Diagnostic tests for HPV infection

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LEARNING OBJECTIVES

Upon completion of this article, the reader will be able to:

1. Understand the role of human papillomavirus (HPV) testing in the diagnosis and prevention of cervical cancer and other HPV-related malignancies.
2. Recognize the standard tests used to identify HPV in the laboratory.
3. Understand the limitations of the various testing modalities for HPV.
4. Identify new and emerging laboratory technology for HPV testing.

Human papillomavirus (HPV) is one of the most common sexually transmitted infections and a necessary cause of important anogenital and other malignancies. HPV is also associated with non-neoplastic diseases, such as common cutaneous warts and recurrent respiratory papillomatosis. HPV-associated cervical cancer is the second most common cancer worldwide in women and the most common malignancy in women in developing countries. More than 450,000 cases are diagnosed each year around the world, leading to almost a quarter of a million deaths.[1]

Anal-cancer is less common but increasing in incidence in men and women. Anal cancer incidence is highest in subpopulations, such as men who have sex with men, HIV-infected men and women, women with cervical pre-cancer lesions, and transplant recipients.[2] It is estimated that all sexually active adults will be exposed to HPV in their lifetime, and studies have shown that as many as 20% of women have HPV-DNA detected in the cervix at any one time.[3] The link between HPV and anogenital malignancies has led to investigation into the use of HPV testing for screening and surveillance of anal and cervical cancers and as a means to identify eligible subpopulations for prophylactic and therapeutic immunization. Although HPV has been associated with other genital (such as penile, anal, perianal, vulvar) and nongenital (such as oropharyngeal and tongue) lesions and malignancies, the focus in this article is the use of HPV testing for cervical cancer.

Biology and classification of HPV

Papillomaviruses are nonenveloped DNA viruses 55 nm in diameter with an icosahedral capsid enclosing a double-stranded, circular DNA genome of 7,900 base pairs.[4] The HPV genome has three functional areas: 1) a noncoding, upstream region that is responsible for regulation of DNA replication and transcription of specific coding sequences or open reading frames (ORFs), 2) the "early" ORF (E1-E7), and 3) the "late" ORF (L1 and L2) regions. The early proteins encoded by the E1-E7 ORFs are associated with HPV-gene regulation and cell transformation, and the two late proteins encoded by L1 and L2 ORFs form the viral shell. Of these, the two most important HPV proteins in malignant-disease pathogenesis are encoded by E6 and E7, which can act synergistically to transform cells from normal to malignant.

The ability of the protein products of E6 and E7 to transform cells relates to their ability to interact with p53 (for E6) protein and retinoblastoma (Rb) protein (for E7), two intracellular proteins that normally regulate cell growth. In normal cells, p53 arrests cell growth after chromosomal cell damage, allowing DNA repair enzymes to work. If E6 binds to p53, the p53 is degraded and permits the accumulation of chromosomal mutations with subsequent genomic instability because of ineffective repair mechanisms. The binding of Rb and E7 has a similar outcome. In normal cells, Rb arrests cellular growth after DNA damage and also induces apoptosis or cellular death. After E7 binds to Rb, there is unchecked cellular growth and chromosomal instability, increasing the probability of malignant change. Other HPV-protein products that may have a role in malignant transformation of cells are E1, E2, and E5.

HPV cannot be grown in tissue culture or in laboratory animals. Nevertheless, current molecular-biology techniques using polymerase chain reaction (PCR) have permitted HPV typing. A distinct HPV type is formally defined as having less than 90% DNA base-pair homology with another identified HPV type. This DNA-level classification is in contrast to the serologic methods used to type other viruses, such as herpes simplex virus (HSV), where the differences in binding of type-specific antibodies to virus envelope protein distinguish HSV-1 from HSV-2.

Currently, more than 80 different HPV types have been isolated, and about 40 of these can cause genital disease. These types can be classified as high-risk HPV types (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82), probable high-risk types (types 26, 53, and 66) or low-risk types (types 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and CP6108) based on the strength of association with malignancy, such as invasive cervical cancer.[5] HPV types 16 and 18 have been the types most commonly isolated in cervical, anal, and penile cancer in most studies to date, but not all infections with types 16 or 18 progress to invasive cancer.[6]

Role of HPV testing for cancer screening

Currently, the cornerstone of screening for cervical cancer is not HPV testing but rather the Papanicolaou (Pap) test. In that method, a sample of exfoliated cells is obtained from a visualized cervix, fixed on a glass slide or collected in a liquid medium, and then analyzed for cytologic abnormalities by a pathologist. If the specimen is adequate, and there is an abnormal result, women can then be referred for colposcopy and treatment. Colposcopy employs a powerful light source and binocular lenses to permit the identification and biopsy of lesions that might have contributed to abnormal cytologic findings. Acetic acid (3% to 5%) is used to assist the colposcopist in the identification of diseased tissue, which appear characteristically white after the application of the dilute solution of acid (acetowhitening). Lesions suspicious for disease are then biopsied to confirm cytologic and colposcopic appearance. Although the sensitivity of Pap tests to detect high-grade cervical lesions is only 55% to 80%, the specificity is over 90%.[7]

HPV testing has been used widely for studies of the natural history and etiology of HPV-related malignancies. It is, however, currently being investigated as an adjunctive or alternative screening modality for cervical cancer. The basis for the use of HPV testing for cervical cancer is rooted in the strong association of HPV infection with invasive cervical cancer and its associated pre-cancer lesions. HPV has been isolated in almost 80% of patients with low-grade cervical pre-cancer lesions, 90% of patients with high-grade cervical pre-cancer lesions, and almost all women with invasive cervical cancers (vs. 16% of normal controls).[8] The sensitivity of HPV DNA testing (84% to 100%) is generally higher than that of Pap smears for detecting cervical pre-cancer lesions, but the specificity is lower (64% to 95%) (Table 1). Self-collected samples (blindly collected) are less sensitive than provider-obtained samples (where the cervix is visualized and sampled directly) for HPV DNA testing.[8] The sensitivity of self-collected cervicovaginal samples for HPV DNA testing, however, is similar to that of Pap tests.

The development of preventive and therapeutic immunizations directed at HPV infected cells may expand the need for systematic HPV testing.

Provided specimens are adequate, cervical Pap tests are classified as "normal" or "abnormal" (Figure 1). The "abnormal" classification includes a range of findings from atypical squamous cells of undetermined significance (ASC-US) and atypical squamous cells: cannot exclude high-grade SIL (ASC-H) to more clearly abnormal findings, such as low-grade squamous intraepithelial lesions (LSIL) and high-grade squamous intraepithelial lesions (HSIL). It is thought that

Figure 1. Incorporating HPV testing in cervical-cancer screening.

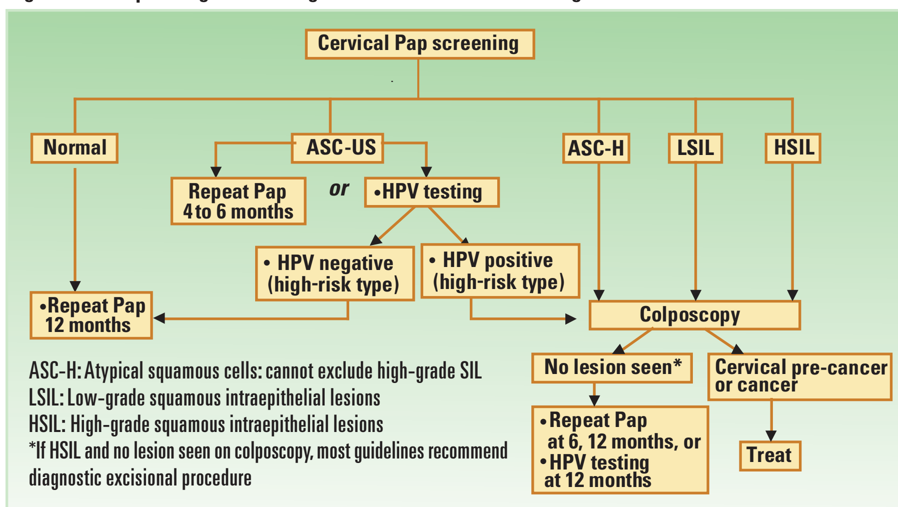

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Table 1.
A comparison of HPV testing vs. Pap testing in cervical-cancer screening.

	HPV test*	Pap test
Portability	Can be self-collected.	Cannot be self-collected. Requires pelvic examination by healthcare provider.
Sensitivity	84% to 100%. Can detect women with false-negative Pap tests.	44% to 88%
Specificity	64% to 95%. Does not detect disease, only infection. HPV infection is transient in many who will never develop a pre-cancerous lesion.	88% to 98%
Interobserver variation	Less	More
Cost	$60	$20 to $40

* Hybrid capture second-generation test (HC2)

HSIL is the true cervical-cancer precursor lesion. At present, HPV testing is cleared by the United States Food and Drug Administration (FDA) for triaging women who have ASC-US on Pap testing.[9] HPV DNA testing is done only if ASC-US is diagnosed. Women who have ASC-US Pap test results and with high-risk HPV types on DNA testing are then referred to colposcopy, while those without high-risk HPV types are seen in one year for a repeat Pap test (Figure 1). Another possible indication for HPV testing, recommended by the American Cancer Society and the American College of Obstetricians and Gynecologists, is its use in combination with Pap tests in women over 30 to increase cervical-cancer screening intervals.[10] If the Pap test is negative and no high-risk HPV types are isolated, the screening interval may be increased to three years. This combined test takes advantage of the high sensitivity of HPV DNA testing and the high specificity of Pap tests. Some have proposed HPV testing as an alternative to the Pap test for primary screening of cervical cancer.[11] There are benefits and disadvantages to this approach. HPV infection may be transient and the majority of women with HPV infection never develop pre-cancer lesions.[12] Many unnecessary referrals to colposcopy may result — particularly in women under 30 years old — and there may be unnecessary psychological consequences with false-positive results. One other proposal for the use of HPV testing is for surveillance following identification and treatment of cervical lesions.[13]

Preparation for laboratory analysis

The performance of HPV testing can be affected by the quantity, quality, and storage of the biological specimen obtained.[14] In general, collection devices (such as cytobrushes) are preferred since they maximize the quantity of the material obtained for future DNA analysis. For blind-sampling methods, such as in anal-cancer screening,[2] Dacron swabs (Baxter Healthcare) are preferable to cotton swabs, because epithelial cells cling to cotton and may decrease the cellular yield. For storage and transport, liquid media — such as a methanol-based fixative (PreservCyt; Cytyc, Boxborough, MA) or the Universal Collection Medium (UCM; Digene, Gaithersburg, MD) — are advantageous because both molecular and pathologic analysis could be performed from the same specimen. Exfoliated epithelial cells, DNA, RNA, and protein could all be preserved using this technology, and materials can be stored for concurrent or future analysis. In one study, cells collected using a methanol-based fixative were able to be used for real-time PCR (RT-PCR) for HPV-16 E6 and E7 one year after storage at –20°C.[15] In another study using a methanol-based fixative, DNA PCR, RNA RT-PCR, and immunohistochemistry were able to be performed on samples stored up to 30 days at room temperature.[16]

Current laboratory methods for HPV detection

There are three principal laboratory methods used for the detection of HPV infection: 1) direct probe methods (such

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Table 2. A comparison of testing modalities used to detect HPV.

	Signal amplification	Target amplification	Target amplification	Target amplification
Example	HC2[29]	PCR MY09/11[30]	PCR GP5+/6+[19]	SPF-PCR[26]
Probes/primers	Mixture of RNA probes	Degenerate primers	Consensus primers	Consensus primers
Test reaction product	DNA/RNA hybrids	450 bp	150 bp	65 bp
Analytical sensitivity (fg)	25 to 75	0.1 to 100	0.5 to10	0.1 to 10
No. of HPV types detectable	13	39	20	43
FDA-approved	Yes	No	No	No
Commercial kit available	Yes	Yes	No	No
Contamination potential	Low	High	High	High
Relationship of HPV to histopathology	No	No	No	No
Quantification of HPV DNA	Yes. Semiquantitative measure obtained.	Possible but not standardized.	Possible but not standardized.	Possible but not standardized.
Can indicate multiple HPV type infection	No	Yes	Yes	Yes

HC2: hybrid capture second-generation test; PCR: polymerase chain reaction; SPF-PCR: short PCR fragment amplification and detection system.

as Southern transfer hybridization and *in situ* hybridization [ISH]), 2) signal amplification (e.g., hybrid capture second-generation [HC2] assay [Digene, Gaithersburg, MD]) and 3) target amplification (PCR variants).[17]

Of the methods used for the detection of HPV quantity and type, most validation and large epidemiologic studies have used the HC2- or PCR-based assays with MY09/11 or GP5/6 consensus primers. Because the HC2 assay does not employ a primary amplification test, it is less affected by cross-contamination and specimen collection — factors which may sometimes make PCR challenging to conduct and interpret (Table 2).

Direct-probe methods

In situ hybridization

In this method, DNA probes are applied directly to target tissue, which, like immunohistochemistry, permits spatial mapping of the target sequences in relation to histopathology. In that way, correlation of HPV presence and disease manifestation can be made. Because this is a generally insensitive and labor-intensive technique, it is not used routinely.

Southern transfer hybridization, dot blot, and filter hybridization are other direct probe methods where target DNA is bound to filter supports before hybridization with DNA probes.[18] These are used primarily in research settings.

Signal amplification

Hybrid Capture HPV DNA assay (Digene, Gaithersburg, MD)

The HC2 assay uses RNA probes specific for the identification of certain high-risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) or low-risk HPV types (6, 11, 42, 43, 44). These long RNA probes are separated into two testing cocktails based on whether high-risk (B) or low-risk (A) types are to be identified. First, the patient's specimen (as whole HPV DNA) is separately hybridized to each of the two testing cocktails. In each case, specific HPV DNA-RNA hybrids are formed. This is then added to a microtiter plate coated with antibodies specific to RNA-DNA hybrids so that the HPV DNA-RNA hybrids previously formed can be captured or immobilized on the plate. Immobilized hybrids are then bound to antibodies conjugated to alkaline phosphatase. Excess antibodies and nonhybridized probes are removed, and a chemiluminescent substrate is added. A luminometer is then used to detect the remaining immobilized hybrids. A semiquantitative measure of the viral load can be obtained based on the intensity of the light emitted by the sample, divided by the light emitted by a positive control (expressed as relative light units) since this is proportional to the quantity of target DNA in the patient's specimen. Often, to reduce cost and time, only high-risk probes are used in clinical evaluation. The HC2 assay is the only method cleared by the FDA for HPV testing of the cervix.[14] Testing of anal specimens for HPV infection is not FDA-cleared.

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Target amplification

Polymerase chain reaction-based assays

PCR technology uses the action of DNA polymerase on specific primers to selectively amplify target HPV DNA. The amplified DNA can be detected by a variety of ways, such as gel electrophoresis, dot blot, or line-strip hybridization. There are more variables in PCR methodology compared to the HC2 assay, and the sensitivity and specificity of the PCR result can vary depending on factors, such as the primer sets, the size of the amplified PCR product, and the DNA polymerase used. PCR can detect between 10 and 100 DNA molecules in a specimen, can produce as many as one million copies from a single double-stranded DNA molecule after 30 amplification cycles, and is the most sensitive method available. Most protocols use consensus primers that target a very conserved region of L1. Type-specific assays that can potentially detect all HPV mucosal types target E6/E7. The consensus primers detect the presence of HPV, and hybridization with type-specific probes can be used to detect as many as 40 individual HPV types. The consensus primers GP5/6 (and the extended version GP5+/6+)[19] and the degenerate primers MY09/11 (and the modified version PGMY09/11)[20] are the most widely used primers. Unlike HC assays, PCR is not FDA-cleared for HPV testing of the cervix, although it is widely used in research settings.

Special issues in the laboratory detection of HPV

Multiple HPV types

Infection with multiple HPV types may have important implications as a marker of persistent disease, multiple (vs. a single) cervical lesions, and progression of low-grade to high-grade cervical pre-cancer lesions. Although there is little evidence for direct molecular interactions between different HPV types in potentiating disease pathogenesis, infection with multiple HPV types may be indicative of host factors important in disease progression, such as attenuated HPV-specific immunity. Unfortunately, the most widely used HPV-detection system, the HC2 assay, does not distinguish between single and multiple infections. The PCR-based methods are able to detect type-specific HPV infection and so can indicate when there are multiple HPV infections. The performance characteristics of the various PCR methods, however, differ in sensitivity and reproducibility, (i.e., GP5+/6+ may detect only 47% of samples with multiple HPV types compared with 90% detected by MY09/11).[21]

Viral load

A high HPV viral load may be associated with an increased risk of development of HPV-related cervical lesions,[22] but the clinical significance of routine viral-load testing remains unclear. Much of this uncertainty stems from the wide variation in testing methodology used in studies. Quantification of HPV DNA can be performed by the HC2 assay (semiquantitatively) or by PCR methods. Adjustment for the number of cells sampled must be made before a valid assessment of HPV viral load is made. There is still no consensus, however, on how best to quantify HPV DNA. Real-time PCR is a promising new technology (see next section).

HPV serological assays

Both IgM and IgG serologic measures of HPV infection are usually obtained using enzyme-linked immunoabsorbent assay (ELISA) technology, targeted to type-specific L1 virus-like particles (VLP). HPV serology is generally type specific and indicates either past or current infection. There may

be a lag time of several months from HPV-associated disease before serology is positive.[23] The sensitivity for HPV VLP ELISAs is 50% to 60% with a specificity of over 90%.[24] Other serologic assays being developed using alternative targets include the "sandwich" ELISA and radioimmunoassays. Because of lack of standardization and reproducibility, HPV serology as a marker of past and/or cumulative exposure to HPV-associated disease is not widely used clinically.

New and future laboratory methods for HPV detection

Real-time PCR

Real-time PCR is a developing technique that has been proven to give a more accurate measure of HPV viral load because a measure of the cellular content in the assay is obtained. There are PCR protocols that use a 5-prime-exonuclease assay and a fluorescent reporter whose signal increases in proportion to the amount of DNA product in the reaction. One nested case-control study[25] demonstrated that cervical-cancer cases had consistently higher HPV-16 viral loads compared to controls and that high HPV-16 viral loads could be detected up to 13 years prior to the diagnosis of cervical cancer. Women with high HPV-16 viral loads had a 30 times higher relative risk of developing cervical-cancer compared to women who were HPV-16 negative. Quantitative HPV PCR using real-time PCR technology is still under active investigation and is not used clinically.

Other methods

The short PCR fragment-PCR (SPF-PCR) system has been developed by Kleter and others[26] as a short PCR fragment and detection system. Sensitivity is improved because, compared with the MY09/11 and GP5/GP6 primer sets, a smaller region of a highly conserved L1 region is targeted (65 bp), and PCR efficiency is inversely proportional to the size of the PCR fragment being amplified. The Amplicor microtiter well plate (MWP) assay (Roche Molecular Diagnostics, Basel, Switzerland) also increases sensitivity to detect HPV infection by amplifying a shorter fragment of the HPV L1 region (170 bp) compared to MY09/11 (450 bp). The assay was only designed, however, to identify high-risk HPV types. A new Hybrid Capture-3 (Digene, Gaithersburg, MD) assay is based on the same technology as the HC-2 assay with identical RNA probes that may hybridize to a patient's HPV DNA, if present. Specificity, however, may be further improved because biotinylated oligonucleotides specific for HPV DNA sequences are used to capture HPV DNA-RNA hybrids (instead of anti-DNA-RNA polyclonal antibody used previously) onto streptavidine-coated wells of a microtiter plate. Other emerging technology is aimed at high-output testing, such as the Rapid Capture System (Digene, Gaithersburg, MD), which uses robots to test as many as 450 specimens in an eight-hour shift. DNA microarray-based tests have the potential to change the HPV-testing landscape dramatically, since thousands of sequences may be analyzed simultaneously with concurrent HPV viral-load analysis and typing.

HPV immunizations and HPV testing

The development of preventive and therapeutic immunizations directed at HPV-infected cells may expand the need for systematic HPV testing. Therapeutic immunizations aug-

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ment the host cell-mediated immunologic response in an already HPV-infected individual. There are a variety of methods but most use HPV E6 and E7 peptides of oncogenic HPV types to activate host T-cells.[27] Prophylactic immunizations use components of the major HPV capsid proteins (L1 alone

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or in combination with L2). These self-assemble into virus-like particles that contain no HPV DNA so are not infectious but induce neutralizing antibodies before the host becomes exposed to HPV infection. The first large randomized, controlled proof-of-concept trial of a HPV prophylactic vaccine was recently published.[28] More than 2,000 women were randomized to receive HPV-16 vaccine with 100% efficacy in preventing HPV-16-related cervical dysplasia. Currently, both therapeutic and prophylactic HPV immunization are type-specific, so that HPV testing would be required to target therapy in the case of future therapeutic testing and to define eligible populations for preventive immunization.

Summary

Human papillomavirus is one of the most common sexually transmitted infections. HPV-associated neoplasms, such as cervical cancer, are significant causes of morbidity and mortality worldwide. Recent advances in molecular biology have facilitated testing methods for HPV infection. Of the various methods, the only test cleared by the FDA for HPV testing of the cervix is the hybrid capture second-generation assay that uses RNA probes specific for the identification of certain high-risk or low-risk HPV types. Currently, the principal cervical-cancer screening modality is the Pap test, but given its higher sensitivity, HPV testing is being incorporated into algorithms for cervical-cancer screening. The development of HPV therapeutic and prophylactic immunization may expand the need for systematic HPV testing to help define eligible subgroups for intervention. □

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