Molecular methods for the detection of human papillomavirus infection: new insights into their role in diagnostics and epidemiological surveillance

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Abstract

Human papillomaviruses (HPVs) comprise more than 180 genotypes. HPV infection is mainly diagnosed by molecular methods.
The aim of our study was to review the main molecular methods used to diagnose HPV infection, underscoring their characteristics.
Several methods have been developed for molecular diagnosis of Papilloma infection, such as those based on PCR technique.
Another commercial non-PCR based diagnostic method is Hybrid Capture test; it is the only commercially available HPV DNA detection test approved by the FDA.
Several Authors have suggested that viral load and E6/E7 transcripts could be used as surrogate markers of persistent HPV infection, being more specific predictors of progressive disease than the simple presence of HPV DNA.
Validating clinical sensitivity and specificity of each technique and improving the interpretation of the results are essential; consequently, there is a clear need for well characterized international quality control panels to compare the various diagnostic methods.
HPV DNA testing could be useful both as a primary screening test, alone or in combination with a Pap smear, for the early detection of cervical cancer precursors, and as triage test to select women with minor cytological abnormalities who will need further follow-up and to predict possible treatment failure in women with diagnosed high-grade intraepithelial lesions who have undergone excisional therapy.
In the next future surveillance for HPV infections, based on these molecular methods, could represent an important step for the development of primary and secondary prophylactic interventions, such as new vaccines targeted to genotypes who might replace those previously prevalent.

Key words: HPV infection, molecular methods, prevention of cervical cancer

Introduction

Only fairly recently, the family "papillomaviruses" (*Papillomaviridae*) became officially recognized by the International Council on Taxonomy of Viruses (ICTV). Human papillomaviruses (HPV) infect only humans. HPVs are highly epitheliotropic viruses, of which more than 180 [1,2] genotypes have been identified so far; they can be subdivided into cutaneous or mucosal categories based upon their tissue tropism [2].

Several genotypes are defined as high-risk genotypes and are associated with a comparatively high risk for invasive disease [3]. In contrast, other genotypes are considered low-risk genotypes, since they are not associated with the development of cervical carcinoma [3].

The analysis of the nucleotide sequence of a number of human HPV has shown the presence of several stretches of DNA that could potentially encode a protein. These stretches of DNA are known as "open reading frames" (ORFs), referring to the possibility of "reading" relatively long segments of the genetic code (400 bases or more) before reaching a termination signal [4]. The
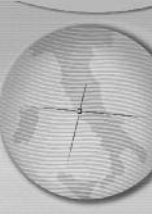
typical HPV genome contains ORFs encoding the structural proteins (L1 and L2), proteins that mediate the viral life cycle (i.e. E1 and E2) and proteins that regulate host-cell DNA replication and transformation (i.e. E6 and E7) [4].

Six ORFs, 85% of the whole genome, encode 6 early (E1, E2, E4, E5, E6 and E7) and two late proteins (L1 and L2). No function has yet been identified for the E3 and E8 ORFs [4].

The nucleotide sequences may be evaluated using sequence databases, as Gene-Bank Database sequences [5]; they are useful in order to study the genomic diversity of HPV sequences (phylogenetic association). HPV types are included in supergroup A, with HPV-6 and HPV-11 (benign types) being referred to as group or clade A10, while HPV-16 and HPV-18, which are the commonest high-risk types for genital cancers, are included in clades A9 and A7 respectively. Furthermore, clade A9 includes HPV-31, HPV-33, HPV-35, HPV-52, HPV-58, while clade A7 includes HPV-39, HPV-45, HPV-59, HPV-68 [3].

Phylogenetic association could explain a potential immunologic cross reactivity [6].

HPV types could be classified based on the strength of their association with cervical cancer; three categories are recognized: high- (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82), probable high- (types 26, 53 and 66) and low-risk (6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 and CP6108) [3].

In the high-risk HPV types, the blockage of cell cycle exit and induction of S-phase in differentiated suprabasal cells is mediated by the E6 and E7 proteins.

Repression by E2 played a role in the down regulation of transcription of the HPV transforming genes E6 and E7. The integration of viral genomes into the cellular genome disrupts E2 expression and leads to an increased expression of E6 and E7 and progression to malignancy. By repressing E6/E7 expression, E2 is able to induce the suppression of cell growth. Detection of E6 and E7 mRNA transcripts has been shown to be of higher prognostic value for the evaluation of the precursor lesions of cervical carcinoma [7].

Furthermore, oncoproteins E6 and E7 are immunogenic, that is able to induce both humoral and cell-mediated responses, which would be ideal for therapeutics [8].

In the last years several diagnostic methods, oriented to the identification of the antigens we mentioned above, have been available; numerous authors have highlighted limits and advantages of them.

On this basis, we reviewed the main, currently used, molecular methods aimed to diagnose HPV infection, highlighting advantages related to each technique.

HPV infection: molecular diagnosis

HPV cannot be grown in conventional cell cultures and serological assays have only limited accuracy and are not commercially available. Consequently, accurate diagnosis of HPV infection relies on viral nucleic acid (DNA and/or mRNA) detection [9].

In particular, detection of high-risk HPV DNA has been shown to be potentially useful as a primary screening test, alone or in combination with a Pap smear, for the early detection of cervical cancer precursors; as triage test to select women with minor cytological abnormalities who will need further follow-up and to predict possible treatment failure in women with diagnosed high-grade intraepithelial lesions who have undergone excisional therapy [9,10].

Several high-throughput methods have been developed for molecular diagnosis of papilloma infection, such as those based on PCR technique. Sensitivity and specificity of PCR are strictly related to the primers pair being used, to the DNA-polymerase, to the extracted DNA quality and purity.

Type specific primers designed to amplify exclusively a single HPV genotype can be used, but in order to detect the presence of HPV-DNA in a clinical sample, multiple type-specific PCR reactions must be performed separately. This method is labor-intensive and the type-specificity of each PCR primer set should be validated.

Alternatively, consensus or general PCR primers can be used to amplify a broad-spectrum of HPV genotypes by targeting a conserved region within the HPV genome. Since the L1 [11] and E1 [12] regions are the most conserved parts of the genome, several consensus PCR primer sets have been developed to amplify these regions:

• GP5+/6+ PCR system [13,14] uses a simple pair of consensus primers to amplify a 140 bp region in the L1 gene allowing the identification of 30 HPV genotypes. This method, characterized by high sensitivity and specificity, is useful in predicting high-grade CIN [15] in spite of documented interlaboratory variation of results [16];

• the MY09/11 system [17] identifies high-risk HPV genotypes by amplifing a 450 bp sequence in the conserved L1 region. The MY09/11 primer set uses degenerate bases in order to reduce variability due to different genotypes. The disadvantage of this design is
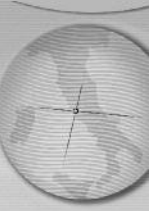
that synthesis of degenerate oligonucleotides is not highly reproducible and can result in a high batch-to-batch variation.

Improved primer design with the development of 2 sets of non-degenerated PGMY09/11 primers has led to improved consistency and better sensitivity for a large number of HPV genotypes [18]. However, when compared to other methods for HPV DNA detection, the MY09/11 system has been shown to generate a large number of discrepant results [19] as well as more multiple HPV positivities [20].

• SPF10 system [21]: primers amplify a 65 bp sequence from a highly conserved region of the viral L1 gene. The use of a defined mixture of non-degenerated primers has the advantage that the oligonucleotides can be synthesized with high reproducibility and PCR is performed at optimal annealing temperatures. The short amplicon allows the test to achieve a high analytical sensitivity, but has the disadvantage of lacking in HPV type discrimination [22].

The analysis of competitive PCR products can be performed in different ways:

a) restriction endonuclease digestion of PCR product followed by analysis of the electrophoretic pattern generated [23];

b) reverse hybridization with type specific probes; this can be achieved using a variety of formats such as line strip assays and micro-titre plates [22];

c) DNA southern blot hybridization [24]; this is not feasible in routine screening tests due to the presence of probes that must be radiolabeled. Biotinylated probes could prove useful in such applications.

d) DNA sequencing [25]; this is not useful for the analysis of possibly coinfected samples.
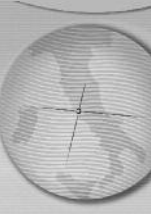
Among the PCR-based commercial kits it could be mentioned:

• Amplicor®
• DuoPap® (Bi-tech)
• Linear Array® HPV test
• INNO-Lipa HPV Genotyping v2
• DNA micro-array chips

1) Amplicor® (Roche), which has been the first commercially available PCR kit using a non-degenerate set of primers targeting the same 170 bp fragment of the L1 gene used in Hybrid Capture II (HC II) to detect 13 high-risk HPV genotypes. This PCR-based test has been demonstrated to be more sensitive than the target signal amplification obtained by HC II [22];

2) DuoPap® (Bi-tech), which is able to identify the main 13 genotypes of high risk-HPV;

3) Linear Array® HPV test (Roche), which allows determination of 37 high and low risk genotypes using oligonucleotide probes after PGMY09/11 PCR amplification;

4) INNO-LIPA HPV Genotyping v2, which is based on the reverse hybridisation principle [26]: part of the L1 region of the HPV genome is amplified using SPF10 primers and denatured biotinylated amplicons are hybridised with specific oligonucleotide probes immobilised on the strip. Results can be interpreted visually; the position is strictly related to the HPV genotype. The INNO-LiPA HPV Genotyping v2 test is able to detect 11 HPV high-risk and 5 HPV low-risk types, while Genotyping Extra, reducing the risk of contamination, can detect 22 HPV high-risk and 6 HPV low-risk types.

5) DNA micro-array chips (HPV Genotyping chips - Biomedlab Company; PapilloCheck® HPV screening–Greiner–Bio–One; Clinical Arrays HPV®– Genomica) have been recently developed for the diagnosis of HPV infection. These methods are all based on a prior target sequence amplification by PCR followed by hybridization using labelled 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. More recently an innovative method based on Luminex microarray technology has been described; it allows high-throughput, simultaneous identification and quantification of several HPV types [27]. HPV DNA is first amplified by PCR and subsequent genotyping is based on hybridisation with type specific oligonucleotide probes coupled to suspended polystyrene beads dyed with various ratios of spectrally distinct fluorophores. Hybridized beads are then passed through a Luminex analyzer in order to determine the spectral signatures indicative of specific HPV genotypes. This liquid bead microarray technique has been shown to have excellent analytical sensitivity, specificity and reproducibility when compared to a reverse line blot based genotyping system [27].

Another commercial non-PCR based diagnostic method is HC 2 test (Digene Corp., Gaithersburg, Maryland, USA) [28]; it is the only commercially available HPV DNA detection test approved by the Food and Drug Administration (FDA) for cervical cancer screening in combination with cytology in women after the age of 30. This test has been demonstrated to have a high sensitivity in detecting high-grade CIN by several cross-

sectional and longitudinal studies [29]. It is a non-radioactive signal amplification method based on the hybridization of the target HPV-DNA to labeled RNA probes in solution. The resulting RNA–DNA hybrids are captured onto microtiter wells and are detected by a specific monoclonal antibody and a chemiluminescent substrate; the intensity of the light signal generated is related to the viral load providing a semi-quantitative measurement of HPV-DNA [30,31]. Two different probe cocktails are used, one comprising probes for five low-risk genotypes 6, 11, 42, 43 and 44 and the other containing probes for 13 high-risk genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68. The test has also been found to detect additional HPV genotypes that cross-hybridise with the probe mixes. Therefore, this test generally distinguishes between the high- and low-risk groups but does not permit identification of specific HPV genotypes. The detection limit of approximately 5,000 genome equivalents makes it less sensitive than PCR and cross-reactivity of the two probe cocktails can reduce the clinical relevance of a positive result [32].

An automated third generation Hybrid Capture assay has been recently introduced but it is based on biotin-labeled oligonucleotide sequences for capture of target regions, in order to effectively reduce the cross-reactivity.

New molecular methods aimed the identification of HPV infection

Several authors have suggested that viral load and E6/E7 transcripts could be used as new surrogate markers of persistant HPV infection; these being more specific predictors of progressive disease than the simple presence of HPV DNA [33-37].

HPV E6/E7 mRNA testing for high-risk types has in fact been found to correlate with the severity of the lesion representing a potential marker for the identification of women at risk of developing cervical carcinoma; viral load has also been shown to be a marker of persistent infection and of active HPV replication [33-37].

These surrogate markers may be determined using commercial tests or "in house" Real time PCR [37].

Several advantages of Real-Time PCR over traditional PCR are described, like quantitative analysis and detection of low copy numbers of HPV DNA; fluorescent signal intensity is detected and correlated with the amount of HPV-DNA copy numbers compared with a standard reference template. A growth curve is recorded according to the amount of initial DNA template.

Currently, it is difficult to identify potential targets, to choose absolute or normalized values for viral load, to discriminate between integrated and episomal DNA, to calculate viral load threshold for every high risk genotype in order to distinguish between transient and persistent infections.

Numerous papers described the diagnostic efficacy of Real-Time PCR method for HPV-16, highlighting that HPV-16 integration into the host genome is already present in some of CIN lesions and that progression to cancer is associated with higher viral load [38].

Furthermore, as E6/E7 oncogene transcripts are found in all cervical carcinomas, several Authors have tried to identify molecular techniques aimed to detect E6 and E7 oncogene transcripts.

They may be detected using Reverse-Transcriptase assay (RT-PCR), rewriting RNA to c-DNA (first strand reaction) and, then, amplifying it (second strand reaction).

NucliSENS EasyQ HPV (BIOMÉRIEUX) is an assay designed to detect E6/E7 mRNA from the high-risk HPV types 16, 18, 31, 33 and 45; it combines NASBA (Nucleic Acid Sequence Based Amplification) amplification and real-time detection [39,40].

Several studies demonstrated that E6 and E7 oncogene transcripts burden during 16 and 18 genotypes infection are strictly associated with the severity of the cervical lesions [37-39,41,42].

The importance of the above mentioned surrogate markers (i.e. viral load and E6/E7 oncogene transcripts) was evaluated in two epidemiological studies performed at the Hygiene and Preventive Medicine Institute of the University of Sassari in collaboration with the Microbiology Department of the University of Milano-Bicocca and the Department of Obstetrics and Gynecology of San Gerardo Hospital [43-45].

The first paper [43,44] was aimed to evaluate the correlation between viral load and cervical lesions severity; the results highlight the potential role of viral load, this being generally found to be strong predictor of precancerous lesions and carcinoma. Five hundred and nighty-three cervical samples were analyzed by an 'in-house' real-time PCR; it was described a good association between HPVs total viral loads and severity of cytopathological/histological findings. There was a significantly higher DNA load for HPV-16 and -31 genotypes in patients with cervical carcinoma when compared to women with normal cytology (p <0.05); by contrast, no statistically significant difference was found for HPV-18 and -33 group. A significant good association between viral load
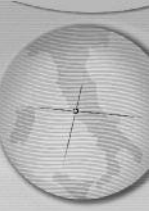
and severity of disease was observed for HPV-31 and HPV-16 (gamma 0.49 and 0.41, respectively; p <0.05); a significant fair to poor association was found in the case of HPV-18 and 33 group. The odds ratio –OR- of high-grade squamous intra-epithelial lesions (HSIL) associated to high viral load of HPV-18 was 8 (95% CI: 1.3-48.2), while the OR associated to high viral load of HPV-16 was 30 (95% CI: 3.8-239.4) [44].

The second paper [45] described the association between oncogene transcripts, detected using NASBA assay and Real-Time PCR, or viral load and cervical lesions severity on 248 cervical samples. The aim of this study was to evaluate the validity and reliability of the above mentioned surrogate markers. It was found a significant association between the two surrogate markers independently from cytology group. Furthermore, the association between the two markers remained statistically significant after stratifying for 16 and 18 genotypes (Fisher's Exact test: p <0.05 and p <0.05, respectively). Markers have a statistically significant association with the results of cytology and histological examination (viral load –VL- OR= 7.2, 95% CI: 3.6-14.5; E6-E7 OR= 6.9 for HSIL, 95% CI: 3.4-13.9). Then, both viral load and oncogene transcripts are good predictors for high-grade lesions (HSIL Receiving Operating Curve –ROC- curve: VL: 0.72, 95% CI: 0.64-0.8; E6-E7: 0.71, 95% CI: 0.64-0.78).

HPV-DNA testing in cervical cancer screening programs

Introducing a cohomprensive PAP-test screening program into a population has the potential to reduce the risk of developing cervical cancer by 60 to 90% within three years [46]. However, meta-analyses suggest that the sensitivity of a single conventional PAP-test for CIN 2/3 or higher is 50 to 60% [47,48]. Low PAP-test sensitivity has been attributed to a combination of poor sample collection, incorrect slide preparation and laboratory interpretation errors [48,49].

There is now considerable scientific evidence that testing for "high-risk" types of HPV is significantly more sensitive than is cervical cytology [50]. Several studies highlighted that sensitivity of HPV-DNA testing is much more reproducible than is cervical cytology; furthermore, it is not influenced by the age as demonstrated for PAP-test whose performance is better in women aged > 50 [51].

The association of both cervical cytology and HPV-DNA testing could be useful in order to increase sensitivity in cervical cancer diagnosis [52].

After the approval of HPV-DNA tests in high resource settings (for istance, US), one of the recent issues has been in what settings should it be utilized alone as the initial screen or in conjunction with cytology. The rationale for using both tests together was that the negative predictive value of the combination of tests is extremely high [52].

However, utilizing both tests in combination could substantially increase the cost of screening unless other public health actions take place.

An alternative approach, identified in Europe in order to decrease the economic burden, could be to utilize HPV-DNA testing alone as the primary screening method and cervical cytology could be used only to determine which HPV-DNA positive women required further workup [51].

Another alternative strategy, called "screen and treat", that was planned for low-resource settings, in which HPV-DNA testing alone is utilized for screening and all HPV DNA positive women undergo treatment, appeared, to be highly effective and inexpensive to cervical cancer prevention [51].

The above-mentioned approach has cost-effectiveness ratios better than traditional cytology and comparable to well-accepted health interventions, such as childhood vaccines [51].

HPV-DNA testing and epidemiological surveillance

Two new approaches for the prevention of cervical cancer have emerged over the past decade: vaccination for the primary prevention of HPV infection in women and the use of molecular methods to detect infection with carcinogenic HPV types, which allow secondary prevention via the identification and treatment of precancerous cervical lesions and early-stage cervical cancers as above described [53]. Countrywide population-based studies of HPV genotype prevalence will be needed in the next future to predict how these two approaches might influence prevention and epidemiology of cervical cancer, and how prophylactic HPV vaccination of women could affect the secondary prevention of cervical cancer. Nowadays, molecular techniques have been used to study prevalence of the infection, prevalent genotypes and groups of age involved in the infection, in order to identify the best strategy for vaccination. After the introduction of cohort-based immunisation in most developed countries, coordination between epidemiological surveillance systems, based on molecular methods, and cancer control programmes will be critical to assess the impact of the vaccine and its benefits [53].
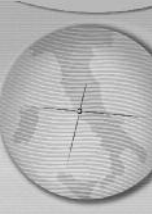
Surveillance for HPV infections in a specific setting could represent an important step for the development of primary and secondary prophylactic interventions, such as new vaccines targeted to genotypes who might replace those previously prevalent; incidence of type-specific and of any oncogenic HPV infections can give early information about potential HPV-type replacement. In order to survey the effects of vaccines on the incidence of HPV infections, it is essential to use appropriate population-based sampling procedures among vaccinated people and among those to whom the vaccine was offered but who did not take part to; furthermore, it is noteworthy that standard reagents for calibration and reproducible analytical assessment of HPV test assays, and other recommended analytical quality control procedures are used in these studies and monitoring activities. The golden standard molecular method, useful to obtain the above mentioned results, should identify not only the high potential carcinogenic effect (i.e. low- or high-risk types) but should discriminate the specific genotype, and if possible, its specific subtype, its viral load and other viral markers associated to negative prognosis [53].

Discussion

The diagnosis of HPV infection has been revolutionized by the development of molecular techniques. Commercializations of molecular biology techniques and cytology processing improvements have led to new products for cervical carcinoma screening. However, molecular techniques also appear to be relatively expensive, in particular those CE marked and commercial versions. This issue hampers the large scale up.

The development of new home-made molecular assays and the implementation in the clinical practice could reduce their cost, but it opens a question on their reliability. Furthermore, as discussed above, economic evaluations showed that screening approaches that use HPV-testing as a primary screening test are more cost-effective than current screening recommendations for both vaccinated and non-vaccinated women [54].

Validating clinical sensitivity and specificity of each new HPV molecular technique and improving the interpretation of the results are essential [55-58]; consequently, there is a clear need for well characterized international quality control panels to compare the various diagnostic methods.

The above-mentioned indications are concordant with those stated by GISCI (Gruppo Italiano Screening del Cervico Carcinoma), suggesting that "*rapid molecular assays should have high sensitivity and specificity, achieving accuracy in the preanalytical and analytical phases…*" [59].

The implications of HPV-DNA detection for patient management purposes needs to be addressed properly [60]. The efficiency of screening strongly depends on predictive value and accuracy of the test.

Currently, several health care providers use qualitative HPV DNA testing for the molecular detection of HPV infection, but it presents numerous analytical pitfalls; in fact, it is not able to distinguish persistent and transient infection, useful for the evaluation of prognosis.

Therefore, detection of E6 and E7 mRNA transcripts together with HPV-load has been shown to be of higher prognostic value for the evaluation of the precursor lesions of cervical carcinoma and for defining risk categories.

In conclusion, the application of HPV DNA testing in conjunction with genotyping is a corner-stone step not only for the correct prediction of the natural history of the infection, but also for a better understanding of epidemiological phenomena like cohort effect, clustering, etc.; moreover, given the substantial genetic heterogeneity of HPVs and the possible clinical relevance of specific subtypes, novel low- or high-density DNA probe arrays (DNA chips) may provide a useful technology for such studies in the future.

Furthermore, the current implementation of vaccination programmes could modify the epidemiological pattern of HPV infection that might be followed by a replacement of the genotypes; the surveillance of such as phenomenon should be monitored through molecular techniques.

On the other hand, because of the high complexity, high costs, and variety of molecular tests, in particular as regards their characteristics in validity (i.e. sensitivity, specifity and predictive values), different approaches are needed for natural history studies, epidemiological surveys, vaccination trials or surveillance in general, and for diagnosis of HPV infections in patients at risk of disease in clinical setting.

Therefore, as a unique gold standard molecular test is not available, now or in the immediate future, new efforts are necessary in the future to reduce the above mentioned disadvantages of molecular tests.

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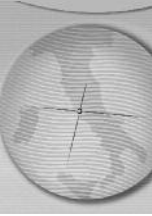
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