

The laboratory diagnosis of genital human papillomavirus infections

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Human papillomaviruses (HPVs) are the etiological agents of several genital cancers, including cancer of the uterine cervix. The detection of HPV infection in genital samples may increase the sensitivity of primary and secondary screenings of cervical cancer. HPV testing may also improve the specificity of screening programs, resulting in the avoidance of overtreatment and cost savings for confirmatory procedures. The major determinants of clinical progression of HPV infection include persistence of HPV infection, involvement of high-risk HPV types, high HPV viral load, integration of viral DNA and presence of several potential cofactors. Signal amplification HPV-DNA detection techniques (Hybrid Capture II, Digene Corporation, USA) are standardized, commercially available, and capable of detecting several high-risk HPV types. They also increase the sensitivity of screening for high-grade lesions in combination with cytology. The sensitivity of these techniques to detect high-grade lesions is higher than that of cytology, but the referral rate for colposcopy is greater. These techniques are approved for the triage to colposcopy of women with cervical smears interpreted as atypical squamous cells of undetermined significance. Triage and screening for cervical cancer using HPV will probably be restricted to women aged 30 years or older because of the high prevalence of infection in younger women. Amplification techniques are ideal for epidemiological studies because they minimize the misclassification of HPV infection status. These techniques can detect low HPV burden infections. Consensus primers amplify most genital types in one reaction, and the reverse hybridization of amplicons with type-specific probes allows for the typing of HPV-positive samples. Consensus PCR assays are currently under evaluation for diagnostic purposes. HPV testing is currently implemented for the clinical management of women.

Key Words: *Cancer; HPV; PCR; Screening; STI*

Cervical cancer is the third most common malignant neoplasm in women worldwide (1). In Canada, 1500 women were newly diagnosed with cervical cancer during the year 2000 (2). Human papillomavirus (HPV) infection is the most common sexually transmitted infection (STI) in North America (1,2). The association between HPV infection and invasive cervical cancer is very strong, specific and consistent, and is independent from other known risk factors, which are now considered to be ancillary causal determinants (3). The strength of the HPV-cervical cancer relationship is even

Le diagnostic de laboratoire des infections génitales causées par les papillomavirus humains

Les papillomavirus humains (VPH) sont les agents étiologiques de plusieurs cancers génitaux, dont le cancer du col utérin. La détection d'infections à VPH dans des échantillons génitaux pourrait augmenter la sensibilité du dépistage primaire et secondaire du cancer du col utérin. Les tests de dépistage du VPH peuvent également améliorer la spécificité des programmes de dépistage, ce qui permet d'éviter les traitements excessifs et des frais d'interventions de confirmation. Les principaux déterminants de progression clinique d'une infection au VPH sont la persistance de l'infection, la présence de types oncogènes de VPH à haut risque, une charge virale élevée, l'intégration de l'ADN viral et la présence de plusieurs cofacteurs potentiels. Les tests d'amplification de Signal pour la détection de l'ADN de VPH (Hybrid Capture II, Digene Corporation, États-Unis) sont standardisés, disponibles commercialement et capables de détecter plusieurs types oncogènes à haut risque. Ces tests augmentent la sensibilité du dépistage des lésions précancéreuses de haut grade, lorsqu'ils sont combinés avec la cytologie. Ils ont une meilleure sensibilité que la cytologie pour dépister les lésions de haut grade, mais augmentent le taux d'aiguillage pour colposcopie. Ils sont approuvés pour le triage en colposcopie des femmes qui ont une cytologie interprétée comme des cellules malpighiennes atypiques à caractère non déterminé. Le triage et le dépistage du cancer du col au moyen des tests de détection de VPH seront probablement restreints aux femmes de 30 ans et plus, étant donné la prévalence élevée d'infections chez les femmes plus jeunes. Les méthodes d'amplification génomique sont idéales pour les études épidémiologiques parce qu'elles réduisent au minimum la mauvaise classification du statut des infections à VPH. Elles permettent de dépister les infections à faible charge de VPH. Les amorces consensuelles amplifient la majorité des génotypes génitaux en une réaction, et une réaction d'hybridation «inversée» des amplicons avec des sondes oligonucléotidiques permet le génotypage des spécimens positifs. Des épreuves de PCR consensuelles sont évaluées en clinique pour leur utilité diagnostique. Les tests de dépistage du VPH sont en voie d'être implantés pour la prise en charge clinique des femmes.

greater than the association between smoking and lung cancer and other well-established causal relationships in cancer (1). Women with normal Papanicolaou (Pap) smears are at increased risk for subsequent squamous intraepithelial lesions (SIL) when HPV-DNA is detected in the uterine cervix (1). When HPV-DNA is detected in women with equivocal or low-grade SIL (LSIL) smears, the risk of progressing to high-grade SIL (HSIL) and cancer is also increased (1). HPV-DNA from high-risk types transforms epithelial cells through molecular events that have been well described elsewhere (3).

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A study conducted in 22 countries disclosed that 99.7% of invasive cervical cancer contained HPV-DNA, indicating that HPV may be a necessary cause of cervical neoplasia (1-3). Due to the central role of HPV in the carcinogenesis of cervical cancer, HPV testing has now been included in current clinical guidelines for the management of cervical disease in women (4).

Because of the use of Pap smear screening programs, the incidence of cervical cancer had declined over the past 50 years but has now stabilized (2,5). However, in a primary screening mode, the sensitivity of the conventional Pap test for detecting cervical cancer precursors taken at any given time has been shown to average 50%. Repeated Pap tests are associated with poor patient compliance with return visits; thus, the conventional Pap test is cost-ineffective for cervical cancer screening. Molecular methods for HPV detection offer promising alternatives as screening tools to improve the sensitivity of conventional cytology to detect prevalent high-grade lesions and provide near 100% negative predictive values. In turn, this could allow for better compliance at lower costs and increased screening intervals safely. Molecular methods for HPV detection are now accepted as tests for the triage of women with abnormal Pap smears for invasive diagnostic procedures as discussed below.

THE BIOLOGY OF HPVs

HPV infects the skin and mucosal epithelia, causing latent and site-specific subclinical and clinical infections. More than 100 HPV types infect humans, 40 of which are preferentially found in the anogenital tract epithelium (2). Genital HPV types are classified into categories based on their association with premalignant and invasive cancer. HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59 and 68 are considered to be high-risk oncogenic types because they are detected in HSIL and invasive cancer (2,3,6). HPV types 6, 11, 42, 43 and 44 are low-risk types mainly associated with acuminated condylomas and less than one-third of cervical LSILs. Analysis of results from several case-control and prospective studies would be useful to better delineate the oncogenicity of novel HPV types. HPV types 16, 18, 31 and 45 are detected in 80% of cervical cancers (2,3). The 7900 base pair HPV-DNA genome is divided into a noncoding region containing regulatory sequences, the long control region (LCR), an early region (E genes) involved in transformation, and a late region (L genes) coding for capsid proteins (7). This genomic organization is conserved among HPV types. Areas of homology among HPV types are located mainly in the E1, E6 and L1 genes and allow for crosshybridization reactions between types or consensus genomic amplification methods (7).

SPECIMEN COLLECTION AND TRANSPORT

Several sampling methods have been used for the detection of HPV infection in the genital tract. Superficial epithelial cells from the ectocervix can be collected by scraping with a spatula. Cytobrushes and Dacron swabs are used to collect cells from the squamocolumnar junction of the cervix, a site where HPV-related diseases develop preferentially (8). The sensitivity of HPV detection is greater when a cytobrush is used rather than a cervical swab (8). Exfoliated cells should then be resuspended in the appropriate transport medium used with the DNA-based methodology for HPV detection (such as the Specimen

Transport Medium [STM] for the Hybrid Capture test, Digene Corporation, USA) or into liquid cytology medium (Preservcyt, Cytoc Corporation, USA or AutoCyte, TriPath Imaging, USA). Protocols for HPV-DNA analysis from cells preserved in liquid cytology media have been published (9,10). Cervicovaginal lavages collect greater quantities of viral DNA than the latter sampling methods but cells from the posterior vaginal epithelium are also obtained. Cervicovaginal lavage is useful in research projects that require large quantities of DNA for the performance of several tests but is seldom useful in diagnostic settings.

Sampling methods that do not require a speculum-assisted examination would facilitate mass screening of underserved populations and improve compliance with cancer prevention programs. Several studies have shown a correlation for HPV-DNA detection often above 80% between cervical samples and vaginal tampons, self-administered vulvar swabs or self-administered vaginal swabs. In one study (11), self-testing for HPV-DNA using vaginal swabs detected 66% (95% CI, 52% to 78%) of high-grade lesions of the cervix compared with 68% using health care personnel-assisted Pap smears in a population of women 30 years or older from South Africa. In two other studies, one from Germany and the other from China, over 90% of HSILs were HPV-positive using either the self-collected or health care personnel-assisted samples for HPV testing. On the other hand, HPV testing in urine specimens has a low sensitivity; in one study (12), only 45% of women with HSIL were identified. Differences in the detection rates and typing of HPV-DNA between physician-obtained and self-obtained samples could be explained in part by differential viral shedding, localized HPV infection, incomplete sample collection or sampling of cells from different sites. Further clinical trials on a large number of women should be conducted to shed insight into the true value of self-obtained versus physician-obtained samples to identify women with disease progression or persistence of HPV infection, and to evaluate improvement in patient compliance and the economic impact of each sampling method.

Correlation between HPV-DNA and histopathology can be done on biopsies with *in situ* hybridization (see below). Polymerase chain reaction (PCR) assays can also be employed on biopsy samples, but the sensitivity of amplification of long DNA fragments is reduced when applied on DNA purified from fixed tissue (13,14).

DIAGNOSTIC TESTS FOR HPV DETECTION

Clinical examination is not sensitive for detecting either latent or subclinical genital HPV infection. HPV-induced cervical lesions are visible when colposcopy is combined with the application of acetic acid solution. Colposcopy is a relatively time-consuming and costly procedure that cannot serve as an efficacious screening tool. High-grade lesions can also escape detection by colposcopy-directed biopsies in up to 3% of women with abnormal smears. Additionally, colposcopy-directed biopsies may fail to detect high-grade lesions and micro-invasive carcinoma.

Microscopy

Koilocytes indicate the presence of productive HPV infection in exfoliated cells and biopsy specimens (15). They are squamous epithelial cells exhibiting perinuclear clearing and increased density of surrounding cytoplasm. Nuclear atypia

(enlargement), hyperchromasia, irregular membranes and double nucleation of the superficial and intermediate cells are the hallmark of productive HPV infection. Many times, the aforementioned changes are less obvious (eg, equivocal), resulting in relatively poor intra- and interobserver reproducibility of these changes for the diagnosis of HPV infection. As well, the sensitivity of infection detection is lower than the molecular methods described below (15).

Current screening protocols for cervical cancer prevention rely on the periodic use of Pap smears to identify women at risk of having precursor cervical lesions or cancer, and for whom further investigation is warranted (5). In contrast to cervical cancer screening, cytology screening of anal precancerous and cancerous lesions is currently not done systematically. Recent studies have demonstrated a high prevalence of SIL and cancer of the anus in men having sex with men and women infected with HIV (16). Increased survival of HIV-infected individuals due to highly active antiretroviral therapy could result in increased morbidity and mortality rates due to unscreened anal cancer caused by HPV. Cytology screening for anal diseases in selected populations of patients should be implemented in Canada.

Cervical SILs include a morphological spectrum of precursor lesions, formerly designated as dysplasia or cervical intraepithelial neoplasia (CIN), some of which can progress into squamous carcinoma (5). The sensitivity of conventional cytology smears in detecting significant cervical lesions ranges from 29% to 80%, a relatively poor level of sensitivity that is partially compensated by repeated testing after short intervals (2,7,17). Liquid-based, thin-layer cytology is a promising alternative that has been shown to improve the sensitivity of conventional cytology for detecting HSIL by 60%, providing an overall sensitivity of 80% (2,5,17). In the Bethesda cytology classification, LSIL combines cytological changes suggestive of CIN I, or mild dysplasia, and HPV-induced morphological changes, while HSIL comprises CIN II and III (moderate and severe dysplastic lesions) and carcinoma in situ (2). Depending on the length of follow-up of untreated HSIL, between 30% and 80% progress to invasive cancer. It should be noted that the most common cytological abnormalities do not correspond to the above categories; these are classified as atypical squamous cells of undetermined significance (ASC-US). Nevertheless, approximately 10% of women with ASC-US Pap tests have HSIL on colposcope-oriented biopsy. As a result, an ASC-US Pap test identifies women at higher risk for HSIL.

Electron microscopy

Nonenveloped icosahedral viral particles can be demonstrated in productive HPV infections, but typing cannot be done with electron microscopy. This technique also cannot detect non-productive precancerous HPV infections, most of which are caused by high-risk HPV types.

Culture

HPV cannot be isolated in cell culture because it requires cell differentiation to complete its replication cycle.

Antigen detection by immunocytochemistry

The use of polyclonal antibodies against the late structural L proteins on biopsies can confirm the presence of HPV-induced morphological changes, but the test is relatively

insensitive. This technique has been replaced by in situ hybridization.

In situ hybridization

Filter in situ DNA hybridization lacks sensitivity and specificity. In situ hybridization on biopsies correlates the detection of HPV-DNA with histopathology. The presence of HPV-induced histologically equivocal lesions in biopsies can be confirmed by in situ hybridization (7). Viral transcription and integration can be studied with this technique in fixed tissue. Because in situ hybridization detects 25 HPV-DNA copies or more per cell, high-grade lesions that often contain lower amounts of viral HPV-DNA are often negative for HPV with this technique (7). PCR in situ techniques are more sensitive but need refinement.

Direct nucleic acid detection tests

Filter-based hybridization assays have been used mainly in research laboratories. The principles of direct hybridization tests have been presented elsewhere (18). Southern blots are fastidious and lengthy procedures that require several steps for the processing of samples and assay completion, are not readily applicable to routine diagnostic laboratories, often require large amounts of sample DNA and use radioactive probes. Furthermore, genotyping of HPV-DNA requires several hybridization reactions with various type-specific probes. Interlaboratory agreement using Southern blot can be as low as 50% (15) because protocols are not standardized, the technique is sensitive to minor changes in reaction conditions, and the interpretation of this test is difficult. Varying the conditions of stringency allows testing for uncharacterized HPV types. Southern blot testing under high stringency conditions is a very specific assay. It can detect as few as 100,000 copies of HPV-DNA per test. Once considered the gold standard for HPV research, Southern blot has now been replaced in research laboratories by PCR assays. The dot blot ViraPap/ViraType test (Digene Corporation, USA) was licensed for diagnostic purposes but could only detect a limited number of HPV types. It is now replaced by the Hybrid Capture system (7).

Signal amplification DNA-based assays: The Hybrid Capture system

Signal-amplification tests can detect lower quantities of DNA than direct methods by amplifying the detection signal without modifying the initial amount of nucleic acids contained in samples (7). The only test approved by the United States Food and Drug Administration for the detection of HPV in exfoliated cells is a signal amplification test, the Hybrid Capture II (HC-2, Digene Corporation, USA) (19). In this test, exfoliated cervical cells are collected with a conical cytobrush provided in the specimen collection kit (Digene Cervical Sampler, Digene Corporation, USA), resuspended in STM and kept at room temperature for up to two weeks. Exfoliated cells can also be obtained with a plastic spatula and an endocervical cytobrush and then resuspended into 10 mL to 20 mL of liquid cytology medium (Preservcyl or AutoCylte) (4). Cells can be preserved in these media at room temperature for a maximum of four weeks. Both collection techniques provide excellent test results (8). Processed samples are treated with sodium hydroxide to denature DNA, hybridized in solution with two mixtures of nonisotopic single-stranded long RNA probes (4). Probe A

mixture detects five low-risk types: 6, 11, 42, 43 and 44, while probe B mixture detects 13 high-risk types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68. Considering the clinical applications of HC-2 discussed below, most laboratories use only the high-risk probe cocktail. Hybridized products are transferred into microplate wells coated with an antibody that specifically recognizes DNA/RNA hybrids. Bound hybrids are quantitated with the addition of an alkaline phosphatase-labelled monoclonal antibody against DNA/RNA hybrids, followed by the addition of a chemiluminescent substrate. The emission of light is measured as relative light units in a luminometer. The intensity of light emitted is proportional to the quantity of bound hybrids. Results are expressed as the ratio of the specimen reactivity to the mean of the positive control. A specimen is considered to be positive if its reactivity is at least equal to that of the positive control.

The HC-2 system has been well-validated, detects several high-risk types in one reaction, uses a convenient format that avoids potential contamination encountered in gene amplification assays, and is not impeded by the presence of interfering substances contained in samples. Unlike cervical cytology, it does not require extensive training, is not subjective and is reproducible with an intra-assay coefficient of variation below 5%. Homogeneous hybridization reactions, as done in HC-2, are faster than the heterogeneous tests discussed above, especially when single-stranded probes are used. These reactions are more applicable to diagnostic laboratories and provide faster results. HC-2 does not control for the quality of sample by measuring cell content. HC-2 can detect as little as 1 pg/mL of target DNA (5000 genome copies per test) (19).

Several studies have evaluated the sensitivity and specificity of Hybrid Capture against histology and/or PCR assays (see below) (4,7,19). A good agreement (kappa values above 0.6) was found in several studies between Hybrid Capture and PCR (19), although the PCR assays used were not standardized (8). These studies have demonstrated some crossreactivity between the high-risk probe cocktail and HPV types not included in the probe mix. The high-risk probe cocktail could react with types 6, 42, 53, 54, 62, 66, 67, 73, CP6108 and CP8061, some of which are low-risk types (8,15,20). Results from several studies on the clinical value of HC-2 were reviewed recently (4). The sensitivity of Hybrid Capture to detect high-grade lesions in these various studies reached 90% (4).

In the near future, HPV-DNA testing could be adapted to an automatic platform, the rapid capture system, which integrates all steps of sample processing and testing (4). The third generation Hybrid Capture system (HC-3) will capture the target DNA sequence by using biotin-labelled oligonucleotide probes, thus reducing the background noise that could be generated by endogenous DNA/RNA hybrids (4). Blocker probes are also included to enhance the specificity of the test. The specificity of HC-3 is improved compared with that of HC-2 (4).

Genomic amplification for HPV detection and typing

PCR is the most powerful tool for the epidemiological investigation of HPV infection or cervical cancer (7). The analytical sensitivity of nucleic acid amplification techniques is usually lower than HC-2 (7,8). PCR is now the gold standard test for HPV research. The natural history of HPV infection has been more clearly defined with PCR. This methodology has overcome the problem of misclassification of HPV status that initially confused the scientific community regarding the role of

HPV in cervical cancer (7). Consensus PCR detects most genital HPV types with great sensitivity. PCR also permits variant analysis of HPV types and allows for the discovery and characterization of novel HPV types. By including a reverse transcriptase step, expression of HPV in clinical specimens can be analyzed with PCR (7). A significant correlation has been found between the level of expression of E6 and the grade of CIN.

Type-specific PCR tests are not a practical means for detecting HPV infection in clinical specimens because of the large number of types involved in genital diseases. Because of the genetic polymorphism of HPV, consensus PCR assays have been devised to amplify in one reaction the majority of known and novel anogenital HPV genotypes. Subsequent typing is accomplished on filters by hybridization with type-specific oligonucleotide probes, homogeneous hybridization reactions with RNA probes in the SHARP system (Digene Corporation, USA), restriction fragment length polymorphism or by DNA sequencing (7). Four consensus assays target conserved sequences in the HPV L1 gene. The GP5+/GP6+, MY09/MY11/HMB01, PGMY09/PGMY11 and SPF1/SPF2 consensus primer sets can amplify a wide spectrum of genital HPV types (7,9,13,21). These assays also now use reverse hybridization for the nonisotopic genotyping of HPV amplicons. Microtitre-plate based generic HPV detection to identify amplicons containing HPV-DNA has also been described with each of these assays. None of the consensus PCR tests is commercialized and standardized for routine clinical use under their current format. In future studies, their performance will need to be compared with Hybrid Capture in clinical trials. The efficiency of the consensus PCR assays described above will depend on the type of sample tested (fixed or fresh tissue), size of amplicon, use of degenerate versus nondegenerate primers, prevalence of multiple type infections, number of primers used in the mixture and sequence variation at the primer sites between types.

The GP5+/GP6+ assay can detect at least 19 different genotypes with an analytical sensitivity of 10 to 200 copies depending on the type tested. These primers detect types 43 and 44 more efficiently than MY09/MY11. Protocols for sample preparation, primer use and genotyping with MY09/MY11 primers have been fully described (13). The degenerate pool of primers MY09/MY11/HMB01 amplifies a broad spectrum of HPV genotypes with varying levels of sensitivity (7,21). It detects as few as 10 copies of viral DNA from frequently encountered genital types. The insertion of nucleotide bases at positions of degeneracy is a random and irreproducible process. The synthesis of degenerate primers does not ensure an equivalent representation of all degenerate primers (21), which could result in lot-to-lot variations in type-specific amplification efficiencies. The new PGMY09/PGMY11 set of consensus primers was designed to eliminate the degeneracies and improve the sensitivity, specificity and reproducibility of L1 consensus PCR. PGMY09/PGMY11 consensus primers comprise 18 different primers derived from the MY09/MY11 primer pair site. These primers amplify a 450 base pair fragment and improve the sensitivity of amplification of genital HPV types over the MY primer pair, including types less efficiently amplified with MY09/MY11 (such as types 35, 52 and 56) (21,22). The SPF system amplifies a 65 base pair fragment with a pool of 10 different primers containing inosine at some positions to optimize amplification of some types (9). The latter two systems

result in greater reproducibility by avoiding the use of degenerate primers. The line blot combined with PGMY primers can genotype 37 types, while the line probe combined with SPF primers can genotype 25 types. Recently, SPF and PGMY systems have been compared for their ability to detect HPV-DNA in genital tract samples (9). The overall agreement for detection of HPV-DNA with both methods was high, with a kappa value at 0.86. When genotyping was considered, over 96% concordance was found between the two assays. Types 42, 56 and 59 were more easily detected with PGMY, while types 31 and 52 were more easily detected with SPF (9). Comparison between the new consensus PCR assays described above and HC-2 will need to be performed to clearly establish the value of PCR and typing in clinical settings.

Detection of integrated HPV genome in human DNA

DNA molecules from high-risk HPV types remain episomal in low-grade lesions but are mostly integrated in the human genome in high-grade lesions and invasive tumours. Integration disrupts the E2 open reading frame, resulting in uncontrolled production of E6 and E7 oncoproteins. Integration of HPV-16 has been shown to precede microinvasion in high-grade dysplasia. Southern blots can demonstrate HPV integration but are not sensitive. Real-time PCR assays have been developed to determine the ratio of episomal to integrated HPV genome by amplifying the E2 and E6 genes (23). The value of these assays to identify women at risk for cervical lesions still needs to be assessed.

Viral load evaluation by real-time PCR

Quantification of viral HPV-DNA in clinical specimens could improve the clinical utility of HPV detection methods (7). Quantitative PCR assays require a control for amplification efficiency and sample inhibition. Few truly quantitative PCR assays have been developed for HPV quantification (24). Real-time PCR assays provide accurate measurements of the initial copy number of target DNA contained in samples by continuously measuring the increments of fluorescence released during the amplification reaction. This monitoring of amplicon synthesis is impossible with conventional PCR. Real-time PCR combines amplification by rapid thermocycling with simultaneous fluorescence detection. Several formats can be used to perform real-time amplification and detection of HPV-DNA: assays based on hydrolysis probes (Taqman assays); assays based on fluorescence resonance energy transfer; and finally, assays using molecular beacons. Real-time PCR assays produce linear results over a wide range of target concentrations. HPV-DNA and a human gene (such as beta-globin) can be amplified for each specimen. The signal obtained can then be compared with reactivity of each concentration from a titration curve of HPV and cellular DNA (24). The signal obtained for HPV-DNA can then be adjusted with the amount of cellular DNA deduced from the signal obtained with beta-globin amplification. Viral load is then expressed as a number of HPV-DNA copies per cell.

Such correction is mandatory to avoid introducing a bias in viral load measurement because more cellular material can be obtained from sampling high-grade cervical lesions, potentially generating an artificially higher HPV viral load (24). Dysplastic cells found in CIN II or CIN III lesions express fewer intercellular adhesion molecules than normal cells and could possibly be sampled more readily than normal cells (24).

A low-stringency PCR assay uses a nonstringent amplification reaction with primers GP5/GP6 that consistently generate a specific band from human DNA. The HPV copy number can also be calculated with the ratio of signals of HPV-DNA to human DNA bands. The role of HPV quantification is currently under study.

HPV variant analysis

An important degree of intratypic diversity has been shown between HPV isolates of the same type (25). HPV variants are defined by a limited number of point mutations that generate in coding and noncoding regions less than 2% and 5% genetic variation, respectively, compared with the prototype isolate (25). HPV variants can be analyzed by single strand conformation polymorphism or PCR sequencing (26). Single strand conformation polymorphism analysis is more convenient for large-scale testing, but is not as discriminatory as sequencing to identify HPV variants (26). Classification of variants can be done through analysis of the LCR where most variability can be encountered. Polymorphism has also been demonstrated in coding regions of HPV including oncogene products E6 and E7. HPV-16 nonprototype variants confer a higher risk than the prototype variant for the development of high-grade or cancerous cervical lesions and anal cancer (25). Some mutations in the E6 gene compared with the European prototype have been associated with persistent HPV infection and anogenital disease, sometimes in correlation with p53 polymorphism (25,27).

Natural variants show different biological properties (25). Nucleotide differences found in some HPV-16 variants result in amino acid changes at the binding site of HPV-16 E7 protein with its cellular target, the pRB protein. Cell-mediated repression of HPV E6-E7 expression is different among HPV variants. In HPV-18 isolates detected in cervical cancer specimens, mutations in the LCR at cellular transcription factor binding sites resulted in reduced DNA-protein binding. Variations in HPV-16 E6 protein can alter an HLA-B7 peptide binding epitope, which could influence immune recognition by cytotoxic lymphocytes and provide a biological mechanism for escape from immune surveillance.

HPV serology

Although serology identifies individuals with current or past infections by HPV, the risk to develop CIN in HPV-16-seropositive women is much lower than the risk measured with HPV-DNA detection. The most widely used and validated serological assay is based on virus-like particles (VLP). VLPs are produced by cloning the L1 gene of HPV into a viral vector with expression of the capsid that self-assembles (15,28). The antibody response against viral proteins is delayed several months after the detection of HPV-DNA (28). Antibody levels are then stable over at least a decade (28). In most studies, the concordance between serological results and HPV-DNA detection was reasonably high (28). Nearly 60% of HPV-16 DNA-positive women are seropositive using VLP enzyme-linked immunoassay (EIA). Overall, serology assays reached a sensitivity level for identification of HPV-infected individuals of 50% (28). The serology assays based on detection of antibodies against capsid protein were found to be type-specific except for strong crossreactivity between types 6 and 11 (28). However, disrupted viral capsids expose epitopes that are cross-reactive between types (28). Type specificity can also be

obtained by blocking experiments with neutralizing monoclonal antibodies V5 for type 16 (28). This epitope is immunodominant and type-specific.

Determinants for seroreactivity against HPV include the number of lifetime sexual partners, HPV viral load and persistence of HPV infection (28). Targets other than the viral capsid proteins, such as E6, E2, E4 and E7 proteins, have been used in different formats, including mainly EIA, Western blot and radioimmunoprecipitation assay (28). Advantages of serology include the ability to measure current and past infections, and the allowance for retrospective studies on serum banks when tissue samples are not available. Serological tests are not presently commercialized or standardized. Serology is a useful tool for epidemiological studies and vaccine trials, but is of limited value for clinical diagnosis.

Quality assurance of HPV tests

Important intra- and interobserver variability has been described with cytology smears. The interlaboratory reliability of a prototype Hybrid Capture assay was evaluated by independent laboratories in one study (29). The accuracy of the various laboratories participating ranged from 83% to 92%. The per cent agreement among laboratories ranged from 87% to 94%, (κ values from 0.61 to 0.83). Results obtained with HC-2 are more reproducible than with the prototype test. Quality control panels are provided by Digene Corporation initially to assess the ability of a laboratory to perform HC-2. No quality controls are available to periodically assess HPV testing with HC-2. If HC-2 becomes widely used for HPV testing, quality control panels will need to be sent regularly to laboratories to monitor quality of testing. The interassay coefficient of variation of VLP serology assays is usually less than 15% (28). Interlaboratory variability and agreement has been found to be good for VLP serology in one study (28).

Excellent intra- and interlaboratory agreement was reported in an international proficiency study on the PGMY-line blot (Roche Molecular Systems, USA) (30). Gene amplification is prone to false-positive reactions due to contamination or to false-negative results because of DNA degradation in the sample or the presence of amplification inhibitors. Procedures to control contamination are well-described, and PCR-based tests have been successfully applied for the clinical diagnosis of STIs. The presence of inhibitors can be monitored by coamplification of an internal control. Inhibition of amplification with PCR has been reported in 6% to 19% of endocervical swab specimens and in up to 45% of urethral swab specimens (31). The Centers for Disease Control and Prevention (USA) recommends monitoring the quality of genital samples submitted for the diagnosis of STIs by evaluating their cellular content. Nucleic acid amplification assays for STIs often include a cellular gene control to assess the quality of genital specimens.

However, the optimal number of cells required to consider a sample adequate for PCR analysis has not been precisely established. The presence of an adequate number of cells can be deduced from viral load measurements with quantitative PCR assays that establishes the HPV copy number per cell in women with and without cervical lesions. Swan et al (24) estimated that genital samples from women without lesions had an HPV-16 viral load of 2.2×10^7 copies per 10^5 cells, while samples from women with lesions had 100 times more HPV-16 DNA copies. Others have demonstrated a lower amount of

HPV-16 in cervical scrapes of women without lesions infected with HPV-16 (5.9×10^5 HPV-16 copies per 10^5 cells) or normal women who did not develop lesions (8.9×10^3 HPV-16 copies per 10^5 cells) (32). In the latter study, women with squamous intraepithelial lesions had HPV loads greater than 10^6 HPV-16 copies per 10^5 cells. This would suggest that at least 10 genital cells are required to detect the lowest HPV-16 viral load measured in specimens with sensitive, type-specific assays. Whether other HPV types infect the uterine cervix at similar copy numbers and whether consensus assays require a higher number of cells for adequate results need to be determined. Quantitative analysis of HPV-DNA in genital samples during the natural history of HPV infection should shed light on the minimal amount of cells required to consider a sample adequate for PCR analysis.

HPV-DNA detection with Hybrid Capture in clinical practice

HPV testing can be used for quality control of cytology smear interpretation (7). For example, a Pap test reported negative and an HC-2 test reported positive could be automatically reviewed to eliminate false-negative cytology due to reading errors. This provides for quality control of cytology. HPV could also be used in the follow-up of women after treatment of cervical cancer precursors to identify those with residual/recurrent disease from those who are disease free (4,19). The primary and secondary screening for cervical cancer are the most important applications of HC-2.

Primary screening of cervical cancer with HC-2

Several studies evaluated the sensitivity and specificity of HC-2 at 84% to 100% and 51% to 89%, respectively, to screen for the presence of high-grade cervical lesions (19). Several studies on the value of Hybrid Capture in primary screening have been conducted in Third World countries, with a few exceptions. These studies reported a greater sensitivity of HPV-DNA testing (85% to 100%) for detecting high-grade lesions than cytology (40% to 78%) (17). In two studies conducted in Costa Rica (20) and South Africa (33) on 8554 and 2944 women, respectively, HC-2 detected 88% of high-grade lesions as a primary screening tool (with referral rates to colposcopy of 12% to 18%), while Pap smears detected 77% of high-grade lesions (with a 7% referral rate).

One Canadian study (34) compared HC-2 with conventional cytology for the primary screening of cervical cancer in 2098 women. Women testing positive in one or both tests and a random sample of women with negative results for both tests were evaluated by colposcopy. The sensitivity of HC-2 was significantly higher than that of cytology for HSIL (90% versus 23%). The combination of both tests had a negative predictive value of 100% for HSIL while referring only 12% of women for colposcopy. In a study conducted in Europe, HC-2 applied on 7932 women attending their routine Pap screening was shown to be more sensitive in detecting high-grade cervical lesions (100%) than conventional cytology (68%) or thin-layer cytology (88%) (35). The positive predictive value of HC-2 may even be greater in women older than 30 years of age because the rate of false-negative smears increases with age, and because the detection of HPV in older women is more meaningful because it more often represents persistent HPV infection. In one study on 2988 women at least 35 years of age, only 5% of women without cervical lesions were infected by HPV (17). The combination of cytology and high-risk HPV-DNA

detection with Hybrid Capture had the highest sensitivity for detecting cervical lesions (7). To better define the role of HC-2 in Canada for primary screening purposes, the Cervical Cancer Screening Trial – a prospective three-arm clinical trial (conventional cytology, HPV testing and liquid-based cytology) on a large number of Canadian women, supported by the Canadian Institutes for Health Research – is being initiated to assess the value of Hybrid Capture compared with conventional and liquid-based cytology for primary screening.

Future studies are needed to determine if the time interval between screening visits can be increased in HPV-negative women with normal cytology smears (17). Patient populations known to comply poorly with medical follow-up could benefit the most from the addition of HPV testing in primary screening strategies, especially in developing countries. Indeed, in several Third World countries, social, cultural and religious attitudes often interfere with gynecological examinations. In such situations, self-sampling for HPV-DNA may be an attractive compromise for cervical cancer screening. Also, in developing countries, molecular testing is easier to establish than high-quality cytology laboratories. Recently, HPV screening and visual inspection after acetic acid application have been found to be more cost-effective than cytology screening in low-resource settings (36).

How should we interpret an HPV-positive test in the absence of a cervical lesion?

If HPV testing is applied for primary screening of cervical cancer, then guidelines on the management of HPV-positive women with a normal cervix have to be proposed. The knowledge of infection by an oncogenic virus could induce emotional distress in women. Many sexually active women and men will be infected by HPV, but most genital HPV infections will be transient (37-39). The prevalence of HPV infection varies from 5% to 40% in sexually active women (2,7). Nearly one-quarter of 20- to 24-year-old Canadian women are infected by high-risk HPV types (39). Incidence of HPV infection is highest in Canadian women 15 to 19 years of age (38). Nevertheless, the mean duration of high-risk HPV infection reached 16.3 months in young women in a study conducted in Canada (37). In up to one-third of HPV-infected women, more than one type will be detected when ultrasensitive assays are used. The highest prevalence of LSIL is found in women 20 to 30 years of age. HSIL is found in women over 30 years of age, and invasive cancer is most often encountered in women over 40 years of age. The clinical impact of detecting HPV-DNA in the absence of a lesion is greater in women over 30 years of age. In this age group, the probability of persistent infection, a strong risk factor for SILs, when detecting HPV-DNA in one sample is greater than in younger women who more often have transient infections. The prevalence of HPV infection drops sharply after 30 years of age (1,2,7). The decreased prevalence of HPV infection after 30 years of age is independent of sexual activity and could be related to the development of an efficient immune response. Being positive for HPV-DNA after 30 years of age could indicate exposure to a new partner or persistence of HPV infection. HPV infection in younger women is often transient; these women should not be alarmed.

In situations where infection persists, HPV induces low-grade lesions one to two years after primary infection. Women with LSIL have a 16-fold increased risk of developing

higher-grade lesions and invasive cancer (7). Up to 25% of women with LSIL will progress to HSIL over four years (7). However, HPV-induced lesions often regress, especially LSIL. Molecular biology studies indicate that HPV-immortalized cells require a second oncogene to reach tumourigenicity in vitro. Several viral, host and environmental factors may thus act as cofactors and induce progression of HPV-induced lesions from precursor diseases to higher-grade lesions. Potential cofactors include host immune status and genetic factors, smoking, high parity, long-term use of contraceptives, nutritional deficiencies and infection by *Chlamydia trachomatis* (1-3). These risk factors have not been incorporated into clear guidelines for the clinician that would help define a group of HPV-infected women at greater risk of disease. The widespread use of HPV testing in young women could result in overdiagnosis and use of colposcopy. The best strategy is to introduce HPV testing into clinical use, considering age along with education to health care providers in order to adequately reassure HPV infected women without lesions. Future screening protocols will most likely include persistence of HPV infection, which will influence the decision to further investigate the presence of cervical diseases.

Secondary screening with HC-2: The triage of women with ASC-US smears to colposcopy

As stated earlier, 10% of women with ASC-US do carry histologically verifiable HSIL (4). In this mode, HC-2 is used to identify high-grade lesions in women whose Pap tests were classified as ASC-US. Because only 31% to 60% of women with ASC-US are infected by high-risk HPV types, could HPV testing identify women with HSIL and avoid unnecessary colposcopy to women with ASC-US smears but without significant lesion (4)?

The landmark multicentre, randomized clinical trial, designated the ASC-US/Low-grade squamous intraepithelial lesions Triage Study (ALTS), supported by the US National Cancer Institute, compared three strategies to detect HSIL in 3488 women with ASC-US smears. The three options included immediate colposcopy, HPV-DNA testing with HC-2 and repeat cytology (40). Results showed that HPV-DNA testing had a sensitivity of 96% for detecting CIN III, while 56% of women were referred to colposcopy. In contrast, colposcopy detected 100% of women with HSIL; however, they all had to be colposcoped. The sensitivity of cytology for detecting CIN III was much lower at 44%, but referred only 8% of women to colposcopy. However, to reach sensitivity rates for detecting CIN III close to those of HPV, 59% of women had to be referred to colposcopy with a cytological threshold of a repeat ASC-US (40). The issue boils down to whether one desires to detect the greatest number of cervical cancer precursors versus subjecting women to unnecessary anxiety due to colposcopic examination. It is the opinion of the authors that from a clinical point of view, it is more important to treat cancer precursors and prevent the development of invasive cancer.

When age was considered, ALTS reported that HPV testing was very sensitive for detecting CIN III in women at least 30 years of age, and resulted in fewer referrals to colposcopy than repeat cytology (41). This lower referral rate of HC-2 may impact on the cost-effectiveness of using HPV-DNA testing in women with ASC-US (41). According to some, reflex HPV testing could save US \$1 billion per year in the management of ASC-US patients (20,33). An evaluation of cost-effectiveness

of the three strategies should be available in the next few years. In the ALTS study, HPV viral load measured with HC-2 did not have any clinical utility due to the important overlap between groups (41). Overall, the clinical significance of quantitative results obtained with Hybrid Capture remains controversial (4,7).

Based on the ALTS results, the significance and management of women with ASC-US Pap tests have been delineated in a recent consensus guideline published in the *Journal of the American Medical Association* in April 2002 (4). Three strategies, namely repeat cytology, immediate referral for colposcopy and reflex HPV-DNA testing, were considered to be equally safe and satisfactory for identifying HSIL in women with an initial ASC-US Pap test. Each of these strategies has advantages and drawbacks. Reflex HPV-DNA testing was the preferred option in the triage of women with ASC-US who provided cellular samples for both cytology and HC-2. This can be performed if liquid-based cytology is used for primary screening for cervical cancer or if all women screened by cytology have a cervical cytobrush sample co-collected during the conventional cytology smear and conserved frozen in STM. Following an ASC-US Pap result, the residual cells in the collection vial or the frozen sample in STM are automatically tested (reflex HPV testing) for high-risk HPV types using the probe B mix. Only those who test positive for high-risk types are sent for colposcopy, while those with negative HPV results are followed with cytology. Colposcopy can thus be avoided for 40% to 60% of women (4). HPV-negative women can be reassured rapidly.

Repeat cytology is widely used but has a low sensitivity (from 67% to 85%) for detecting HSIL and must, therefore, be repeated several times to increase its sensitivity. This introduces a delay in diagnosis and could also result in a lower compliance with follow-up and greater anxiety. Also, women with repeat ASC-US cytology must be referred to colposcopy (4). This happens in approximately 70% of the cases (42). Theoretically, immediate colposcopy informs the clinician on-site of the presence of disease; however, in real life practice, colposcopy does not have a sensitivity of 100% in distinguishing abnormal from normal tissue, even in expert hands. Colposcopy-oriented biopsy is uncomfortable to painful, expensive and could lead to overdiagnosis and overtreatment. A better identification of women at higher risk for significant lesions with HPV testing could reduce the burden imposed on colposcopists by these referrals and avoid unnecessary procedures on women without HSIL (4). Considering only women 30 years of age and older for HPV-DNA testing will increase the specificity of HPV detection. For women who had colposcopy and did not have a lesion confirmed at biopsy, HPV testing can be repeated at 12 months with referral to colposcopy for women testing positive for high-risk HPV (4). In such cases, women have persistent HPV infection (two positive HC-2 tests) and should be followed annually with cytology because they are at high risk for developing HSIL within five years. HPV-DNA testing is not recommended for immunosuppressed women because the majority of these women are infected by HPV.

As summarized in several review articles (2,4,19,17), studies have evaluated the role of HC-2 for the triage of women with ASC-US to colposcopy. Discussion of these studies is beyond the scope of this article. Results of sensitivity and specificity of

HC-2 in detecting high-grade lesions ranged from 89% to 96% and 74% to 85%, respectively. The negative predictive value of HC-2 was often high at $\pm 98\%$. HPV testing is more sensitive than cytology to detect CIN II and higher grade lesions (42). Similar results on the sensitivity of HC-2 to detect high-grade lesions were reported for European women with borderline Pap smears (43). In Canada, a community-based trial comparing HC-2 and Pap smear in women with ASC-US or LSIL showed a sensitivity of 88% for HC-2 compared with 56% for Pap to detect HSIL (44). Loss to follow-up was greater in the cytology group (33% versus 17%). HPV-DNA testing may therefore allow for a quicker triage of women with ASC-US and more efficiently retain participants into a screening program. A repeat Pap smear referred an equal number of women to colposcopy as HPV testing (44). As explained above, the follow-up of HPV-positive women older than 30 years of age without cervical lesions will have to be addressed in the future. Persistent infection is more common in this age group than in younger women, where transient infections are more common, and persistence is associated with the development of significant lesions (1).

Secondary screening: Is there a role for HC-2 in the triage of women with LSIL or HSIL smears to colposcopy?

The management of women with HSIL at cytology is widely accepted and does not require HPV testing (4). Five per cent to 20% of women with LSIL on cytology smears have high-grade cervical disease at colposcopy. Could HPV testing for high-risk HPV types help identify women with Pap smears showing LSIL who harbour a high-grade lesion? The ALTS trial showed that HPV testing by HC-2 does not help in the triage of women to colposcopy with LSIL, because 83% of women with LSIL had HPV infection with high-risk types (10). However, HC-2 could be useful in the follow-up of women with LSIL smears and negative colposcopic and histological findings. In this group of women, repeat cytology smears at six and 12 months or repeat HPV-DNA testing once at 12 months may be appropriate (4). If the repeat tests are positive, then the individuals should be referred back to colposcopy.

CONCLUSION

Prospective cohort studies and clinical trials will help further define the role of HPV testing and the clinical algorithm required for its use in primary screening settings. The role of HPV testing in primary screening looks promising for Third World countries and needs to be defined in developed countries by well-designed clinical trials. HPV-DNA testing is now well-accepted and included into clinical practice for the triage of women with ASC-US to colposcopy. The role of the recently developed nonisotopic consensus PCR assays needs to be defined in comparison with Hybrid Capture in well-designed studies before they can be considered in clinical practice.

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