Review

Molecular diagnosis of human papillomavirus (HPV) infections

Anco Molijn*, Berhard Kleter, Wim Quint, Leen-Jan van Doorn

DDL, Fonteynseweglaan 5, 2275 CX Voorburg, The Netherlands

Received 26 July 2004; accepted 6 December 2004

Abstract

Human papillomaviruses (HPVs) comprise more than 100 genotypes. The mucosal types can be divided into high-risk and low-risk (LR) types depending on the associated disease risk. HPV infection is mainly diagnosed by molecular methods, since reliable serological tools are not available and culture of the virus is not possible. Accurate molecular diagnostic techniques that can be used to inform patient management and follow-up after treatment are now available for detection and identification of HPV. The diagnosis of HPV infections in patients at risk of disease in a clinical setting requires a different approach from that used for epidemiological studies, vaccination trials and natural history studies. This review describes the different molecular methods available for HPV detection and genotyping and their possible clinical utility.

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Keywords: HPV-DNA; Molecular diagnostics; Genotyping; Type-specific PCR; Broad-spectrum PCR; Reverse hybridization

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Abbreviations: ASCUS, atypical squamous cells of undetermined significance; CIN, cervical intraepithelial lesion; HPV, human papillomavirus(es); HR-HPV, high-risk HPV; LR-HPV, low-risk HPV; hc2, Hybrid Capture II; HSIL, high-grade squamous intraepithelial lesion; LBA, line blot assay; LiPA, line probe assay; LSIL, low grade squamous intraepithelial lesion

* Corresponding author. Tel.: +31 70 340 1670; fax: +31 70 340 1671.
E-mail address: Anco.molijn@ddl.nl (A. Molijn).

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1. Introduction

Infection with human papillomaviruses (HPVs) can cause warts on cutaneous epithelium, while in the anogenital region these viruses can cause both genital warts and various forms of cancer in men and women (Bernard, this volume). The main interest in HPV relates to its causative role in cervical cancer, one of the most common cancers in women, with an annual incidence of almost half a million and a mortality rate of approximately 50% (Parkin et al., 2001).

The development of cervical cancer is considered to be a multistep process, where HPV is necessary but in itself an insufficient cause (Walboomers et al., 1999; Steenbergen et al., this volume). Disease can only develop when there is persistent HPV infection of the cervical epithelium. Cervical cancer is a rare complication of infection with high-risk HPV (HR-HPV), but every abnormal or dysplastic lesion of the cervix is potentially malignant and may develop into cervical cancer over time. Abnormal cervical epithelial cells can be detected microscopically following Papanicolaou (Pap) staining of conventional cervical smears or of the more homogeneous cell suspension from liquid cytology medium. This forms the basis of cervical screening programmes for detection of women at risk of disease progression (Cuschieri and Cubie, this volume). Molecular detection of HPV provides a different approach to screening and patient management.

The HPV virion has a double-stranded, circular DNA genome of approximately 7900 bp, with eight overlapping open reading frames, comprising early (E), and late (L) genes and an untranslated long control region. The L1 and L2 genes encode the major and minor capsid proteins. The capsid contains 72 pentamers of L1, and approximately 12 molecules of L2. The early genes regulate viral replication and some have transformation potential (Steenbergen et al., this volume).

At present, 118 HPV genotypes have been classified according to their biological niche, oncogenic potential and phylogenetic position (de Villiers et al., 2004; Bernard, this volume). Both mucosal (anogenital and oral) and cutaneous (skin) HPV types are distinguished and high-risk and low-risk (LR) genotypes are defined, depending on their association with cervical carcinoma or associated precursor lesions. HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82 are considered oncogenic or high-risk genotypes, while types 26, 53, and 66 are probably oncogenic (Munoz et al., 2003). An HPV isolate is described as a new genotype if the L1 sequence differs by more than 10% from any previously known HPV genotype. Within a genotype, subtypes and variants can be distinguished, which differ 2–10% and maximally 2%, respectively (de Villiers et al., 2004). As intra and intergenomic recombinations are rare, genotypes can be reliably classified by analysis of only part of the viral genome (Chan et al., 1995). The choice of a genomic region used for typing of viral isolates is important and must show sufficient discriminatory power for intertypic variation to distinguish a wide range of different genotypes, while intratypic variation (i.e. heterogeneity within the same genotype) should be limited to permit reliable identification.

1.1. Diagnosis of HPV infections

HPV cannot be grown in conventional cell cultures, and serological assays have only limited accuracy (Dillner, 1999). 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. Consequently, accurate diagnosis of HPV infection relies on the detection of viral nucleic acid.

Studies of HPV prevalence in various populations worldwide have shown a wide range of positivity rates (Bauer et al., 1991; Munoz, 2000; Wheeler et al., 1996). In general, however, the prevalence of HPV is higher in young women compared to women over 30 years (de Roda Husman et al., 1995; Evander et al., 1995). The majority of HPV infections in young women resolve spontaneously, most frequently within a 24-month period (Moscicki et al., 1998). The heterogeneous outcome of epidemiological studies may be due to several important factors. First, there appear to be marked differences in HPV prevalence in different populations with respect to age, frequency of cytological abnormalities and diversity of HPV genotypes. Secondly, multiple sampling and HPV-DNA detection techniques have been used, with different sensitivity and specificity, which may impact significantly on detection rates.

The natural history of HPV infection, including mode of transmission of the virus, development of persistent infection, clearance of the virus and interaction with the immune system is only partially known. At present, there is no established definition of a persistent HPV infection. One study suggested that women with mild or moderate dyskaryosis should only be referred for treatment after a persistent HPV infection of at least 6 months (Nobbenhuis et al., 1999). However, detection of HPV-DNA in consecutive samples should include genotyping or even analysis of molecular variants to confirm persistence of the same virus over time (Mayrand et al., 2000).

1.2. Detection of HPV-DNA and identification of HPV genotypes

HPV-DNA can be detected in cervical smears and biopsy specimens by various methods, of which in situ hybridization is complementary to cytology. This method is based on the use of labeled probes that specifically hybridize to intracellular HPV-DNA. Although the sensitivity of this method is limited, it permits localization of HPV infection in the sample and possible co-localization with other markers (Sato et al., 1998). Identification of HPV genotypes would require the use of type-specific probes in multiple in situ hybridization experiments. Alternatively, HPV-DNA can be directly isolated
from clinical samples and detected by Southern blot or dot spot hybridization. However, such approaches are insensitive (Kuypers et al., 1993; Melchers et al., 1989), labor intensive and unsuitable for high throughput screening. Therefore, nucleic acid amplification methods have been developed to increase the sensitivity as well as the specificity of HPV-DNA detection.

1.3. Sample collection and nucleic acid isolation for HPV-DNA analysis

HPV-DNA assays can be performed using the same specimen as used for cytological examination, which is an important logistic aspect of routine clinical testing. However, a cervical scrape is only a small sample of the cervical epithelium and sampling errors may influence cytology grading. Only a portion of the cervical cell suspension is used for DNA isolation with only a fraction of the isolated DNA being used for specific DNA detection. Therefore, if a specimen only contains a limited number of HPV-DNA copies, sampling errors may produce inconsistencies even in a sensitive assay. Furthermore, the outcome of a HPV-DNA assay can vary depending on the menstrual cycle (van Ham et al., 2002; Harper et al., 2003). This not only has consequences for determining HPV-DNA presence or absence, but also could influence the accuracy of HPV detection, particularly when multiple HPV genotypes are present at different concentrations. A recent study revealed that analysis of cervical scrapes as well as biopsy specimens from the same patient yielded comparable, but not identical HPV genotyping results (Quint et al., 2001). Thus, sampling errors should always be taken into account.

Stability of the sample during transport and storage is also important. The viral nucleic acid must be preserved to avoid false-negative results caused by degradation by endogenous endonucleases. This is especially important when analyzing HPV-RNA transcripts. To assess the integrity of genomic DNA in the specimen and its suitability for molecular analysis, adequate controls, such as β-globin gene amplification or spiking of the sample with known positive material, are crucial. Several commercially available sampling kits, originally intended for cytology (e.g. PreservCyt, Cytyc Corp.) adequately preserve nucleic acids for molecular diagnosis even after prolonged storage at ambient temperatures (Sherman et al., 1997). A wide variety of methods is available for DNA extraction, the choice of which is dependent on origin and quality of the clinical material tested and the diagnostic test used, which should be thoroughly validated for specific laboratory needs.

2. HPV nucleic acid detection

2.1. Signal amplification systems

Signal amplification of ISH is possible using tyramide signal amplification (TSA; Zehbe et al., 1997), but the threshold remains low and consistent detection is difficult to achieve. Consequently, despite the interest of cytologists, the method has not been adopted for large scale HPV testing.

The Hybrid Capture II system (hc2, Digene Corp., USA) is a non-radioactive signal amplification method based on the hybridization of the target HPV-DNA to labeled RNA probes in solution (Bozzetti et al., 2000; Lorincz, 1996). The resulting RNA–DNA hybrids are captured onto microwell plates and are detected by a specific monoclonal antibody and a chemiluminescent substrate, providing a semi-quantitative measurement of HPV-DNA. Two different probe cocktails are used, one comprising probes for five low-risk genotypes 6, 11, 42, 43 and 44 and the other containing probes for 13 high-risk genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68. This assay has become the standard in many countries, is widely used in clinical studies, and has FDA approval. However, hc2 has some limitations. It distinguishes between the high-risk and low-risk groups but does not permit identification of specific HPV genotypes. The detection limit of approximately 5000 genome equivalents, makes it less sensitive than PCR (Cope et al., 1997; Smits et al., 1995) and cross-reactivity of the two probe cocktails (Castle et al., 2002; Poljak et al., 2002) can reduce the clinical relevance of a positive result. Nevertheless, hc2 has been widely used in clinical trials worldwide and has been shown to be robust and reproducible as a screening assay (Castle et al., 2004a). Trials of the automated third generation Hybrid Capture assay were recently reported (Castle et al., 2004b).

2.2. Target amplification systems

PCR is the most widely used target amplification method, using a thermocycling process and employing oligonucleotide primers flanking the region of interest to amplify DNA in the presence of a thermostable DNA polymerase. Two approaches for detection of HPV-DNA by PCR are relevant.

2.2.1. Type specific PCR versus broad-spectrum PCR

Type specific primers designed to amplify exclusively a single HPV genotype can be used, but to detect the presence of HPV-DNA in a single sample, multiple type-specific PCR reactions must be performed separately. This method is labor-intensive, expensive and the type-specificity of each PCR primer set should be validated. Alternatively, consensus or general PCR primers can be used to amplify a broad-spectrum of HPV genotypes. Such primers target a conserved region in different HPV genotypes. Since the L1 region is the most conserved part of the genome, several consensus PCR primer sets are aimed at this region (Hildesheim et al., 1994a) (Fig. 1). General primers in the E1 region have also been described (Tieben et al., 1993) and several other broad-spectrum PCR primers were reported, but have not been extensively used in clinical situations. Three different designs of general PCR primers can achieve broad-spectrum detection of HPV-DNA. The first incorporates one forward and one reverse primer, followed by probes with different fluorochromes or chemiluminescent substrates.
Fig. 1. Outline of the HPV-DNA genome, presented in a linear form. The position of the early (E), late (L) genes and the untranslated region (UTR) is indicated, as well as the positions of the four most widely used primer sets CPI/II (Tieben et al., 1993), MY09/11 (Hildesheim et al., 1994), GP5+/6+ (Jacobs et al., 1997), SPF10 (Kleter et al., 1998) and Roche Amplicor HPV assay with their respective amplimer sizes (the precise location of the primers, used in the Roche assay is unknown). The 291 bp fragment used for formal classification of HPV genotypes (Chan et al., 1995; de Villiers et al., 2004) is shown in the L1 region.

2.2.2. Real-time PCR

Real-time PCR can also be used to detect HPV-DNA. Type-specific PCR primers can be combined with fluorescent probes for real-time detection (Josefsson et al., 2000; Tucker et al., 2001; Ylitalo et al., 2000) although multiplexing several type specific primers within one reaction can be technically difficult. Broad-spectrum PCR primers have also been used in real-time PCR (Strauss et al., 2000; Cubie et al., 2001) but are less amenable to quantitation than a type-specific primer system. Due to the sequence heterogeneity of different HPV genotypes, genotyping of PCR products from broad-spectrum PCR requires a mixture of probes and since these will all have different hybridization characteristics, standardization is difficult (Hart et al., 2001).

2.2.3. RT-PCR

It is also possible to look for specific viral RNA by incorporating a reverse transcriptase (RT) step before PCR amplification. Although the vast majority of HPV detection strategies used for epidemiological studies and clinical management have, thus far, been DNA based, detection of expression of HPV oncogenes may have significant clinical value. For example, Lamanecq et al. (2002) developed a real-time RT-PCR for HPV 16 E6/E7 transcripts and suggested that it may be more specific for the detection of symptomatic infections. Wang-Johanning et al. (2002) also described an HPV 16 E6/E7 quantitative real-time RT-PCR and found that expression increased coordinately with severity of the lesion. There is currently one commercially available RNA based HPV assay, the PreTect HPV Proofer (Norchip AS Klokkarstua, Norway).

This assay incorporates NASBA amplification of E6/E7 mRNA transcripts prior to type specific detection via molecular beacons for HPV16, 18, 31, 33 and 45. Initial data, on the prognostic value and specificity for underlying disease, is promising (Kraus et al., 2004; Lie et al., 2004), but the...
clinical value of this method compared with DNA based assays remains to be determined in large-scale prospective studies.

The physical state of the HPV genome has also been explored as a potential diagnostic marker. Integrated virus is associated with a neoplastic phenotype/high grade disease, where loss of the regulatory E2 protein on integration results in up-regulation of oncoproteins E6 and E7. Detection of integrated HPV can be performed by identification of viral-cellular fusion transcripts such as the APOT technique (Klaes et al., 1999) and by ligase mediated PCR (Luft et al., 2001) with detection of integrate-derived HPV transcripts showing a high specificity for high-grade disease and cancer. However, as application is currently restricted to identification of types 16 and 18, they are at present more appropriate for epidemiological studies.

2.3. Detection and analysis of amplification products

PCR amplimers can be detected easily by standard agarose gel electrophoresis. However, subsequent sequence-specific analysis considerably increases both the sensitivity and specificity of the assay. Several methods have been developed for this purpose.

2.3.1. PCR and restriction fragment length polymorphism (PCR-RFLP)

After amplification, the sequence composition of a PCR product can be investigated by restriction enzymes. Digestion of PCR products with restriction endonucleases generates a number of fragments, which can be resolved by gel electrophoresis, yielding a particular banding pattern. This method is straightforward but labor-intensive. More importantly, the method depends on availability of restriction enzymes capable of detecting specific mutations. Consequently, detection of multiple HPV genotypes, present in different quantities in a clinical sample by PCR-RFLP is usually complex and the sensitivity to detect minority genotypes is limited (Grce et al., 2000).

2.3.2. Hybridization analysis of PCR products

A common way to investigate the sequence of PCR products is hybridization with one or more oligonucleotide probes. Type-specific PCR products can be confirmed with corresponding type-specific probes. The original method is Southern blotting, where a PCR product is electrophoresed prior to transfer to a membrane that is subsequently hybridized to a labeled probe (Kuypers et al., 1993). However, Southern blotting is labor-intensive and not suitable for routine application. Therefore, alternative hybridization formats have been developed.

2.3.3. Microtiter plate hybridization

To increase the throughput of a diagnostic assay, hybridizations to oligonucleotide probes can be performed in microtiter plates (Jacobs et al., 1997; Kleter et al., 1998; Kornegay et al., 2001). Biotin labeling of one of the primers generates labeled PCR products that are then captured onto streptavidin-coated microtiter wells. Double-stranded DNA is denatured under alkaline conditions and the unattached strand is removed by washing. A labeled oligonucleotide probe is added, which hybridizes to the captured strand. Hybridization can be detected following binding of conjugate and substrate reaction. The Roche Molecular Systems Amplicor HPV MWP assay was recently described. This method is based on the detection of 13 high-risk genotypes by a broad-spectrum PCR in the L1 region, amplifying a fragment of approximately 170 bp. The heterogeneous interprimer region is detected with a cocktail of probes for high-risk genotypes. Preliminary data suggests this assay is more sensitive than hc2 for detection of the same HR-HPV types (21st International Papillomavirus Conference, Mexico, February 2004), although further work is required in prospective cohorts to assess whether this increased sensitivity is a benefit. An advantage of this method is the high throughput of the microtiter format. Therefore, this method is suitable for distinguishing HPV-DNA positive and negative samples as a first step in HPV diagnosis.

2.3.4. Direct sequence analysis of PCR products

Rapid sequencing methods of PCR products are also now available for high throughput, thus permitting application in routine clinical analysis (Arens, 2001). However, sequence determination is not suitable when a clinical sample contains multiple HPV genotypes. Sequences, which represent a minority species in the total PCR product, may remain undetected. In turn this may underestimate the prevalence of infections with multiple HPV genotypes, with important consequences for vaccination or follow-up studies (Kleter et al., 1999). This was confirmed in a recent study from our group comparing sequence analysis of SPF10 PCR products with reverse hybridization in 166 HPV-positive cervical scrapes. Compatible HPV genotypes were found in all samples. Direct sequence analysis detected multiple types in only 2% of the samples, while reverse hybridization found multiple types in 25%. The presence of multiple HPV genotypes is a common phenomenon in many patient groups. Up to 35% of HPV-positive samples from patients with advanced cytological disorders and more than 50% of HIV-infected patients (Gonçalves et al., 1999; Levi et al., 2002) contain multiple HPV genotypes, whereas multiple genotypes are less prevalent in carcinoma patients (Kleter et al., 1999).

The genotype can be deduced from an HPV sequence by two methods. First, the sequence can be used to interrogate a sequence database using a homology search. Extensive databases are available on the Internet and can be freely accessed at http://www.ncbi.nlm.nih.gov. BLAST software (Altschul et al., 1990) permits fast homology searches of a sequence within a continuously updated sequence database. Secondly, phylogenetic analyses can be performed. The novel sequence can be used in a multi-sequence alignment with a set of known HPV sequences, representative of different
HPV genotypes. Based on the sequence alignment, a phylogenetic tree can be constructed, providing a graphical representation of the evolutionary relationships between the detected sequence and reference sequences, and a genotype can be deduced. It should be noted that formal classification of genotypes is entirely based on sequence analysis of the viral genome, whereas genotyping of clinical samples is performed by analysis of only a limited, but representative part of the genome.

2.3.5. Reverse hybridization

Reverse hybridization provides an attractive tool for simultaneous hybridization of a PCR product to multiple oligonucleotide probes. This method comprises immobilization of multiple oligonucleotide probes on a solid phase and addition of the PCR product in the liquid phase. Hybridization is followed by a detection stage.

The most frequently used reverse hybridization technology comprises a membrane strip containing multiple probes immobilized as parallel lines, called line probe assay (LiPA); line blot assay (LBA) or linear array (LA). A PCR product is generated, usually using biotinylated primers. The double-stranded PCR product is denatured under alkaline conditions and added to the strip in a hybridization buffer. After hybridization and stringent washing, the hybrids can be detected by addition of a streptavidin-conjugate and a substrate, generating colour at the probe line, which can be visually interpreted. This method permits multiple HPV type detection in a single step and requires only a limited amount of PCR product. An example of the HPV LiPA (Kleter et al., 1999; Melchers et al., 1999; Quint et al., 2001) is shown in Fig. 2.

Alternative reverse hybridization methods for HPV and genotyping are the line blot assay using PGMY primers (Gravitt et al., 1998; Coutlee et al., 1999; Vernon et al., 2000; Gravitt et al., 2001; Laxcano-Ponce et al., 2001) and reverse line blot for GP5+/6+ (van den Brule et al., 2002). HPV-DNA micro arrays work on the same principle (Klaassen et al., 2004; Park et al., 2004). Reverse hybridization methods are particularly useful for the detection of type specific infections and multiple genotypes.

3. Clinical utility of molecular HPV diagnosis

The development of highly sensitive DNA detection assays over the past years has revolutionized the diagnosis of HPV and allowed various crucial aspects of HPV infections to be studied. However, diagnostic test results should be interpreted with care and require careful laboratory validation (Daniel et al., 2000; Schiffman et al., 1995; Castle et al., 2004a; Cubie et al., submitted for publication). There is a clear need for well characterized international quality control panels to compare the various diagnostic methods.

The implications of HPV-DNA detection for patient management need to be further assessed. Recent studies have shown that the prevalence of HPV-DNA and of multiple HPV genotypes in the same patient is higher than expected. Also, the efficacy of large community-based HPV screening studies depends on the accuracy and predictive values of the diagnostic assays used. To identify women with an increased risk for cervical neoplasia, it is clear that detection of HPV-DNA alone is insufficient and novel algorithms are being developed which combine cytological screening and HPV-DNA analysis, to optimize the positive and negative predictive values for development of disease (Cuzick et al., 1999; Nobbenhuis et al., 2001b).

Accurate HPV genotyping is essential for adequate classification of patients into low-risk or high-risk groups. Furthermore, preliminary evidence suggests that the presence of multiple HPV genotypes may reflect repeated exposure.

![Fig. 2. Outline example of the reverse hybridization HPV line probe assay. (A) Amplimers are denatured and hybridized to probes immobilized as parallel lines on a strip. After stringent washing, the hybrids are detected by enzyme-conjugated streptavidin and a substrate, yielding a color reaction. (B) Hybridization patterns which can be interpreted visually after alignment with a probe line template from specimens containing single or multiple HPV genotypes.](image-url)
and may relate to increased risk for disease progression (Bachtiary et al., 2002; van der Graaf et al., 2002). However, this is controversial as the evidence is inconclusive (Chaouki et al., 1998; Rousseau et al., 2001).

Recently, the results of a HPV16 VLP-based vaccine trial indicated that the development of type-specific antiviral therapies or vaccines requires the introduction of suitable algorithms for detection and genotyping of HPV (Koutsy et al., 2002). These methods are also necessary for accurate follow-up during clinical trials, monitoring of antiviral or surgical treatment as well as triage and management of patients.

To address cervical cancer detection worldwide and assess the geographic distribution of HPV genotypes, extensive epidemiological studies are required. Given the substantial genetic heterogeneity of HPV and the possible clinical relevance of specific subtypes, specific molecular tools will be required. Novel low- or high-density DNA probe arrays (DNA chips) may provide a useful technology for such studies.

4. Conclusions

Two different pathways can be envisaged for the use of molecular HPV diagnostics (Snijders et al., 2003). The first application is aimed at identifying women at risk of developing cervical cancer, either in community-based screening programs or in the clinical setting. Here, a highly sensitive HPV detection assay will greatly overestimate the proportion of women who have low-grade cytological abnormalities. A less sensitive or a quantitative assay might be more effective in identifying women at risk of progression, thus improving both negative and positive predictive values (Schlecht et al., 2000; Cuschieri and Cubie, this volume). It is also important that the assay detects only high-risk genotypes, which are associated with a significantly increased risk for cervical carcinoma.

The second area for HPV testing includes vaccination trials, epidemiological and natural history studies. In contrast to the clinical application, highly sensitive and reproducible assays, which assess the broadest possible spectrum of HPV genotypes, are required. The aim of such studies is to obtain a maximum of information about HPV status in populations and to monitor the course of infections in detail. For instance, during vaccination studies, this will help determine vaccine efficacy and possible cross-reactivity with other HPV types. A single HPV assay with an adjustable cut-off for detection and linked to a genotyping method, would be ideal for both areas. At present, such a method does not exist, and testing algorithms are composed of combinations of different and complementary assays.

References


