

Molecular detection methods of human papillomavirus (HPV)

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ABSTRACT: Human papillomavirus (HPV) testing can identify women at risk of cervical cancer. Currently, molecular detection methods are the gold standard for identification of HPV. The three categories of molecular assays that are available are based on the detection of HPV DNA and include (1) non-amplified hybridization assays, such as Southern transfer hybridization (STH), dot blot hybridization (DB) and in situ hybridization (ISH); (2) signal amplified hybridization assays, such as hybrid capture assays (HC2); (3) target amplification assays, such as polymerase chain reaction (PCR) and in situ PCR. STH requires large amounts of DNA, is laborious and not reproducible, while ISH has only moderate sensitivity for HPV. The sensitivity of the HC2 assay is similar to that of PCR-based assays, with high sensitivity being achieved by signal rather than target amplification. PCR-based detection is both highly sensitive and specific. Since PCR can be performed on very small amounts of DNA, it is ideal for use on specimens with low DNA content. In the future, with the advance of technology, viral DNA extraction and amplification systems will become more rapid, more sensitive, and more automated. (Int J Biol Markers 2009; 24: 215-22)

Key words: HPV, Molecular detection, PCR, Hybridization

INTRODUCTION

Human papillomavirus (HPV) infection has a global distribution and has been identified as the leading etiologic agent for cervical cancer and its precursors in adulthood (1). Different HPV types can cause a wide range of infections, including common warts, genital warts, recurrent respiratory papillomatosis, low-grade and high-grade squamous intraepithelial lesions (SILs), and cervical cancer. Cervical cancer remains the second most common cancer among women worldwide, with an estimated 493,000 new cases and 274,000 deaths in 2002. Cervical cancer is most prevalent in developing countries, where 80% of the cases occur, and it accounts for at least 15% of all female cancers.

HPVs can be classified into cutaneous and mucosal types (2). Cutaneous types infect the squamous epithelium of the skin and produce common, plantar and flat warts, which usually occur on the hands, face and feet. Specific cutaneous types are also detected in *Epidermodysplasia verruciformis*, a rare familial disorder which is related to the development of large cutaneous warts that can progress to skin cancer (3). Mucosal types infect the mucous membranes and can cause cervical neoplasia in adults as well as anogenital warts in both children and adults.

Mucosal HPVs are classified into high-risk and low-risk types. High-risk HPV types have been implicated in the development of SILs and their progression to cervical cancer (4, 5). To date, 15 HPV types have been classified as high risk and these include HPV-16, 18, 31, 33, 35, 39,

45, 51, 52, 56, 58, 59, 68, 73 and 82. HPV-16 and 18 are considered to be the most frequent HPV types worldwide and are responsible for approximately 70% of all cervical cancer cases (6, 7). Low-risk HPVs have been associated with benign warts of oral and urogenital epithelium in adults as well as children, and are only rarely found in malignant tumors. Different HPV types vary in tissue distribution, oncogenic potential and association with anatomically and histologically distinct diseases.

HPVs are double-stranded DNA viruses that comprise a remarkably heterogeneous family of more than 130 types (4, 8). HPV is a small virus of 55 nm in diameter, which consists of the viral genomic DNA and its coat. The viral genome is double-stranded circular DNA of nearly 8000 base pairs and encodes 8 proteins: E1, E2, E4, E5, E6, E7, L1 and L2. The early proteins E5, E6 and E7 are involved in cell proliferation and survival, with E6 and E7 playing a key role in HPV-associated carcinogenesis. The early proteins E1, E2 and E4 are involved in the control of viral gene transcription and viral DNA replication. The coat of the virus is made up of 2 proteins, the major one being L1 and the minor component L2. The coat proteins assemble into structures known as capsomeres and 72 of these come together to form the spherical coat.

It is generally accepted that HPV E6 and E7 function as the dominant oncoproteins of high-risk HPVs by altering the function of critical cellular proteins. Expression of the E6 and E7 proteins as a consequence of viral integration is paramount to the establishment and maintenance of the tumorigenic state. In addition, expression of E6 and E7 increases the genomic instability of the host cell, thus

accelerating malignant progression (9). E6 and E7 target important cellular growth regulatory circuits including the p53 protein and the retinoblastoma tumor suppressor protein Rb, respectively. HPV E6 has been shown to interact with and enhance the degradation of p53, which plays an important role in cell cycle control and apoptosis in response to DNA damage, while HPV E7 disables the function of Rb. During the last decade, it has been demonstrated that HPV E6 and E7 interact with both host cell targeting and a plethora of key host cellular proteins that are involved in apoptosis and malignant cellular transformation (10).

HPV testing was recently introduced into clinical practice with the aim of identifying women at risk of cervical cancer (Tab. I). HPV testing is recommended in the triage of women with atypical squamous cells of undetermined significance (ASCUS) (11). The use of HPV testing in the follow-up of women after local treatment of cervical intraepithelial neoplasia (CIN) is also strongly supported by clinical evidence (12). Screening prior to vaccination could identify women who have already been exposed to HPV and thus have reduced benefit from their vaccination. However, financial barriers prevent the prescreening of all women by HPV testing. HPV testing has also been proposed as a useful tool for primary cervical screening and the management of women with low-grade SILs. However, recent evidence is insufficient to support HPV testing instead of conventional cytology (13-15). It is likely that after the introduction of vaccination against HPV, the role of HPV testing for triage for low-grade SILs and primary cervical screening will be re-evaluated.

Several molecular assays are available for the detection of HPV infection in tissue and exfoliated cell samples, and they present different sensitivities and specificities. They

can be divided into: (1) target amplification assays/PCR, (2) direct hybridization assays, and (3) signal amplified hybridization assays.

TARGET AMPLIFICATION ASSAYS/PCR

Polymerase chain reaction (PCR) is the most widely used method for amplification of nucleic acids. PCR-based detection of HPV is both highly sensitive and specific. The chemistry of this assay relies on a thermostable DNA polymerase which recognizes and extends a pair of oligonucleotide primers that flank the region of interest. Finally, the viral DNA is sufficiently amplified in vitro to generate an adequate amount of target, which is then directly visualized on an agarose gel. Theoretically, PCR is able to detect 1 copy of a target sequence in a given sample. In practice, the sensitivity of the PCR-based method is about 10-100 HPV viral genomes in a background of 100 ng cellular DNA. Since PCR can be performed on very small amounts of DNA (10-100 ng), it is ideal for use on specimens with low DNA content.

Generally, HPV detection by PCR can be performed either by type-specific primers, designed to exclusively amplify a single HPV genotype, or by consensus/general PCR primer pairs, designed to amplify a broad spectrum of HPV genotypes. Unfortunately, the use of multiple type-specific PCR reactions in order to detect HPV-DNA in a single sample has the disadvantage of being costly and time-consuming, and the primers usually have to be confirmed by others (16-18). The use of general primers is much more convenient. Usually, general primers identify a conserved region among different HPV genotypes, such as the L1 (19) or E1 regions (20). However, most laboratories utilize consensus primers directed to the conserved L1 region.

There are numerous consensus PCR primers that can be used. The GP5+/6+ pair is aimed at the L1 conserved region, but it is 100% complementary to just a few HPV genotypes. The mismatching produced between the consensus PCR primers and the various HPV genotypes is overcome by setting a low annealing temperature at the PCR reaction (21-23). Other consensus primers are a mixture of many different oligonucleotides and do not have to be used at a lower annealing temperature. One example of these primers is the MY09/11 set (24-27).

Moreover, a combination of various non-degenerate primers which target the identical location of the viral genome can be applied. Combined primers usually contain inosine, which matches with any nucleotide. Primer sets belonging to this category have the advantage of being highly reproducible; moreover, PCR can be performed at optimal annealing temperatures. PGMY, SPF10 (27), LCR/E7 (28), as well as a combination of the MY11 and GP6+ primers are examples of combined primers (29).

TABLE I - RECOMMENDATIONS OF THE USE OF HPV TESTING IN CLINICAL PRACTICE

1. Primary cervical screening

- HPV testing and Pap smear co-testing increases sensitivity of primary cervical screening

- HPV testing is not recommended to replace Pap smear as an initial screen

2. Cervical screening of women with low-grade SIL

- HPV testing can increase the sensitivity of Pap smear to detect "high-risk" women with low-grade SIL who require colposcopy

3. Post-treatment follow-up of SIL

- HPV testing can detect residual disease following treatment of SIL earlier than Pap smear

4. Cervical screening prior to vaccination

- HPV testing prior to vaccination is not recommended

SIL, squamous intraepithelial lesion

Another point of consideration is the size of the amplicon to be produced after the PCR. Generally, the efficiency of a PCR reaction is inversely proportional to the size of the amplicon. Various treatments of the clinical samples, before or during the DNA extraction procedure, usually result in low quality of the DNA and/or DNA degradation. Therefore, the PCR primers that produce a small amplicon tend to be more efficient than those responsible for larger amplicons (28, 30, 31).

When the PCR is complete, the amplicon sequence can be examined in various ways. One involves the digestion of the amplicon with specific restriction endonucleases, an approach known as HPV restriction fragment length polymorphism (RFLP), followed by agarose gel electrophoresis analysis. However, RFLP often results in a number of fragments which are difficult to interpret. This becomes more evident when multiple infections are present (31-37).

PCR products can also be detected with a mixture of type-specific probes, for example in an enzyme immunoassay (EIA) (23). One good example is the HPV oligonucleotide microarray (HPVDNAChip, Biomedlab Co.), which contains 22 type-specific probes; 15 of the high-risk group (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 and 69) and 7 of the low-risk group (6, 11, 34, 40, 42, 43 and 44). Briefly, the PCR product is hybridized onto the chip, and after a washing step, hybridized signals are visualized with a DNA chip scanner. The sensitivity of this assay has been reported to reach 94.9%, enabling this application to be considered a diagnostic tool with significant advantages since it can discriminate the HPV genotype and identify multiple infections (38, 39). Ideally, a larger number of HPV type-specific oligonucleotides could be spotted on the chip, although this method requires the presence of expensive equipment and may not be suitable for many laboratories. A similar assay was recently released by Gen-Probe Inc., called the APTIMA(R) HPV Assay. This assay distinguishes 14 high-risk HPV genotypes in an amplified HPV nucleic acid. In particular, it identifies the E6 and E7 mRNA sequences, produced at higher levels when HPV infection progresses toward cervical cancer (40).

Sequencing reactions of double-stranded PCR products can be performed directly with the GP6+ primer, using commercially available kits such as the BigDye Terminator cycle sequencing kit (Applied Biosystems, CA, USA). Subsequently, the DNA sequences obtained from the patient samples can be compared to the GenBank sequences by using the BLAST program at the National Center for Biotechnology Information website. Thus, the sequencing reaction can now be applied in routine clinical analysis (41). Unfortunately, in many patients the presence of multiple HPV genotypes, visualized as multiple peaks at the sequencing electrophoregram, is very common and represents a major obstacle in the determination of

the DNA sequence. On the other hand, the frequency of multiple HPV infections can be underestimated when DNA sequences that represent a minority in the total PCR product remain undetected (42). One such example is the study by Levi et al (43), which compared the sequence analysis of SPF10 PCR products with reverse hybridization in 166 HPV-positive cervical scrapes. Well-matched HPV genotypes were found in all samples. With the former assay, multiple types were detected in only 2% of the cases, whereas the latter assay was capable of detecting multiple genotypes in 25% of the samples.

Multiple HPV genotypes are found in up to 35% of HPV-positive patients with advanced cytological disorders and more than 50% of HIV-infected patients (43, 44), whereas multiple genotypes are less prevalent in carcinoma patients (42). The genotype from an HPV sequence can be deduced through alignment with a set of known HPV sequences, using the BLAST software (45) of a genome database (e.g., <http://www.ncbi.nlm.nih.gov>). The complete genomes of various papillomaviruses have been fully sequenced. In order to designate a new type, the L1, E6 and E7 ORFs must differ by more than 10% from the closest type known. Differences of 2-10% lead to the definition of a new subtype, whereas differences of less than 2% define intratype variants (46).

Real-time PCR techniques have been developed to quantify HPV-DNA in clinical samples (47-49). Real-time PCR has the following advantages: 1) it is capable of detecting the viral load; 2) with the use of different fluorochromes which emit fluorescence as the PCR reaction proceeds, the reactions can be performed in multiplex, thus amplifying simultaneously different nucleic acid targets; 3) using a 7-log dynamic range to extrapolate viral load/concentration in the standard curve, it is possible to detect nucleic acids even at very small concentrations, which would not be detected by conventional PCR (47, 48). Finally, real-time PCR is extremely reproducible, rapid and pertinent in a clinical setting.

Novel real-time PCR methods have recently been released and are capable of being used as high-throughput screening tools. One such example is the GenID real-time PCR assay, the amplification of which is based on the L1 region of HPV. The assay is designated to detect the non-integrated copies of HPV. The assay's calibrators are designed to detect ~10,000 copies/reaction (~100 infected cells). Amplification is balanced over the genotypes, which is important to achieve optimal clinical sensitivity. Detection of high-risk HPVs (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68), low-risk HPVs (6, 11, 42, 43 and 44/55), and internal controls is carried out in the same reaction tube using 3 different color-compensated dye channels (50). However, the exclusive optimization of this assay for the LightCycler 2.0 instrument (Roche) can be regarded as a weakness. Unlike other HPV tests, the newly released CE-marked Abbott RealTime High Risk

HPV assay can detect the 14 highest-risk HPV genotypes and, in the same procedure, identify women infected with the HPV-16 and HPV-18 genotypes. The assay can rapidly identify HPV-infected patients at risk of cervical cancer by combining 2 diagnostic tools in one test: HPV high-risk screening and viral genotyping.

Apart from TaqMan oligo-probe technologies, SYBR Green-based real-time PCR assays utilizing the GP5+/6+ primers have also been used for HPV quantification with high specificity and sensitivity, whereas the results showed excellent concordance with the enzyme immunoassay-reverse line blot and sequencing assays (51).

It seems that the importance of HPV viral load detection with real-time PCR remains vague so far, since viral load values are an average summed over many infected and uninfected cells. The production of a high HPV viral load can be observed during severe disease rather than being the cause thereof (52).

Although the majority of HPV detection strategies are DNA based, the detection of the expression of HPV oncogenes may have significant clinical value. This can be performed by reverse-transcription real-time PCR. Several groups have correlated the HPV-16 and HPV-18 E7 transcript levels with the severity of cervical lesions (53, 54).

Moreover, Cattani et al (55) detected E6/E7 RNA transcripts in 18.2% of HPV DNA-positive patients with normal cytology. The rate of detection increased gradually with the grade of the observed lesions, suggesting that testing for HPV E6/E7 transcripts is a useful tool for screening and patient management, providing more accurate predictions of risk than DNA testing.

The PreTect HPV-Proofer (NorChip AS, Klokkestua, Norway) is a commercially available RNA-based HPV assay that incorporates the NASBA amplification of E6/E7 mRNA transcripts prior to type-specific detection via molecular beacons for the following HPV types: 16, 18, 31, 33 and 45 (56, 57). However, large-scale prospective studies need to be performed in order to better determine the clinical value of this assay.

Finally, a protocol for the amplification of papillomavirus oncogene transcripts (APOT) from cervical specimens has been proposed that allows the distinction of episomal from integrated HPV mRNAs (58). In most cervical carcinomas, HPV genomes are integrated into host cell chromosomes in order that transcribed mRNAs can encompass viral and cellular sequences. In contrast, in early preneoplastic lesions, HPV genomes persist as episomes, and derived transcripts contain exclusively viral sequences. Thus, detection of integrated-derived HPV transcripts in cervical swabs or biopsy specimens by the APOT assay points to advanced dysplasia or invasive cervical cancer, where loss of the regulatory E2 protein on integration results in the upregulation of the oncogenes E6 and E7. However, since the assay is based on RT-PCR protocols it has not readily been adapted for use in routine diagnostic testing.

DIRECT HYBRIDIZATION ASSAYS

Southern blot hybridization (SBH) and in situ hybridization (ISH) can be used, but they have serious defects such as low sensitivity, delay, and the need for possibly large amounts of highly purified DNA (59). Of the direct probe methods, ISH is the least specific for HPV detection (60-62) (72% for condylomatous lesions and 30% for invasive cancer cells). Recently, new ISH assays have emerged, showing better results. The INFORM HPV 3 (Ventana Medical Systems) test can detect 13 types of oncogenic HPV (16, 18, 31, 33, 35, 45, 51, 52, 56, 58, 59, 68 and 70). This assay was shown to correlate with PCR-based assays. Despite the favorable correlation, however, the assay detected significantly fewer HPV-positive cases among patients with cervical carcinomas than PCR assays (63). Another novel ISH assay, the HPV-CARD assay, was shown to have high analytical sensitivity and a high signal-to-noise ratio, to allow quantification of HPV-infected epithelial cells, and to distinguish between HPV physical states (64).

SIGNAL AMPLIFIED HYBRIDIZATION ASSAYS

The most reliable signal amplified hybridization assay is the FDA-approved Hybrid Capture 2 (HC2) test (65). This method is based on 13 RNA probes specific for the corresponding number of high-risk HPV types (HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68). The assay's sensitivity is excellent since it can detect HPV-16 DNA of a concentration down to 1 pg/mL, which corresponds to $\sim 10^5$ viral gene copies. Nevertheless, there has been some crossreactivity of the various RNA probes with HPV types not represented in the probe mix (66). However, it has been proven that the HC2 assay scores significantly more positive samples than ISH and GP5+/6+ PCR (67).

Hybridization of the biotin-labeled PCR products to oligonucleotide probes in streptavidin-coated microtiter plates has been shown to increase the efficiency of diagnostic assays (23, 28, 68). One such assay is the Roche Molecular Systems Amplicor HPV MWP assay, which is capable of detecting 13 high-risk genotypes by a broad-spectrum PCR in the L1 region, producing an amplicon of ~ 170 bp. The sensitivity of this method appears to be superior to that of the HC2 assay for the detection of the same high-risk HPV types. Moreover, the high throughput of the microtiter format gives the Amplicor HPV MWP assay a further advantage, making it appropriate for the distinction between HPV-positive and HPV-negative DNA samples.

However, since both the HC2 and Amplicor tests only differentiate between infection with 1 of 13 high-risk HPV genotypes and no high-risk HPV infection, neither allows for the individual identification of specific genotypes, nor

do they identify multiple genotypes possibly involved in infection. This is an unfortunate weakness, as recent studies have provided evidence for a difference in oncogenic potential between the different high-risk HPVs (69), arguing for the importance of HPV genotyping in screening and triage (70-73).

Furthermore, the CLART HPV 2 system is based on a low-density microarray that detects infections and coinfections of up to 35 of the most relevant HPV genotypes; 21 high risk (16, 18, 26, 31, 33, 35, 39, 43, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, 85 and 89) and 12 low risk (6, 11, 40, 42, 44, 54, 61, 62, 71, 81, 83 and 84). The diagnostic sensitivity and specificity of this system can reach 98.2% and 100%, respectively (74).

The simultaneous hybridization of a PCR product to multiple oligonucleotide probes can be performed by reverse hybridization. The first step of this method consists of the immobilization of the probes on a solid phase, then the PCR product is added in the liquid phase and this is followed by the detection of the signal produced. The most frequently used reverse hybridization assays are the line probe assay, line blot assay (Roche Molecular Systems, Alameda, CA, USA) (75-78), and linear array (Roche Molecular Systems). These methods are judged advantageous in their ability to rapidly genotype HPVs present in samples with high sensitivity and specificity (24, 79-82). Castle et al (83) compared the linear array with the line probe assay and found that the former was more analytically sensitive, resulting in increased detection of individual genotypes and of multigenotype infection. The findings translated into a more clinically sensitive but less specific test for CIN3 or worse in a population of women enrolled in a low-grade SIL triage study (ALTS) because of an ASCUS Pap-test result.

Reverse hybridization assays are especially useful for detecting type-specific infections and multiple genotypes. Alternative reverse hybridization assays for the detection of HPV and its genotyping are the line blot and reverse

line blot assays, using the PGMY (24, 84-87) and GP5+/6+ (88) primer sets, respectively. HPV DNA microarrays are based on the same principle (89).

CONCLUSIONS

Molecular assays are the gold standard for HPV identification. If one would like to pinpoint each method's specific characteristics, the extremely high sensitivity and specificity of PCR should be mentioned, along with the need for only small amounts of DNA template. The HC2 assay is equally sensitive, with the difference that its sensitivity is achieved by signal rather than target amplification. Southern blot hybridization on the other hand requires large amounts of DNA template, is difficult to apply in routine practice and not always reproducible, whereas *in situ* hybridization is not always as sensitive as the PCR and HC2 methods.

Further investigation is required to clarify the role of molecular HPV testing in current primary cervical screening programs. In the future, the introduction of a fast, cheap and reliable test for the molecular detection of HPV in clinical practice is expected to improve the Pap-test sensitivity for the early diagnosis of cervical HPV infection. With the advancement of technology, viral DNA extraction and amplification systems will become more rapid, more sensitive, and even more automated.

Conflict of interest statement: None declared.

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Received: August 19, 2009

Accepted: October 14, 2009