HPV Infection and Detection

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Abstract. Human papillomavirus (HPV) can be transmitted by sexual activity and direct skin-to-skin contact. The virus accounts for more than 90% of cervical cancer cases. Target amplification and signal amplification are two detection methods for HPV diagnosis. COBAS 4800 and the Hybrid Capture (HC2) system are two examples of target amplification and signal amplification techniques, respectively. According to the cohort study done in Hong Kong, detection assays have better concordance with samples in the more severe lesion. While COBAS 4800 has greater specificity in targeting high-grade lesions, HC2 has better sensitivity. This review aims to discuss HPV infection, and detection techniques and point out possible improvements.

Keywords: Human papillomavirus; infection; detection.

1. Introduction

From the *Papillomaviridae* family, human papillomavirus or HPV is small in size. The viral genome consists of double-stranded DNA [1]. Above 80% of females and 90% of sexually active males are suspected to be infected with HPV of any type at least once during their lives. While the virus is mainly transmitted between individuals via sexual transmission, people of all ages can also be infected through direct contact of skin or mucosa with HPV-positive individuals [2]. However, the study reported that the body's immune system can eliminate 66% of the virus in 12 months, and the percentage raises to 90% within two years [3]. Although not all people infected by HPV will show the symptom or any clinical signs, and the human immune system has the ability to expel the virus within months or years, the risk of having related diseases like cervical cancer increases with daily habits such as smoking [4]. Individuals with low-risk HPVs (LR-HPVs) are likely to develop cutaneous or anogenital warts, and high-risk HPVs (HR-HPVs) might cause anogenital or oropharyngeal cancers. According to the investigation, HPV leads to more than 90% of cervical cancer cases [1]. In 2020, the number of new cervical cancer was found in nearly 604,000 people, and the number of death was about 342,000 cases around the world. It makes the virus to be the most frequent pathogen account for female cancers [1, 5]. Therefore, a regular, sensitive detection of susceptible populations or people at high risk of infecting HPV is necessary for finding potential carriers and thus controlling the transmission. This review is going to discuss HPV infection, related diseases, and HPV diagnosis technologies and suggest improvements to existing detection methods.

2. The mechanism of HPV infection

2.1. HPV structure

The general diameter of the papillomavirus is about 52 to 55 nm, and its genome is composed of double-stranded DNA circulating inside a non-enveloped icosahedral capsid. HPV genome has 8,000 base pairs, and it is divided into three parts. These parts are distinguished into early, late, and non-coding regions. The early region consists of E1-6 early genes while the late region contains L1-2 late genes. Besides, the non-coding region can be named as non-coding long control region or upstream regulatory region (LCR or URR). The enhancer and promotor DNA sequences responsible for controlling viral and cellular genes transcription and replication can be found in the LCR, the region located between E and L. E and L regions are also the only coding regions in HPV genome where the opening reading frames (ORFs) can be found [6, 7].

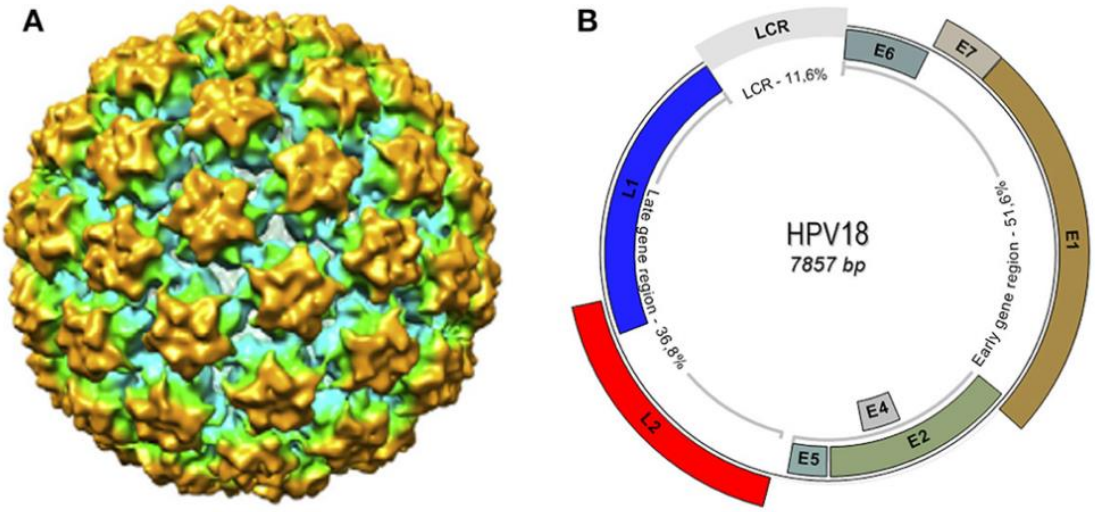

Figure 1. HPV structure (A) The reconstruction of HPV 18 with the cryo-electron microscopy; (B) HPV 18 genome (adapted from Kombe Kombe et al. 2021) [1]

Most of the region in the HPV genome encodes a specific protein that has a crucial role in interfering with the host cell's replication cycle and establishing HPV infection. Proteins encoded by the E region are essential participants in viral replication, whereas proteins encoded by the L region are critical in virion assembly. The E1 gene is responsible for producing the protein that is used to start or prepare for viral genome replication by the host's cellular DNA replication. In HPV genotypes 6, 11, and 16, protein E2 cooperates with E1 for initiating and regulating viral replication and transcription. Despite viral DNA replication, protein E2 plays a pivotal role in promoting or preventing transformation and apoptosis of the host cell since inactivating E2 will promote E6 and E7 gene expression, and thus it facilitates the replication of infected cells along with the viral DNA sequence and avoid the cell apoptosis. E4 protein can distort the shape of keratin as it mostly appeared later in the viral cycle when viral DNA has been amplified extensively. The protein coded by E5 also takes place in viral genome replication but also helps the virus to stay away from the host's immune system. Protein E6 will interact with the cell's protein p53 while E7 will interact with retinoblastoma (pRb), which triggers p53's proteolysis and pRb's inactivation. The proteolysis and inactivation of p53 and pRb mark the invasion of the cell, inhibiting the cell's normal replication cycle. Major capsid proteins are encoded in gene sequence L1, and minor capsid proteins are encoded in the L2 region. Since E6 and E7 mRNAs are present throughout disease or cancer development, the DNA test targeting E6 or E7 mRNA is a possible way to show the infection status but hard to distinguish the lesion of progression from regression [8].

2.2. HPV infection

HPV has an unusual mechanism in that its replication is restricted in keratinocytes found in stratified epithelium including both the skin's outermost layer and certain mucosal membranes. In the surface tissue, keratinocytes will undergo the process named cell differentiation in which they are specialized and change from flattened stem cells to the hard barrier on the surface, which provides protection away from pathogen invasion and unfavorable water outflow. The HPV infection cycle is firmly based on the cell differentiation of keratinocytes, and less-differentiated basal keratinocytes are the primary targets of the virus at the beginning. Therefore, when the part of the basement membrane is exposed to epithelial wounds or surface abrasions, there is a chance for HPV to invade stratified epithelium tissue [9]. After reaching keratinocyte stem cells through microtraumas, HPV attaches to the basal cell as L1 protein interacts with the cell's extracellular receptor on the surface. The specificity of this interaction is mainly determined by L1, but the prior study suggested that the receptor HPV bound to is highly conserved and extensively located on the surface of the cell. Although the detailed order of HPV entry is not fully revealed yet, most of the investigation pointed

out that heparan sulfate, the glycosaminoglycan, plays a role in HPV's primary attachment in the extracellular matrix [10]. Also, lamim-5 is another possible receptor for HPV entry as it has a higher affinity with the virus compared to heparan sulfate. On the other hand, the alpha-6-integrin receptor on the plasma membrane is considered to be the secondary receptor that also plays a role in virus penetration following heparan sulfate proteoglycans (HSPG). After interactions between capsid proteins and HSPG, the virus experiences proteolytic cleavage after the conformational change; the change also allows the virus to bind to the secondary receptor and hardly to the primary receptor, triggering internalization in the end [11].

Afterward, to establish the viral infection, HPV is transported into the intracellular space within a membrane-closed vesicle of endosomes. The virion is likely to be engulfed by the cell via clathrin- or caveolin-mediated endocytosis; it is the route frequently applied by non-enveloped viruses [12]. The viral uncoating can hardly be found within 8 to 15 hours after the initial interaction between the virus and cell [9]. L2 capsid protein helps viral genome release by breaking the endosomal membrane with the cationic cell-penetrating peptide [13]. The viral DNA excluding the L1-only capsids genome will go through the host's other cellular components including the Golgi apparatus and endoplasmic reticulum. Eventually, it reaches to cell's nucleus and establishes viral genome replication [9, 12].

After trafficking, plasmid, vegetative, and productive replication are three possible methods for virus reproduction. The plasmid replication contains two stages: amplification and maintenance stages. The amplification includes 50-400 diploid genomes. Later, successive progeny groups have an unchanged number of couples in the maintenance stage. In vegetative replication, the HPV gene expression initiates as keratinocyte stem cells differentiate from the basal membrane. But at the beginning of the infection cycle, viral DNA in the form of extra-chromosomal fragments experiences episomal replication, and few viral copies are made to render the host cell recognizable from the normal cell. Gene expressions of L1 and L2 only appeared after massive viral genomes are produced when basal keratinocytes become mature, forming the intermediate or outmost layer of the stratified epithelium; the new HPV formation completes as the cell differentiation process [6]. The replication of capsid proteins L1 and L2 is suspected to avoid the immune response by the host's immune system since the superficial layer has comparatively less inspection from the system [14]. Unlike other non-enveloped viruses that also infect animals, HPV applies the mechanism of desquamation as the way to release progeny viruses produced instead of killing the host cell through the lytic cycle; the desquamation without significant inflammatory response is relatively more obscure, which also marks the completion of reproductive replication [6].

3. HPV related diseases

After viral infection, not all individuals will express obvious symptoms. The type of HPV is determined by the sequence of the viral capsid, which mainly consists the major capsid protein L1. About 200 HPV types are identified; different HPV types are usually responsible for different diseases, and many of them are carcinogenic while others are able to develop warts around the human body [7, 15].

3.1. Wart

Cutaneous warts are a well-known symptom caused by HPV since Greek and Roman times. In general, most of the warts are benign. The lesion is usually associated with a complete dermis layer's hypertrophy. The skin thickens, folds, and increases the horny layer, and the condition further develops into acanthosis, papillomatosis, and hyperkeratosis. In the end, part of the skin loses its original, flattened shape. Warts can be spread in both direct and indirect ways as individual contact with the skin of another HPV-negative person or objects that have previously been contaminated by an infected person. The infection in the oral region is possible via the transmission or autoinoculation from one's finger or knees, which is common among children playing contact games. After the treatment, the reappearance of lesions is still possible, and it is mostly caused by the remaining virus

besides old warts. The immunological control of skin warts is commonly associated with the functional humoral or cell-mediated immune system. Cytotoxic T cells and NK cells play key roles in disease regression. The immune protection is type-specific, and the later infection with the same type of HPV will awake the adaptive immune response and antibodies to work against the reinfection [15].

Common warts can be found in various parts of the body but are mostly concentrated on the hand, finger, foot, elbow, and knee. Warts in these regions tend to be out-growing, making a part of the skin to be outstanding from the nearby surface. They grow in multiple numbers of nodules with a hard surface, and their shapes are not regular but vary from one region to another. Some small warts look like papules while other large ones with hyperkeratotic and fissured lesions have cauliflower-like characteristics. HPV 2 and HPV 4 are mainly contributed to the development of common warts whereas small warts are usually caused by HPV 4 [15].

Condylomata acuminata and venereal warts are commonly known as genital and anal warts, which are also marks of genital HPV infection. Anogenital warts are less concentrated with a cauliflower-like shape located on mucosal surfaces. The wart's size varies from small papule to large lesion while its color can be white, pink, brown, or red [15, 16]. Pregnant women with anogenital warts will have a lesion that grows as the length of pregnancy increases, but after delivery, warts usually enter the regression period [15]. The anogenital warts are typically spread among people through sexual transmission, and 90% of anogenital warts are contributed to LR-HPV 6 and HPV 11 [17]. People with anogenital warts might suffer from itching, fissuring, and bleeding. Most genital warts can recover in 6 months both with or without medicine and clinical treatment, but recurrence does happen which might weaken the further treatment [15, 16]. The infection may be asymptomatic, but people without any symptoms may transmit the virus to their partners. Most people are suspected to be infected at once, especially when people start to involve in sexual behaviors. Since HPV types associated with warts are different from cancers, having genital warts does not indicate the urgent requirement for cervical cancer screening [16].

3.2. Cancer

Unlike warts that can be identified through physical observation, cancers usually require more in-depth detection like molecular diagnosis and pap test for cervical cancer [18]. The study proved that HPV nucleic acid sequence can be found in about 99% cervical cancer cases while 70% of global cases of cervical cancer are caused by HR-HPV 16 and 18. In a precancerous state, the detection using a colposcope is necessary to see the cervical lesion clearly by enlarging the image of epithelium tissue. The degree of the lesion can be classified by the extension or the burden of disorganization of epithelium from the superficial to the normal surface. When the region of uncontrollable cells grows above the epithelium surface but its length is lower than one-third of the basal epithelium, it is considered cervical intraepithelial neoplasia grade 1 (CIN1); if the size further extends to about two-thirds and greater than two thirds, it becomes the stage of CIN2 and CIN3, and the lesion is the instant precursor of cervical cancer. The lesion eventually develops into cervical cancer when unregulated cells grow invasively, disrupt the basal membrane, and bring the infection to the dermis [15]. As the lesion develops into CIN2 and 3, there is only a 40% and 33% chance for the infection to be eliminated by itself. Therefore, immediate treatment and regular cervical checkups are important to suppress the lesion and prevent it from developing into cancers [16].

4. HPV detection methods

The cultivation of HPV is limited, so the detection of HPV infection mostly relies on molecular biology detections. Although clinical and serological detections are able to find the presence of the virus in a given sample, techniques targeting viral nucleic acids provide a more accurate and type-specific result [19, 20]. Target and signal amplification are two parts of molecular-biology techniques for HPV diagnosis [19].

4.1. Target amplification

The sample used for cytology can also be applied to HPV DNA detection, but its size is usually small, and only a fraction of the sample DNA is extracted for further diagnosis. Polymerase chain reaction (PCR) is one common method used for amplifying nucleic acid. Generally, the heat-resistant DNA polymerase targets desired pair of oligonucleotide primers. After 30 rounds of the amplification cycle, a DNA molecule fragment can be expanded into a billion DNA molecules. GP5+, GP6+, PGMY09, and PGMY1 are examples of consensus primers required by PCR detection for HPV nucleic acid presence and types. HPV genotypes can be copied at one time through the reaction. The primer mainly focuses on the L1 region, which codes for capsid protein and has lasted for generations without significant mutation. The HPV type of the sample is then identified by restriction-fragment length polymorphism (RFLP) or other methods like direct sequencing [20].

Type-specific PCR is available for detecting chosen HPV genotype. Since in each trial, the technique only amplifies the given genotype, it is difficult to check the presence of HPV infection including all viral types. As a result, additional trials are required to generate a more complete view of potential HPV infection in the sample. Also, before performing type-specific PCR, the manipulator should ensure primers applied are identified and correspond to specific HPV genotypes. Moreover, the cost of this technique and the labor force is huge. Still, type-specific PCR can apply to real-time PCR by adding fluorescent probes [21].

Real-time PCR assay provides a more sensitive detection for HPV infection. Using the technique, it is possible to find out the viral load and deal with quantities [20]. Many studies have supported that the technique is capable of estimating the change of high-grade cervical lesions by measuring HR-HPVs in the sample from a cervical smear [19]. Through fluorochromes that have the ability to generate fluorescence, the reaction can be done several times targeting disparate nucleic acid. The 7-log dynamic range makes the prediction of HPV nucleic acid possible. Real-time PCR is practical for its immediate result and imitable procedures for clinical usage [21].

Abbott real-time PCR assay targets HR-HPVs using the homogeneous technique, which is a type of qualitative PCR. It detects HPV 16 and 18 at the same time. Also, Abbott real-time PCR is suspected to detect other genotypes including 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68. Using this technique, it is attainable to identify single or more infection genotypes from a liquid sample containing cervical cells. In another technique, COBAS® 4800 system, samples will be automatically prepared. The technique is able to verify 14 genotypes. A single tube is required to separate genotypes 16 and 18, but the other 12 types including 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 can stay in a pool. COBAS® 4800 is remarkable for its efficiency and operability. Primary specimens are allowed, and the test result is available after 4 hours. The test matches the international standard for clinical usage and screening test, making it a reliable detection method to be used [20].

4.2. Signal amplification

Hybrid Capture (HC2) is one of the liquid-phase amplification methods. It is accessible in both commercial and clinical practices. The stretches of single-stranded RNA or RNA probes are applied for performing hybridization. It is designed for targeting HR-HPVs with genotype 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. For the clinical sample, it will be firstly denatured by heat and alkaline. Peroxide-labeled antibodies are used for detecting more HPV types using multiple probes. The analytical sensitivity test has shown that HC2 is less sensitive than techniques found under target amplification. Since multiple probes are utilized in the detection process, it is hard to show a high-resolution typing result [19]. Also, the technique tends to have a lower specificity as it sometimes unintended ignored certain HPV types [22].

Under morphological signal amplification, DNA in situ hybridization (ISH) is another way to detect HR-HPVs. The detection is done with histological assistance on cytological slides. The fluorescence or substrate coloration with specialized microscopy is necessary in ISH detection. The signal amplification process becomes important when small numbers of the virus are present and the probe doesn't respond to all of them. The commercial ISH with indirect biotin-streptavidin applied is

proved to be insufficient for testing cervical lesions at a later stage. Catalyzed reporter deposition is another option, but it is costly when dealing with intensive test requirements [19].

5. Application of HPV detection methods

In Hong Kong, the cohort study has done on 6,345 females with various HPV diagnosis tests including HC2 and COBAS 4800 tests, which aimed to make a comparison. People were randomly chosen to be in the control group or intervention group. While HC2 detection showed 506 (7.97%) positive results, COBAS 4800 showed 555 (8.75%) positive results out of 6,345 test samples. Overall, both detection methods received 284 positives and 5,568 negative cases, which contributed to a total 92.23% agreement between the two tests. From various cytology groups researching the HPV prevalence, two tests had no remarkable difference in testing HR-HPVs, but they appeared in females without HPV infection. Through the study focusing on HC2 and COBAS 4800 tests in various histology conditions, the overall agreement on test results between the two methods was 74.30% [23].

Table 1. The comparison of COBAS 4800 and HC2 tests in detecting various histology conditions The concordance analysis between COBAS 4800 and HC2 positive results associated with specific histology (adapted from Liu et al. 2022) [23]

Histology (N)	Cobas				HC2	Overall agreement (95% CI)	Kappa (95% CI)	P value*
	HPV16+ (%)	HPV18+ (%)	12 OHR+ (%)	HR-HPV+ (%)	HR-HPV+ (%)			
Normal (186)	5 (2.69)	6 (3.23)	41 (22.04)	52 (27.96)	102 (54.84)	70.97% (64.44-77.49)	0.443 (0.342-0.560)	<0.001
CIN1 (288)	12 (4.17)	9 (3.13)	86 (29.86)	107 (37.15)	165 (57.29)	75% (70.0-80.0)	0.518 (0.428-0.608)	<0.001
CIN2+ (28)	2 (7.14)	0	21 (75.0)	23 (82.14)	26 (92.86)	82.29% (77.83-100.74)	0.523 (0.0-1.0)	0.250
CIN3+ (19)	2 (10.53)	0	13 (68.42)	15 (78.95)	17 (89.47)	89.47% (75.67-103.27)	0.612 (0.0-1.0)	0.500
Overall (502)	19 (3.78)	15 (2.99)	148 (29.48)	182 (36.25)	293 (58.37)	74.30% (70.48-78.13)	0.509 (0.445-0.577)	<0.001

Abbreviations: N, number of cases; CIN, cervical intraepithelial neoplasia; CIN2+, CIN2 and worse; CIN3+, CIN3 and worse; CI, confidence interval

*: McNemer test

The remarkable discrepancy did not happen in testing CIN 2+ and CIN 3+ but in females with normal or low-grade HPV infection. Specifically, compared to COBAS 4800, HC2 received more positive results in detecting samples with the normal or lower lesion (COBAS 4800: 27.96% and 37.15%; HC2: 54.84% and 57.29%, $p<0.001$). However, COBAS 4800 showed greater congruence of positive results with severe lesions (COBAS 4800: 67.33% and 65.94%; HC2: 46.41% and 44.62%). Therefore, COBAS 4800 demonstrated a better specificity than HC2 in dealing with CIN 2+ and CIN 3+ cases; on the other hand, HC2 tests tend to have a moderately better sensitivity [23].

6. Conclusion

From the study in the Chinese cohort study, COBAS 4800 (target amplification) and HC2 (signal amplification) have overall similar test results with 92.23% accordance. Both methods are suitable for cervical disease diagnosis in that there was no significant divergence in diagnosing HR-HPV infections. Besides, the investigation suggested that co-testing is essential for the primary detection of HPV infection, which ensures a more accurate testing result [23]. Also, most detection assays tended to have lower concordance with normal or low-grade lesion cases. The accuracy of positive testing in females with HPV infection gradually increased with the abnormality of the disease: 22% for normal or non-infection cases, 68% for low-grade lesions, and 84% for greater than CIN 1+ cases [24]. To improve the technique, the HC2 test needs to work on raising the specificity of high-grade HPV infection testing or CIN 2+ and CIN 3+ cases. Other techniques should focus on raising sensitivity and accuracy in the diagnosis of lower lesion cases, which could help to reduce needless colposcopy. To make a more comprehensive view of the advantage and disadvantages of each detection method, the validation study needs to be done in a larger population. Also, the working efficiency of each method was not added to the consideration [23].

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