between different HPV types, several authors have proposed the use of general primers for their simultaneous detection.^{24, 28, 29} This approach seems more convenient than using specific primers for each HPV²³ or a mixture of such primers in a single tube.³⁰

In this study, a set of general primers already described by Evander and Wadell was employed;²⁴ these are complementary to sequences spanning the E1-E7 region of HPV genomes. This region is highly conserved among different genotypes and is not usually affected by integration into cellular DNA. Unlike the previous authors, who checked the specificity of the amplified products by Southern blot hybridisation, we exploited selective sequence recognition by restriction endonucleases. The amplified DNA was digested by several different endonucleases to assign it to a specific HPV genotype. Only in the case of *AccI* were some drawbacks encountered, such as the presence of extra-numerary bands in some experiments. However, these bands clearly represented partial digestion products and are shown here to demonstrate that identification was not affected, if the molecular complexity of the generated fragments was carefully checked. The restriction digestion profiles showed that the amplification bands visualised by ethidium bromide staining were specific for each HPV type.

The use of Southern blotting, followed by hybridisation with specific probes, is a very effective procedure for the detection and identification of amplified sequences. However, although it is more sensitive than ethidium bromide staining, it is not practical for the routine examination of a large number of clinical samples. Restriction typing, such as the one described here, is, at least in theory, more specific than hy-

bridisation probing, either with Southern or dot blot which depend critically upon the stringency conditions used in the experiment. Further problems can arise from false positive hybridisation signals caused by partial identity between the probe and the amplified DNA. When working with HPVs, such an event is not at all rare, because of the high degree of sequence homology shared by many HPV genotypes. Restriction typing has other advantages; being easy to perform and adaptable, it can provide the clinician with a large body of information, e.g., by using either *NdeI* or *AccI*, "oncogenic" and "non-oncogenic" HPVs can readily be distinguished, a feature which is already clinically relevant. Alternatively, it is possible to type within "oncogenic" HPVs with *AccI*, and also to discriminate between HPV-6 and HPV-11 (*TaqI*). Finally, all six HPVs tested can be simultaneously typed with a single enzyme (*SspI*, table).

This approach, of PCR amplification followed by

restriction endonuclease typing, could be very useful, provided that enough amplified DNA is produced. Our results with clinical samples have demonstrated the effectiveness and specificity of the method when the amplification product is clearly detected by ethidium bromide staining. A higher sensitivity is warranted when working with clinical samples containing just a few cells with a low copy number of HPV genomes. Different general primers, eventually coupled in a nested PCR,²¹ could solve this problem.

It has to be emphasised that HPV-16 and -18 are the HPVs most frequently associated with genital carcinoma; therefore, their detection should be the primary goal of any method devised to reveal and type HPV.

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