Laboratory Methods for Detection of Human Papillomavirus Infection

2

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Contents

2.1 Introduction

The diagnosis of human papillomavirus (HPV) infection can be inferred from morphological, serological, and clinical findings. In productive infections, such as warts, virus particles of about 50 nm diameter can be detected by electron microscopy and by immune detection of the virus capsid proteins (L1, L2). Immunological detection of HPV in human cells or tissues has been hindered by three main factors: (a) the late, capsid proteins are only expressed in productive infections; (b) the early proteins are often expressed in low amounts in infected tissues; and (c) the lack of high-quality, sensitive, and specific antibodies against the viral proteins. Antibodies generated against bovine papillomavirus (BPV) late proteins have been widely used because of the observed cross-reaction with HPV late proteins; however, they have low sensitivity and fail to discriminate between HPV types, which is essential for disease risk determination. Detection of HPV early proteins is even more complicated due to the low expression levels generally observed in cells or tissues derived from HPV-positive lesions. Antibodies against E6 or E7 are available but their use is mostly restricted to in vitro assays including the direct visualization in cells or tissues (immunohistochemistry) or in protein extracts (Western blots and immune precipitation assays), not always with consistent results.

HPV cannot be propagated in tissue culture and hence in most cases its accurate identification relies on molecular biology techniques. With a double-stranded DNA genome of about 8,000 base pairs (bp) in length and a well-known physical structure and gene organization, tests of choice for detecting HPV from clinical specimens are based on nucleic acid probe technology. Direct detection of HPV genomes and its transcripts can be achieved with hybridization procedures that include Southern and Northern blots, dot blots, in situ hybridization (ISH), Hybrid Capture (HC; formerly DIGENE Co., now QIAGEN, MA, USA), and DNA sequencing (reviewed in Iftner and Villa, 2003). A variety of signal-detection procedures are available that can further increase the sensitivity of these assays. The only procedure potentially capable of recognizing all HPV types and variants present in the biological specimen is DNA sequencing of the viral genome, either after cloning into plasmids or by direct sequencing of a polymerase chain

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reaction (PCR) fragment. This method, however, is presently labor-intensive and requires expensive equipment. Moreover, direct sequencing of specimens containing multiple HPVs awaits further developments.

For HPV genome analysis, hybridization in solid phase, such as Southern blot for DNA and Northern blot for RNA molecules, is an excellent procedure that can generate information with quality, but is time-consuming and requires large amounts of highly purified nucleic acids. Moreover, it requires well preserved, ideally full-size molecules, and therefore cannot be done with any biological specimen, particularly those derived from fixed tissues in which DNA degradation is often observed. It is also technically cumbersome and time-consuming, not being amenable to large-scale population studies. ISH is a technique by which specific nucleotide sequences are identified in cells or tissue sections with conserved morphology, thereby allowing the precise spatial localization of the target genomes in the biological specimen. One great advantage of ISH is that it can be applied to routinely fixed and processed tissues, which overcomes the relatively low analytical sensitivity of this method. This can be increased by combining PCR to ISH, a procedure known as in situ PCR (Nuovo et al., 1992). ISH has been used to detect messenger RNA (mRNA) as a marker of gene expression, where levels of viral proteins are low (Stoler et al., 1997). The major limitations of the method are the potential for error in HPV typing because of probe cross-hybridization. However, recent improvements have made it possible for this method to be widely used for HPV DNA and RNA detection in tissues, with high sensitivities and specificities. Viral DNA and RNA can also be detected by a series of assays based on PCR. In this case, the viral genomes are selectively amplified by a series of polymerization steps, which result in an exponential and reproducible increase in the HPV nucleotide sequences present in the biological specimen (Iftner and Villa, 2003). A summary of the characteristics and usefulness of different HPV detection assays is presented in Table 2.1.

Studies conducted during the last two decades have clearly demonstrated the role of HPV infections and risk of cervical neoplasia in women (Bosch et al., 2008). In men, however, this knowledge is just starting to accumulate. An accurate definition of infection and identification of HPV-associated diseases in the male genital tract is critical (Dunne et al., 2006; Partridge and Koutsky, 2006). Moreover, the natural history of HPV-related diseases and transmission of HPV between individuals cannot be fully understood without the establishment of proper measures of the prevalence and incidence of HPV infections in male subjects. The dynamics of HPV infection in special populations, including immune-deficient individuals, can also be improved with precise measures of HPV exposure including both HPV DNA and serology. This chapter discusses the different technologies available for detecting HPV infections. Moreover, the differences in anatomical sites and types of specimen collection in men are highlighted.

2.2 HPV DNA Methods

Presently, the two methodologies most widely used for genital type detection that have equivalent sensitivities and specificities are Hybrid Capture version 2 (HC2) and PCR with generic primers. Both assays are suitable for high-throughput testing, with automated execution and reading, which is a necessary step to be considered for use in large epidemiological studies and in clinical settings.

HC2 is based on hybridization in solution of long synthetic RNA probes complementary to the genomic sequence of 13 high-risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) and five low-risk (6, 11, 42, 43, 44) HPV types, which are used to prepare high (B) and low (A) probe cocktails that are used in two separate reactions. DNA present in the biological specimen is then hybridized in solution with each of the probe cocktails allowing the formation of specific HPV DNA-RNA hybrids. These hybrids are then captured by antibodies bound to the wells of a microtiter plate that specifically recognize RNA-DNA hybrids. The immobilized hybrids are detected by a series of reactions that generate a luminescent product that can be measured in a luminometer. The intensity of emitted light, expressed as relative light units, is proportional to the amount of target DNA present in the specimen, providing a semiquantitative measure of the viral load. HC2 is currently available in a 96-well microplate format, is easy to perform in clinical settings, and is suitable for automation (Lorincz and Anthony, 2001). Furthermore, HC2 does not require special facilities to

Table 2.1 Characteristics of HPV test technologies

	Test	Analytical	Clinical	Comments
		Sensitivity/specificity	Sensitivity/specificity for CIN3/cervical cancer	
Based on cell morphology	Pap smear/tissues	Not applicable	Low/high	Limited because of their low-sensitivities Highly dependent on sampling and tissue preservation Cannot type HPV
	Colposcopy	Not applicable	Moderate/low	
	Visual inspection	Not applicable	Low/low	
Detection of HPV proteins	Immunocito/histochemistry[a]	Low/high	Low/low	
	Electron microscopy[a]	Low/high	Low/low	
	Western blots[a]	Low/high	Low/moderate	
Detection of HPV genomes				
Direct methods	Southern blot[a,b]	Moderate/high	Moderate/high	
	ISH[a,b]	Moderate/moderate	Moderate/moderate	
	Dot blot	Low/high	Low/high	
Signal amplification	HC[c,d,e]	High/high	High/high	
Target amplification	PCR[c,d,e]	High/high	Very high-high/high-moderate	
	Real-time PCR[d,e]	Very high/high	Very high[f]	
Detection of anti-HPV antibodies				
	ELISA peptides	Low/low	Low/low	
	VLP	Moderate/high	Low/low	
	Fused E6/E7	High/moderate	Low-moderate/high	

[a]Technically cumbersome and/or time-consuming
[b]Requires DNA and tissue preservation
[c]Less dependent on sampling; can be done in crude samples
[d]Suitable for high-throughput testing and automation
[e]Provides viral load information
[f]No data available

avoid cross-contamination, because it does not rely on target amplification to achieve high sensitivity, as do PCR protocols. Often only the high-risk cocktail is used, and this reduces the time and cost of the test. The U.S. Food and Drug Administration recommended cutoff value for test-positive results is 1.0 relative light unit (equivalent to 1 pg HPV DNA per 1 ml of sampling buffer).

A newly developed HC assay uses RNA probes as in HC2, but has been developed to be used in low-resource settings (*careHPV*, QIAGEN NV). It is designed to be a rapid test, able to detect 14 HPV types in about 2.5 h, and can be performed outside a specialized laboratory by staff with minimal training. The ability of the *careHPV* test to detect premalignant lesions was found to be 90% in a large study conducted in the rural area of China (Qiao et al., 2008).

The sensitivity and specificity of *PCR-based methods* can vary, depending mainly on the primer set, the size of the PCR product, the reaction conditions and performance of the DNA polymerase used in the reaction, the spectrum of HPV types amplified and the ability to detect multiple types, and the availability of a type-specific assay. PCR can theoretically produce one

billion copies from a single double-stranded DNA molecule after 30 cycles of amplification. Therefore, care must be taken to avoid false-positive results derived from cross-contaminated specimens or reagents. Several procedures are available to avoid this potential problem in using PCR protocols for HPV DNA detection.

The most widely used PCR protocols employ consensus primers that are directed to a highly conserved region of the L1 gene, since they are potentially capable of detecting all mucosal HPV types. Among these are the single pair of consensus primers GP5/6 and its extended version GP5+/6+ (de Roda Husman et al., 1995; Jacobs et al., 1995) as well as the MY09/11 degenerate primers and its modified version, PGMY09/11 (Gravitt et al., 2000). Full distinction of more than 40 types can be achieved by hybridization with type-specific probes that can be performed in different formats, and restriction fragment length polymorphism analysis by gel electrophoresis (Bernard et al., 1994), dot blot hybridization, line strip assays, and microtiter plates that are amenable to automation. Recent developments include the Amplicor HPV test kit (Roche Diagnostics, CA, USA) designed to amplify with non-degenerate primers a short fragment (170 bp) of the L1 gene of 13 high-risk genotypes. A PCR-based linear array HPV product, which exploits the pGMY09/11 amplification system and is capable of identifying 37 HPV genotypes, including all high- and low-risk genotypes in the human anogenital region, is also commercially available (Linear Array HPV Genotyping Test, Roche Diagnostics).

Another pair of consensus primers is available that amplifies a smaller fragment (65 bp compared to 150 bp for the GP primers and 450 bp for MY09/11) of the L1 gene. This short PCR fragment (SPF)-PCR is designed to discriminate between a broad spectrum of HPVs in a reverse line blot hybridization (LiPA) (Kleter et al., 1999). Tests that rely on shorter fragments of the viral genome are considered to be more sensitive and amenable for less-preserved specimens. The SPF and GP5+/6+ systems are widely used in epidemiological studies and have being adapted to formats amenable for high-throughput testing. A fast and reliable HPV typing method has been developed using nonradioactive reverse line blotting (RLB) of GP5+/6+ PCR-amplified HPV genotypes. In this way 40 HPV-positive clinical samples can be simultaneously typed for 37 HPV types (14 HR and 23 LR types) (van den Brule et al., 2002).

Recently, PCR protocols based on a 5′-exonuclease assay and real-time detection of the accumulation of fluorescence were developed and named real-time PCR. Compared to other assays, such as HC, this is considered to be an accurate method of estimating viral load, while controlling for variation in the cellular content of the sample by quantification of a nuclear gene. Several studies have shown that the risk of developing cervical neoplasia is associated with higher copy numbers of different HPV types (Gravitt et al., 2003; Moberg et al., 2004; Schlecht et al., 2003). However, there are inherent differences in the assays to determine viral load that could obscure the interpretation and clinical relevance of the results obtained. Further studies to evaluate the clinical relevance of viral load are warranted.

Testing for the presence of more than one HPV type in the biological specimen is preferentially done by PCR-based methods, since HC2 does not discriminate between HPV types. In general, it seems that PCR systems using multiple primers such as PGMY09/11 and SPF-PCR are more robust for detecting multiple infections than systems using single consensus primers such as GP5+/6+. This may especially be true in cases of mixed infections where one type is present in large amounts, but all types present could be identified by very sensitive reverse line blot assays or linear arrays, as described above.

2.3 HPV RNA Methods

HPV RNA is being considered as an important target for the molecular diagnosis of HPV infections. Testing for viral RNA aims to evaluate the HPV genome expression (and hence their activity in the infected cells), unlike HPV DNA assays that detect only the presence of viral genomes. The rationale is that the presence of transcripts of the HPV oncogenes E6 and E7 is a more accurate and specific marker of cells at risk or already transformed by high-risk HPVs. HPV 16 E6 and E7 transcripts can be detected with high sensitivity in clinical specimens by employing PCR-based methods including reverse transcriptase PCR (RT-PCR) (Sotlar et al., 1998), quantitative RT-PCR, and real-time PCR (Lamarcq et al., 2002). Recent studies have shown that testing for E6/E7 transcripts of HPV types 16, 18, 31, 33, and 45 with an RNA-based

real-time nucleic acid sequence-based amplification assay (NASBA; PreTect HPV-Proofer; Norchip, Norway) was more specific in detecting individuals that developed high-grade cervical disease than HPV DNA detection by PCR with GP5+/6+ consensus primers (Molden et al., 2005). Moreover, detection of such oncogenic transcripts identified which HPV high-risk infections persisted without having to perform repeat testing (Cuschieri et al., 2004). Another important application for HPV RNA studies has been suggested by Klaes et al. (1999), who developed a method (*APOT*, amplification of papillomavirus oncogene transcripts) to differentiate between episomal and integrated HPV oncogene transcripts. The rationale behind this method is that in cervical cancers HPV genomes are often integrated into the host chromosomes, while in normal and premalignant tissues the viral DNA is usually kept as episomes. Using this assay, they were able to show a strong correlation between detection of integrated high-risk HPV transcripts and presence of high-grade cervical neoplasia. The main problem with these techniques is that RNA is a much more labile molecule than DNA, and therefore less available in most biological specimens depending on the time and type of storage conditions. Therefore, there is great interest in collection media capable of preserving both DNA and RNA molecules. These include collection media which contain methanol, shown to preserve both the cell morphology and integrity of DNA, RNA, and proteins (Cuschieri et al., 2005; Nonogaki et al., 2004).

2.4 Serological Assays

At present there is no agreed standard methodology for serological assays that measure antibody acquired in a present or past HPV infection, although virtually all reported studies employ enzyme immunoassays (Konya and Dillner, 2001). Before neutralizing antibody assays were made available (Pastrana et al., 2004), most serological assays were type-specific HPV VLP ELISA (Carter et al., 2001). More recently, an automated multiplex assay based on the use of Luminex beads was developed for the detection of different serotypes with the same sensitivity and specificity achieved in the single-type assays (Dias et al., 2005).

Standardized methodologies that measure total serum antibody, neutralizing antibody, and type-specific antibody concentrations will be necessary. Not all of these assays will be routine, but if and when employed they must be standard and consistent. These assays will require the establishment of an International Standard(s) with an arbitrarily assigned unit measure or international units (IU). These issues were recognized by the World Health Organization (WHO), who established collaborative studies to evaluate reference reagents for type-specific HPV serologic assays (http://whqlibdoc.who.int/hq/2004/WHO_IVB_04.22.pdf and Ferguson et al., 2006). Importantly, about half of the individuals exposed to HPV never develop measurable titers of antibodies, although this scenario will change drastically as HPV prophylactic vaccination is implemented in different areas around the world. This information is and will be predominantly known in women, which underscores the need to better understand the natural course of HPV infection and corresponding immune responses to HPV in men (Svare et al., 1997).

It is very important to stress that the analytical sensitivities and specificities of HPV tests vary largely, depending on the assay characteristics, the type and quality of the biological specimen, and the type and quality of the reagents employed, including the use of different DNA polymerases that affect test performance. Moreover, caution should be used to interpret such comparisons, because the assays differ in their ability to detect different HPV types either as single or multiple infections. In general, there are good to excellent agreement rates between tests performed with HC2 and generic-PCR employing MY09/11 and GP5+/6+ systems. This emphasizes the availability of several robust HPV tests. Nevertheless, standardized methods and validated protocols, reagents, and reference samples should be available to assure the best test performance in different settings.

Although the analytical sensitivities of some HPV detection assays can be very high, and therefore valuable for addressing the burden of HPV infections epidemiologically, their corresponding clinical significance is not so evident (Snijders et al., 2003). This is because several HPV infections do not persist and therefore do not lead to clinically relevant disease. Anogenital HPV infections are very common in young, sexually active populations, including both women and men. However, in the latter, the natural history of persistence and disease development is largely unknown.

2.5 Detecting HPV Infection in Men

Results from different studies of HPV infections in men are not always consistent and vary considerably according to the anatomical site sampled, the type of collection, and the HPV DNA test used to ascertain HPV presence and type (Baldwin et al., 2003; Dunne et al., 2006; Giuliano et al., 2008b, c; Nielson et al., 2007). Among a series of clinical and histopathological techniques, PCR has emerged as the most sensitive method to define HPV infection in men. Indeed, this is the methodology being used in large cohort studies of the natural history of HPV infection and risk of neoplasia in men (Giuliano et al., 2008a; Svare et al., 2002) (see also Chaps. 1, 5, and 6).

Specimen Collection Site: Single and multiple anatomical sites of the male genitalia are often sampled. They include the penile shaft, coronal sulcus/glans (including the prepuce in uncircumcised men), scrotum, urethra, as well as urine and semen (Aguilar et al., 2006; Benevolo et al., 2008; Fife et al., 2003; Giuliano et al., 2008a; Nielson et al., 2007). It has been shown that specimens from the urethra and semen contribute little toward the analysis of HPV DNA prevalence and that specimens should be collected from the shaft, glans, and scrotum in a combined sample (Giuliano et al., 2007).

Collection Method: Another most important aspect when analyzing HPV infection in men is the variability attributable to the collection method. Several methods to obtain samples from the male genitalia have been described. They include the removal of exfoliated cells from the penis surface either by direct scraping with a swab or brush (dry or pre-wetted in saline) (see also Sect. 3.5 in Chap. 3) or by first abrading the skin with a nail file followed by removal of cells with a swab (Weaver et al., 2004). This in fact is the method that is being applied to collect cells from the penis of men included in a large clinical trial for the quadrivalent HPV L1 VLP prophylactic vaccine (Giuliano, 2007). Recently, a systematic study has shown that skin cells exfoliated with a swab can be used reliably for HPV testing (Flores et al., 2008). Moreover, HPV prevalence results obtained from self-collected samples in men show good agreement with clinician-collected samples, which may be considered a suitable alternative for studies on HPV transmission (Hernandez et al., 2006; Ogilvie et al., 2008).

In summary, several methods to detect HPV infections are available. The choice of a particular method is very much dependent on its analytical as well as its clinical sensitivities and specificities. In the male genitalia, HPV detection is further complicated by the low amount of DNA obtained from exfoliated skin cells, which highlights the importance of validating the collection and detection method of choice. There are sufficient and validated tools for performing studies of anogenital HPV infections in men, and results are rapidly accumulating. This experience should contribute to accelerate our knowledge about the natural history of HPV infection and risk of anogenital disease in men.

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