



Recent Advances in Human Papillomavirus Detection and Genotyping

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Authors' contributions

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Review Article

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ABSTRACT

Worldwide, cervical cancer is the second-most common cancer and a leading cause of cancer-related deaths in developing countries. Epidemiological and biological studies have identified Human papillomavirus (HPV) as etiological agent for cervical cancer. Currently, it's widely accepted that 40 among 200 genotypes of HPV considered as high risk are implicated in the development of other human cancers. The diagnosis of cervical cancer and precancerous lesions is mainly based on cytological screening that have largely contributed to reduce the prevalence of cervical cancer in both developed and developing countries. Although this screening has decreased the incidence of cervical cancer, cytological techniques lack sensitivity and HPV-related cervical disease, including premalignant and malignant lesions, continues to be a major burden on health-care systems. The current trend in cervical cancer screening is to improve the sensitivity of screening with new methods and to propose new algorithms for diagnostic and early therapeutic decisions. Recent achievement in molecular approaches have emerged in clinical practices, are characterized by high sensitivity, specificity and the short time required to perform the procedure, which explain the great interest given to these techniques for HPV testing. These techniques are widely used for HPV

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detection and/or genotyping, they are based on signal amplification methods (hybridization techniques in liquid phase) or target amplification methods (gene amplification by PCR). This review paper was edited to summarize the main molecular techniques used in HPV testing and recent advances for HPV detection and genotyping.

Keywords: HPV; cervical cancer; detection; genotyping; molecular testing.

1. INTRODUCTION

Four decades have passed since HPV were recognized as the etiologic agent of cervical cancer development [1-3]. Until now, more than 200 HPV genotypes have been identified but the interest is focused only on genital HPV (40 genotypes) that are associated with precancerous and cancerous lesions of the cervix [4]. The phylogenetic tree of Papillomaviruses contains 16 genus, and among them five genus (Alpha, Beta, Gamma, Mu and Nu) contain HPV infecting the anogenital mucosa [5].

Worldwide, HPV were detected in cervical lesions and also in cases with normal cytology. According to their association with invasive cervical cancer, HPV were divided into low-risk genotypes (including types 6, 11, 40, 42, 54, and 57) and high-risk genotypes (including types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, and 68). It's widely reported that HPV16 and 18 are the most prevalent HPV genotypes associated with cervical cancer. However, there are geographical differences in the distribution of HPV types amongst populations [6].

Currently, there's evidence that HPV infection is a sexually transmitted infections, the peak incidence of HPV infection mainly occurs about 20-25 years, and fortunately 60 to 80% of these viral infections are transitory and eliminated by the immune system in 8 to 24 months [7,8].

At the present time, cervical lesion diagnosis is mainly based on cyto- and histopathological analyses [9]. Cytology diagnosis, especially using the Pap smear screening test, has largely contributed to decreasing mortality and has been very successful in lowering the incidence rate of cervical cancer in countries with high coverage and good quality control [10]. However, conventional cytological and histological diagnosis is even more problematic, because it is difficult to do well, exhibit a low sensitivity and quality sample interpretive errors due to the

manipulators subjectivity of the reading of slides, leading to a greater number of interpretive errors [11]. Various efforts were made to overcome these problems and although it is now more common for laboratories to use the thin prep Pap test, this method is not widely used in small hospitals or clinics because of the need for greater technical expertise and equipment. Thus, most laboratories around the world do not have access to these methods.

Therefore, there is a pressing need for new methods that will allow for both rapid detection of HPV and for viral genotyping to identify patients who are infected with high oncogenic HPV. To address these issues, a number of efforts around the world were made and are under way to develop new molecular methods for HPV detection and genotyping. This review paper will summarize the main molecular techniques used for HPV screening and genotyping, and will focus on intrinsic characteristics (sensitivity and specificity) [12] and the practical limitations for their adequate and efficient use in cervical cancer and precancerous lesions management.

2. HISTORY

The widespread adoption of the Pap test (commonly named cervical smear test), as a screening tool for cervical cancer, is arguably one of the most significant advances in public health of Human history. It has been seventy four years since the birthday of this technique, developed by Papanicolaou in 1941. In the late 1990s, liquid based cytology (LBC) was born as an alternative method of Pap test. Currently, as with many processes used, technology and automation have been introduced in an effort to improve the efficiency and accuracy of the Pap test. In 1998, the U.S. Food and Drug Administration (FDA) approved the AutoPap as a computerized instrument to replace Humans in reading Pap smear slides. The Fig. 1 summarize the main techniques used for HPV diagnosis [13,14].

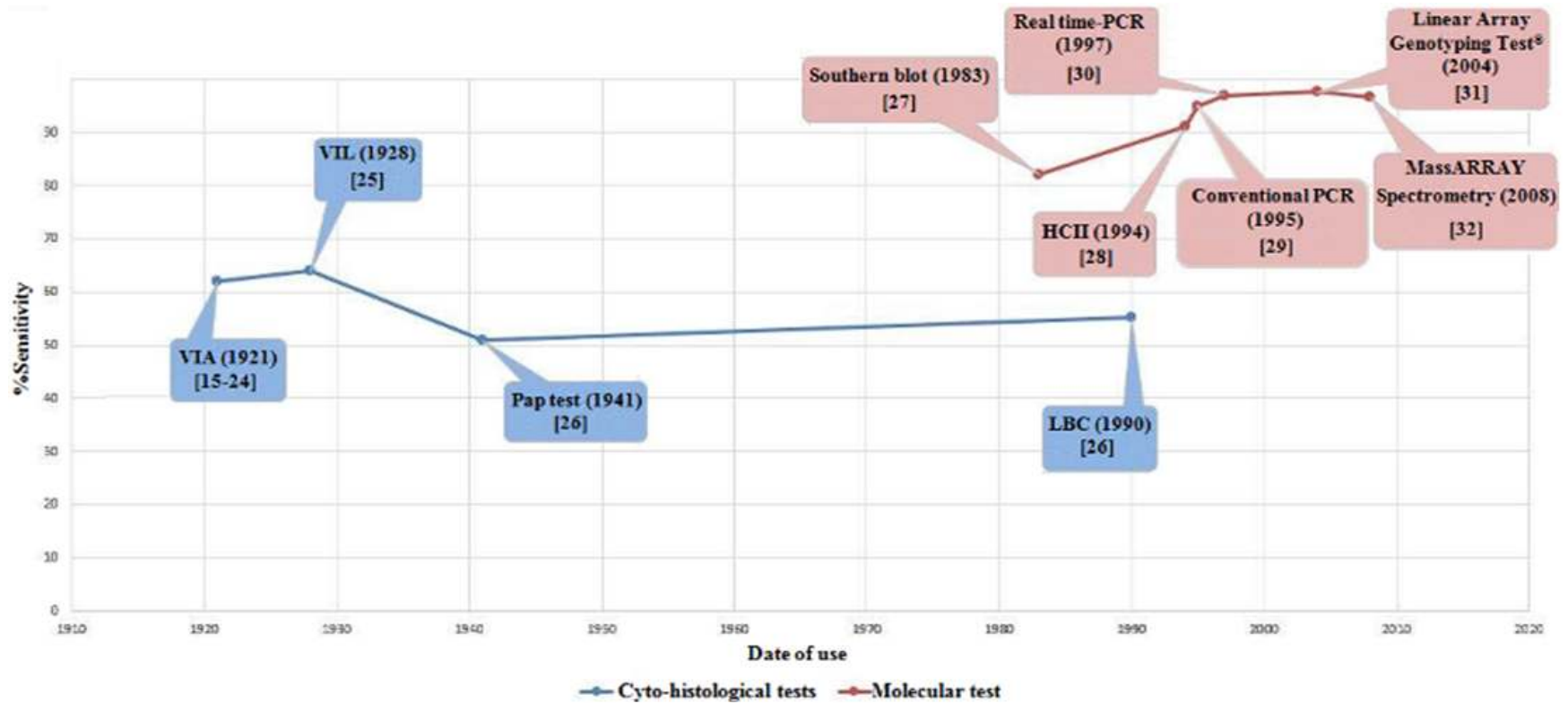


Fig. 1. Chronological representation depending on the sensitivity of different cyto-histological and molecular technologies appeared in the 20th and 21st century

VIA: Visual inspection with acetic acid; VIL: Visual inspection with lugol's iodine; Pap test: Papanicolaou test; LBC: Liquid based cytology; HCII: Hybrid Captur II.

* Sensitivity of each test is given as a mean of various published data

In 1924, the gynecologist Hans Hinselmann, constructed in collaboration with the optical instrument company Leitz, a colposcope that provides three-dimensional images. Hinselmann had found out that when he applied dilute acetic acid on the cervix (VIA: Visual Inspection with Acetic Acid), zones of abnormal proliferation of cervical cells became visible as white patches. Around 1928, a Vienna gynecologist, Walter Schiller, proposed to replace acetic acid with lugol's iodine (VILI: Visual Inspection with Lugol's Iodine). After Lugol's iodine application, normal cervical cells containing glycogen will uptake iodine and exhibit a brown color, while proliferating abnormal cells cannot store glycogen and remain white on a dark background [33].

Even the discovery of the HPV and its etiological role in cervical cancer development was reported in 1976 and 1977 [34,35] and confirmed in 1983 [36], the interest on HPV as biomarker of cervical cancer diagnosis was confirmed after the discovery of high oncogenic HPV by Zur Hausen in 1984. During last decades, molecular techniques have evolved rapidly both at conceptual and methodological levels. Their introduction in medical diagnosis have opened a new era for better management of Human and Animal diseases and brought new keys for targeted therapies. Evolution of these molecular techniques had began by the immunohistochemical staining set up in 1982 [37], non-amplified nucleic acid hybridization methods, such as Southern blot assay in 1983 [38], filter *in situ* hybridization in 1985 [39] and dot blot hybridization in 1986 [40]. In 1989, serological assays to detect antibodies to HPV capsid or functional protein antigens have also received attention as investigational tools in epidemiological and clinical studies [41,42]. In 1993, the immunoassay-based nucleic acid hybridization techniques such as the Hybrid Capture™ (HC) assay have revolutionized the HPV detection in cervical cancer and precancerous lesions [43]. In 1991s and after the emergence of Polymerase chain reaction amplification (PCR) [44], various PCR based techniques were developed and are applied for HPV detection, genotyping and quantification. These techniques are mostly based on PCR and/or hybridization, and require internal control (eg. β -globin) used to assess specimen quality and identify specimens containing factors that inhibit the amplification process.

3. DETECTION OF HPV PCR WITHOUT SPECIFYING THE TYPE

HPV cannot be grown *in vitro* [45], and serological assays have only limited accuracy [46]. Indeed, after an HPV infection, antibodies produced by the humoral immune response against the major capsid protein, remain detectable for many years, and therefore it's not possible to differentiate between present and past infections [46].

Considerable progress has recently been made in developing novel approaches and tools, especially molecular methods (commercial and 'in-house'), for direct and accurate detection and genotyping of HPV in clinical specimens.

Different techniques have been developed to detect the presence of HPV in clinical samples without specification of the HPV genotype(s). These techniques are used as a first step in the HPV genotyping process or are designed to detect some HPV genotypes, mostly high risk HPV, for efficient cervical lesion diagnosis [47,48].

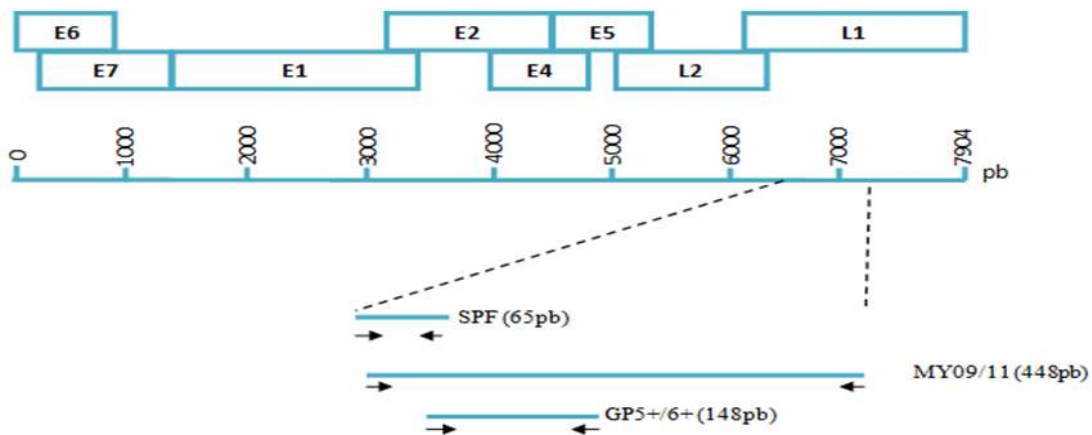
3.1 Detection of HPV with PCR Using Consensus Primers

The amplification of DNA by PCR is now a standard technique in molecular biology [49]. In the first years following the original description of the method [50], an enormous number of publications appeared describing refinements of reaction parameters and variations of PCR for fragment enrichment, cloning, mutation detection, *in vitro* mutagenesis DNA sequencing and a host of other uses [51-55]. One of these uses of this technique in cervical cancer screening is searching for HPV DNA.

The wide diversity of 200 genotypes of HPV has led to develop a system of universal primers with broad spectrum, allowing the amplification of many known and unknown HPV genotypes [56]. These universal primers need to target a region highly conserved among different HPV genotypes and that is not likely to be deleted or modified in the event of integration of the viral genome into the genome of the host cell. The L1 region is the most conserved region of the viral genome and being sufficiently remote from the E2 region (most common breakpoint in case of integration) [56,57]. Several broad-spectrum primers systems were designed within this region, according to different strategies (Fig. 2, Table 1).

Table 1. Characteristics of the different primers systems with broad spectrum available for the PCR amplification of HPV [58]

Name of the primer system	Target area of the viral genome	Location on the HPV genome	Average size of the amplicon
MY09/11 and PGMY09/11	L1	6722-7170	448 bp
GP5/GP6 and GP5+/GP6+	L1	6764-6904 6761-6909	140 bp for GP5/6 148 bp for GP5+/6+
SPF ₁₀	L1	6582-6645	65 bp

**Fig. 2. Diagrammatic representation of the locations of MY09/11, GP5+/6+ and SPF10 general primer sets on L1 region of HPV genome in the HPV L1 gene**

Initially, HPV detection was made by PCR consensus using MY09/11 primers [59]. These primers were in a conserved region of the L1 open reading frame with the intent of amplifying in a single reaction 5 HPV types; 6, 11, 16, 18, and 33; whose sequences are known and, presumptively, other genital HPVs with shared sequence homology in this region. The chosen regions were not entirely homologous even among the five original HPV types, and positions with nucleotide base heterogeneity were accommodated by inclusion of degenerate base sites. The resultant degenerate primers comprised a mixture of 24 unique oligonucleotide sequences [60]. This approach has the disadvantage that the synthesis of primers is not reproducible. Synthesis of a mixture of oligonucleotides with degenerate base sequences relies on the presumed random addition of one of two or more nucleotide bases at the position of degeneracy. Random insertion is not a controlled process and leads to a mixture of oligonucleotides that may vary from manufacturing batch to another, so that each batch of primers must be carefully tested and validated in terms of amplification efficiency of each HPV genotype [61].

To improve the reproducibility and sensitivity of the MY09/11 HPV amplification system, a set of oligonucleotide pools, PGMY09 and PGMY11, based on the same primer binding regions used for MY09/11 [47], was developed. In this approach, virus types were grouped together by sequence homology in each of the two primer binding regions, and from these groupings, a set of 5 upstream oligonucleotides comprising the PGMY11 primer pool and a set of 13 downstream oligonucleotides comprising the PGMY09 primer pool were designed (PGMY09/11 primer system) [61,62]. This strategy has the advantage of high reproducibility of synthesis and to use an optimal temperature of hybridization during the PCR cycles, avoiding non-specific amplifications.

GP5 and GP6 primers were selected from the HPV L1 region on the basis of sequence information from HPV 6, 11, 16, 18, 31 and 33, and was found to amplify target DNA of at least 27 mucosotropic HPV genotypes under conditions that allow mismatch acceptance [44]. To offset the mismatches with other HPV types, PCR is performed with an annealing temperature of the primers below the optimum temperature

[56,63]. PCR efficiency was improved using GP5+/6+ primers corresponding to GP5/GP6 primers extended in their 3' ends with short highly conserved sequences [64]. Indeed, increased primer length contributes to a more efficient amplification probably by increasing the stability of the primer template complex [64].

Interestingly, many research groups use nested PCR for HPV detection. In fact, the use of MY09-MY11 and either GP5-GP6 or GP5+-GP6+ primers in a nested PCR assay has been shown to increase the overall sensitivity compared to that of each primer pair alone.

During last decades, amplification and detection of HPV from genital samples demonstrated the ability of the used primers to amplify a spectrum of more than 30 genital HPV types, albeit with various levels of sensitivity. Moreover, the strength of this general primer-mediated PCR method has been further substantiated by the detection of HPV DNA in 95 to 100% of cervical cancer cases [62]. However, in case of multiple infections, these broad-spectrum primers are theoretically able to amplify all HPV types present in the same reaction. However,

competition phenomena occur between the different matrices and efficiency's PCR will not often equal for each HPV types present in sample, and therefore some HPV are amplified preferentially [62,65].

3.2 Detection of HPV with DNA Hybridization in Liquid Phase

HPV DNA was firstly detected by southern blotting, *in situ* hybridization and dot blot hybridization, requiring large amounts of purified DNA and using mostly radioactive probes [66]. Because these techniques have several limitations, considerable progress has been made in developing novel approaches and tools. The technology progress has allowed the development of new substrates for signal amplification [66]. The main kits used are reported in Table 2. These tests are based on the hybridization of the target HPV-DNA to labeled RNA probes in solution (Fig. 3). When hybridization occurs, the reaction product is visualized by hybridization immunoassays reaction, this strategy is called also "aggregate" or "cocktail".

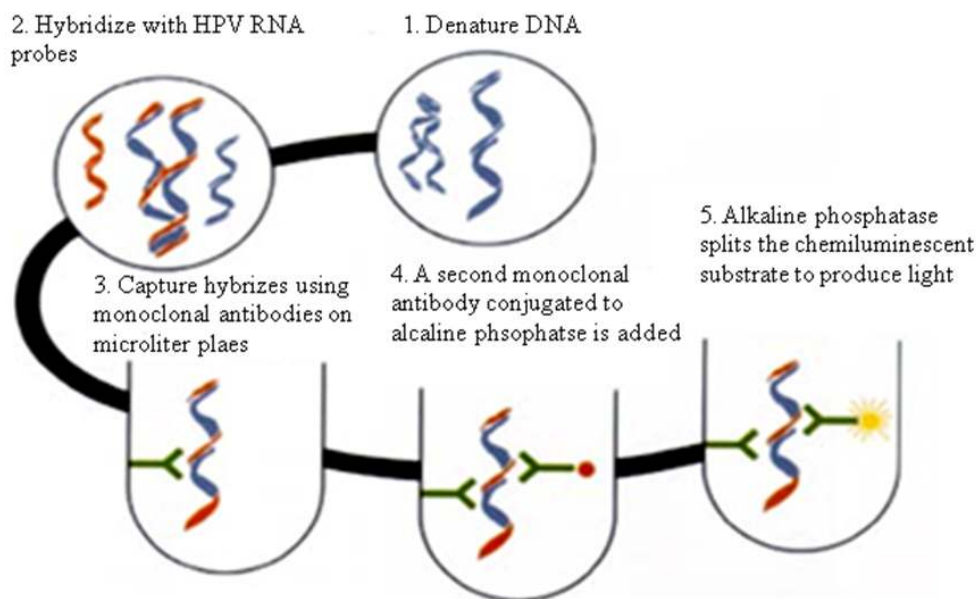


Fig. 3. Hybridization in liquid phase

The denatured HPV DNA in the biological sample is hybridized with the RNA probe, and specific RNA-DNA hybrids are formed. These hybrids are captured by antibodies bound to the wells of a microtiter plate that recognize specifically RNA-DNA hybrids. The immobilized hybrids are then reacted with alkaline phosphatase-labelled anti-DNA-RNA monoclonal antibodies and the plate is subsequently washed. The bound enzyme conjugated antibodies are incubated with a chemiluminescent compound. Dephosphorylation of this substrate produces light that is measured by a luminometer. The results are analyzed by a software program. The intensity of emitted light, expressed as relative light units, is proportional to the amount of target DNA present in the specimen, providing a semi-quantitative measure of the viral load

In these tests, the main advantage is the use of specific antibodies for hybrids detection and the amplification of hybridization signal using chemiluminescence or fluorescence labeling, offering high specificity and sensitivity for HPV detection. The main tests used worldwide are Hybrid Capture II® (HCII: Digene Corp., USA) and Cervista® HPV HR (Hologic Inc., Marlborough, MA, USA). These tests have showed high sensitivity in the detection of cervical cancer, and CIN III and CIN II lesions [67]. The two tests share the detection of HR HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68. HCII is able to detect also LR HPV, in particular 6, 11, 42, 43 and 44, whereas Cervista® can also detect the HR HPV 66.

3.3 Detection of HPV with DNA Hybridization after DNA amplification

Direct hybridization has showed serious defects [68]. The main disadvantage raised on the low sensitivity and the need of large amounts of highly purified DNA [69]. To increase the sensitivity of the tests, many laboratories have combined DNA Hybridization with PCR amplification. Mostly, primers used for DNA amplification are 5'biotinylated and after hybridization with specific probes corresponding PCR products harboring the biotin could be detected by an immuno-enzymatic assays (Fig. 4). The main tests used for HPV detection

after PCR DNA amplification by PCR are reported in Table 3.

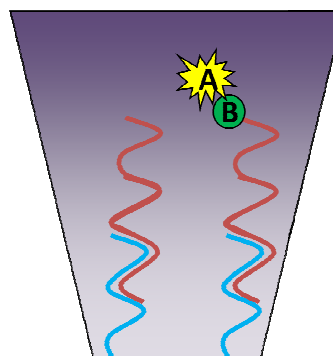


Fig. 4. Immuno-enzymatic assay for detection of PCR amplification

The main test used for HPV detection using DNA hybridization and PCR amplification is the AmpliCor HPV test kit (diagnostics Roch), which is based on the detection of 13 high-risk HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) by a broad spectrum of PCR products (Fig. 5) [70]. The heterogeneous interprimers region is detected with a cocktail of probes for high-risk genotypes. A study has been conducted by Sandri and coll., showed that the HC2 assay and the AMPLICOR HPV test give comparable results, and are both being suitable for routine use [71].

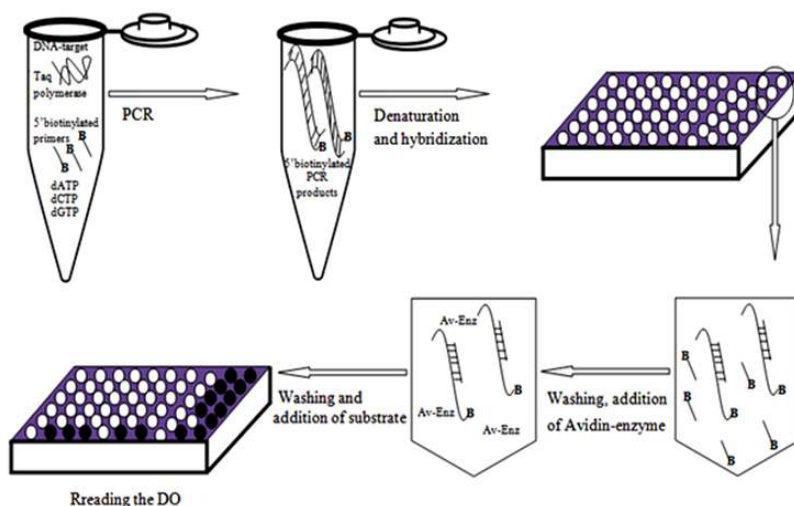


Fig. 5. Schematic representation of the AmpliCor protocol for HPV DNA detection

After the amplification of the 165 bp fragment from the HPV L1 gene using biotin labeled primers, PCR product is denatured and hybridized to the specific probe at the well bottom. The PCR product specifically bound to the specific probe is detected through biotin by a streptavidin-conjugated antibody with HRP (horse-radish peroxidase) and visualized by color reaction of the substrate containing hydrogen peroxidase and color substance as 3,3',5,5'-tetramethylbenzidine

Table 2. Kits used for HPV detection with signal amplification

Test Kit name	Provider	Hybridization	Revelation	HPV detected	Approval Level	Sensitivity	Specificity	PPV
Hybrid Capture II®	Qiagen	microplate with RNA probes	Chemi-luminescence	High risk: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68 Low risks : 6, 11, 42, 43 and 44	FDA approved and CE-IVD	92.3%	19.5%	73.8%
Test Probe set® RUO	Qiagen	microplate with RNA probes	Chemi-luminescence	High risk: 16, 18, and 45	CE-IVD			
Cervista® HPV HR	Hologic	microplate	Fluorescence	High risk : 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68	FDA approved and CE-IVD	84.5%	72.7%	

Table 3. Main tests used for HPV detection after PCR amplification

Test Kit name	Provider	Amplification	Hybridization	revelation	HPV detected	Approval Level	sensitivity	specificity	PPV
Cobas 4800 test	Roche diagnostics	Specific primers	On tube		16 , 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68		95.2%	24.0%	37.6%
Amplicor HPV TEST®	Roche diagnostics	PCR with primers PGMY09/11	On microplate	Colorimetric	High risk: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 et 68	CE-IVD	95.2%	96.5%	96.1%
Abbott Real Time High-Risk HPV®	Abbott	Amplification by RT PCR with primers GP5+/GP6+	On microplate	Fluorescent fret probes	High risk: 16a, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68	CE-IVD	93.3%	27.3%	38.2%
BDTM HPV-GT	Becton Dickinson	Consensus primers PCR	On microplate	Fluorescent	High risk: 16, 18, 45, 31a , 51a , 52a , (33, 58), (59, 56, 66) and (35,39, 68)	FDA approved and CE-IVD	95.0%	24.2%	37.8%

4. GENOTYPING TECHNIQUES OF HUMAN PAPILLOMAVIRUS

In some cases, there is a real need of identifying the HPV genotype(s) present in the biological samples. Genotyping techniques are widely used to:

- Estimate the prevalence of specific types in a given population to address the epidemiological profile of HPV infection [72,73];
- To evaluate the importance of single and multiple infections [74-76];
- To accurately measure the persistence of infection for each virus type (in primary screening, triage of ASC-US and follow-up after conization) [77,78].
- To estimate a risk of progression to high-grade intraepithelial lesions of the cervix and cancer (in the case of infection with high risk genotypes, especially types 16 and 18) [79];
- To provide eligibility criteria for women who want to benefit from HPV vaccination [80];
- To provide more information for better management of the infection.

Generally, genotyping techniques are based on DNA amplification by PCR followed by different approaches that can identify specifically the HPV type that may be present in a biological sample. Moreover, these techniques are able to highlight HPV infection caused by multiple HPV genotypes. In this regard, the challenge was to detect each HPV type with an identical sensitivity to avoid the identification of a preferred type with respect to another.

4.1 Genotyping by Type Specific PCR

Type specific PCR is an alternative technique that could be used to detect HPV genotypes using specific primers [81]. This approach is based on HPV genome polymorphisms, mainly E6 and E7 [81]. This technique is a highly sensitive method that is easy to interpret and can characterize virus types in cases of multiple infection [82-83]. However, this technique is time consuming and needs many PCR tests for each sample [84]. This disadvantage has been partially overcome by the use of real time PCR. In fact, in this technique, the use of different fluorochromes that emit fluorescence, as the PCR reaction proceeds, the reactions can be performed in multiples and can amplify different nucleic-acid targets [85]. Selecting virus types to

be tested should be based on epidemiological and prevalence studies, as there is a wide variation in the genotype distribution in different regions around the world [86].

4.2 Genotyping by Sequencing

Worldwide, genotyping by sequencing is presented as a gold standard method [87], since it gives the complete sequence of the L1 region that defines the different HPV genotypes. However, HPV genotyping is performed by on-line BLAST algorithm of a hypervariable L1 region [88]. This hypervariable region of 34- to 50-base sequence including the GP5+ priming site was selected as the signature sequence for routine HPV genotyping [89]. Nucleotide sequences were aligned and compared with those of known HPV types available through GenBank by using the online BLAST 2.0 software server (<http://www.ncbi.nih.gov/blast>). In accordance with established guidelines, a nucleotide sequence was assigned to an HPV type if it corresponded with a known HPV genotype by >90% [90,91].

This method can detect the majority of HPV genotype, present in the sample, and can also detect HPV variants (mainly types 16 and 18) whose distribution varies according to geographical areas [92]. However, the main limitation of the genotyping by sequencing is to diagnose multiple infections [93]. Moreover, it is also the most labor-intensive and costly technique [94].

4.3 Genotyping by Hybridization with Immobilized Probes

These kinds of techniques are based on the hybridization of amplified HPV DNA with specific probes immobilized on strips [95]. Usually, HPV DNA is amplified with biotinylated primers and hybridized on strip containing a set of specific probes of different HPV genotypes. The hybrids are visualized by colorimetric reaction with the biotin group present on the amplicon (Fig. 6). In each strip, a positive control is included to validate DNA extraction, PCR amplification and hybridization steps. Comparison of obtained bands to the reference strip will identify HPV genotypes [95].

Currently, the two commercial assays most widely used, are the Linear Array Genotyping Test® (Roche Diagnostics) and is INNOLiPA HPV Genotyping Extra® test (Innogenetics) [96].

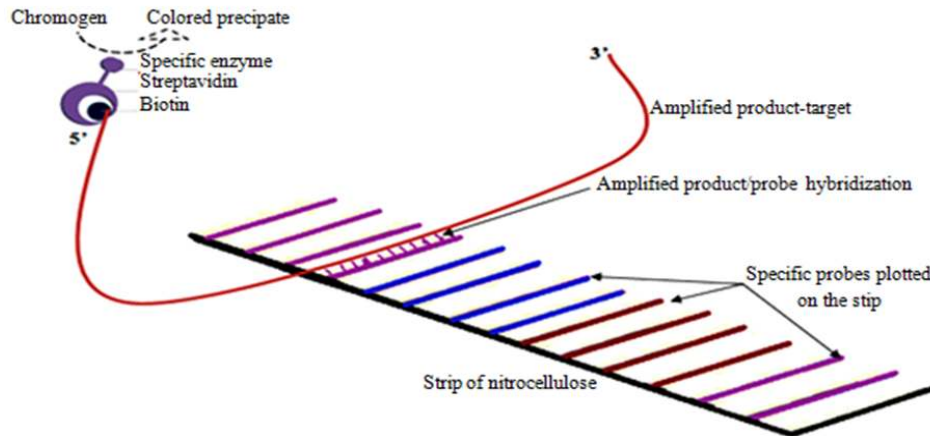


Fig. 6. Reverse line blot hybridization by using probes immobilized on strips

After the amplification of the 65 bp fragments from HPV L1 gene by using biotin labeled SFP 10 primers, the PCR products mixed with different probes firmly anchored to the membrane, finally the revelation of staining indicating hybridization is done by a chemiluminescence reaction

Linear Array Genotyping Test® consists of an amplification of extracted DNA by PCR with biotinylated consensus primers PGMY09/11, allowing the production of biotinylated amplicons of 450 bp which will be denatured and hybridized with specific probes immobilized on strips. The hybrids will be revealed by addition of streptavidin coupled to peroxidase: streptavidin binds to the biotin and the addition of peroxidase substrate causes the formation of a blue precipitate in strip form. In this test [96], the β -globin is used as a positive control. In INNOLiPA HPV Genotyping Extra® test, the extracted DNA is amplified by PCR with biotinylated consensus primers SPF10, allowing the production of biotinylated amplicons of 65 bp [97]. The hybrids will be revealed by addition of streptavidin coupled to alkaline phosphatase: streptavidin binds to the biotin and the alkaline phosphatase will transform the chromogen substrate (BCIP NBT) giving a purple precipitate on strip form [97]. In this test, the HLA-DBP1 is used as a positive control. The INNOLiPA HPV Genotyping Extra® test can identify 28 HPV genotypes: 18 high-risk HPV, seven low-risk HPV, and three unclassified [97].

4.4 Genotyping by DNA Chip

Recent development using chip technology was also applied for HPV detection and genotyping. In this technique, the primers are labeled with fluorescent markers. The PCR products are hybridized with probes already blotted onto a chip, and after a washing step, hybridized signals are visualized with a DNA chip scanner (Fig. 7)

[98]. This technique is simple, sensitive and rapid. It also allows to detect different types in multiple HPV infections. At present, the two main kits of Genotyping by DNA chip are PapilloCheck® (Greiner Bio One) and Clart HPV2® (Genomica).

The Papillo Check® kit (Greiner Bio-One GmbH, Frickenhausen, Germany) is a method for detection and genotyping 24 HPV: 18 HR-HPV and 6 LR-HPV [99]. This is based on PCR on the amplification by PCR of a 350 bp fragment within the E1 region, followed by the hybridization of amplicons obtained with specific probes immobilized on chips. Each HPV type is detected by a specific probe, present in 5 replicates on the DNA chip. The bound DNA is then marked with a fluorescent molecule which is excited at a given wavelength. Hybridization is then scanned and analyzed using specific software. Four internal quality controls are included in each ship: control orientation of the chip, control hybridization of adequate sampling, control of PCR amplification using the human *ADAT1* gene, and control of the rich collection [100].

The CLART® Human Papillomavirus 2 (Genomica, Madrid, Spain) methodology allows the detection and genotyping of 35 HPV: 18 HR-HPV and 17 LR-HPV. This technique uses biotinylated consensus primers that amplify a 450 bp fragment within the HPV L1 region. Co-amplification of an 892 bp region of the FTR gene and a 1.202 bp fragment of a transformed plasmid provides a control to ensure DNA extraction adequacy and PCR efficiency.

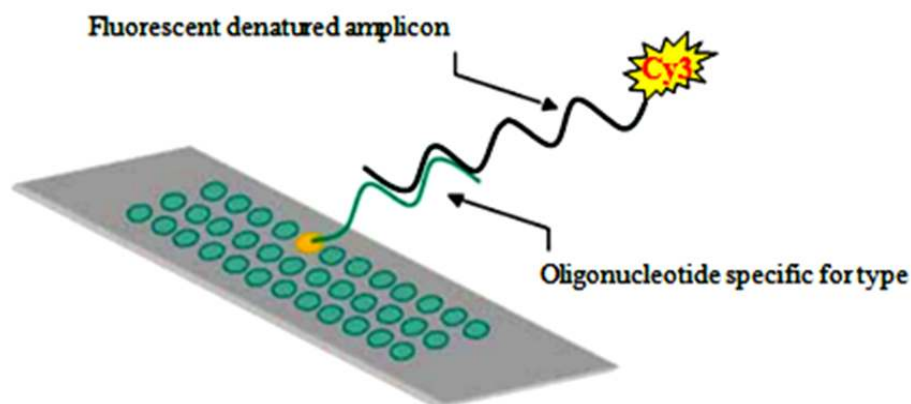


Fig. 7. Schematic representation of a DNA chip

After amplification of the targeted gene, amplicons are hybridized on the blade forming hybrids amplicon-probe specific to an HPV type, and then visualized by fluorescence in the scanner

Amplicons are detected by hybridization in a low-density microarray containing triplicate DNA specific probes loaded on chips. Revelation of hybrids is performed by addition of a streptavidin/peroxidase and the substrate, leading to the formation of a precipitate. An automated reader is used to identify HPV genotypes [101].

4.5 Genotyping by Luminex Technology

A number of bead-based HPV genotyping assays have now been developed with the Luminex platform using different combinations of primers, PCR conditions and statistical analyses [101-105]. In these techniques, an array of suspension technology, xMAP®, is used. The principle is based on the use of polystyrene microbeads coupled with specific oligonucleotide probes (Fig. 8). After amplification of HPV DNA with fluorescent primers, the amplicons are denatured and then hybridized on dyed microspheres labeled with fluorophores specific to each HPV genotype [106].

However, not every laboratory has access to a Luminex instrument, which currently can only be used to analyze Luminex bead sets combined with a single target fluorophore. Recently, flow cytometry, as a common platform in clinical laboratories, has been adapted to analyzing generic multiplexed bead arrays. Usually, two lasers are used, the first one to detect the presence of an HPV amplicon, the other to detect the color of the microsphere and hence the genotype HPV [107].

4.6 Genotyping by Mass Array Spectrometry

High technological development, like mass array spectrometry, was also applied to HPV genotyping [108]. This technique utilizes a three-step process composed of competitive PCR, primer extension (single iPLEX assay), and matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) separation of products on a matrix-loaded silicon chip array. The iPLEX assay is based on multiplex PCR followed by a single-base primer extension reaction [108]. After the PCR, remaining nucleotides were deactivated by shrimp alkaline phosphatase (SAP) treatment, the single-base primer extension step was performed, and the primer extension products were analyzed using MALDI-TOF MS (Fig. 9). The mass array spectrometry is a fully automated high throughput method that can allow to detect 15 High risk HPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 73) in 2,000 samples in about 11 h. In addition, this test incorporates an internal oligomer standard for quality assurance which can be used also for viral load quantitation [109,110].

4.7 DNA Fragment Analysis with Capillary Electrophoresis

DNA fragment analysis with capillary electrophoresis appears as an effective and powerful technique to identify amplified DNA fragments [111,112]. It offers a good specificity of detection and it's ideally suited for handling small amounts of sample material. It has been used to

type many micro-organisms such as Mycobacteria [113], Enterococci [114] and HPV [115].

The target amplification used by specific reverse primers, were designed on L1 gene and fluorescently labeled; these primers were used on two multiplex PCR with one common forward primer, the lengths of products were revealed by capillary electrophoresis. Based on the length of the fragments, we can know the corresponding HPV genotype; also this technique allows to determine the presence of multiple infection [115].

Table 4 summarize the characteristics of the main kits widely used for HPV genotyping, including HPV genotypes detected, the primers used for DNA Amplification, the sensitivity and when available specificity and positive predictive value (PPV).

4.8 Genotyping by Detection of E6/E7 mRNA

Recent advances in molecular biology and RNA preservation have lead to the development of molecular tests based on the detection of HPV RNA. These tests are based on the study of E6/E7 mRNA of HR HPV reflecting the initiation and maintenance of the precancerous or

cancerous state. For a long time, these tests were limited for technical consideration related to sample preservation, RNA extraction, storage and manipulation. During the last decade, powerful analytical techniques were developed. The main techniques are Nucleic Acid Sequence Based Assay (NASBA) and Transcription-Mediated Amplification (TMA).

The NASBA method allows the analysis of the specific expression of E6 / E7 mRNA from 5 HR HPV: 16, 18, 31, 33 and 45. This test is based on a one step isothermal process for amplifying single-stranded RNA molecules combined with real-time detection of target RNA using fluorescent probes type Beacon (hybridizing to single stranded molecules). This technique provides a rapid test, highly specific and easy to use for HPV detection and genotyping [116,117].

The TMA test detects E6 / E7 mRNA of fourteen HR HPV (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68). In this technique, two enzymes (reverse transcriptase and RNA polymerase) are used for RNA amplification. After the TMA amplification, detection is performed by the HPA (Hybridization Protection Assay), using a specific DNA probe labeled with a molecular detector: Acridinium ester, emitting a chemiluminescent signal with appropriate reagents [118].

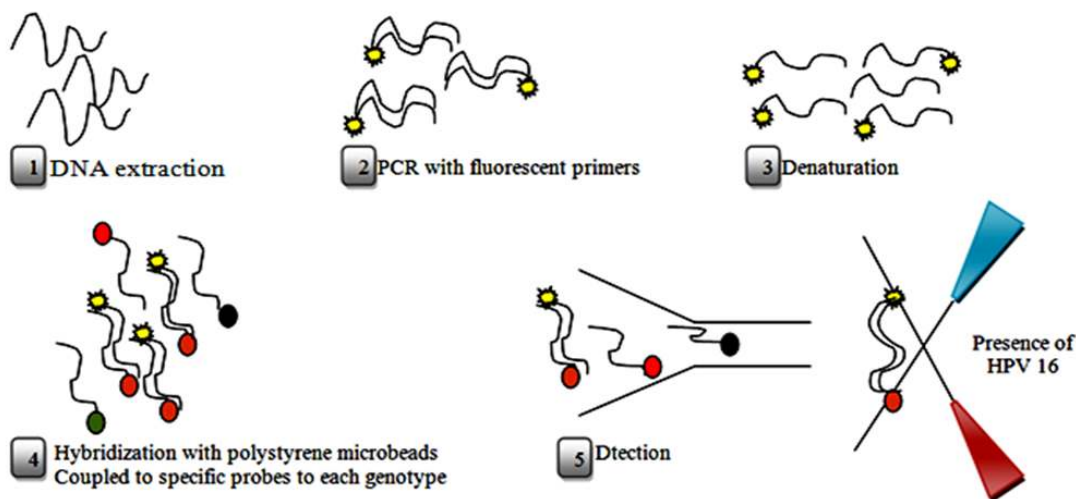


Fig. 8. Schematic overview of HPV genotyping of GP5+/6+-PCR products by bead-based multiplex HPV genotyping

After DNA denaturation and PCR reaction with fluorescence primers, PCR product will be denatured and hybridized with microbeads coupled to specific probes to each HPV genotype, finally the HPV types presented on the samples will be detected thanks to the lasers

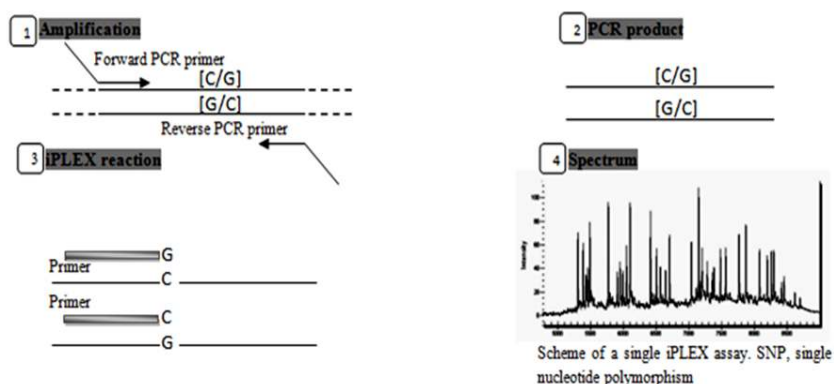


Fig. 9. Different steps of mass array spectrometry

After DNA amplification by PCR, remaining nucleotides were deactivated by shrimp alkaline phosphatase (SAP) treatment, the single-base primer extension step was performed, and the primer extension products were analyzed using MALDI-TOF MS. Individual Competitors for 15 HPV types were designed and mixed with extension primers of each HPV type targeted. As each HPV type had its own respective competitor into one multiplexed reaction, analysis confirmed that there were only the peaks of competitors for each type that was not present in the sample and thus excluded any possibility of contamination across the group of types targeted in the multiplexed reactions performed

Table 4. Main tests used for HPV genotyping

Test Kit name	Provider	Amplification	HPV detected	sensitivity	specificity	PPV
Linear Array Genotyping Test®	Roche diagnostics	Consensus primers PCR PGMY	HPV-LR:6, 11,40, 43, 44, 54, 70, 69, 71, 74 et HPV-HR : 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82	97.6%	93.9%	73.9%
INNO-LIPA Genotyping Extra®	Innogenetics	Consensus primers PCR GP5+/GP6+	HPV-HR :16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 et 82 and HPV-LR 6, 11, 40, 43, 44, 54, 70 and 3 non classified types : 69, 71, 74.	92.5%	35.1%	19.1%
Papillo Check®	Greiner Bio One GmbH	Consensus primers E1 PCR fragment of 350 pb	HPV-LR: 6, 11, 40, 42, 43,44, HPV-HR 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, 82	96.4%	96.3%	-
Mass ARRAY Spectrometry	Sequenom Inc., San Diego, CA	consensus MY09/MY11	HPV-HR 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 73	96.6%	-	-
HPV CLART 2 Genomica assay	Genomica, Madrid, Spain	consensus MY09/MY11	HPV-HR :16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 et 82 and HPV-LR 6, 11, 40, 43, 44, 54, 61, 62, 70, 71, 72, 81, 83, 84, 85 and 89	85.1%	92.6%	66.9%

5. QUANTIFICATION OF HPV DNA

Recently, a new categorization of HPV infections based on serial measurements of type-specific viral load was described, and an underlying mechanism based on differential cervical basal cell division after infection was proposed [119]. There is evidence that viral load address the utility of predicting the progression or severity of disease [120-123]. Indeed, the quantity of virus detected in smears could be related to the grade of the lesion and be a prognostic factor [119]. Currently, molecular techniques can monitor changes in viral load during the infection and the progression of the disease.

The advent of real-time PCR allows to provide a precise quantification of HPV DNA (number of DNA copies) as compared to internal standards [124]. The simultaneous detection and quantification of the target DNA are possible by measuring "in real time" the emitted fluorescence. The intensity of the fluorescence is proportional to the quantity of amplicons synthesized and therefore the number of targets (HPV genome) initially present in the sample [124].

6. TECHNIQUES AVAILABLE TO IDENTIFY VIRAL INTEGRATION

When HPV infection occurs, the viral DNA is present in the cell as an episomal form and the infection resolves in the great majority of cases due to host immune response [125-127]. Persistence of infection favours viral integration in the cell genome which, together with other factors, can progress to HSIL and cancer [128]. During HPV-DNA integration into the host cell DNA, the viral genome usually breaks at E1 and/or E2 open reading frames (ORFs), whereas the E6/E7 ORFs and long control region remain intact [128]. The main methods used to identify the HPV DNA integration are Southern blot hybridization and Real time PCR.

6.1 Southern Blot Hybridization

This technique initially used for accurate and specific detection and genotyping of HPV in specimens, was also used to determine the physical status of the HPV genome in the host cell [129]. However, this technique lacks sensitivity and therefore requires large amounts (5-10 µg) of highly purified and well preserved DNA, limiting the utilization of this technique on DNA from premalignant lesions or cervical

exfoliated cells [129]. Southern blot hybridization can also be used in the semi quantitative evaluation of viral load by comparison to a known amount of viral DNA [130].

6.2 PCR-Based Methods

Recently, many PCR-based methods have been developed to identify integrated HPV DNA in the host cell genome, namely ligation mediated PCR (DIPS-PCR) [131], the amplification of papillomavirus oncogene transcript (APOT) test [132], and Restriction Site PCR (RS-PCR) [133].

The APOT assay, an RNA-based amplification of viral-cellular fusion transcripts specific for HPV integration, is less laborious than the DNA based integration detection assays (RS-PCR and DIPS-PCR). However, the main problem rely on using RNA, that require fresh frozen material with proper RNA quality, and is therefore less available in most biological specimens, depending on the time and type of storage conditions [134].

Basically, DNA from cervical samples is amplified by quantitative real-time PCR for the E2 and E6/E7 ORFs with their respective primer-probe pair. The physical status of HPV DNA is evaluated by calculating the ratio between the levels of E2 and E6/E7 HPV genes. An E6/E2 ratio of 1.2 or greater is suggestive of HPV DNA integration [135]. Samples with suggested HPV DNA integration (E6/E2 ratio ≥ 1.2) could be subjected to a RS-PCR to identify the viral integration site into the human genome [133].

7. CONCLUSION

During last decade, HPV testing has opened up a new frontier in cervical cancer screening. Its higher sensitivity offers a number of advantages, including, most importantly, the potential of reducing cervical cancer rates while reducing the number of screens in a lifetime necessary to achieve this goal.

A broad spectrum of HPV tests were developed around the world, typically detect a wide spectrum of HR-HPV and LR-HPV. Some of them are limited to provide information on the presence of the virus, whether other tests allow to determine the exact genotypes associated with the lesion. Some tests can be conducted in a basic molecular biology laboratory and other Overall, HPV testing has a high sensitivity but

has a low specificity and their utilization in combination with cytology techniques is crucial.

Guidelines for the use of HPV testing were largely discussed in women under or more than 30 years, for CIN2/3 diagnosis and in case of borderline/ASC-US cytology, and many algorithms were developed.

HPV testing has significant potential added value for clinicians and cervical cancer control officials:

- a. Earlier diagnosis leads to close monitoring of the patient for adequate management of the disease and improved patient outcomes.
- b. HPV testing can play a critical role when considering the significant inter-observer variability found in the sub-classification of squamous lesions which is of great importance in the management guidelines of abnormal Pap smear results
- c. HPV testing is also a reliable tool in the surveillance of patients treated for uterine cervix cancer allowing for differential diagnosis between a true relapse and post-radiotherapy atypical cells with an optimisation of the medical resources available.

HPV testing can provide substantial savings

- i. For the patient (earlier diagnosis, improved outcomes, reduced health-care costs, monitoring could be planned every 5 years in case of HPV negative results);
- ii. For the health care provider (earlier definitive diagnosis, focused diagnostic testing, close monitoring, optimum patient care);
- iii. For the public health program (abbreviated period for potential sexual transmission, focused contact investigations).

To achieve implementation of HPV testing around the world, that we believe will have benefic on the cervical cancer and other related diseases diagnosis, some points are raised on:

1. Development of new recommendations and screening algorithms incorporating these technologies, specifying the respective roles of HPV testing and integrating the potential changes in the disease management
2. HPV testing use in less economically developed nations

3. How to best utilize the information we have about HPV to reduce the risk of cervical cancer in developing and developed countries.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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