Detection of human papillomavirus in vulval carcinoma using semi-nested PCR and restriction enzyme typing: a rapid and sensitive technique

N H Cartwright, L J Sant Cassia, A J Easton, A G Morris

Abstract

Aims—To develop a highly sensitive technique for the reliable detection and typing of human papillomavirus (HPV) DNA in clinical tissue.

Methods—A two step, semi-nested PCR was used with primers spanning the L1 region of the HPV genome and capable of detecting HPV DNA of all known HPV types. The clinical samples were typed by digestion of the 412 base pair PCR product with *Rsa* I, generating unique fragments for each HPV type. Thirteen samples were screened by this method, including nine vulval carcinoma samples and four wart samples from the penis and vulva.

Results—Experiments using DNA extracted from HPV DNA positive cell lines—that is, CaSki (HPV type 16) and HeLa (HPV type 18) established that the technique could detect as few as 50 HPV copies and that the predicted *Rsa* I fragments from HPV types 16 and 18 were generated. The predicted 412 base pair fragment was observed for all 13 clinical samples subjected to semi-nested PCR. *Rsa* I digestion of the product of the second round of PCR permitted the positive identification of the HPV type in most cases.

Conclusions—This technique provides an effective and rapid means of detecting HPV DNA, in most cases providing the HPV type. High risk HPV types were always detected in the nine vulval carcinoma samples analysed. The amount of tissue available from the biopsy specimens was small, confirming the sensitivity of the method.

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Human papillomavirus (HPV) infection is associated with several conditions, ranging from carcinoma to wart-like lesions, with certain HPV types preferentially associated with specific clinical outcomes. HPV types 6 and 11 are associated with benign genital warts¹⁻³ and laryngeal papillomas⁴ and are classed as low risk for the development of malignant tumours. Severe genital dysplasias and carcinomas are most frequently associated with HPV types 16 and 18, and these are classed as high risk virus types. HPVs infecting the genitourinary tract are associated with sev-

eral anogenital cancers, including carcinomas of the vulva,⁵⁻¹⁰ cervix¹¹⁻¹⁵ and penis.¹⁶⁻¹⁸ HPV infection is now recognised as a major aetiological factor in these conditions. Sensitive detection and typing of HPVs, involved in both genital and cutaneous conditions, are essential factors in determining correlation between infection and disease.

The requirement for diagnostic techniques becomes increasingly necessary as the frequency of HPV infection becomes more evident. The detection of a wide range of HPV types is important for the determination of the prevalence of virus infection in clinical tissue. The polymerase chain reaction (PCR) has been the method most commonly used for the amplification of viral DNA in clinical tissue.¹⁹⁻²² The most widely used PCR involved the use of DNA primers that span a sequence of the HPV genome (L1 region) that is homologous to most, if not all, HPV types known, permitting the detection of a wide range of HPV types. It has been suggested that the sensitivity of this PCR may be limited and so a technique with greater sensitivity would be an advantage.^{23, 24} For conditions such as vestibular vulvitis,²⁵⁻²⁸ which in some cases are associated with HPV infection, a sensitive and reliable test is needed.

The identification of the HPV type is also important and has been carried out previously using methods such as oligonucleotide probe hybridisation^{29, 30} and Southern blot analysis³¹ in conjunction with PCR. Diagnostic restriction enzyme analysis, which does not involve the use of radioactivity, has frequently been used to identify HPV types in clinical tissue.^{4, 32} Here, we report the use of a semi-nested PCR technique to detect HPV DNA in small amounts of clinical material, in conjunction with restriction enzyme analysis to identify the HPV type present.

Methods

SAMPLE SELECTION AND ISOLATION OF DNA

Thirteen patient samples from the Walsgrave Hospital, Coventry, were included in the study. Nine of the patients had extensive vulval carcinoma. Four small biopsy samples from genital warts were also tested. Samples were placed on ice in RPMI 1640 immediately after removal.

DNA was extracted from the fresh tissue samples by finely chopping the material and incubating the samples overnight in fresh RPMI 1640 medium containing final concentrations of DNase (0.04 g/ml), hyaluronidase (0.04 mg/ml) and collagenase (2 mg/ml). The suspension was filtered and the supernatant

Department of
Biological Sciences
University of Warwick
Coventry CV4 7AL

Correspondence to:
Dr Alan Morris or
Dr Andrew Easton.

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centrifuged to pellet the cells. The pellet was washed twice in phosphate buffered saline (PBS) and the cells were lysed in Hirt's solution (10 mM Tris (hydroxymethyl)methylamine, 0.5% SDS, 20 mM EDTA, pH 7.4) for a minimum of six hours at 37°C. Following phenol/chloroform extraction and ethanol precipitation, the DNA was resuspended in sterile distilled water and stored at - 20°C.

PCR AMPLIFICATION

Amplification reactions were carried out using heat stable Taq polymerase.³³ Reactions for the first round PCR contained aliquots of the clinical samples, control DNA (U138MG) or no DNA (water negative control) in 50 µl of solutions containing 10x PCR buffer, 5 mM MgCl₂ (GibcoBRL, Paisley, UK), 0.25 mM of each dNTP (dATP, dTTP, dGTP, and dCTP; Promega), 2 units Taq polymerase (GibcoBRL) and 15 pmoles/l of each of the consensus PCR primers MY09 (5'⁷¹⁷⁰CGTCCMARRG-GAWAGTGATC₇₁₅₁3') and MY11 (5'⁶⁷²²GCM-CAGGGWCATAAAYAATGG₆₇₄₁3').¹⁹ The subscripts indicate the nucleotide positions in HPV type 16 DNA to which the oligonucleotides anneal. They are degenerate in several positions to allow for complementarity to each of the HPV types. (M denotes A plus C; R denotes A plus G; W denotes A plus T; Y denotes C plus T.)

DNA from clinical samples was subjected to 30 cycles of amplification with a denaturation step at 94°C (30 seconds), an annealing step at 54°C (30 seconds) and a 72°C elongation step (30 seconds). The reaction was completed with a five minute elongation step at 72°C. This procedure was designed to generate a fragment of approximately 450 base pairs in length from most HPV types. The amplified DNA product spans nucleotides 6722-7170 in HPV type 16 and the corresponding regions of other genital HPVs.

In the semi-nested second round reaction, the primer MY09 was used in conjunction with a new second primer, R1 (5'⁶⁷⁵⁸CAATTATT-TGTTACTGTGGTAGA₆₇₈₀3') to reamplify the material generated in the first round, thus generating a 412 base pair fragment. An aliquot of the first round reaction product was added to a fresh PCR mix and subjected to a further 30 cycles of amplification. The conditions used were the same as previously but using an annealing temperature of 56°C. This fragment was a suitable size for restriction enzyme typing. To reduce the risk of contamination, all manipulations were carried out in a class 2 microbiological hood, separate pipettes were used for the PCR reagents and DNA. Negative controls were set up first, followed by the samples and finally the positive controls.

RESTRICTION ENZYME TYPING

PCR amplified double stranded DNA was specifically cleaved using the restriction endonuclease Rsa I, as recommended by the manufacturer. Analysis of all available sequences of the HPV L1 region indicated that this enzyme generated characteristic restriction fragments from the purified 412 base pair product of the

Table 1 Rsa I restriction fragments predicted for the 412 base pair (bp) fragment from a number of HPV types. All HPV sequence data available from the GenBank database were analysed to generate this table

HPV type	Restriction fragment sizes (bp)
6	162, 149, 101
8	240, 172
11	216, 136, 34, 26
16	311, 70, 31
18	136, 125, 85, 38, 28
30	326, 86
31	378, 34
33	236, 103, 39, 34
34	186, 162, 59, 5
35	338, 42, 32
40	327, 85
45	339, 45, 28
52	402, 10
53	403, 9
56	412
57	412
58	349, 63
59	412

semi-nested PCR for most HPV types and could distinguish between different types, as indicated in table 1.

Results

SEMI-NESTED PCR AMPLIFICATION OF CLINICAL SAMPLES

The sensitivity of the semi-nested PCR technique was investigated using DNA extracted from HPV DNA positive HeLa (HPV type 18) and CaSki (HPV type 16) cell lines. The results showed that the technique could detect HPV DNA in as few as 50 copies of the HPV genome (data not shown).

Figure 1 shows the results from eight of the vulval carcinoma samples examined. As can be seen with the first round PCR on the upper section of fig 1, only the positive controls generated the expected 450 base pair fragment. The samples (fig 1, lanes 5-12) gave no amplified product. The lower section of fig 1 shows the second round semi-nested PCR. Fragments of the predicted size of 412 base pairs were seen in both of the positive controls and in all of the samples, indicating that all of the samples contained HPV DNA. This result illustrates the increased sensitivity of the semi-nested PCR technique, and confirmed that the semi-nested technique could detect HPV DNA where the standard technique failed. The additional vulval carcinoma sample and four wart biopsy specimens were also subjected to semi-nested PCR and found to contain HPV DNA (data not shown).

RESTRICTION ENZYME TYPING OF CLINICAL SAMPLES

Restriction enzyme digestion of the PCR product generated from the second round with Rsa I was carried out in order to identify the HPV types present in the clinical samples. The digestion patterns of some of the more common HPV types likely to be encountered are shown in table 1. An additional enzyme(s) can be used to clarify the virus type further where necessary. In most of the clinical samples positive identification of the HPV type present was achieved. Figures 2 and 3 illustrate the digestion patterns produced from the eight vulval carcinoma samples shown to contain

Figure 1 A 1.5% (w/v) agarose gel illustrating the results of semi-nested PCR amplification of eight vulval carcinoma patient samples. First round PCR primers MY09 and MY11 generated a 450 base pair (bp) fragment. Second round PCR primers generated a 412 bp fragment. Lane M = marker; lanes 1 and 13 = negative water controls for first and second round, respectively; lanes 2 and 14 = U138MG cell line DNA negative control; lanes 3 and 15 = HeLa DNA positive control; lanes 4 and 16 = CaSki DNA positive control; lanes 5–12 = first round PCR on samples 1–8; lanes 17–24 = semi-nested PCR on samples 1–8.

Figure 2 Restriction digestion patterns of positive controls and vulval carcinoma samples 1–3. Lane M = marker; lane 2 = HeLa control; lane 3 = CaSki control; lanes 4–6 = samples 1–3. All digests were electrophoresed on a 2% (w/v) agarose gel.

Figure 3 Restriction digestion patterns of vulval carcinoma samples 4–8. Lane M = marker; lanes 2–6 = samples 4–8. All digests were electrophoresed on a 2% (w/v) agarose gel.

Table 2 Typing of HPV DNA from clinical samples

Sample number	Sample type	HPV type
1	Carcinoma resection	16
2	Carcinoma resection	16, 18
3	Carcinoma resection	16, 18, U*
4	Carcinoma resection	18
5	Carcinoma resection	16, 18
6	Carcinoma resection	16
7	Carcinoma resection	16
8	Carcinoma resection	16
9	Carcinoma resection	16
10	Penile wart biopsy	16
11	Vulval wart biopsy	6, 16
12	Penile wart biopsy	6, 16
13	Penile wart biopsy	16, U*

*Unknown HPV type(s).

HPV DNA in fig 1. Figure 2 also shows the digestion patterns produced from the positive controls: HeLa (lane 2) and CaSki (lane 3). Samples 1 (fig 1, lane 4), 6 (fig 2, lane 4), 7 (fig 2, lane 5), and 8 (fig 2, lane 6) generated a pattern corresponding to that generated by the HPV type 16 positive CaSki cell line, indicating a single HPV type 16 infection in these samples. Sample 4 (fig 2, lane 2) gave the same digestion pattern as that seen with HeLa cell line DNA (HPV type 18) indicating a single HPV type 18 infection.

Samples 2 (fig 1, lane 5) and 5 (fig 2, lane 3) contained HPV types 16 and 18 DNA, while sample 3 (fig 1, lane 6) generated a pattern not seen in the other samples or predicted from known sequences of HPV. DNA fragments produced by this sample corresponded to both HPV types 16 and 18, and possibly another HPV type.

The restriction patterns found with some of the samples were more complex than others. The remaining five samples were also digested with Rsa I (data not shown). Sample 13 gave a digestion pattern that corresponded with the pattern produced by CaSki cell line DNA (HPV type 16) but also yielded fragments not corresponding to a known HPV type. However, the unidentified pattern was not the same as the unique fragments produced by sample 3. A summary of the HPV types identified in the five samples is shown in table 2, together with the results from patient samples 1–8 (figs 2 and 3).

Discussion

The semi-nested PCR method has been used to detect various types of viruses in clinical specimens.^{34 35} The sensitivity of the semi-nested technique has previously been compared with single step PCR for detection of Dengue virus and it was shown that the two step system was more sensitive than the one step one.³⁵ Our results show that semi-nested PCR is capable of detecting as few as 50 copies of HPV DNA in the HeLa cell line (data not shown) and the increased sensitivity of the semi-nested PCR technique can be seen in fig 1, where the first round PCR (equivalent to the L1 consensus PCR usually used to detect HPV DNA) failed to detect virus DNA in clinical samples. All of the clinical samples screened by semi-nested PCR were found to be positive for HPV DNA, while in the first round most of the samples were negative, confirming the greater sensitivity of the two step PCR method.^{34 35}

The fragment generated from the semi-nested PCR was an acceptable size for restriction enzyme analysis. Using Rsa I, restriction endonuclease analysis permitted the positive identification of HPV types in the clinical samples screened. High risk viruses were always detected in the nine vulval carcinoma samples. These results agree with previous findings that high risk viruses such as HPV types 16 and 18 are found in conditions such as vulval and cervical carcinoma, and therefore are the expected HPV types.^{7 12 15} The most common HPV type found in the vulval carcinoma samples was type 16. This agrees with the work of Hording *et al.*⁷ HPV type 6 was detected in two of the wart biopsy specimens, which also agrees with previous findings where low risk HPV types are mainly associated with benign infections.¹⁻³ The technique developed here could be used to detect HPV DNA in a wide range of tissue types. On the whole, the method is robust and relatively fast (two days). The overall method is sensitive and avoids the use of radioactivity. Its main advantage is its sensitivity, permitting its use where only small quantities of tissue are available—for example, vestibular vulvitis.²⁵⁻²⁸

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