

Review Article

Exploring Molecular Diagnostic Approaches for HPV

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A B S T R A C T

Introduction: Human Papillomavirus (HPV) is implicated in various anogenital cancers, with developing nations experiencing higher infection rates. Since HPV cannot be cultured in vitro, molecular diagnostic techniques are critical for its detection.

Aim: This study aims to explore and evaluate the various molecular diagnostic approaches available for detecting HPV, emphasising their utility in identifying high-risk HPV strains linked to cervical cancer.

Methods: We review a range of molecular diagnostic techniques, including target amplification methods like Polymerase Chain Reaction (PCR), Reverse Line Blot, Real-Time PCR, and various other advanced HPV DNA testing methods like COBAS HPV Test, Xpert HPV, and Anyplex II HPV 28. Signal amplification techniques such as Digene Hybrid Capture 2 and CARE HPV are also discussed for their efficacy in detecting HPV.

Conclusion: Molecular diagnostic approaches have significantly advanced HPV detection, providing high sensitivity and specificity, particularly for high-risk HPV strains. These techniques are advantageous in the early diagnosis and management of cervical cancer, particularly in regions with high HPV prevalence. Further refinement and accessibility of these methods are essential for global cervical cancer control.

Keywords: Human Papillomavirus, Cervical Cancer, Molecular Diagnostics, COBAS HPV Test, HPV DNA Testing, Signal Amplification

Introduction

HPV represents a broad family of double-stranded DNA viruses which will infect the cells of the epithelium. These viruses are classified into five distinct groups— α , β , γ , μ , and ν —comprising around 225 different types.¹ Persistent infection of HPV is a major sexually transmitted disease (STD) and is linked to over 5% of all cancers globally. A significant number of women who are diagnosed with a high-risk HPV strain are highly sensitive for developing

cervical cancer within 3 to 5 years, even though about 90% of HPV infections resolve or become inactive within 1 to 2 years.² Cervical cancer has lower mortality rates than occurrence rates worldwide, with a ratio of 57% between the two (GLOBOCAN 2020). Adenocarcinomas are the second most common type of cancer after squamous cell carcinoma.

Harald zur Hausen initially reported HPV's role in the initiation of cervical cancer in the 1970s following which

many molecular, epidemiological, and clinical observational studies were conducted and implicated HPV as an etiological agent in several anogenital cancers like cervical cancer. The occurrence and prevalence of various pathologies caused by this virus vary according to its regional conditions, genotype, the study population as well and the anatomical site sampled. Generally, developing nations have higher rates of HPV infection and diseases associated with it. For the diagnosis of HPV, we have to rely on various immunological and molecular biology techniques as the virus cannot be propagated in tissue culture.

There are about 200 HPV types, and their whole genome sequencing has been characterised to date. HPV can be categorised into 2 prime phylogenetic genera, the α -HPV & the β -HPV. They are mucosal and cutaneous infective respectively.³ Based on the lesion that mucosal HPV causes—whether benign or potentially malignant—they are further classified into ‘low’ and ‘high’ risk categories. The β -genus cutaneous forms, such as HPV5, have been linked to non-melanoma skin cancer in immunocompromised and immunosuppressed people².

Methods

A comprehensive search was conducted in major international databases such as Scopus, Web of Science, Medline (PubMed), and EMBASE to gather all relevant articles that provide the latest insights into the structure and genome of HPV, as well as its pathogenesis, diagnostic approaches, types, testing methods, and various amplification techniques. This search focused on studies not thoroughly covered in previous reviews. We examined publications from January 1985 to March 2020, restricting the language to English. The search included the following keywords: “Human Papillomavirus,” “molecular diagnostics,” “cervical cancer,” “PCR,” “Real-Time PCR,” “COBAS HPV Test,” “HPV DNA testing,” “signal amplification,” and “HPV detection.”

Structure and genome of HPV

Non-enveloped double-stranded DNA viruses make up all forms of HPV. The HPV genomes are circular and 8 kilobase pairs in size. The structure as well as function of the HPV genome are broadly classified into three components (i) The Early Region (ii) The late region (iii) The URR (Upstream Regularity Region).³

The E region, consisting of E1, E2, E4, E5, E6, and E7 open reading frames (ORFs), encodes nonstructural viral proteins crucial for viral replication, trans-activating viral transcription, and fostering proliferation and cellular transformation. The L2 and L1 ORFs, which encode major as well as minor capsid proteins as well as structural viral proteins, make up the late gene region, or L.⁴

Almost 10 per-cent of the genome is the URR, encompassing the L1 ORF and the start codon for E6. The URR controls early gene transcription, cellular tropism, and viral amplification. It also “contains recognition sites of DNA for the host and factors of viral transcription. The URR, which has a region of keratinocyte-specific enhancer right next to the gene promoter, illustrates the importance of host cell tropism for the expression of viral genes along with the life cycle.⁵ For assessing genetic heterogeneity among various types of HPV, the early region and URR are useful, as they express variability in gene expression.⁶

At the viral origin of replication, the E1 & E2 proteins form heterodimers to initiate bidirectional genome synthesis. Before the late promoter of differentiation-dependent activation as well as the genome amplification, the E2 protein sustains viral genomes at low copy numbers by blocking early promoter activity.^{7,8} The ORF of E5 encodes a transmembrane protein that is linked to late gene virus life cycle events, likely plays a role in cell signalling, and interacts with the growth factor of platelet-derived along with the growth factor of epidermal affect cellular proliferation.⁹

Eight main proteins are encoded by the majority of genomes, six of which are found in the ‘early’ region and two in the ‘late’ region. These key proteins participated in the cell cycle, replication, and transcription of the HPV virus, as well as in cell signaling, apoptosis regulation, and the structural and immunological alteration of the infected cell. Throughout the infectious cycle most of the major proteins are expressed and less at late times. More than one isoform of the E6 protein is expressed in HPV¹⁰

HPV pathogenesis

Infection with the HPV virus mainly occurs through micro lesions of skin and mucosa and infects the basal layer cells that can proliferate. Following infection, the cells divide and the population of cells spreads across. Some progeny cells move into suprabasal cell layers to differentiate, activate viral genes, replicate viral DNA, and generate capsid proteins. The formed viral particles are ultimately liberated to the surface, where they may go on to infect other tissues. The progression of lesions in the HPV-infected cells occurs as follows. Infected “cells progress to LSIL (low-grade squamous intraepithelial lesion) and HSIL (high-grade squamous intraepithelial lesion) followed by carcinoma *in situ*, and ultimately invasive cancer¹¹

In the basal layer cells, there is restricted expression of the ‘early’ genes (like E5, E6, and E7) which leads to enhanced proliferation and lateral expansion of the infected cells. When progeny viral particles enter suprabasal cells, the expression of the ‘late’ viral gene initiates, leading to the circular viral genome replication and the structural protein synthesis. After completion, the viral particles

are assembled and discharged into the mucosa or higher layers of the epidermis.¹²

The 'early' viral genes mainly possess proliferation-stimulating activity. In the course of early infection, E5 plays a crucial role in stimulating cell growth. It forms a complex with the colony-stimulating factor-1 (CSF-1) receptors, and platelet-derived growth factor- β (PDGF- β), as well as epidermal growth factor¹³. Additionally, it keeps cells from dying after DNA damage¹⁴. The E5 protein is not required for the final stages of HPV-mediated carcinogenesis because as the infected lesions develop into cancer, the episomal viral DNA integrates with the "DNA of host cell, and a major genome portion, together with the coding region of E5, is eliminated¹⁵. E6 & and E7 proteins play a crucial role in the invasive transformation of the infected cells by degrading the p53 gene and RB gene respectively. The two viral proteins act synergistically in cell immortalisation and malignant transformation.¹⁶ The E6 oncoprotein binds to P53, leading to its degradation by forming a complex with E3 ubiquitin-protein ligase E6AP. It also blocks the genes of transcription of tumour-suppressive. The degradation of the RB gene activates the transcription factor E2f, leading to the initiation of S-phase genes, which induces the proliferation of cells and increases viral gene transcription.¹⁸ The increased expression of the E2f transcription factor leads to improved expression of p16INK4a (p16) that controls the essential transition of the G1-S phase.¹⁹ Low-risk HPV" have lower affinity in targeting the RB gene compared to HR HPV implicating their difference in causing carcinoma.

Diagnosis

Persistent infection of the cervical epithelium with HPV causes dysplasia development and carcinoma of the cervix. Other factors like age, immune status, and gene mutations also determine the development of dysplasia or carcinoma.²⁰

Papanicolaou staining, applied to conventional cervical smears or suspension of cells from the medium of liquid cytology, can detect abnormal epithelial cells of the cervix and form the basis for cervical carcinoma screening programs for detecting carcinoma development among women high at risk. For the control of cervical cancer worldwide carcinoma screening is the best method. The primary screening and diagnostic method is the pap smear but it has limitations like low specificity leading to repeated screening at short intervals and in developing countries it remains beyond the economic resources of a country.²¹ Furthermore, women's participation in cytology-based screening programmes may be restricted by cultural obstacles and client attitudes (issues). Worldwide research has unequivocally demonstrated that HPV is the cause of

all cervical carcinomas; hence, direct HPV detection in cervical specimens may be utilized in addition to or as a substitute for cytology-based screening tests.²² The new WHO guidelines suggest that an HPV DNA-based test be used instead of imaging inspection using acetic acid (VIA). For both cytology and HPV-DNA testing healthcare provider is needed to obtain the sample but HPV-DNA testing is simpler. Additionally, the WHO recommends utilizing self-collected samples for HPV DNA testing.

Types of HPV DNA testing methods

The HPV cannot be cultured in laboratory settings. Therefore, the diagnosis of HPV infection mainly depends on molecular technologies which detect HPV in cervical and vaginal samples of both younger and older age group women.²³ Based on whether amplification of nucleic acid is used or not in the technique it is broadly divided into nucleic acid probe tests and amplification-based tests. The amplification techniques are further classified as (i) Target amplification (ii) signal amplification (iii) probe amplification. At present the amplification of the target and signal along with non-amplification methods are utilized in HPV detection.

Target amplification methods

It is a method that produces concentrated samples of a certain genetic sequence by duplicating DNA fragments from a particular gene sequence.²⁴

PCR

PCR is the most commonly used method. It occurs as a cyclic three-step reaction in which a master mix of chemicals is placed into the reaction vessel and the biochemical process occurs in an automated thermocycler finally, the amount of targeted DNA doubles with each thermocycler²⁵ L1 consensus primers PCR systems mainly GP5+/6+ [50-52] and MY09/11 were used about 20 years ago. To ascertain whether a certain sequence is present in the sample, type-specific PCR necessitates multiple PCR cycles.²⁶ Several available kits for target amplification are discussed below.

Real-time PCR

Type-specific primers are paired with fluorescent probes for real-time PCR to enable precise quantification of the virus contained in the sample and real-time detection.²⁷ It can be challenging technically to multiplex many type-specific primers in a single reaction during real-time PCR. It is challenging to standardise the probe mixture used in broad-spectrum PCR genotyping of different HPV genotypes since each probe will have a unique hybridisation characteristic. The commercial real-time PCR Geno ID kit. This kit takes less than three hours to complete and identifies 5 and 14 low-risk and high-risk types, respectively.^{28,29}

Reverse line blot and linear array

The line blot assay, exclusively for research use, originates from the HPV genotyping test known as the Linear Array. A membrane strip is coated with probes specific to several HPV types. The results of PCR are then hybridised into the strip, allowing for visual identification. To well as identifying 27 distinct HPV types, the extended version of this assay includes 11 low-risk types (61, 62, 64, 67, 69 to 72, 81, 82, and 89).²⁶ PGMY primers are also included in the Linear Array method, a widely used technique for HPV genotyping employing strips for hybridization. Fourteen of the 37 HPV types that this assay may identify are high-risk genotypes. There is currently research being done on automation techniques to reduce the subjectivity involved in reading the outcomes of a linear array because the results can be interpreted differently when assessed visually, particularly in specific areas of hybridisation strip where a visible band may be present.²⁸

INNO-LiPA HPV Genotyping Extra

This process of simultaneously hybridising a PCR result to many oligonucleotide probes is an appealing technique. The procedure involves immobilizing several probes of oligonucleotide as parallel lines on a membrane strip, generating a PCR product with biotinylated primers, denaturing the double-stranded result in a highly alkaline, and then adding it to the strip in a hybridisation solution. Following rigorous washing and hybridisation, the hybrids can be identified by adding a substrate and a streptavidin conjugate, which results in clearly interpretable purple/brown bands near the probe line. This test detects 24 HPV strains, including high-risk and low-risk.³⁰

AMPLICOR-HPV

In this test, 13 HR types of HPV can be detected. It requires only a minute amount of sample 250 microliters which is added to 96 -microwell plate.³¹ Amplicor HPV does not detect the specific genotype of HPV, it can detect only HPV. It is also not FDA-approved.

PapilloCheck

It is a DNA array-based test. This test can detect and identify simultaneously twenty-four types of low as well as HR HPV. It employs PCR-based amplification using E1 gene-specific primers, which is “followed by DNA chip hybridisation containing immobilised HPV oligoprobes.³² This method allows for the testing of 12 samples simultaneously.

Multiplex HPV Genotyping Kit

The Kit is combined with PCR products to create this fluorescent bead array, which is based on PCR technology. One β -globin probe, one control probe, and twenty-six populations of beads are linked to the 24 HPV probes in this bead mix. Afterward, the hybridised PCR product

is read on the Luminex analyser after being labelled with R-phycoerythrin marked streptavidin. This test can identify 24 HPV types that are high- and low-risk.³³ High sensitivity makes it suitable for large-scale epidemiological investigations and routine HPV testing.³⁴

COBAS HPV test

The HR-HPV L1 gene is the target of this automated real-time PCR assay, which also uses the human beta globulin gene as an internal control. This assay detects 14 HR-HPVs simultaneously and also gives specific genotypic information for HPV16 &18. Oligonucleotide probes labelled with 4 different fluorescent dyes are used for the detection of the amplicon during thermocycling. This is an FDA-approved method. Within a duration of 5 hours, 94 samples can be tested.³⁵

Xpert HPV

It is a multiplex PCR test that amplifies target DNA from fourteen HR HPV strains in multiplexes within a single examination. takes place within a cartridge. Types of HPV 16 and HPV 18/45 are particularly identified by Xpert HPV in two different detection channels. A pooled result of the test indicates 11 additional HR types (31, 33, 35, 39, 51, 52, 56, 58, 59, 66, and 68). It targets the E6/E7 segment of the viral DNA sequence in patient specimens from HR HPV (Human Papillomavirus). Its total processing time is one hour.³⁶

BD HPV on clarity assay

This method targets the E6/E7 segment of viral DNA and can detect 14 HR types of HPV simultaneously. TaqMan DNA probes, which have a fluorescent dye at the 5-prime end and a quenching molecule at the 3-prime end of the oligonucleotide, are used to detect the target DNA. The BD Viper LT System detects amplification fluorescently in four optical channels.³⁷

Anyplex II HPV 28

The detection method is unique and the PCR is multiplex real-time. It is a semi-quantitative DNA PCR that can detect 19 HR-HPV and 9 LR HPV types. With the use of this test, risk may be immediately stratified according to viral load and genotype. It can be used for screening, follow-up, and post-treatment situations. It is CE-marked not FDA approved³⁸

The APTIMA HPV

This test, known as transcription-mediated amplification (TMA) capture, measures the expression of “E6/E7 mRNA in a single measurement by capturing the target after cell lysis, followed by transcription-mediated amplification as well as probe hybridization protection. This assay detects HPV E6/E7 mRNA from fourteen HR HPV types as a pool in

cervical" samples. This test has received FDA consent for usage in screening women as young as 21 years old who have ASC-US Atypical Squamous Cells of Undetermined Significance (ASC-US) on their Pap tests, as well as for screening women as old as 30 years old as a supplement to Pap testing.³⁹

ONCO E6 cervical test

This assay detects E6 oncoprotein along with the HPV infection detection so it is useful in indicating the existence of cervical pre-cancerous or cancerous lesions. Detection of the E6 protein provides a means of identifying the transition from infection toward cancer. It utilises a lateral flow assay format with monoclonal antibodies for HPV 16/18 E6. It is a CE-marked test.

CLART HPV 2

This is a low-density microarray visualisation multiplex PCR experiment that is followed by genotype amplicon-specific hybridisation. The test contains two internal controls: (i) amplification control (plasmid) used for the control process in every tube and (ii) DNA control (human CTFR gene) is used for sample abundance. Thirteen HR genotypes and twenty-two LR genotypes are detected. Automated test result reading is crucial for high-throughput screening setups.

Single amplification techniques

HPV can be detected using signal-amplified methods such as branching DNA and hybrid capture. Instead of amplifying the target DNA using PCR for detection, this approach amplifies the chemiluminescent or fluorescent signal.⁴²

Digene hybrid capture 2 assays

This is an FDA-approved assay. It uses the principle of nucleic acid hybridisation and detection by chemiluminescence on an antibody-coated Microtiter well. It is the most commonly utilised assay in clinical laboratories and is known as the gold standard method for the diagnosis of HPV.

When specimens containing HPV-DNA are hybridised with an RNA probe specific to HPV, a DNA: RNA hybrid molecule is created. Antibodies that bind DNA: RNA hybrids are coated on the microplate well, which holds the hybrid molecules on the plate. The hybrid molecules are bound by antibodies conjugated to alkaline phosphatase, and a signal is observed upon adding a chemiluminescent substrate.⁴³ This assay can detect only HPV but not its specific genotype. it detects thirteen HR-HPV types.

CARE HPV

This technique involves the combination of full-length RNA probes through hybridisation, coupled with chemiluminescence signal amplification for detection. it can detect 14 HR types (16,18,31,33,35,39,45,51,52,56

,58). This test is developed by Qiagen for mainly use in developing and underdeveloped countries and it is planned to be utilised by slightly trained participants. It has a short running time of 2 hours and 30 minutes which allows clinicians to follow up with the patient in the same visit. It is more affordable and 90 percent accurate.⁴⁴

Cervista HPV HR

This is a special signal amplification method that uses Invader technology that uses 2 simultaneous isothermal reactions. In this method, both DNA probes containing a sequence-specific region and an invader probe bind with specific target sequences in the HPV DNA molecule. A 5' oligo flap is released from the probe using proprietary enzymes.⁴⁵ Each molecule results in the breakage of several probes, which amplifies the signal. In the meantime, each flap is associated with a FRET (Fluorescence Resonance Energy Transfer) probe to function as an invader oligo. A fluorescent molecule is present in the FRET probe near a quencher molecule. Another cleavage process takes place upon the flap's contact with the FRET probe, liberating the fluorophore from its quencher. It produces a luminous signal that may be seen.⁴⁶ This assay has increased specificity and it is FDA approved. CERVISTA HPV 16/18 works on the same principle and detects HPV 16 & 18.

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Discussion and Future Direction

Women at the highest risk of cancer in impoverished nations frequently have less access to the knowledge and resources they need to protect themselves from infection. A wide range of social, cultural, and clinical factors impact their ability to participate in different preventive initiatives. Therefore, it is important to have a complete grasp of the complexity of HPV testing before incorporating tests into different prevention strategies.

The following are some circumstances in which testing of HPV-DNA could eventually be employed in cancer prevention programs: 1. as a means of triage of women who have tested positive in a pap smear for atypical squamous cell carcinoma. 2. As a means of surveillance of women after treatment for high-grade dysplasia 3. Can be used as a primary screening method for high-grade dysplasia in older women (women age >35).

WHO's updated guidelines recommend using an HPV DNA-based test instead of a VIA or cytology test. Though many HPV detection tests are available only a few are FDA approved. It has been anticipated that next-generation sequencing technology, in association with RT-PCR, will be crucial in the future for differentiating between various HPV strains. In addition to HPV type, new diagnostic biomarkers with high specificity for cervical premalignant lesions will become essential.

Conclusion

Some more recent technologies for HPV testing are developing as researchers look for ways to make them more widely applicable in different contexts. Diagnostic, clinical, and social implications of HPV testing are to be learned and considered for its role in screening programs. HPV DNA detection methods will become a viable substitute for extensive cytological screening programs if they are demonstrated to be affordable, practicable to use, and widely accepted.

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