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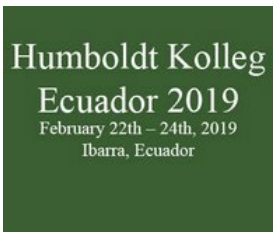

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REVISION/REVIEW

Prevention of cervical cancer development through early detection of HPV using novelty molecular applications

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ABSTRACT

Human Papillomavirus (HPV) is the predominant cause of cervical cancer worldwide. The infections with HPVs 16 and 18 have a high oncogenic risk for cancer development. Besides, the genes E6 and E7 encode viral oncoproteins associated with infection. New molecular techniques for HPV detection, show important advantages such as high sensitivity, recognition capacity, reliability, among others. These techniques allow the standardization of new protocols associated with the detection in a variety of substances and samples. The stretch relationship between the virus and the disease open a new field to study early detection of the HPV infection. Additionally, less concentration of the sample is needed. Considering the significance of the detection, the present paper explains five novelty molecular applications for the prevention cervical cancer and early detection of HPV such as RNA *in situ* Hybridization for the detection of HPV E6/E7, genosensors, electrochemical DNA biosensor, PCR-based urine assay and a semen assay for detection of HPV. All the methods related to DNA samples could be used for both genders, there are more acceptable and easy to collect.

Keywords: human papillomavirus, HPV, E6/E7 mRNA, in situ hybridization, genosensors, electrochemical biosensor, urine assay, semen assay.

INTRODUCTION

Human papillomavirus (HPV) refers to a group of 150 types of double-stranded DNA virus of which 40 infect genital

mucous . DNA virus encodes 8 genes with two functional regions the early (E) and the late (L). In addition, it presents a long control region (LCR), which includes most of the regulatory elements associated with viral replication and transcription. The genes E6 and E7 encode the major viral oncoproteins associated with the development of cancer[2]. Although, the most common female cancer is cervical cancer and its principal causal agent is HPV. Thirteen types of high-risk oncogenic HPV have been identified, among which HPV-16 and 18 are the most frequently detected in cervical samples. In the case of Ecuador, there were 1612 cases detected[3]. HPV develops premalignant and malignant lesions on the cervix that means a high risk to produce cervical cancer. Novelty molecular methods have been used as a good alternative to analyze samples of cervical lesions, urine, and semen. These new molecular methods can provide high sensibility, specificity and best recognition capacity using less sample compared with conventional ones[4]. Initially, molecular methods were based on target, signal, and probe amplification, such as polymerase chain reaction (PCR). Besides, new molecular applications like biosensors can integrate basic molecular techniques such as Southern blot, hybridization, and PCR that are more sensitive and reliable to early detection of HPV and prevention of cervical cancer[5].

These methods are of particular interest for the early detection of HPV infection from low-risk lesions that can become carcinogenic. Besides, they avoid the problems of false negatives of cytological techniques that are most commonly used[6]. Thus, the anticipated determination of cervical cancer biomarkers (E6/E7 and HPV-DNA viral load quantification) helps in the early detection of HPV. Consequently, the need to use new molecular methods is an important tool for the detection of infected cells by HPV. This improvement allows the early detection of positive HPV-AR in women that have more risk to present cervical lesions and cancer thanks to an early closer control[3]. The purpose of this literature review is to show new molecular methods on the detection and prevention of cervical cancer by HPV and related biomarkers. To examine the effectiveness of these methods on improving diagnosis in women, experimental studies published are reviewed. Overall, the molecular methods have been found to be an effective method to early detection of HPV. First, this review outlines RNA in situ hybridization to the detection of HPV E6/E7 mRNA. Second, major insights into genosensors development for the early detection of HPV. Third, novel electrochemical DNA biosensor. Fourth, detection and genotyping of HPV-DNA through a PCR-based urine assay. Finally, the molecular approach of semen to probe for the detection of HPV.

Since conventional diagnosis to new techniques

The oldest technique of identification of HPV is Pap smear, it consists in the identification of abnormalities from a sample of tissues of the cervix[3]. Next, this LBC consists of a device in the form of a brush that collects a more homogeneous sample than Pap smear. This characteristic allows better absorption of the sample improving sensitivity. However, it is much more expensive[8]. On the other hand, there is another highly used technique that is called Visual Inspection with Acetic Acid (VIA). It is the economic one, but with limited resources, it also has limited specificity[9]. There are also other techniques used nowadays such as southern blot, dot-blot hybridization, in situ hybridization and radiolabeled nucleic acid hybridization assays[10]. Even Though these techniques have high-quality, they require a big amount of sample and have low specificity. Improvements in molecular biology methods can accurate the detection and typification of HPVs[11].

RNA *in situ* Hybridization for the detection of HPV E6/E7 mRNA

In situ hybridization (ISH) is a technique employed to screen a specific segment of nucleic acids in heterogeneous cell populations such as tissue samples. The base of this technique is the adequate preservation of nucleic acid within a histologic sample that can be recognized through a reporter molecule. This molecule is joined to a complementary strand which attaches to the nucleic acid. The detection of the reporter molecule allows to localize RNA sequence and assess the degree of gene expression[12]. HPV E6/E7 oncoproteins act on the development of cervical cancer and their overexpression can be measured as E6/E7 messenger RNA (mRNA) transcripts. RNA in situ hybridization

(RNA-ISH) is a method with high sensitivity and specificity to detect HPV E6/E7 mRNA through the localization of individual cell signals in clinical samples[13].

Pandey, Bhosale, & Mahimkar in 2018 used RNAscope® assay kit that is an RNA *in situ* hybridization technology based on simultaneous detection of E6/E7 mRNA of seven high-risk HPV subtypes (HPV 16, 18, 31, 33, 35, 52 and 58). First, tissues pass through a process of fixation within formalin and paraffin embebition. Then, each tissue was divided into 3 sections. Consequently, the first section was used for an HPV test, the second section to positive control for Ubiquitin C (UBC) and the third section to negative control for bacterial gene dapB. Additionally, the reporter molecule used was diaminobenzidine present in Brown Reagent Kits (RNAscope®), which produce a strong spot when a target gene expression is detected.

In relation to the evaluation of treated samples, the researchers used the UBC test to evaluate the presence of hybridizable RNA. If existed with insufficient RNA quality, the UBC slide was negative and the sample was disqualified. The evaluation of the dapB test was performed in cases where staining is nonspecific. Finally, the negative cases with weak staining were taken for HPV scoring, and a positive HPV test result was determined by the punctate staining inside the cytoplasm or nucleus of the malignant cells[13].

Genosensors for the early detection of high-risk HPV DNA related to cervical cancer development

The viral attack that comes associated with a high risk of cervical cancer starts with the entering of HPVs 16 and 18. These viruses begin with the formation of E6 and E7 proteins, whose interfere in the cell function and promotes the formation of malignant keratinocytes. Eventually, the uncontrollable growth of the cell is used in order to avoid cell death, these infected cells are used for the identification of HPV and in some cases, the viral concentration is minimal[14]. Biosensors appear as a solution to the previously described problem, its principal benefit starts with the early detection of the viral infection. Genosensors are molecular tools that allow dynamic monitoring of the event of hybridization, in this case, for HPV-16 and 18. This technology is a DNA-based genosensory detection method and it is focused on the early detection of HPV precancer[11].

This genosensors can be classified by the nature of their diagnosis. There are electrochemical, optical, fluorescent, piezoelectric, magnetic, aptameric and immunosensors. Electrochemical biosensors were first described by Vernon, et.al. in 2003[15]. This bioelectronic device allows the identification of 21 types of HPV. It uses a gold electrode that has assembled a monolayer of oligonucleotides that are immobilized and also they are specific for HPV genotypes identifying through ferrocene labeling[15]. These sensors were improved through the past of the years, with variation in the composition of the electrode by addition of some nanomaterials in order to increase specificity. This approach helps to improve the identification of the more common or dangerous members of the HPV family[11].

Additionally, there are others like fluorescent sensors that provide *in situ* evidence of the existence of an HPV strain through the use of organic nanocrystals as a label for the quantification of HPV genotypes[16]. Also, the manipulation of the nanocomponents of this biosensor allows the optimization of infection detection. On the other hand, magnetic biosensors are a multilayered system based in specific biological probe immobilization that interacts with its counterpart sample with HPV traces[17]. Finally, piezoelectric biosensors are newly developed from the electrochemical and optical transducers that detect nucleic acids through a sequence-specific technique. These sensors are constructed around a piezoelectric crystal as a substrate that will accumulate electrical charge due to the mechanical stress produced[18]. Besides, Surface Acoustic Waves (SAW) is based on Bis-peptide nucleic acid (Bis-PNA) probe for the detection of HPV DNA through the caption of the double-stranded target DNA[19].

Novel Electrochemical DNA biosensor for the detection of HPV

An electrochemical DNA biosensor is a device that combines biological and detector components. In this case, the

biological component is a sDNA which is going to hybridize with other materials of the biosensor provoking electrochemical changes that are going to be detected. Changes in light emission or absorption, mass transport, proton concentration, among others, generate a specific signal that is transformable into a quantifiable response[20,21]. In general, DNA biosensors present many advantages such as rapid detection, low cost, portable, good sensitivity and selectivity, compatible with mass production, and less complicated to use compared to other nucleic acid assays[20].

Jampasa et al. (2018), develop and fabricate a new electrochemical DNA sensor based on sandwich hybridization to detect HPV-16 and 18 simultaneously. The sensor contains two signaling probes, the first one is an unlabeled acpcPNA capture probe called P1. This is a peptide backbone with favorable characteristics because it can form antiparallel hybrids with a complementary target DNA with high affinity and specificity. The second signaling probe is the anthraquinone (AQ) modified acpcPNA called AQ-P2. The sensor is based on the immobilization of P1 onto a Cys/gold-modified electrode through N-terminal lysine residue using a cross-linking agent. The AQ-P2 was designed with the objective of partially complement upstream (ASU) or downstream (ASD) positions on the target DNA sequence previously hybridized by P1 probe. This guarantees the electron transfer between the surface of the electrode and the labeled DNA molecule, provoking electrochemical changes in the biosensor. To prove the efficiency of the biosensor, scientist use cervical cancer cell lines that contain HPV-16 and 18, obtained from the Human Genetics Research Group[22,23].

New PCR-based urine assay for HPV-DNA detection and genotyping

This method does not require the collection of cervical samples, only a urine sample is needed. A urine assay is a non-invasive self-sample collection method that is more acceptable and easier to collect than a traditional biopsy of cervical samples. Last investigations use MY09/11 as primers for the detection of HPV DNA[24]. Tanzi et al. (2013) use a new set of primers in the same region and position as MY09/11 consensus primer pair. Consequently, there was an improvement in the sensitivity and an increase in the number of HPV genotypes detected by PCR.

This new PCR assay was tested in cervical and urine samples of HPV infected women (high-risk group) to prove its efficacy. Urine samples from 107 infected women were collected in sterile containers. The DNA extraction was performed from these samples using commercial methods. The purity and concentration of each sample were evaluated using a spectrophotometer. The detection of HPV in DNA samples was performed by PCR amplification using a degenerate primer pair ELSI-f and ELSI-r. Each PCR run includes positive and negative controls. PCR products were subjected to restriction fragment length polymorphism genotype analysis. Amplified products were treated with 3 different digestion solutions. The molecular weight of fragments generated allowed the identification of new genotypes. 31 different HPV types were identified in urine samples in comparison with 29 HPV types identified in cervical samples. HPV-51 and 73 were detected only in cervical samples whereas HPV-39,59, 69 and 85 were identified exclusively in urine samples[25].

The molecular approach of semen probe for detection of HPV

All the previous tests for HPV detection were focused on women. According to the Centers for Disease Control and Prevention in 2018, men are highly involved in the process of infection and there is not currently approved test for HPV. Moreover, in this technique, infected samples are easy to collect and open the field for the detection of HPV exclusively in male[26]. For the performance of the probe, DNA was extracted from the total semen obtained from processing it. They obtained an aliquot of the total semen sample and an aliquot of semen obtained by the swim-up procedure (SU fraction) to perform detection and genotyping. The kit used was the QIAamp Mini Kit (Qiagen). This probe uses ten microliters of DNA for PCR-reverse transcriptase hybridization process that are combined in INNO-LiPA HPV Genotyping Extra (Fujirebio Europe) according to manufacturer's instructions. This allows the performance of specific probes for 16, 18, 44, 31, 73 HPV genotypes. The total semen and the SU fraction are processed through lysis to obtain all the components of the semen through the Cell Culture DNA kit (Qiagen) according to the manufacturer's instructions with some modifications. When they were in the tuning phase of the

protocol, each step was monitored for cell and sperm lysis[27].

Molecular application for HPV detection	Reference	Number of HPV genotypes detected	HPV genotypes detected exclusively compared with cervical samples	Qualitative features
RNA *in situ* Hybridization for the detection of HPV E6/E7 mRNA	Pandey, Bhosale, & Mahimkar (2018)	Seven high-risk HPV subtypes (HPV 16, 18, 31, 33, 35, 52, and 58)	E6/E7 mRNA	-High specificity -Good sensitive
Novel Electrochemical DNA biosensor for the detection of HPV	Jampasa et al. (2018)	Two (HPV-16 and 18)	None	-Rapid detection -Low cost -Portable -High affinity -High specificity -Good sensitivity -Good selectivity - Compatible with mass production
New PCR-based urine assay for HPV-DNA detection and genotyping	Tanzi et al. (2013)	31 different HPV types	HPV-39, 59, 69 and 85	-Non-invasive -Self-sample collection
The molecular approach of semen probe for detection of HPV	Capra et al. (2019)	16, 18, 44, 31, 73 HPV genotypes	None	-Non-invasive -Self-sample collection

Table 1. Comparative table of molecular methods for HPV detection

CONCLUSION

In response to HPV as the most important factor generating cervical cancer, the investigation of new efficient detection mechanisms based on molecular techniques helps to reduce the time of detection of the disease, increasing efficiency of diagnosis. The existent literature indicates new molecular methods on the detection and prevention of cervical cancer that nowadays are more effective than conventional ones and they are highly used in medical applications. RNA *in situ* hybridization allows the localization of E6/E7 mRNA sequence and quantification of the degree of gene expression. In addition, the molecular techniques discussed in this paper such as biosensors promise to be a rapid and efficient detection mechanism.

The electrochemical DNA biosensor base on sandwich hybridization is able to detect HPV-16 and 18 simultaneously with high affinity and specificity using a single strand of DNA from cervical cancer cell lines. On the other hand, the PCR-based urine assay is a non-invasive self-sample collection method that uses a new set of primers in PCR in order to detect 31 different HPV types than the 29 HPV types identified in cervical samples. HPV-39,59, 69 and 85 were identified exclusively in urine samples. Besides, techniques such as detection of HPV in semen are a step forward in the detection of HPV due to the fact that previously the detection tests were directed towards women and there was no approved detection test for individuals of the male gender. All the methods related to DNA samples could be used for both genders, there are more acceptable and easy to collect. Finally, the standardization of these techniques can be used at industrial scale in the medical field. Additionally, the demand for better molecular tools will increase the great scale production of these molecular applications.

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