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Detection of human papillomavirus in cervical cell specimens by hybrid capture and PCR with different primers⋆

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The purpose of this study was to compare hybrid capture assay with PCRs using different primers for the L1, E6-E7 regions for the detection of human papillomavirus (HPV) genome. One hundred twenty-five cervical smears with normal (n = 42) and abnormal (n = 83) cytology were investigated. Those at high-risk for HPV were studied by hybridization antibody capture assay and PCR with the pU-1M/pU-2R primers. Target DNA from the HPV L1 region was amplified by SPF10 primer set and home-PCR with MY09/MY11 primers. The presence of HPV DNA in cervical smears was detected by SPF10 (in 72% of cases), MY09/MY11 (58%), hybrid capture (55%) and pU-1M/pU-2R (39%). Results obtained with the SPF10 and MY09/MY11 consensus primer sets as well as hybrid capture and pU-1M/pU-2R specific for high-risk types differed significantly (χ^2, $P<0.0005$). The correlation between assays with the use of SPF10 and MY09/MY11 was 86% and between hybrid capture and the pU-1M/pU2R technique — 78%. In 49% of samples HPV DNA was detected by the four methods, whereas in 12% only by the SPF10 primers. The most sensitive technique was found to be PCR with the use of SPF10 primers, while the most specific — the MY09/11 PCR method. It seems that home-PCR with MY09/MY11 primers could be applied in screening tests.

Keywords: papillomavirus, HPV detection, PCR, hybrid capture

INTRODUCTION

Human papillomavirus (HPV), the major cause of cervical cancer and cervical dysplasia, is a member of the *Papillomaviridae* family of DNA viruses. So far, 118 types have been identified according to their biological niche, oncogenic potential and phylogenetic position (de Villiers *et al.*, 2004). There are about 40 HPV viral types that are commonly found in the genital tract. They are classified in the *Alphapapillomavirus* genus (Fauquet & Mayo, 2005).

HPV's nonenveloped capsid has an icosahedral symmetry, containing 22 capsomers with a diameter of 52–55 nm. The HPV virion contains circular, double-stranded DNA. The HPV genome codes for only eight early open reading frame proteins (E1-E8, with E3 and E8 function unknown) and two late open reading frame proteins (L1 and L2) on a single strand of DNA (Lowy & Howley, 2001). The late proteins L1 and L2 are the major and minor capsid proteins of the virion. The DNA and amino-acid sequences are highly conserved between HPV types, especially of the L1 protein. The early genes of the HPV genome code for proteins E1 and E2 which are responsible for viral replication and transcription, and E4 seems to aid virus release from infected cells. The E6 and E7 genes are invariably expressed in tumours and are sufficient to induce proliferation and immortalize cells in culture. Expression of E6/E7 is required continuously to maintain the prolifera-

tive state of the cells (zur Hausen, 2000). The protein products of these genes interfere with the normal function of tumour suppressor genes. HPV E6 interacts with p53, and E7 binds to retinoblastoma protein, leading to tissue proliferation. HPV types can be classified according to various criteria, e.g. their tissue tropism, oncogenic potential and phylogenetic classification.

On the basis of epidemiological studies of the frequencies of certain types of intraepithelial cervical lesions and cervical cancer, the anogenital HPV types can be generally categorized, according to their oncogenic potential, as being either high-risk or low risk (Bosch *et al.*, 2002; Muňoz *et al.*, 2003).

Papillomaviruses cannot be grown in conventional cell culture, and serological assays have only limited accuracy. As infection with HPV is followed by a humoral immune response against the major capsid protein (Dillner, 1999), with antibodies remaining detectable for many years, serology is not suitable for distinguishing present and past infections. Thus accurate diagnosis of HPV infection is based on molecular methods, including hybridisation (e.g. hybrid capture, Southern and dot blot hybridisation) and PCR (Manos *et al.*, 1989; Low *et al.*, 1990; Cox *et al.*, 1995; Jacobs *et al.*, 1997; Clavel *et al.*, 1999; Gravitt *et al.*, 2000). Various PCR-based methods have been described for the identification of HPV genotypes. Individual genotypes can be detected by type-specific PCR primer sets. However, these require the performance of multiple parallel assays for each sample, and type-specific PCR primers have not been reported for each HPV genotype. Alternatively, general PCR primer sets can be used, permitting simultaneous amplification of a broad range of HPV genotypes. Recently a novel general primer set, designated SPF10, amplifying a fragment of only 65 bp of the L1 region of the HPV genome was developed (Kleter *et al.*, 1998). The aim of the present study was a comparison of the hybridization antibody capture assay *versus* PCRs using different primer sets for the L1, E6/E7 regions for the detection of the HPV genome. The choice of these primer sets was determined by the fact that they are most commonly used in HPV detection.

MATERIALS AND METHODS

Cervical smears were obtained from 83 women (40 ± 15 years old) with low grade (LSIL, n = 44) and high grade (HSIL, n = 12) squamous intraepithelial lesions and cervical cancer (Ca, n = 27). All women were diagnosed by cytological examination. The diagnosis of patients with HSIL and Ca was also confirmed by histopathological methods. Cervical cancer patients were hospitalised in the Centre of Oncology, M. Sklodowska-Curie Memorial Institute in Krakow (Poland) and none of them had radiotherapy before material collection.

The control group consisted of 42 women 28 ± 5 years old undergoing preventive examinations before planned pregnancy who did not display atypical squamous cells of the cervix. All cervical smears obtained before the cervical biopsy specimens were taken with a cervical brush and collected to specimen transport medium (Digene Diagnostic, USA) and stored frozen at –70ºC until tested. Samples were then thawed and divided into portions for hybridisation and PCR methods.

Hybrid capture HPV assay (Digene Diagnostic, USA). This test is a signal-amplified solution hybridisation antibody capture assay that utilizes chemiluminescent detection (Lorincz, 1996). Specimens containing the target DNA hybridize with a specific HPV RNA probe cocktail. The resultant RNA:DNA hybrids are captured onto the surface of a tube coated with antibodies specific for hybrids. Immobilized hybrids are then reacted with alkaline phosphatase-conjugated antibodies specific for the RNA:DNA hybrids, and detected with a chemiluminescent substrate. The substrate is cleaved by the bound alkaline phosphatase. The intensity of the light emitted denotes the amount of target DNA in the specimen. In our study, the RNA probe mix for the detection of high-risk HPV types (HPV 16, 18, 31, 33, 35, 45, 51, 52, 56) was used following the supplied instructions.

PCR. DNA was isolated using Genomic DNA Prep Plus kit (A&A Biotechnology, Gdynia, Poland). Cell samples were lysed in a buffer with proteinase K (1.25 mg/ml) and chaotropic salts. Addition of ethanol caused DNA to bind when the lysate was spun through a silica membrane in a microcentrifuge tube. Following washing to remove contaminants, DNA was eluted in 10 mM Tris/HCl, pH 8.5 (preheated to 75°C). PCR was performed in a final reaction volume of 50 µl containing 10 µl of the isolated DNA sample.

General PCR primers (MY09/MY11, SPF10) to amplify a broad-spectrum of HPV genotypes and pU-1M/pU-2R primers to amplify high-risk HPV DNA were used separately. Table 1 presents the primers used for the PCR technique and the reaction conditions for each primer pair.

Each experiment was performed with separate positive (CaSki cells) and negative (H$_2$O) PCR controls. Analysis of the PCR products was performed by electrophoresis in 3% agarose gel. To ensure adequate DNA preparation, PCR amplification of β-globin gene was performed in a separate reaction, resulting in a 110 bp product (Saiki *et al.*, 1985).

The analytic sensitivity of the methods used determined by analyses of serial dilutions of CaSki-

Table 1. HPV primers used in PCR

Primers	Localization of sequence	Product size	PCR protocol	PCR conditions	References
MY 09 MY11 (consensus)	HPV L1	450 bp	50 mM KCl, 6.5 mM MgCl$_2$, 200 μM DTP, 2.5 U *Taq* polymerase, 50 pmol of each primer	94°C/30 s 55°C/60 s 72°C/30 s 40 cycles	Manos *et al.*, 1989
SPF10 (consensus)	HPV L1	65 bp	50 mM KCl, 10 mM Tris/HCl, pH 9.0, 0.1% Triton X-100, 0.01% gelatin, 200 μM dNTP, 1.5 U Ampli *Taq* Gold DNA polymerase, 100 pmol of each primer	94°C/9 min 94°C/30 s 52°C/45 s 72°C/45 s 72°C/5min 40 cycles	Kleter *et al.*, 1998
pU-1M pU-2R (specific for high risk HPV 16, 18, 31, 33, 35, 52, 58)	HPV E6/E7	228–268 bp	50 mM KCl, 10 mM Tris/HCl, pH 8.3, 1.5 mM MgCl$_2$, 200 μM dNTP, 2.5 U *Taq* polymerase, 100 pmol of each primer	94°C/30 s 55°C/2min 72°C/2min 30 cycles	Fujinaga *et al.*, 1991

cell HPV-16 DNA (Meissner, 1999) revealed that hybrid capture had a sensitivity limit of 50 000 HPV-16 genomes/reaction, PCR using pU-1M/pU-2R – 300 genomes/reaction, MY09/MY11 primers — 250 genomes/reaction, SPF10 — 10 genomes/reaction (Cavuslu *et al.*, 1996; unpublished authors' data). The results were analysed by the Student's *t*-test and χ^2 test.

RESULTS

HPV DNA was detected in 90 of 125 cervical smears by at least one of the following methods: hybrid capture and PCR with three different primers (pU-1M/pU-2R, MY09/MY11, SPF10). Looking at the results of all the methods, only SPF10/PCR showed all HPV positive results. HPV infection was present in 9 (21%) women with normal cytology (control group) and significantly more frequently in patients with LSIL — 43 (98%), those with HSIL — 11 (92%) and with cervical cancer — 27 (100%). Results of the comparison of the methods used are shown in Table 2.

Significant differences were found between the results of the assays performed with the four different methods (χ^2, *P*<0.0005) except for the techniques of hybrid capture and PCR with primers MY09/MY11. SPF10 PCR was found to be the most sensitive and MY09/11 PCR the most specific method. The correlation between PCR with the universal primers MY09/MY11 and SPF10, as well as hybrid capture and PCR with pU-1M/pU-2R primers specific for high-risk HPV types was 86% and 78%, respectively. It was also demonstrated that PCR results with the use of universal primers MY09/MY11 correlated in 86% with the hybrid capture method which detected the high-risk types.

A positive result of HPV infection was obtained in 49% of women when all four test methods were used. When only three test methods were used the positive result was obtained in 26% of women and for two test methods only — 13%. In 12% of examined women the infection could be detected only with one technique — the SPF10 PCR (Table 3).

Table 2. Comparison of positive results obtained by different methods (hybrid capture and PCRs) in women with different cytological diagnoses

Detection method*	No. of HPV-positive women with various cytological diagnoses				No. of HPV(+)	Sensitivity %	Specificity %	χ^2 test
	Normal	LSIL	HSIL	Ca				
SPF 10	9	43	11	27	90	100	66	*P*<0.0005
MY09/MY11	4	34	9	25	72	80	100	
HC	4	37	10	18	69	92	68	*P*<0.0005
pU-1M/pU-2R	0	21	8	20	39	65	93	

*HC, hybrid capture assay; PCR with primers: SPF10, MY09/MY11, pU-1M/pU-2R

Table 3. Concordance of HPV positive results obtained in parallel with hybrid capture assay and PCR with three different sets of primers

No. of methods	Detection methods*	No. of HPV (+)	%
four	HC + pU-1M/pU-2R + MY09/MY11 + SPF10	44	49
three	HC + pU-1M/pU-2R + SPF10	1	26
	HC + MY09/MY11 + SPF10	18	
	pU-1M/pU-2R + MY09/MY11 + SPF10	4	
two	HC + SPF10	6	13
	MY09/MY11 + SPF10	6	
one	SPF10	11	12

*HC, hybrid capture assay; PCR with primers: pU-1M/pU-2R, MY09/MY11, SPF10

DISCUSSION

HPV infection is closely linked to the development of squamous cell benign and malignant neoplasms of the lower genital tract, in both men and women. This relationship can be estimated at many levels, as shown by epidemiological, clinical and molecular data. Thus, the development of methods for simple, rapid and accurate detection HPV has a central role in many strategies designed to reduce the risk of cervical cancer. In this study we applied the hybrid capture test (approved by FDA for diagnostic use), the PCR technique with the use of commercially available primers (pU-1M/pU-2R and SPF10), and home-PCR with the MY09/MY11 primers. Hybrid capture is a simple technique that could be readily automated for large-scale use if required. There is only a slight risk of contamination and false-positive results, since no DNA amplification step is necessary. In the hybrid capture method, a probe detecting the high oncogenic potential types was applied so the results could be compared with the results of a more sensitive method, PCR with primers detecting high-risk types, pU-1M/pU-2R. This assay did not result in more cases of HPV infections being detected. In 20 women, the result obtained with the use of the hybrid capture technique was not confirmed by PCR amplification with the use of pU-1M/pU-2R primers. A possible explanation for this could be that the probe used in the hybrid capture assay had a broader spectrum of types it could identify (HPV 45, 51, 56 additionally). So, the next step of this research was the application of universal primers for the region L1 which could detect both high and low oncogenic potential HPV types. The results of the PCR assay with the use of MY09/MY11 primers compared to the results of hybrid capture technique were found to be similar (69 and 72 patients HPV-positive, respectively). Other universal primers

which detected a 65 bp fragment localized inside the sequence amplified by the MY09/MY11 primers, were also applied. The best results were obtained by means of this method. HPV infection was detected in 90 (72%) of examined women. Our previous, unpublished data suggest that SPF10 PCR is the most sensitive technique for HPV 16 (which occurs latently in the CaSki cell-line) detection. Therefore it may be suggested that this method is the most sensitive one also for other HPV types. The differences between the results of PCR with either MY09/MY11 or SPF10 primers could also be explained by the differences in the ranges of HPV types detected. According to other authors, the SPF10 primer can detect 57 different types of HPV whereas the MY09/MY11 primers distinguish only 25 of them (Gravitt *et al.*, 2000; Perrons *et al.*, 2005).

In the Perrons *et al.* (2002) study, HPV DNA was amplified from 80% of samples by SPF10 and from 42% only by MY09/MY11. They obtained concordant positive results in 42% of samples while in 38/100 samples only the SPF10 primer detected HPV DNA. In our study the results were compatible in as many as 80% of the samples tested (72/90).

Comparison of the hybrid capture method and PCR with various primer demonstrated that in most cases (75%) positive results could be achieved by means of three or four methods. This suggests that the high-risk types dominated in the examined tissue samples. Only in 12% of samples the positive result was obtained with the use of only one method, the most sensitive one detecting the broadest of spectrum HPV types. However, the high cost of HPV detection with the SPF10 PCR method limits the clinical applicability of this assay in screening studies. Therefore it seems that home-PCR with MY09/MY11 primers, the sensitivity of which is comparable to the hybrid capture method, is a good solution for screening examinations.

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