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Detection and typing of human papillomaviruses in mucosal and cutaneous biopsies from immunosuppressed and immunocompetent patients and patients with epidermodysplasia verruciformis: a unified diagnostic approach

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Abstract

***Aim*—To develop a unified diagnostic approach for the detection of human papillomavirus (HPV) DNA in skin and mucosal biopsies from both immunocompetent and immunosuppressed individuals using a degenerate polymerase chain reaction (PCR) method.**

***Methods*—The sensitivity and specificity of three published degenerate primer sets (HVP2/B5 and F14/B15; MY09/MY11; CP62/69 outer and CP65/68 nested primer pairs) were evaluated in PCR reactions with serial dilutions of 12 representative cloned HPV types. This combination of primers was then used to detect HPV DNA in 49 benign and malignant lesions of cutaneous and mucosal origin from immunosuppressed, immunocompetent, and epidermodysplasia verruciformis (EV) patients, and compared with detection rates using single primer sets alone.**

***Results*—The observed sensitivity of MY09/MY11 and CP62/69+CP65/68 was high for mucosal and EV HPV types, respectively. The sensitivity of all primer sets for cutaneous types was low, but nonetheless the use of this combination of primers allowed HPV DNA detection in all of the benign warts analysed. Several mixed infections were also identified. A high prevalence of HPV DNA was similarly detected in squamous cell carcinomas from immunocompromised patients; the HPV types found were exclusively EV related.**

***Conclusions*—The use of a combined degenerate primer PCR approach considerably improves HPV DNA detection over individual primer sets and allows detection of mixed infections. The findings may help explain the discrepancies in published reports relating to HPV DNA detection in benign and malignant skin lesions. Further modifications to this method are in progress which should significantly improve comprehensive HPV detection and typing for diagnostic purposes.**

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In recent years there has been a considerable increase in the number of identified human papillomavirus (HPV) types. There are currently 77 distinct types based on DNA sequence homology, but several groups have now described and partially characterised novel sequences predicting the existence of many more.[1–4] Historically, the HPV have been grouped according to the location and clinical context from which they were initially isolated, giving rise to the terminology "cutaneous," "mucosal," and "epidermodysplasia verruciformis" (EV) types.[5] Subsequent phylogenetic analyses based on sequence information have broadly reflected this clinical classification.[6]

Accumulating epidemiological and experimental data strongly support the notion that certain so called "high risk" mucosal HPV types such as HPV 16 and 18 have carcinogenic potential and are causally related to anogenital carcinoma.[7 8] Similarly, although the nature of the association is not clear, certain cutaneous and EV HPV types are found in a high percentage of non-melanoma skin cancers from patients with the rare inherited skin condition epidermodysplasia verruciformis[9]. Such patients appear to be genetically predisposed to HPV infection and are at greatly increased risk of developing squamous cell carcinomas (SCC) on sun exposed sites compared with the general population.[10] More recently, a high prevalence of HPV DNA, particularly of cutaneous and EV HPV types, has been demonstrated in non-melanoma skin cancers which develop in many immunocompromised renal transplant recipients.[1–4 11] A similar spectrum of HPV DNA has also been detected in otherwise healthy individuals with skin cancer, although at a lower frequency ([3] and Berkhout RJM: 14th International Papillomavirus Conference, Quebec City, 1995).

The large number of HPV genotypes identified during the past decade has posed a challenge to the development of simplified HPV DNA detection systems. Most methods currently used rely on the amplification of DNA by the polymerase chain reaction (PCR).[12] However, the considerable sequence diversity of HPV types reduces the range of viruses detectable by type specific PCR primer sets and limits investigators to amplifying small numbers of closely related types. This has led to considerable discrepancy in the prevalence

and type of HPV reported in different tissues by different investigators, particularly in skin lesions.[13 14] To address this problem, degenerate PCR has recently been developed[15]; degenerate primers are essentially mixtures of many primers with nucleotide differences at several positions which render them complementary to the target DNA of different HPV types, enabling detection of a broader range of HPV types, usually at the expense of reduced sensitivity. Using this technique the combined studies from several groups suggest that a broad range of cutaneous, mucosal, and EV HPV types may be found in the skin, particularly in immunosuppressed individuals, and that more than one HPV type may be detected in individuals lesions.[1-4 11] Nonetheless, discrepancies still exist in these data, which most likely reflect the differing sensitivities and spectrum of HPV types detected by the particular set of degenerate primers used.[13 14]

To examine this, we have compared the sensitivity and specificity of established degenerate primer pairs for HPV detection in the skin and have assessed the practical value of using a combined panel of primers to increase sensitivity and specificity. Forty nine mucosal and cutaneous biopsies from 36 immunosuppressed and immunocompetent patients, including four patients with EV, were analysed for HPV DNA using this approach.

Methods

HPV PLASMIDS

Plasmid clones containing the complete genomes of HPV- 2, 3, 4, 5, 6, 8, 10, 11, 16, 31, 41, and 57 were used as representative of cutaneous (HPV-3, -4, -10, and -41), mucocutaneous (HPV-2 and -57), high risk mucosal (HPV 16 and 31), low risk mucosal (HPV-6 and -11), and EV (HPV-5 and -8) HPV types.

TISSUE SAMPLES

Forty nine biopsies were analysed from 36 patients, including immunocompromised renal and bone marrow transplant recipients (14), immunocompetent individuals (18), and patients with EV (4). The lesions comprised viral warts (22), anogenital carcinomas (5), cutaneous squamous cell carcinomas (19), basal cell carcinomas (2), and normal skin (1). All biopsies were obtained with informed consent. One half of each sample was snap frozen and stored at −70°C and the other half was paraffin embedded, sectioned, and stained with haematoxylin and eosin for histological examination.

DNA EXTRACTION

DNA from frozen tissue was extracted by a standard proteinase K/phenol–chloroform–isoamyl alcohol extraction technique.[15] As a control for cross contamination between samples, identical buffer solutions which did not contain tissue were simultaneously subjected to an identical purification process.

PCR PRIMERS

Four established oligonucleotide primer pairs which have previously been used in the detection of HPV in both benign and malignant skin lesions were evaluated. These primers are all located within the conserved L1 (major capsid protein) open reading frame of the HPV genome. Although not formally documented, these primer pairs appear to have somewhat different specificities and sensitivities.[1 2]

The degenerate primer pair HVP2/B5 was described by Shamanin *et al* to detect HPV from all groups with the exception of the phylogenetic clade comprising HPV-4, -48, -50, -60, and -65 for which the primer pair F14/B15 were used.[1 16] The primer MY09/MY11 was originally designed by Manos *et al* for the detection of HPV in genital lesions[17] but has also been widely used to analyse skin lesions.[18 19] Finally, Berkhout *et al* have described several pairs of nested primers which were designed to detect EV HPV types.[2 4] Of these, one particular set comprising CP62/69 as an outer primer pair and CP65/68 as an internal nested pair was chosen for the purposes of this study since it was considered to be particularly sensitive for detecting HPV in skin biopsies from both immunocompetent and immunosuppressed individuals (Berkhout RJM: 14th International Papillomavirus Conference, Quebec City, 1995).

PCR AMPLIFICATIONS

Amplification reactions were performed as previously described for each primer set on a Perkin-Elmer 480 thermal cycler (Perkin-Elmer, Warrington, Cheshire, UK).[1 2 16 17] Initially, the sensitivity and specificity of each primer set was determined by PCR amplification of 10-fold serial dilutions of each HPV plasmid (ranging from 0.001 fg to 100 ng of plasmid DNA) in the absence and presence of a background of 100 ng of human placental DNA (Sigma).

For the subsequent analysis of clinical samples, 100–200 ng of cellular DNA was used as template in each first round PCR reaction. For each set of reactions, negative controls for reagents (water only) and DNA (human placental DNA) were included and processed in the same way as the lesional samples throughout all PCR steps, as were the negative controls for DNA extraction. None of the negative controls was positive for HPV. Four HPV plasmids (0.01 pg) containing the genotypes of HPV-2, -4, -5, and -6 served as positive controls. Before amplification with the HPV primers, each sample was amplified with β globin primers to confirm adequate preservation of DNA.[20]

SEQUENCE ANALYSIS

Amplified PCR products that appeared as a visible band after ethidium bromide staining were purified after separation in a 2% agarose low melting point gel (QIAquick Gel Extraction Kit, QIAGEN, Germany) and directly sequenced by fluorescent dideoxynucleotide chain termination cycle sequencing (ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit, Perkin-Elmer) with both forward and reverse primers on a Perkin-Elmer 377 ABI Prism automated sequencer.

Table 1 Detection of cloned human papillomavirus (HPV) DNA by each of the degenerate primer sets

Group	HPV plasmid	HPV DNA detected (copies/cell) *			
		HVP2/B5	F14/B15	MY09/11	CP62–69
Mucocutaneous	2	50	n/t	5000	–
	57	500	n/t	5×10^5	–
Cutaneous	3	500	n/t	5×10^5	–
	4	5×10^4	5–50	–	–
	10	500	n/t	500	–
	41	5	n/t	5×10^5	–
Mucosal	6	50	n/t	0.05	–
	11	50	n/t	0.05	–
	16	50	n/t	0.05	–
	31	50	n/t	0.05	–
EV	5	500	n/t	–	0.0005
	8	5000	n/t	5×10^5	0.0005

*10-fold serial dilutions of HPV plasmid were amplified in a background of 100 ng human placental DNA (see text) and the copies per cell calculated. The absolute amount of HPV plasmid detected ranged from 0.1 fg (equivalent to 0.0005 copies/cell) to 100 ng (equivalent to 5×10^5 copies/cell).
EV, epidermodysplasia verruciformis; –, negative; n/t; not tested.

Table 2 Results of human papillomavirus (HPV) typing with each degenerate primer set

Patient	Immune status	Lesion	Site	Results of HPV typing with each primer set			
				HVP2/B5	F14/B15	MY09/MY11	CP62–69
1	ICP	Wart	Anal canal	57	–	57	–
2	ICP	Anal Ca	Anal canal	55	–	55	–
3	ICP	Wart	Genital	57	–	–	–
4	ICP	Wart	Mouth	32	–	32	–
5	ICP	Wart	Genital	–	–	6	–
		Wart	Genital	–	–	6	–
6	ICP	Vulval Ca	Vulva	44	–	44	–
7	ICP	Vulval Ca	Vulva	–	–	–	–
8	ICP	Vulval Ca	Vulva	–	–	–	–
9	ICP	Wart	Anal canal	–	–	6	–
10	ICP	Cervical Ca	Cervix	16	–	16	–
11	ICP	Wart	Skin	3	–	–	–
		Wart	Skin	57	–	57	–
		Wart	Skin	57	–	57	–
		Normal	Skin	–	–	–	–
12	ICP	Wart	Skin	27	–	–	–
13	ICP	Wart	Skin	10	–	10	–
14	ICP	SCC	Skin	–	–	–	RTRX2
		SCC	Skin	–	–	–	–
15	ICP	SCC	Skin	–	–	–	–
16	ICP	SCC	Skin	–	–	–	–
17	ICP	SCC	Skin	–	–	–	–
18	ICP	SCC	Skin	–	–	–	–
19	RTR	SCC	Skin	–	–	–	–
		SCC	Skin	–	–	–	20
		SCC	Skin	–	–	–	–
		SCC	Skin	–	–	–	–
20	RTR	SCC	Skin	–	–	–	5
21	RTR	SCC	Skin	–	–	–	–
		SCC	Skin	–	–	–	–
22	RTR	SCC	Skin	–	–	–	23
		SCC	Skin	–	–	–	23
23	RTR	SCC	Skin	–	–	–	25
		SCC	Skin	–	–	–	–
24	RTR	SCC	Skin	–	–	–	21
25	RTR	BCC	Skin	–	–	–	–
26	RTR	BCC	Skin	–	–	–	–
27	RTR	SCC	Skin	–	–	–	21
		Wart	Skin	–	–	–	21
28	RTR	Wart	Skin	27	–	27	–
29	RTR	Wart	Skin	–	–	11	20
30	RTR	Wart	Skin	–	–	11	RTRX1
31	RTR	Wart	Skin	–	–	–	36
32	EV	Wart	Skin	19	–	19	19
33	EV	Wart	Skin	57	–	–	20
		Wart	Skin	57	–	–	20
34	EV	Wart	Skin	2	–	–	5-rel
35	EV	Wart	Skin	20	–	–	20
36	BMT	Wart	Skin	2	–	–	21

–, negative; BCC, basal cell carcinoma; BMT, bone marrow transplant recipient; Ca, carcinoma; ICP, immunocompetent patient; rel, related; RTR, renal transplant recipient; SCC, squamous cell carcinoma.
RTRX1 and RTRX2 denote HPV sequences available in the GenBank database for which the full genome sequence has not yet been published (locus name HPVRTRX1 and HPVRTRX2 respectively; accession numbers L38918 and L38919 respectively).

The nucleotide sequences obtained were analysed using the Sequence Navigator computer software (Macintosh). Sequences of 150 bases or more with fewer than 5% unidentified bases were processed. The forward and reverse complement sequences were aligned and homology of the consensus sequence was compared with those of known HPV types available through the GenBank database (National Center for Biotechnology Information, National Institutes of Health, Bethesda, Maryland, USA) using the GCG Blast programme. In accordance with established guidelines, a nucleotide sequence was regarded as an HPV type if it shared over 90% homology with a known type, and a related type if the sequence homology was between 75% and 90%.[13]

Results

SENSITIVITY AND SPECIFICITY OF INDIVIDUAL PRIMER PAIRS

Analysis with cloned HPV (table 1) showed that different primer pairs had distinctly different sensitivities and specificities. The primer pair HVP2/B5 detected all HPV types within the given concentration. Generally, the copy number detected by HVP2/B5 was high (10 pg to 10 ng, or 5 to 5×10^4 copies/cell) which is likely to be a consequence of the high degree of degeneracy of these primers.[1 16] Despite this, HVP2/B5 was able to detect the majority of the cutaneous and mucocutaneous HPV types in particular at a lower copy number than the other primer pairs. This was complemented by the F14/B15 primer pair which was able to detect HPV-4 at low copy number. MY09/MY11 was the most sensitive in detecting mucosal HPV types, detecting these HPV types in the femtogram range in our hands (10 fg or 0.05 copies/cell). Although capable of detecting cutaneous HPV types as well, this was usually at higher copy number than that achieved by HVP2/B5 and F14/B15. Finally, the nested primers CP62/69+CP65/68 were exquisitely sensitive for EV HPV types but were unable to detect cutaneous or mucocutaneous HPV within the study concentration range. Some cutaneous and mucosal HPV types were detected in the first round PCR reaction (data not shown), but after the nested reaction only EV HPV types were finally identified.

ANALYSIS OF CLINICAL LESIONS

All virus warts from both skin and genital sites were HPV DNA positive using this combination of primers, improving upon the detection rate achieved by the individual primer sets alone (15/22 with HVP2/B5 + F14/B15; 12/22 with MY09/MY11; and 10/22 with CP62/69+CP65/68) (table 2). Of the 27 lesions in which only one HPV type was detected overall, this was identified by all three primer sets in just one case, and with two primer sets in 10 cases. However, in 16 of these singly infected lesions, only one primer set successfully detected HPV. In six warts, all from immunosuppressed individuals, codetection of two distinct HPV types was possible by using this combination of primer sets.

The differential specificities and sensitivities of the various primer pairs formed the basis for such an identification of multiple infections within single lesions. For example, HVP2/B5 detected the EV-HPV type-20 in patient 35 but not in patient 33 in whom mucocutaneous HPV-57 was identified, while CP62-69 detected HPV-20 in both lesions. The HPV plasmid dilution experiments provide the most likely explanation for this apparent discrepancy; it is clear that within the dilution range tested HVP2/B5 is able to detect HPV-57 whereas CP62-69 is not, while data from the HPV-5 and -8 plasmids tested would suggest that both primer sets are able to detect HPV-20. Thus it is likely that in patient 33 HPV-57 was present in higher copy number than HPV-20 and was therefore preferentially detected by HVP2/B5, whereas CP62-69 was able to detect HPV-20 only. By the same token, the single infection with HPV-20 in patient 35 was detected by both primer sets.

Discussion

For a meaningful epidemiological assessment of the presence of HPV in skin and mucosal lesions it is necessary to employ a method which is not only sensitive but is also able to detect and reliably type a wide range of HPV genotypes. Our data show that for consistent detection of HPV, established degenerate primer sets used alone will underdetect HPV DNA in both skin and mucosal lesions. A combined primer panel allows more comprehensive detection of a broader range of HPV types than is possible with the individual primer sets, and is also valuable in allowing the identification of multiple HPV types within single lesions. This was confirmed by both the evaluation of cloned HPV DNA and the analysis of clinical lesions. It is notable, however, that the combined primer pairs employed were highly sensitive for EV-HPV types (CP62/69 and CP65/68), lower for mucosal types (MY09/MY11), and lower still for cutaneous types (predominantly detected by HVP2/B5). This suggests that mucosal and cutaneous HPV DNA may still be underdetected by this method, especially in SCC where copy number may be low.

It is notable that none of the clinical specimens analysed was positive with the primer pair F14/B15, even though the HPV plasmid dilution experiments confirmed that the sensitivity of F14/B15 was relatively high. One possible explanation for this observation is simply that none of these lesions contained the limited range of HPV types detected by F14/B15, namely HPV-4, -48, -50, -60, and -65. These HPV types are most commonly found in skin warts from immunocompetent patients and although such lesions were included in this study, the numbers were small. We anticipate that the use of this particular primer set would be justified if a large number of such lesions were examined. An alternative explanation is that these HPV types were present but at a copy number below the lower limit of detection possible with F14/B15.

Despite these limitations, this unified approach allowed the detection of HPV DNA in 100% of mucosal and cutaneous warts analysed. While we cannot exclude the possibility that the combined panel of primers with their differing but complementary specificities may still not be detecting *all* HPV types present in all lesions, the level of detection attained using this approach, particularly in cutaneous warts, improves substantially upon the results of previous studies; degenerate PCR and other methodologies have failed to detect HPV DNA in up to 40% of cutaneous viral warts.[1 21 22] Although all viral warts analysed in this series were found to harbour HPV DNA, a considerable number of other lesions (predominantly non-melanoma skin cancers) were HPV DNA negative. One explanation for this is that these lesions did not harbour HPV DNA. While it is beyond the scope of this study to comment on the significance of HPV prevalence and type at a pathogenic level, many of these HPV negative lesions are non-melanoma skin cancers in which a role for HPV is far from well established. Alternative explanations include the presence of unidentified HPV types, novel HPV types, and undetectably low copy numbers of HPV.

Of particular importance in the development of a method suitable for detecting HPV in the skin, we have observed striking differences in the HPV types detected in lesions from immunocompetent individuals compared with immunosuppressed and EV patients. The HPV types harboured by viral warts in immunocompetent individuals strictly reflected the site of the lesion; single infections with mucosal and mucocutaneous types (HPV-6, -16, -32, and -57) were found in lesions derived from anogenital and oral sites, and cutaneous and mucocutaneous HPV types (HPV-3, -10, and -57) in warts removed from glabrous skin. However, in cutaneous warts from EV and immunosuppressed transplant patients, mucocutaneous (HPV-2 and-57), and EV HPV types (HPV-5, -9, -19, -20, -21, -36) were found in addition to several potentially novel EV related types—HPV 5 related and the previously described RTRX1 and RTRX2.[2] Particularly notable was the identification of mixed infections within individual warts from this group of patients. These data thus highlight the diverse spectrum of HPV types which may be encountered in skin lesions from immunosuppressed individuals, and emphasise the crucial importance of employing an HPV detection method which is sufficiently comprehensive and which allows identification of multiple HPV types within single lesions and also of potentially novel HPV types.

CONCLUSIONS

In summary, the unified approach to HPV detection and typing described allows relatively rapid and comprehensive screening for HPV DNA in a wide range of cutaneous and mucosal lesions. It may therefore be particularly relevant in the descriptive epidemiology of skin lesions from both immunosuppressed and EV patients where preliminary data indicate

the presence of a diverse range of HPV types. It is clear, however, that such a strategy will require further modifications. In particular, cutaneous HPV types are not detected to a sensitivity equivalent to that of mucosal and EV types, and the precise sensitivity of this methodology for the detection of mixed HPV infections has yet to be formally established; studies designed to address these issues are currently under way. Finally, our observations underscore the need to interpret results of PCR based studies relating to HPV DNA detection in benign and malignant skin lesions[23] in the light of the limitations of the approach used.

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