9 HPV Infection: Pathogenesis and Detection

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9.1 Introduction

Human papillomavirus (HPV) infections are one of the most common sexually transmitted infections (STIs) across the world. There are more than 150 types of HPV, of which about 40 are known to affect humans [1]. HPV can be subgrouped into cutaneous types and mucosal types based on the tissue tropism. Cutaneous types infect keratinizing epithelium, e.g., skin of hands/feet, and are linked to warts (plantar warts, flat warts, common warts) on the hands, face, and feet. The mucosal types infect nonkeratinizing epithelium, e.g., mucosa of anogenital tract, oral mucosa, conjunctiva, and respiratory tract [2]. The mucosal HPV types are further classified into high-risk and low-risk types, based on whether they cause cancerous or benign changes in the tissues affected [3].

The high-risk oncogenic types are linked to cervical, vaginal, vulvar, and anal cancer in women and penile, anal, and oropharyngeal cancer in men [4]. The low-risk non-oncogenic types are responsible for warts and other benign pathologies in both sexes (Table 9.1).

Although HPV infection is easily acquired, most infections are subclinical and transient [5]. Detection of HPV infection in the asymptomatic population is by screening or when persistent HPV infection causes clinical manifestations like warts or cancer. Since HPV infection is not a notifiable infection (unlike HIV or certain other STDs), its true incidence is difficult to estimate, but the prevalence of asymptomatic infection varies from 2 to 44%, depending on the population and region [6].

Table 9.1 Mucosal HPV types

Mucosal HPV	HPV types	Associated diseases
Low-risk	6, 11	Low-grade cellular changes; genital warts (condylomata acuminata, smooth papules, flat papules, keratotic warts); lesions on oral, upper respiratory, upper gastrointestinal, and ocular sites; recurrent respiratory papillomatosis
High-risk	16, 18, 31, 33, 45, 52, 58	High-grade cellular changes, anogenital (i.e., cervical, vulvar, vaginal, anal) and oropharyngeal cancer

HPV infection is common in the general population and also found in immune-compromised people. The virus is transmitted by direct contact with an infected tissue or through fomites. In general, the infection resolves spontaneously within 1–5 years. This chapter is focused on the pathogenesis of HPV infection and how it can be detected in the asymptomatic population.

To better understand the pathogenesis of HPV infection, one needs to be familiar with the structure and life cycle of the virus. This is also important so that we may know how and when to detect HPV.

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9.2 HPV: Structure and Life Cycle

9.2.1 Structure of Human Papillomavirus

It is a non-enveloped DNA virus, having an icosahedral outer shell composed of L1 and L2 capsid proteins and a single molecule of double-stranded, circular DNA as its genome. The HPV genome is functionally divided into three regions [7]:

1. A noncoding upstream regulatory region (URR) or long control region (LCR). This regulates DNA replication by controlling the transcription of the "early" and "late" regions through enhancer and silencer sequences.
2. An "early" region which includes the genes E1, E2, E3, E4, E5, E6, E7, and E8. The expression of these is important for determining whether an HPV infection would be active or latent or would undergo malignant transformation. The early region is involved in viral replication and oncogenesis.
3. A "late" region, which encodes the L1 and L2 structural proteins for the viral capsid.

The vital functions of these genes in the early and late regions in the HPV genome are outlined in Table 9.2.

Table 9.2 Functions of the HPV genome

Gene	Function
E1	Viral DNA replication
E2	Modulation of viral transcription, DNA replication, and segregation of viral genomes
E3, E8	Unknown
E4	Controlling virus maturation and release of virions (productive viral infections)
E5	Enhances transforming activity of E6/E7
E6	Oncoprotein; interaction with p53 protein
E7	Oncoprotein; interaction with pRB protein
L1	Major capsid protein
L2	Minor capsid protein

9.2.2 Life Cycle of HPV

HPV infection in humans has a predilection for the mucosa, e.g., genital HPV has a predilection for the genital mucosa and skin, and its replication is closely linked to the replication and differentiation process of the host cell. There is some evidence to indicate that due to the anatomical, histological, physiological, and immunological features of the transformation zone, this is a vulnerable site of entry of the HPV infection [8].

The life cycle of HPV can be divided into two phases [9]:

1. Maintenance phase: infection enters the body through sexual intercourse and begins as an infliction of the basal layer of the stratified squamous epithelium. It is presumed that the infection requires microtrauma or abrasion of the epithelium to enter. The basal layer of the stratified squamous epithelium contains stem cells, which divide periodically, with one daughter cell migrating upward to undergo terminal differentiation and the other remaining in the basal layer as a slow-cycling, self-renewing population [10]. Once the infection gains access to the basal layer, a productive infection begins, wherein the viral genome is maintained in the basal cells at a stable level with a low copy number. This is also the reservoir to develop a viral wart [11]. Viral DNA replication is supported by early HPV genes, and the cells infected with HPV can be sustained in the lesion for a long period. These infected daughter cells migrate upward to the surface, and viral late gene products (L1 and L2) are produced, which causes the viral DNA to be packaged into capsids, and progeny virions are released to reinitiate infection in sexual contacts. In benign lesions caused by HPV, viral DNA is located extrachromosomally (episomal DNA) in the nucleus.

   At this stage, though there is amplification of the viral genome, the viral DNA is yet to integrate in the host genome, and the infection

Table 9.3 HPV infection and its clinical correlates

Variables	hr HPV infection	hr HPV persistence	hr HPV persistence	hr HPV persistence
Type of HPV infection	Transient/latent hr HPV infection	Productive hr HPV infection	Transforming hr HPV infection	Transforming hr HPV infection
CIN (Richart)	Normal	CIN1	CIN2/CIN3	Invasive carcinoma
Dysplasia (WHO)	Normal	Mild-moderate dysplasia	Moderate-severe dysplasia, CIS	Invasive carcinoma
Cytology (Bethesda)	NILM	ASCUS-LSIL	HSIL	Invasive carcinoma

is a "productive infection," which cytologically and histologically corresponds to LSIL and CIN1, respectively [12]. As there is no cellular transformation in this vegetative phase of the HPV life cycle, the body can still clear this infection. This is outlined in Table 9.3.

2. Differentiation-dependent phase: in a small percentage of women with persistent HPV infection, the virus integrates with the host genome, leading to what is called a "transforming infection." Once the integration of HPV DNA is complete, it results in deletion and loss of expression of E2 region [13]. This interferes with the function of E2, which in the normal course of events is to downregulate the transcription of the E6 and E7 genes. This leads to an increased expression of E6 and E7 genes. The overexpression of early genes results in increased production of the E6 and E7 proteins. This results in increased proliferation of the squamous epithelium. This cytologically and histologically corresponds to HSIL and CIN2/3, respectively. This is depicted in Table 9.3. At a molecular level, the host cell machinery is used by the HPV to encode for viral proteins. Overexpression of viral early genes E6 and E7 in proliferating cells alters the viral life cycle [14]. The suprabasal differentiated cells reenter into S-phase of the cell cycle caused by the early proteins E6 and E7. This activates the host replication machinery needed for amplification of viral genomes for virion synthesis. There is a continuous mode of DNA replication, DNA amplification to high copy number, capsid synthesis, and viral assembly. The virus copy number increases from 50 to 200 copies to several thousands of copies per cell [9].

9.3 Pathogenesis of HPV Infection

Most sexually active men and women will acquire the infection at some point in their lives, but majority of them will clear the infection without manifesting any symptoms. At 12 months, 66% of all infections and at 24 months 90% of all infections are cleared by immune-competent individuals [15, 16]. This also means that 10% of the women will have a persistent infection that can predispose to cancer.

The incubation period of the infection, i.e., time from acquiring the infection to the development of clinical manifestations, can be anywhere from weeks to months for genital warts, months to years for cervical abnormalities, and years to decades for cervical cancer. This is a variable process continuing back and forth from clearance of HPV infection, persistence of HPV infection, progression to precancer, regression of precancer, and progression to invasive cancer based on the immune status and other risk factors of the patient (Fig. 9.1). Certain conditions like immunosuppression, older age, and multiple partners increase the risk of persistent infections. It is difficult to distinguish persistent HPV infection from clearance followed by reinfection, although reinfection with the same HPV type appears to be uncommon [9]. Also, when the levels

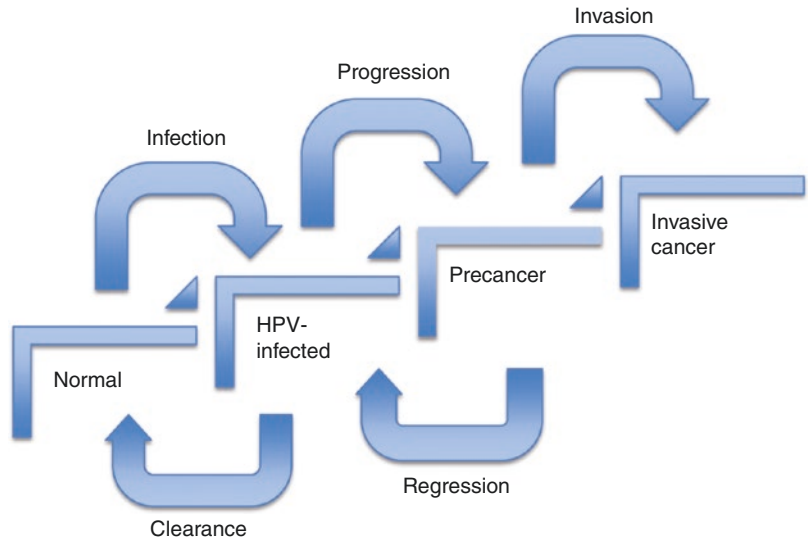

Fig. 9.1 HPV infection cycle

are below the threshold of detection of assays due to viral latency, the HPV infection may go undetected [17].

In precancerous changes due to persistent HPV infection, CIN1 and some CIN2 lesions show relatively low levels of E6 and E7 expression (in which the viral genome replicates episomally), whereas invasive cancer, CIN3, and some CIN2 lesions have high levels of E6 and E7 expression (due to integration of viral DNA into the host cell genome) [18].

The frequency of integration varies between the high-risk HPV types. For example, HPV 16, 18, and 45 are found more often in the integrated state compared to HPV 31 and 33. Because of this, precancers induced by the former HPV types progress to invasive cervical cancer much faster compared to the latter [19].

In addition, two features in the HPV life cycle indirectly contribute to carcinogenesis. First, replicative phase of HPV occurs in differentiated epithelial cells, which are not usually involved in DNA synthesis as they have exited the cell cycle [20]. This means that the viral DNA replication and assembly occur in a cell that will be terminally differentiated and die by natural causes. As a result, the virus lies dormant for many months to years, during which time, host defense mechanisms apparently remain unaware of the pathogen and the immune response generated is insufficient to eliminate the virus, thus developing persistent infection. As the terminal keratinocyte is already programmed to die, HPV replication and release do not cause cell death and inflammation and thus escape recognition. Also, due to the absence of viremia, cell lysis, necrosis, or any other inflammatory signals, there is an inadequate humoral immune response [21]. The viral infectious cycle is confined to the intraepithelial compartment, thus there is no viremia or spread through blood and lymphatics, and thus cellular immunity is not activated. But since they require the host machinery and cellular enzymes to replicate while at the same time maintaining differentiation, they require the help of E6 and E7 proteins. However, any disruption in this process can lead to immortalization of cells. Even so, integration is not a normal part of the HPV life cycle. Integration of high-risk HPV genomes represents a noteworthy event associated with progression from preneoplastic lesions to invasive cancer [22].

Second, the site of DNA replication within the epithelium in high- and low-risk HPV types differs. In low-risk HPV, DNA replication is initiated in the less differentiated cell population where elements of the cellular DNA replication machinery are still present. In high-risk HPV, this replication occurs in more differentiated cells and thus requires more forceful priming of the cell division machinery [20].

Thus, in a transforming infection, protein products of viral genes result in altered expression of cell cycle and DNA repair regulators, enhancing the oncogenic potential of the infection by immortalization, genomic instability, and malignant transformation. Essential to the pathogenesis of HPV is the role of oncoproteins E6 and E7 which are described below.

Role of E6 The E6 protein binds and degrades p53, which is a tumor-suppressor gene product, thus resulting in inhibition of apoptosis [23]. This anti-apoptotic activity of E6 is of critical significance in the development of cervical cancer, as this compromise in the process cellular DNA damage repair allows the accumulation of secondary mutations to go unchecked and predisposes to cancer [24]. The degradation of p53 or blocking of its function by E6 consequently inhibits the apoptotic signaling that would destroy the HPV-infected cell in the usual course of events. The E6 protein is able to disrupt both extrinsic and intrinsic pathways to facilitate a protective environment and prevent cell death.

Low-risk HPV E6 proteins do not bind p53 at detectable levels and have no effect on p53 stability in vitro. The high-risk HPV E6 in addition to binding p53 has another function that is important for immortalization. This is the ability to activate the expression of the catalytic subunit of telomerase (hTERT). Thus, the E6 protein is able to promote the maintenance of the telomere, through the action of telomerase [25].

Role of E7 The E7 protein drives cells into S-phase as it binds and inactivates the retinoblastoma protein (pRB), which is a tumor-suppressor protein, thus resulting in progression of the cell cycle [26]. This results in increased cellular DNA synthesis and cell proliferation. The E7 protein from low-risk HPV types has lower affinity to pRB. It also results in upregulation of p16 expression. These lead to uncontrolled cellular proliferation which is no longer controlled at G1/S transition.

Malignant progression occurs when additional mutations are accumulated over time, e.g., ras or fos genes. This is also why cervical cancer occurs many years after the initial HPV infection, indicating that although HPV infection is essential for initiation of the process, the culmination to cancer occurs only when host genome mutations accumulate over time [27].

Infection with HPV causes cellular growth, thereby increasing the demand for nutrients and oxygen. To overcome this, angiogenesis is induced by increased activity of hypoxia-inducible factor-1 (HIF-1) and its target genes. It is this activity which is responsible for persistence of infection with HPV [28].

The increase in cellular proliferation and genomic instability leads to increased amount of damaged DNA which cannot be repaired, thus transforming cells into malignancy. Supplementary to this chromosome instability, other potential mechanisms for carcinogenesis are methylation of viral and cellular DNA, telomerase activation, and immunogenetic factors. Both humoral and cellular responses can be elicited by oncoproteins E6 and E7, and so they can play an important role in therapeutics [29].

Supplementary Role of E5 HPV infection also leads to formation of tetraploid cells by inducing cell fusion and failure of cytokinesis, thus causing aneuploidy [15]. Tetraploid cells formed by accident cannot undergo normal mitosis and thus are unresponsive to p53-induced apoptosis. This chromosomal instability favors integration of HPV genomes, further leading to generation of viral-cellular fusion transcripts and expression of the E6-E7 genes. Thus, the role of E5-induced cell fusion is in the early stage of development of HPV-associated cervical cancer, rather than tumor maintenance [30].

The endogenous interferon response, which is important in activating the innate immunity, is inhibited by infection with high-risk HPV. In the absence of this, the adaptive immunity is also not activated, effectively creating an HPV antigen-tolerant milieu. There is decreased expression of pro-inflammatory cytokines (IL-1, IL-6, TNF-α, and TGF-β) and increased expression of anti-inflammatory cytokine (IL-10) [31]. Development

of HPV-specific T-cell response is also suppressed due to the downregulation of major histocompatibility complex (MHC) I expression [32].

9.4 Detection of HPV Infection

After acquiring the infection, it can be detected by the commercially available tests for a period of up to 1 year [33]. More than 90% of cervical and anal cancers are caused by HPV. Approximately 70% of vulvar, vaginal, and oropharyngeal cancers are also linked to infection with HPV [34].

HPV infection is diagnosed using clinical (warts) and molecular evidence of infection. Immunological evidence of HPV infection is difficult due to the innate life cycle of HPV wherein late (capsid) proteins are only expressed in productive infections and early proteins are expressed in low amounts in infected tissues and lack of a robust antibody response to the viral infection.

Since the HPV cannot be grown in tissue culture, its detection is dependent on the detection of viral nucleic acids (DNA or RNA) using molecular techniques like nucleic acid probe technology and DNA sequencing. In addition to knowing whether high-risk HPV (hr HPV) infection is present, there is also an increasing need to know the type of hr HPV infection (genotyping). The potential advantages of genotyping are:

1. Certain HPV types are more linked to certain cancers, e.g., HPV 18 to adenocarcinoma (AC) and adenocarcinoma in situ (AIS) and HPV 16 to both squamous cell carcinoma (SCC) and AC [35].
2. Some infections have multiple HPV genotypes, and knowing the proportion of each is sometimes relevant.
3. Genotyping is crucial after rolling out a vaccination program to know the efficacy of prophylactic vaccines in reducing the prevalence of infection types covered by the vaccine.
4. In the clinical scenario, HPV genotyping may have prognostic significance in monitoring the response to treatment (LEEP/cryotherapy). The same strain of HPV in the posttreatment follow-up smear as in the pretreatment sample may indicate inadequate removal or inability of the body to clear the infection (more severe consequences), while a different strain may indicate reinfection (less severe consequences).
5. Multiple HPV genotypes are found in about a third of the HPV-infected patients and in half of those with HIV positivity [36]. Multiple genotypes are less common in those with carcinoma.

There are three main types of detection methods currently in use—non-amplified hybridization assays, signal-amplification techniques, and target amplification techniques [37]. These are discussed in detail below and outlined in Table 9.4.

I. Non-Amplified Hybridization Assays These include Southern blot for DNA, Northern blot for RNA, dot blot hybridization, and in situ hybridization (ISH). There are some disadvantages with the first three of these methods that is why they are not commonly used. Mainly, it is the requirement of large amounts of purified DNA to perform analysis, labor-intensive process, poorly reproducible and having only moderate sensitivity [38]. They cannot be run on fixed tissues where the DNA has degraded. ISH, on the other hand, can be run on processed and fixed tissues. It identifies specific

Table 9.4 Types of HPV tests

Technique of HPV testing	Commercially available tests
Non-amplified hybridization assay	Southern blot, Northern blot, Dot blot, In situ hybridization
Signal-amplification assay	HC2 (and care HPV), Cervista HPV
Nucleic acid amplification assay	Amplicor, PapilloCheck microarray, Clinical arrays HPV test, INNO-LiPA, Linear Array HPV genotyping, Luminex microarray (MCHA), real-time PCR (Abbott RT-PCR, Cobas 4800, GenoID), genome sequencing, CLART HPV 2, HPV E6/E7 mRNA (PreTect Proofer, NucliSENS EasyQ HPV, APTIMA HPV)

nucleotide sequences with conserved morphology in cell or tissue sections, thereby determining the spatial location of target genomes in the specimen [39]. The sensitivity of ISH can be improved by combining it with PCR (known as in situ PCR) [40]. ISH can be used to detect mRNA as a marker of gene expression [41].

II. Signal-Amplification Techniques These can be liquid phase or morphological techniques and are based on the technique of amplification of signals generated by DNA/RNA hybrids and DNA in situ hybridization, respectively. These are non-PCR-based tests. Two commonly used tests are HC2 and Cervista HPV.

a. Hybrid Capture (HC2®) (Qiagen, USA, *formerly Digene Co.*): It is FDA approved and detects DNA from 13 hr HPV to 5 lr HPV types at sensitivity of 5000 copies of HPV genome per reaction well using the technique of chemiluminescence to detect RNA/DNA hybrids [42]. It uses RNA probes complimentary to the genomic sequence of 13 hr HPV and 5 lr HPV types, which are used to prepare high- and low-probe cocktails. DNA present in the specimen is hybridized with each of the probe cocktails leading to the formation of RNA/DNA hybrids. These bind to the antibodies in the reaction well that are programmed to bind the RNA/DNA hybrids. The final detection is using a luminometer to detect the intensity of emitted light by the hybrid product. The amount of light (in relative light units—RLU) gives an indirect/semiquantitative measurement of the amount of hr HPV DNA present in the specimen. Usually only the high-probe mix is used (as it more clinically relevant) which reduces the cost of the test. More than or equal to 1 RLU (which equals 1 pg of DNA per ml of buffer) is considered a positive test. It is performed in a 96-well microtiter plate, which can run many automated samples in one go. Another advantage is that it is not susceptible to cross-contamination, as it does not use PCR to amplify the DNA [43]. This assay distinguishes between hr HPV and lr HPV but is not able to tell the specific type of HPV (genotyping). Genotyping is important, as the risk of high-grade changes is 10–15% with HPV 16/18 and less than 3% for all other hr HPV types combined [44].

   A recently developed low-cost assay, for use in low-resource countries (*careHPV*®, Qiagen NV), uses RNA probes (like HC2) to detect 14 hr HPV types. It is a rapid point-of-care test that takes about 2.5 h to perform and does not require specialized laboratory or staff. It was clinically validated in a large study in rural China and found to have an accuracy of 90% in detecting premalignant lesions [45].

   To reduce cross-reactivity, an automated third-generation Hybrid Capture assay has been introduced recently. It is based on biotin-labeled oligonucleotide sequences for capture of target regions [46].

b. Cervista® HPV (Hologic Inc., Marlborough, USA) detects the presence of 14 hr HPV types (Cervista® HPV HR and Genfind® DNA Extraction) with or without individual genotyping for HPV 16/18 (Cervista® HPV 16/18). It is more sensitive than HC2 and has a lesser false-positive rate [47]. It is also FDA approved.

III. Target Amplification Techniques These are PCR-based assays that amplify the target segments of HPV DNA and aid detection of hr HPV types and specific genotypes. This assay relies on a thermostable DNA polymerase, type-specific or general/consensus primers, which bind to and replicate the area of interest. After several cycles of replication, the viral DNA is amplified sufficiently in vitro to allow it to be visualized. This amplification can be real time (which can help to quantify viral load) using reverse hybridization techniques. Theoretically, PCR can detect a single copy of the target sequence in the reaction tube as it is highly sensitive and specific. In practice, the sensitivity is 100–1000 genome equivalents per reaction tube (i.e., 10–100 HPV genomes in a background of 100 ng of cellular DNA). Due to this PCR can be performed even in specimens that have a low DNA content. PCR has the advantage of generating one billion copies

after just 30 cycles of replication, from a single double-stranded DNA molecule [38]. Detection of HPV using PCR can be done using primers. Primers can be of three types:

1. Type-specific primers (which will amplify specific HPV genotypes): they are more expensive and time-consuming than using consensus primers.
2. General/consensus primers: these are based on the conserved regions common to all HPV genotypes, generally the long control region L1 or E6/E7 regions [48, 49]. Commonly used consensus primers are GP5/6 pair, and its extended set GP5+/6+ pair aimed at L1 region and MY09/11 degenerate primers and its modified version, PGMY09/11 [50]. The disadvantage of using degenerate primers like MY09/11 is a large batch-to-batch variation and poor reproducibility [51]. The modified primers, PGMY09/11, have more consistency and better sensitivity for a large number of HPV genotypes [46].
3. Combined primers: contain inosine which matches with any nucleotide and can target the location of the viral genome with accuracy and reproducibility.

In any PCR reaction, the efficiency is inversely proportional to the size of the amplicon or PCR product. The smaller the size of amplicon, the more is the efficiency of the PCR, especially when the DNA sample is degraded or low in quantity [52]. In addition, the sensitivity and specificity of the individual PCR-based method depend on the primer set, reaction conditions, function of DNA polymerase, HPV types amplified, and the ability to detect multiple or specific types of HPV. To reduce the false-positive rates from DNA contamination, strict PCR protocols need to be followed. Once the PCR is run and the products are amplified, they can be analyzed by restriction fragment length polymorphism (RFLP) and plated on agarose gel [53]. Another way is to use type-specific probes using enzyme immune assay (EIA). The PCR product is hybridized onto the chip and read using a DNA chip scanner. This has a high sensitivity (more than 90%) and can be used to identify the type of HPV as well as multiple infections [54]. However, the disadvantages are the false-negative results if multiple subtypes of HPV are present in the sample with low copy number. Because multiple infections are common, PCR may not detect all the HPV genotypes [55]. To overcome this problem, a simple technique of PCR-RFLP can allow the HPV to be genotyped, at a lower cost and effort [56]. It can also detect multiple HPV types in the same infection and differentiate them into lr HPV and hr HPV. Amplicor® HPV MWP assay (Roche Molecular Systems) was the first commercially available PCR kit using nondegenerate primers, which detected 13 hr HPV without individual genotyping by amplifying a short fragment (170 bp) of the L1 gene using primers. It is more sensitive than HC2 and amenable to high-throughput testing [57].

In addition to DNA, viral mRNA can also be amplified using reverse transcriptase PCR or nucleic acid sequence-based amplification (NASBA). For example, detection of E6/E7 mRNA from the hr HPV types can indicate high specificity for detection of high-grade lesion. One such commercially available assay is the APTIMA® HPV assay, which identifies mRNA from 14 hr HPV types [58]. The PreTect HPV-Proofer (NorChip AS, Norway) uses NASBA amplification of E6/E7 mRNA prior to type-specific detection of 5 hr HPV types.

Various target amplification techniques are:

a. PapilloCheck® microarray analysis: It uses a DNA chip and allows for parallel analysis of multiple DNA samples. Currently, the main role of microarray analysis is in gene expression profiling and mutation analysis [59]. PapilloCheck® is a commercially available microarray chip which detects 24 genotypes of high- and low-risk HPV using a chip scanner. Its advantage is identification of hr HPV and lr HPV for screening [60]. However, it cannot detect HPV types 35 and 53 and is expensive to run [61].
b. Clinical Array Technology (CLART)® HPV kit (Genomica SAU, Spain): It is a commercially available test kit, which uses the human

cystic fibrosis transmembrane conductance regulator (CFTR) gene plasmids to check the PCR method and DNA integrity, detecting 35 hr HPV types [62].

Both these methods are based on a prior target sequence amplification by PCR followed by hybridization using labeled type-specific oligonucleotide probes fixed on a chip/slide or solid support. After DNA hybridization, an automatic detection system can be used to determine the possible presence of up to 42 different HPV genotypes [46].

c. INNO-LiPA® (LiPA HBV GT; Innogenetics NV, Ghent, Belgium): In this test part of the L1 region of the HPV genome is amplified using SPF10 primers. This short PCR fragment (SPF-PCR) is designed to discriminate between a broad spectrum of HPVs in a reverse line blot hybridization (LiPA) which is interpreted visually. The position of the blot is related to the HPV genotype. The INNO-LiPA HPV Genotyping v2® test detects 11 hr HPV and 5 lr HPV, while Genotyping Extra® can detect 22 hr HPV and 6 lr HPV types [63]. It can be used on cervical swab specimens but is less efficacious than real-time PCR [64].
d. Linear Array® HPV Genotyping (Roche Molecular Diagnostics, Pleasanton, USA): It is a PCR-based assay using PGMY09/11 amplification system coupled with reverse line blot hybridization. It can detect 37 HPV types including the 15 most common hr HPV types. This is done using the Auto-LiPA instrument (Innogenetics, Ghent, Belgium), which uses colored signals on strips than can easily be interpreted as per the Linear Array® reference guide [65].
e. Microplate colorimetric hybridization assay® (MCHA) (Boehringer Mannheim, Germany)/ Luminex microarray technology: It allows high-throughput, simultaneous identification and quantification of 6 hr HPV types using PCR-based technology and colorimetric hybridization with type-specific probes attached to dyed polystyrene beads. These are then passed through a Luminex analyzer in order to determine the spectral signatures indicative of specific HPV genotypes [66]. It has a high analytical sensitivity, specificity, and reproducibility for identifying HPV 16/18 as well as 31/33/45 and somewhat less for HPV 39. Probes for other HPV types can be added [67].
f. Real-time PCR: "In-house" real-time PCR can detect viral load as well as HPV genotypes from a very small concentration of nucleic acids using fluorescence to detect the HPV. It can simultaneously amplify different nucleic acid targets. It can be run on both tissue samples and cellular slides. It is rapid, reliable, sensitive, specific, and validated for use for screening in high-throughput testing [68]. Examples of real-time PCR tests are:
   – Abbott® RT-PCR, which detects HPV 16/18 as well as other 12 high-risk genotypes (pooled) [69]. It serves a dual diagnostic purpose of hr HPV screening and viral genotyping in the same test.
   – Cobas® 4800 (Roche Molecular Systems, Pleasanton, USA) HPV test also uses real-time PCR to detect 14 hr HPV types from a single sample. It detects HPV 16, HPV 18, and HPV 12 pooled hr HPV types [70]. It is easy to use and gives rapid results (within 4 h), making it suitable for screening. It is reliable and clinically validated to detect hr HPV and for ASCUS triage [71]. It is FDA approved.
   – GenoID® real-time PCR assay, which amplifies the L1 region of HPV and detects non-integrated copies of HPV. It detects 15 hr HPV and 6 lr HPV with a sensitivity of 100 infected cells [72].
g. HPV genome sequencing: There are two techniques for this. The first is the traditional Sanger technique [73] and its subsequent modification using fluorescent dyes (both of which have not been clinically validated), and the second is using pyrosequencing. This technique can be applied to any source of DNA or RNA that can be amplified by PCR, be it fresh or formalin-fixed. This latter method has several advantages over the former. It is easy to decipher readout in real time, inexpensive, rapid, and quantitative [74].

h. CLART® Human Papillomavirus 2 (Genomica, Madrid, Spain): It can detect 35 HPV DNA types in a semiquantitative manner using PCR technology. It is a highly sensitive (98%) and specific test (nearly 100%) [75].
i. HPV E6/E7 mRNA: Because of the role of E6/E7 mRNA in cervical carcinogenesis, their detection has a stronger correlation with cervical disease than detection of HPV DNA alone [76]. There are three commercially available assays:
   – PreTect® HPV-Proofer assay (NorChip AS, Norway): It has high sensitivity and specificity and is based on real-time PCR (real-time nucleic acid sequence-based amplification assay (NASBA)) [77]. It detects E6/E7 mRNA from 5 hr HPV types (HPV 16/18/31/33/45).
   – NucliSENS® EasyQ HPV (bioMerieux): It also detects E6/E7 mRNA from 5 hr HPV types (HPV 16/18/31/33/45) [78].
   – APTIMA® HPV assay (Gen-Probe, San Diego, USA) which detects HPV E6/E7 mRNA from 14 hr HPV types. Thus it is more sensitive than PreTect Proofer assay [79]. In addition, it is fully automated, does not cross react with lr HPV, has lower limits of detection, and can predict advancement of disease more accurately. It is FDA approved.

Detecting E6/E7 mRNA has been found to be more specific in detecting individuals that develop high-grade disease rather than HPV DNA detection by PCR with GP5+/6+ consensus primers [80]. There is a significant association between E6/E7 oncogene transcripts and severity of disease on cytology and histology, for both HPV 16 and 18. Also, this identifies persistent hr HPV infections without having to perform repeated testing [81].

In addition, one can determine the HPV DNA viral load and markers for HPV DNA integration as markers for HPV infection.

1. *HPV DNA Viral Load*: It can be determined using quantitative real-time PCR and semi-quantitatively using HC2. It rises as the severity of disease on cytology and histology worsens [82]. The best correlation is seen with HPV 16 infection [83]. There is a positive correlation of HPV 16 and 31 DNA load with severity of disease and a fair to poor correlation of HPV 18 and 33 DNA with severity of disease [84]. Viral load declines with therapy and can guide further management. High viral load production occurs in severe disease but is the effect rather than the cause [85].
2. *HPV DNA Integration*: In the initial phase of infection, HPV DNA lies extrachromosomally in the cell. However, during the process of integration, it becomes intrachromosomal. This causes changes in the expression of several viral genes, all toward one aim of persistence and dissemination of infection [86]. Viral integration occurs earlier than even morphological changes, such that viral integration may not always correspond to high-grade lesion as the molecular events predate clinical events [87]. HPV integration is detected using real-time PCR, which can calculate the ratio of E2 and E6/E7 genes. A 1:1 ratio indicates integration [88]. Other methods are FISH and PCR. In the former fluorescent probes are used for TERC gene and MYC gene loci [89]. One of the key functions of E6 is activating telomerase reverse transcriptase (TERT) gene expression on chromosome 5p. Telomerase is located at the ends of chromosomes. It has a structural RNA component (TERC) that serves as a template during telomere elongation and a catalytic subunit (hTERT) that has reverse transcriptase activity. Its purpose is stabilizing and repairing repeated DNA sequences at telomere end of chromosomes [90]. Telomerase can be detected using quantitative RT-PCR. Increase in the catalytic subunit activity is seen in cervical carcinogenesis [91]. PCR can also be used to detect pure integrated DNA, using type-specific E1 and E2 primers but without the site of integration [92].

A protocol for the amplification of papillomavirus oncogene transcripts (APOT) from cervical specimens can allow differentiation of episomal DNA from integrated DNA. In normal cells and early dysplasia, the HPV genomes are episomal,

while in advanced dysplasia and invasive cancer, they are integrated into the host cells. Using APOT, a strong correlation between detection of integrated high-risk HPV transcripts and presence of high-grade disease was seen [93]. However, because this technique tests for RNA, and RNA is more labile than DNA, the time and type of specimen storage also influence the results and amount of RNA available for analysis. Collection media that can preserve both RNA and DNA integrities, such as methanol-based liquid media, are preferred [94].

9.5 Which Test to Choose?

The ideal test should have high sensitivity to detect all infections as well as good specificity to avoid unnecessary anxiety to all women who have screened positive. The first is achieved by having a PCR-based test, which has a high sensitivity, and using amplification primers which can detect all variations and integrations in the hr HPV genome and specifically the E6/E7 region which is the most preserved [95]. Low specificity of such testing is then overcome by using secondary triage with E6/E7 mRNA analysis or HPV viral load quantification. In an epidemiological study to know prevalence of HPV or efficacy of vaccination, tests with high sensitivity are used, as the offset in specificity is not important in these settings.

In general, there is good correlation between HC2 and PCR using consensus primers. While testing for more than one HPV type in the biologic specimen is preferentially done by PCR-based method, HC2 is more accurate in HPV detection as low- or high-risk groups and does not distinguish between individual HPV types. Both are suitable to high-throughput testing and automation.

9.6 Samples for HPV Detection

For target amplification methods, which are PCR based, the specimen needs to be such where the nucleic acids are not degraded or exposed to PCR-inhibiting organic solvents. In the LBC media, preservation of nucleic acids occurs, and media like the Universal Collection Medium (UCM) by Qiagen (Digene) do not contain large amounts of organic solvents. Formalin-fixed and paraffin-embedded specimens may have nuclear degradation but no exposure to PCR-inhibiting substances.

For detecting mRNA, special media that preserve the integrity of mRNA as well as DNA (PreservCyt LBC) are needed so that mRNA can be analyzed through RT-PCR and NASBA. Also, mRNA can be detected on biopsy specimens and extracted through special techniques.

For signal-amplification methods, e.g., HC2, the specimen can be both cervical smears and biopsy specimens transported in Specimen Transport Medium (STM) by Qiagen (Digene). This medium destroys cell morphology and causes release of nucleic acids without the use of additional solvents [96]. Cervical smears can also be collected in ThinPrep Medium by Cytyc. This medium preserves cell integrity and requires additional solvents to cause cell lysis and release of DNA. A middle ground between the two is UCM that has the advantages of both. Leftover fluid after preparing an LBC slide can also be used to detect HPV DNA using HC2.

Although the analytic sensitivity of some HPV detection tests is very high (thus useful to know prevalence of HPV infection), its corresponding clinical significance is not. This is because all HPV infections may not persist or lead to clinically relevant disease [97].

Detection of HPV has certain advantages as compared to other methods of screening. First, its high sensitivity and negative predictive value means that very frequent screening can be avoided. Second, detection of HPV DNA in a woman over the age of 30 years puts her at higher risk of developing CIN, and thus appropriate management can be instituted early. Third, the testing itself is objective and automated [98].

9.7 Conclusion

Thus, a deep understanding of the life cycle of HPV and its role in cervical carcinogenesis is important to understand the advantages and limitations of detection methods for HPV. Despite

several tests being available, there is no gold standard molecular test. There is a need for testing to become more rapid, automated, low-cost, and easily accessible in all resource situations.

Key Points

- Human papillomavirus (HPV) is one of the most common sexually transmitted infections with more than 150 subtypes, subdivided into low-risk (non-oncogenic) and high-risk (oncogenic) types.
- Low-risk HPV 6/11 cause majority of genital warts; high-risk HPV 16/18 cause 70% of all HPV-associated cervical cancers, with 31/33/45/52/58 accounting for another 10%.
- Most HPV infections, whether low-risk or high-risk, are asymptomatic and transient and resolve spontaneously without any clinical sequelae.
- In persistent infection, unique characteristics of the HPV genome make it a suitable pathogen to infect and transmit disease.
- E6 and E7 protein expression plays a major role in carcinogenesis induced by persistent HPV infection.
- In early precancer (CIN1), the viral genome is episomal (extrachromosomal), while in late precancer (CIN2/3) and invasive cancer, the viral DNA integrates into the host cell genome.
- HPV cannot be grown in culture; hence detection relies on molecular methods.
- Two basic techniques in use are signal amplification (HC2) and target amplification (PCR based).
- Several tests are available but only five are US FDA approved. These are *Digene Hybrid Capture® 2*, QIAGEN (hr HPV and lr HPV); *Cervista® HPV HR* and Genfind® DNA Extraction Hologic (14 hr HPV types); *Cervista® HPV 16/18 Hologic* (HPV 16/18); *Cobas® 4800*, *Roche* (14 hr HPV, specifically HPV 16/18); and *APTIMA®*, *Gen-Probe*, Hologic (E6/E7 mRNA of 14 hr. HPV types).
- In a clinical setting, tests with high sensitivity and good specificity are preferred, with secondary triage by a test which has high specificity. In a population-based/epidemiological setting, tests with high sensitivity are adequate.

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