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Published in:
APMIS

DOI:
10.1111/j.1600-0463.2010.02621.x

Publication date:
2010

APMIS 118: 520–528

DOI 10.1111/j.1600-0463.2010.02621.x

Methods for HPV detection in exfoliated cell and tissue specimens

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Snijders PJF, Heideman DAM, Meijer CJLM. Methods for HPV detection in exfoliated cell and tissue specimens. APMIS 2010; 118: 520–528.

Given the causal involvement of high-risk human papillomaviruses (HPVs) in cervical cancer and a subset of squamous cell carcinomas of other anogenital regions as well as the oropharynx, much attention has been focused on the development and application of HPV detection assays. HPV detection assays are almost exclusively based on the detection of viral nucleic acids, mostly viral DNA. The HPV detection methods that are nowadays in use can broadly be subdivided into target amplification methods and signal amplification methods. In this review, several principles of various methodologies are explained and examples of some commonly used HPV detection assays are given. In addition, attention is paid to the use of HPV assays for detecting clinically meaningful HPV infections, i.e. infections related to (pre)cancerous lesions, e.g. cervical cancer screening purposes. For the latter, it is important that HPV tests are clinically validated according to validation strategies as outlined in guidelines.

Key words: HPV; HPV detection methods; clinical validation; cervical cancer; head and neck cancer.

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The large group of human papillomaviruses (HPVs) can roughly be subdivided into two subgroups, one comprising the types that infect the skin (so-called cutaneous HPV types) and the other comprising types that infect the mucosa (mucosal HPV types). Phylogenetically, cutaneous and mucosal HPVs form distinct trees, reflecting their sequence divergence. This review deals with mucosal HPVs only. As HPVs cannot be cultured easily, almost all HPV detection assays currently in use rely on the assessment of viral nucleic acids, mostly DNA. Originally, Southern blot and Northern blot hybridizations were the methods of choice for HPV DNA and RNA detection, respectively. These methods, however, require large amounts of input material, are time consuming and in principle can only detect one HPV type at a time. In addition, they are not applicable to routine formalin-fixed tissue specimens that typically contain cross-linked, severely degraded nucleic acids. The HPV detection methods that are nowadays in use can broadly be subdivided into target amplification methods and signal amplification methods (1). Target amplification methods utilize nucleic acid polymerases, target-specific oligonucleotides ('primers'), and a mixture of the four (deoxy)ribonucleotides to amplify a specific nucleic acid sequence up to a level at which it can be easily detected using one of the many read-out systems that are available. The most commonly used target amplification-based method is the polymerase chain reaction (PCR), a 'cyclic' exponential DNA amplification method, which makes use of repetitive cycles of temperature switches to accomplish

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consecutively target DNA denaturation, primer annealing and primer extension by a thermostable DNA polymerase. Multiple HPV PCR assays with different read-out systems coupled thereto have been developed.

Although DNA is the amplifiable target in the PCR, mRNA can also be amplified if the reaction is preceded by a reverse transcriptase step to first generate a cDNA strand from the mRNA template, an assay referred to as reverse-transcriptase (RT-)PCR. In addition, isothermal amplification methods have been developed, such as nucleic acid sequence-based amplification (NASBA), which are conducted at a constant temperature approaching the optimum of the polymerase(s) involved. Given the lack of temperature switches, these methods are particularly suited to amplify mRNA because for the latter, no heat denaturation step is required to make the target accessible to specific oligonucleotides (2).

Signal amplification methods are based on an initial hybridization step of nucleic acids in the specimen with target-specific probes in liquid phase or *in situ* on cells or tissue slides, after which the signal (i.e. the hybridization event) is amplified and ultimately visualized with one of the various available methodologies.

Here, different methodologies for mucosal HPV testing are described using examples of HPV tests that have been published and/or are commercially available. It should be realized that this is far from a fully comprehensive overview of all mucosal HPV detection assays that are currently available. In addition, we will discuss recent important findings on distinguishing clinically relevant HPV infections, i.e. infections reflecting clinically meaningful (premalignant and malignant) disease caused by the virus, from clinically irrelevant infections, such as transient and/or coincidental infections.

HPV PCR METHODS AND READ-OUT SYSTEMS FOR DETECTING PCR PRODUCTS

Given the fact that there exist many different HPV types, about two decades ago, a major challenge was to develop PCR assays that can detect multiple different HPV types in one assay run (so-called broad-spectrum PCR assays). This was accomplished by selecting consensus primers directed against highly conserved sequences of the HPV genome, almost exclusively within the L1 or E1 open reading frames. The first consensus PCR assays that resulted from this effort were the IU/IWDO (3), MY09/11 (4) and GP5/6-PCR (5) systems. From the latter two, which target 450 base pair and 150 base pair regions within L1, respectively, second-generation, modified versions (i.e. PGMY (6) from MY09/11 and GP5+/6+-PCR (7) from GP5/6-PCR, respectively) were deduced. Today, these assays still belong to the most commonly used HPV detection assays. By utilizing the conserved L1 sequences targeted by MY09 and GP5+ primers, an ultrasensitive short-fragment PCR assay, the SPF10, was subsequently developed that amplifies a 65 base pair region (8). Despite overall sequence conservation in the viral L1 and E1 regions, these assays still had to deal with some degree of inter-type heterogeneity at the nucleotide level, which precluded the selection of single primer pairs that fully match corresponding sequences of a broad spectrum of HPVs. Therefore, to ensure broad-spectrum HPV detection, consensus primer assays either use low-stringency PCR conditions to allow some degree of mismatch acceptance between primers and target sequence (as used in GP5/6-PCR and GP5+/6+-PCR), degenerate primers with nucleotide variations at ambiguous base positions (as used in MY09/11), primers with the non-specific base analogue inosine at ambiguous base positions (as used in IU/IWDO), combinations of the latter two (as used in SPF10), or sets of overlapping (multiplex) primers (as used in PGMY).

During the last years, many novel PCR-based HPV detection assays have been described, including those that target other conserved regions within viral L1 [such as Roche Amplicor, Branchburg, NY, USA; (9)] or E1 [such as PapilloCheck (Greiner Bio-one , Frickenhausen, Germany; 10)] regions. In addition, modifications on existing broad-spectrum PCR systems were conducted, aiming at better targeting HPV types, which did not react that efficiently with the original assay. Examples of the latter are multiplex variants of GP5+/6+-PCR such as BSGP5+/6+-PCR (11), the Abbott RealTime High Risk HPV test Abbott, IL, USA; (12) and MGP PCR (13). More recently, multiplex

assays have also been developed that use primers targeting different viral regions of different HPV types, rather than a conserved region (e.g. 14).

Equally important for the final assay outcome is the read-out system used to detect the PCR products. In fact, the variability in read-out systems is almost as high as the variability in PCR systems. Most read-out systems are based on hybridization of PCR products to oligonucleotide probes targeting internal regions flanked by both primers, either or not in a so-called 'reverse' hybridization format. In the latter format, oligonucleotides are immobilized on a solid support, hybridized to (biotin-)labelled PCR products and ultimately visualized via colorimetric or fluorescent staining procedures.

One commonly used read-out system involves an enzyme immunoassay (EIA) staining procedure. To accomplish this, one of the PCR primers is labelled with a biotin group, and after capturing of biotinylated PCR products to streptavidin-coated microplate wells, the immobilized PCR products are hybridized with cocktails of e.g. digoxigenin-labelled oligoprobes specific for the HPV types of interest. This is followed by an incubation with an antibody/enzyme conjugate that catalyses a colour reaction upon substrate addition, which is detectable in an ELISA reader. EIAs using oligoprobe cocktails representing high-risk and/or low-risk HPV types are used as primary read-out systems for the GP5+/6+-PCR [i.e. GP5+/6+-PCR-EIA (15)], the SPF10 [DEIA (DDL, Voorburg, the Netherlands; 8)] and the Roche Amplicor assay (9). Although EIA-based read-out assays are useful for high-throughput analyses, they are less suited for genotyping because for that purpose, multiple microplate wells have to be loaded and scored per PCR product, each representing a single HPV type. Apart from being laborious, this requires high quantities of PCR product and often more than a single PCR reaction is needed to generate a sufficient product for comprehensive genotyping. Therefore, more recently, reverse hybridization techniques have been introduced for genotyping purposes. As solid support for oligoprobe immobilization, strips, filters, microarrays and microsphere (Luminex, Austin, TX, USA) beads are used. Examples of strip formats are the Linear Array HPV genotyping (Roche) assay for PGMY products (16), LiPa (DDL) for SPF10 products (17) and the Digene HPV genotyping RH test (Qiagen, Hilden, Germany) for GP5+/6+-PCR products (18). An example of a filter-based assay is the reverse line blot for GP5+/6+-PCR products (19). The hybridized PCR product is detected on strips by a colorimetric reaction (16–18) and on filters by chemiluminescence (19), the latter allowing repeated usage of the same filter. In microarray-based reverse hybridization genotyping methods, such as the CLART HPV2 (Genomica, Madrid, Spain) and PapilloCheck assays, hybridized oligonucleotide probes spotted on microarray slides or at the bottom of a tube are visualized either using fluorescent dyes (PapilloCheck) or following coloured substrate deposition (CLART HPV2).

A reverse hybridization-based HPV genotyping tool that also deserves attention involves a flow cytometry-based method, using Luminex colour-coded microsphere beads. The different microsphere beads contain two spectrally distinct fluorochromes present in various concentrations in such a way that a spectral array is created encompassing up to 100 different microsphere sets with specific spectral addresses. In this system, each type-specific oligonucleotide probe is covalently attached to a specifically coloured microsphere bead set. During analysis, individual microspheres are interrogated by two lasers. The first laser excites the fluorochromes within the microsphere and allows identification of the microsphere set, and consequently the attached type-specific probe. The second laser excites a reporter fluorochrome coupled to the hybridized PCR product and allows quantification of the PCR product. Luminex bead-based suspension array methods developed for genotyping of GP5+/6+-PCR products include the MPG assay (20) and the Digene HPV genotyping LQ test (Qiagen; 21).

Finally, read-out systems coupled to real-time PCR formats using (mixtures of) type-specific probes labelled with fluorescent dyes, or CYBR green are increasingly used. Recent examples of such formats include the commercial Abbott RealTime High Risk HPV test (12) and the Roche Cobas 4800 HPV test (22). Although real-time PCR formats are ideal for quantification, the multiplicity of this technique, and thereby genotyping possibility, is limited

because the current generation of real-time thermocyclers cannot distinguish many different fluorescent dyes. Hence, broad-spectrum real-time formats are currently limited to the detection of HPV types as a pool (i.e. group detection) plus simultaneous genotyping for a restricted number of types, such as two types (HPV 16 and HPV 18) in case of the Abbott RealTime High Risk HPV and Roche Cobas 4800 HPV test formats.

In principle, all aforementioned PCR assays are applicable to exfoliated, cervical scrape samples as well as frozen tissue samples. Given the fact that DNA in formalin-fixed, paraffin-embedded tissue specimens is usually degraded to fragments smaller than 250 base pairs because of fixative-induced cross-links, only PCR assays generating relatively short PCR products can be reliably applied to this material. It will be obvious that the various PCR and read-out assay combinations display variable analytical properties, particularly in terms of sensitivity for the detection of the various mucosal HPV types.

HPV mRNA detection methods

Given the fact that sustained viral oncogene E6 and E7 expression is essential for the initiation and maintenance of the transformed phenotype induced by mucosal high-risk HPVs, several assays have been developed for the specific detection of high-risk HPV E6/E7 mRNA. These include many type-specific RT-PCR assays that often were designed to detect transcripts spliced within E6, so-called E6* mRNAs, which easily can be distinguished from coincidentally co-amplified viral DNA (23). RT-PCR assays can be applied to fresh-frozen specimens or samples in which RNA is well preserved, such as liquid-based cytology samples of cervical scrapings. Despite the fact that formalin-fixed tissue contains degraded nucleic acids, viral mRNA can be amplified from formalin-fixed material as long as the amplified products are not longer than about 100 base pairs and proper quality controls are used to check the integrity of the mRNA (23, 24).

Recently, isothermal mRNA amplification methods such as NASBA and transcription-mediated amplification (TMA) have also been developed for HPV E6/E7 mRNA analysis. These assays utilize a reverse transcriptase to generate cDNA first, RNase H to degrade the RNA template (in case of NASBA) and a T7 RNA polymerase to produce multiple RNA copies from the cDNA. The PreTect HPV Proofer assay (Norchip, Klokkarstua, Norway) and related NucliSENS EasyQ HPV v1 (Biomerieux, Boxtel, the Netherlands) assays are examples of NASBA assays that detect E6/E7 mRNA of five high-risk HPV types (i.e. HPV 16, 18, 31, 33 and 45) in a real-time format using different molecular beacon probes in two assay runs to allow genotyping (25, 26). The Aptima HPV assay (Gen-Probe, San Diego, CA, USA) uses TMA technology to amplify E6/E7 mRNA of 14 high-risk HPV types and chemiluminescence detection of amplified products without genotype distinction (27). All these assays use quality controls to check for the integrity of the mRNA in the specimens. The isothermal amplification assays are particularly applicable for HPV mRNA detection in liquid-based cytology samples.

SIGNAL AMPLIFICATION METHODS

The best known technique in the category of liquid-phase signal amplification methods is the FDA-approved Hybrid Capture 2 (HC2; Qiagen, Gaithersburg, MD, USA) assay (28). The high-risk HPV HC2 assay uses a mixture of full-length RNA probes representing 13 HPV types (i.e. HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) to hybridize to HPV DNA in heat-alkaline-denatured samples. DNA–RNA hybrids are subsequently captured in microplate wells coated with antibodies that specifically recognize DNA–RNA hybrids. HPV DNA present in the samples is detected by peroxidase-labelled antibodies recognizing the RNA/DNA hybrids, and visualized by chemiluminescence. The analytical sensitivity of this method is 1 pg/mL of cloned HPV16 DNA, which corresponds to approximately 10^5 HPV16 genome copies. A more recent liquid-phase assay that is also FDA approved is the Cervista HR HPV test (Hologic, Madison, WI, USA) targeting 14 high-risk HPV types (29). This system makes use of a Cleavase enzyme that first cleaves a type-specific oligonucleotide probe when it

overlaps an invader oligonucleotide at the target DNA recognition site. The resulting 5′-portions of the cleaved probes can subsequently bind to a universal hairpin fluorescence resonance energy transfer (FRET) oligonucleotide creating another invasive structure that is recognized as substrate by the Cleavase enzyme. The enzyme then cleaves the FRET oligonucleotides between the fluorophore and quencher molecule, which results in the production of a fluorescence signal. The probes are present in large molar excess and rapidly cycle on and off the target sequence so that many cleaved 5′-portions are generated per target sequence that in turn create new substrates for the Cleavage enzyme when bound to FRET oligos. Both HC2 and Cervista assays are specifically designed for HPV detection in cervical scrapings.

In addition to the above-mentioned liquid-phase methods, HPV DNA *in situ* hybridization (ISH) methods have been developed for cytological (30, 31) and histological slide preparations (32). This can be achieved by fluorescent detection or coloured substrate deposition and bright field microscopy. A commercially available HPV ISH system (Ventana Inform HPV, Tuczon, AZ, USA) uses an indirect biotin-streptavidin method, which at present lacks sufficient sensitivity to detect all high-grade cervical lesions (31). As an alternative, tyramide signal amplification, also known as catalysed reporter deposition (CARD), can be used both in fluorescent (32) and bright-field (33) applications. Also for the CARD method, a commercially available system exists (i.e. Dako GenPoint, Glostrup, Denmark).

Various mucosal HPV tests described in the previous paragraphs that are currently in use are listed per category and assay principle in Table 1. Most of these assays only detect (subsets) of high-risk HPV types.

DETECTION OF CLINICALLY RELEVANT HPV INFECTIONS

Recent randomized controlled cervical screening trials have shown that high-risk HPV testing on cervical scrapes can be used as a primary tool for cervical screening, given its superior sensitivity and negative predictive value for cervical precancer compared with cytology (34–37). To make a distinction between HPV test performances for outcome measure '(pre)malignant disease' vs outcome measure 'HPV presence', the terms 'clinical sensitivity and specificity' and 'analytical sensitivity and specificity' were introduced. Herein 'clinical' refers to relevant disease-related HPV infections and 'analytical' to any HPV infection (38). We currently know that various HPV detection methods differ in their clinical performance of detecting HPV-related premalignant disease (38). As an example, a comparison of the high-risk HPV GP5+/6+-PCR-EIA with the analytically more sensitive SPF10-LiPa on cervical scrapings of women with normal cytology revealed that application of the latter did not lead to an increase in clinical sensitivity for cervical precancer or cancer, but instead to a substantial decrease in clinical specificity compared with that of the GP5+/6+-PCR-EIA (39). Thus, detection of very low viral copy numbers in cervical scrapings does not reflect (risk of) high-grade cervical disease, but the presence of clinically irrelevant infections instead. In practice, application of ultrasensitive HPV assays would result in a high false-positivity rate (i.e. detection of high-risk HPV positives without high-grade disease) and consequently many redundant and unnecessary follow-up procedures. On the other hand, insufficient analytical sensitivity would result in an unacceptably low clinical sensitivity for high-grade disease, as has been shown for a commercially available DNA ISH assay (31). In fact, the high-risk HPV methods that were successfully applied in large randomized controlled screening trials are the high-risk HPV HC2 and GP5+/6+-PCR-EIA assays. Both assays showed an optimal balance between clinical sensitivity and specificity and are considered clinically validated for screening purposes.

Recently, guidelines for HPV test requirements for cervical screening purposes have been formulated (40). These guidelines describe a clinical validation strategy for candidate HPV tests that are aimed for cervical screening purposes. The validation strategy is based on clinical equivalence analysis of the candidate assay relative to a clinically validated reference HPV test (i.e. HC2) by non-inferiority testing using samples that originate from a population-based screening cohort. It involves assessment of

Table 1. Overview of discussed methodologies that are nowadays in use for mucosal human papillomavirus (HPV) detection

Category	Target	Assay principle	Method	Reference
Target amplification	DNA	PCR: end-point hybridization read-out	PGMY-Linear array (Roche)	16
			SPF10-LiPa (DDL)	17
			GP5+/6+-PCR-EIA[1]	15
			MGP PCR-Luminex	13
			BSGP5+/6+-PCR-MPG	20
			Digene HPV genotyping RH test (Qiagen)	18
			Digene HPV genotyping LQ test (Qiagen)	21
			Amplicor (Roche)	9
			PapilloCheck (Greiner-Bio-one)[2]	10
			CLART HPV2 (Genomica)	–
		Real-time PCR-based	Abbott RealTime High Risk HPV test	12
			Cobas 4800 HPV test (Roche)[3]	22
	mRNA	RT-PCR	HPV 16 E6*I RT-PCR-EIA	23
		NASBA	PreTect HPV Proofer assay (Norchip)	25
			NucliSENS EasyQ HPV v1 (Biomerieux)	26
		TMA	Aptima HPV assay (Gen-Probe)	27
Signal amplification	DNA	Liquid phase	Hybrid Capture 2 (Qiagen)[1]	28
			Cervista HR HPV (Hologic)	29
		In situ	Ventana Inform HPV (Ventana)	31
			Dako Gen point (Dako)	–

[1]Clinically validated for cervical screening purposes.
[2]Partially clinically validated for cervical screening purposes according to validation criteria described in Meijer et al. (40) when assay read-out is restricted to 14 high-risk HPV types.
[3]Partially clinically validated for cervical screening purposes according to validation criteria described in Meijer et al. (40).

clinical sensitivity for ≥CIN2, clinical specificity for ≥CIN2 and assay reproducibility. Using this approach, candidate HPV DNA tests can be validated for application in cervical screening programmes without the necessity of performing large, prospective screening trials (41). It is of note that so far, to the best of our knowledge, hardly any of the abovementioned HPV detection assays have, next to HC2 and GP5+/6+-PCR-EIA, proved to fulfil these validation criteria. Exceptions are the PapilloCheck and Cobas 4800 HPV tests, which fulfilled the clinical sensitivity and specificity criteria, the first assay in case scoring of high-risk HPV positivity is restricted to 14 high-risk HPV types (11; D.A.M. Heideman, A. Hesselink, C.J.L.M. Meijer, P.J.F. Snijders unpublished data).

For tissue specimens also, the discussion on distinguishing clinically relevant from clinically irrelevant HPV infections has emerged. This particularly holds true for head and neck cancers, more specifically oropharyngeal squamous cell carcinomas, which are known to be aetiologically heterogeneous. One subset of these tumours can be attributed to excessive smoking and alcohol use, and the other to high-risk HPV, with HPV 16 being the predominant type (42). However, reported HPV prevalence rates in these tumours are highly variable, and it has become clear that HPV DNA detection by PCR in these specimens does not *per se* indicate a causal association (43). Similar findings have been described for other cancers with a heterogeneous aetiology, such as penile cancers (44).

The fact that the detection of HPV DNA by PCR in cancer tissue may not directly be linked to a causal relationship in case of head and neck cancers initially became evident after a comprehensive analysis of a series of frozen tissue specimens (45). Only a subset of tumours in which HPV16 DNA could be detected by PCR displayed E6/E7 mRNA expression, as assessed by RT-PCR. The HPV16 DNA-positive tumours with E6/E7 transcripts could be genetically distinguished as a specific subgroup (46). Ever since, several efforts have been made to detect biologically relevant HPV infections in formalin-fixed tissue specimens of head and neck cancer. In principle, detection of E6/E7 region mRNA by short-fragment RT-PCR seems highly sensitive and specific for clinically

meaningful HPV infections (23, 24). However, as this method is technically more challenging, alternative, easier assays have been proposed (44). These include $p16^{INK4A}$ immunostaining, a highly sensitive but not 100% specific marker for transforming HPV infections, and DNA ISH assays, being highly specific, although less sensitive (23, 24). To reach the highest sensitivity and specificity for clinically relevant diseases, diagnostic algorithms that first use $p16^{INK4A}$ immunostaining, followed by consensus high-risk HPV GP5+/6+-PCR or HPV DNA ISH assays seem most promising (23, 47, 48). Further validation studies are necessary to determine which algorithm is most accurate in detecting clinically relevant HPV infections in tumour tissue specimens and most reliable in assessing prognosis and performing patient stratification for future clinical trials that test HPV-targeted therapies.

In summary, there exist nowadays many different HPV tests that all have their own technical, analytical and clinical properties. The choice for an HPV test for a given purpose should therefore not solely be based on practical considerations, but also on the intention for its use. In particular, when aiming at the detection of clinically relevant HPV infections, it is of utmost importance to use a clinically validated HPV detection assay.

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