A Simple HPV 18 Detection Method Based on Ultra Specific Primer Immobilized on Glass Slides

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This study was carried out to develop a simple and inexpensive method for detection of Human papillomavirus (HPV 18) based on irreversible immobilization of ultra specific primer on silanized glass slides. This method is revealed by Blue Green Loading Dye I (LGC) and compared with conventional polymerase chain reaction (PCR) for endocervical samples. The new method was tested in 40 DNA samples with pre-cancer uterine lesions of women treated in Hospital of Recife PE, Brazil. DNA samples were extracted using Wizard Genomic DNA Purification Kit according to manufacturer's instructions. The samples were tested for HPV 18 by conventional PCR (PCRc) and the products visualized on 1.2% agarose gel, with LGC under UV 260 nm. After that the positive and negative samples to HPV

18 were tested by immobilization method and the results visualized with LGC under UV 260 nm. Both PCRc and immobilization method showed high degree of correlation (95%), whereas comparison between PCRc and immobilization method showed good correlation (100%). PCRc is widely known for detection of HPV because of its high sensitivity and efficiency, but due to high cost it is not yet standardized for use in public health laboratories. In our study, the single-stranded DNA immobilized method on a glass slide was effective in screening for HPV revealed by Blue Green and may be an alternative method for diagnosis of HPV once it offers a fast, and easy handling. *J. Clin. Lab. Anal.* 27:143–147, 2013.

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INTRODUCTION

Human papillomavirus (HPV) is the generic name of a group of virus of different types. As HPV is transmitted sexually, it can cause the emergence of high-risk genital lesions as precursors of malignant tumors, especially cancer of the cervix and penis. It is a common virus in young sexually active individuals. HPV types 11, 40, 61, 70, 72 have low risk for developing cervical cancer and are associated with benign lesions whereas the types 16, 18, 31, 33, 35, 39, 45, 51, 58, 59 are HPV causing high-grade lesions cervical intraepithelial neoplasia (CIN II and III) and cancer. The types 16 and 18 show greater carcinogenic potential in comparison to others, and the persistent infection of these types is the main cause for the development of cervical cancer and its precursory lesions (1, 2). With 4,800 fatali-

ties and 18,430 new cases (3), cervical cancer is the fourth leading cause of death in women in Brazil. The molecular diagnostic techniques such as hybrid capture, (4,5) in situ hybridization, among others, have been a major advances in early diagnosis of cervical cancer associated to HPV. Currently 44% of cases are cancer precursor lesion, called

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in situ or noninvasive, can be completely cured if treated (3) with such techniques. Brazil has made advances in this process. But despite all the progress in the HPV detection process, it is expected that 75% to 85% of the global population is infected (1). Despite the sensitivity, reliability, and effectiveness of molecular techniques, it is not yet possible to introduce these techniques in Brazilian public health because of their high cost. It is therefore necessary to research and develop alternative methods for rapid and inexpensive detection of HPV in order to standardize the laboratory in public health for assisting the early diagnosis of cervical cancer. The study of the immobilization of biomolecules on solid supports such as porous glass, clay, silica, and others has allowed the development of methods for rapid and inexpensive diagnosis of some diseases such as dengue, HPV, etc. Biomolecules immobilization is a great tool with many advantages. In the case of diagnostic methods, we can highlight advantages regarding stability in the response, reduced costs of analysis, and also its reusability (6). Nowadays, there is a growing interest in developing new process of immobilization of DNA on glass or other materials for use in new diagnostic targets. An example is the technology used in the microarray that can detect the genetic material and is based on hybridization between target DNA sequences, molecules derived from a biological specimen that are marked by fluorochromes, and sequences of DNA probes, molecules that represent the genes of interest. In this case, the probes are immobilized on a solid matrix that can consist of a layer of silica or glass, a solid substrate of nylon, and others (7).

Given the above-mentioned conditions, this study aimed to develop a new more stable and simple diagnostic method for HPV 18. This method comprised the immobilization of specific oligonucleotide sequences in a silanized glass slide and compared the detection of HPV 18 by the method of immobilization with the results obtained by the PCR in endocervical samples of women presenting to a public hospital of the metropolitan region of Recife-PE, Brazil.

MATERIALS AND METHODS

Patients

Forty samples of vaginal secretions from women with lesions of low and high grade Atypical squamous cells of undetermined significance (ASCUS) Atypical glandular cells of undetermined significance (AGUS) to cervical cancer and treated at the Center for Women's Health Institute of Integrative Medicine Professor Fernando Figueira (IMIP), Recife-PE, were analyzed. The study was previously approved by the local Research Ethics (No. 355/08) and informed consent was obtained from all patients.

Extraction Genomic DNA

DNA extraction of endocervical samples was done using the kit Wizard Genomic DNA Purification Kit (PROMEGA Corporation Biotechnology Madison, USA). After extraction, the samples were treated with RNase and then maintained at a temperature of -20°C .

Silanization Glass Slides

Slides of approximately 0.5×0.3 mm were dipped in a solution of sulfuric acid and hydrogen peroxide (1:3 v/v) for 90 s and then silanized with a coupling agent 3-aminopropyl-triethoxysilane 3APTS (SIGMA-Aldrich Corporation, Chemical and Biochemical Products, St. Louis, USA) in anhydrous acetone and left in the oven for 8 hrs at 100°C , then washed with deionized water and dried at 37°C .

Immobilization DNA on Silanized Surface

The primer specific for HPV 18 (5'- GCC AGC ACA GCA GGA ACT ACC-3') was immobilized in different concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 pmol/ μl) in water on silanized glass surface. The slides were immersed in a solution of 10 μl primer in each above-mentioned concentration for 20 min, washed with deionized water three to four times, dried and tested with DNA samples from HPV-positive and -negative women.

Hybridization

In response to HPV 18, DNA/HPV (double-stranded) was extracted from endocervical samples of infected women. For separation and subsequent hybridization of the DNA strands, it was heated in a water bath for 2 min at 95°C and immediately hybridized with the DNA primer immobilized on glass slides. The slides were washed with distilled water to remove unbound DNA. The double-stranded DNA was revealed with 0.1 $\mu\text{g}/\text{ml}$ of "Blue green loading dye I" (LGC Biotechnology, Brazil), a dye that has the property of binding preferably the hydrogen bonds by complementation of DNA strands, emitting a fluorescence blue light on UV 260 nm.

Quantification Hybridized DNA on Silanized Slides with Immobilized Primer

Samples hybridized on glass slides were quantified in a Fotoanalisador (Model Vitor II). This equipment measures the intensity of light emitted by fluorescence and in this case, the Blue Green Dye that emits the blue color with the hybridization of complementary strands was used. For this test, different concentrations of the primer HPV 18

were used: 0.2, 0.4, 0.6, 0.8, and 1 pmol/ μ l and 0.5 μ l of DNA samples of patients diluted in 10 μ l deionized water, in a concentration of 60 ng/ μ l. The quantity of 0.5 μ l of DNA samples from patients was hybridized with concentrations of 0.2, 0.4, 0.6, 0.8, and 1 pmol/ μ l primers immobilized on the glass slide. The measurement of the intensity of light produced by the blue green was performed by Fotoanalizador (Model Victor II). It was observed that the fluorescence intensity was proportional to the concentration of immobilized DNA and hybridized with DNA samples (data not shown). Slides that had hybridized viral DNA, emitted a blue color in the presence of Blue Green Dye when viewed on a UV transilluminator. The slides not having viral DNA from HPV 18 did not emit fluorescence (data not shown).

Scanning Electron Microscopy (SEM)

Microscope images were obtained using a scanning electron microscope (Philips XL-30 FEG) and all images were captured at 15 kV. For images, the glass slides containing the immobilized biomolecules were previously metalized. The slides were mounted on stubs with carbon tape and subjected to a step of bonding to the device Bal-tec SCD 0.50 Sputter Coater, where a 20-nm-thick film of gold is coated onto the surface of the slides.

Statistical Analysis

Data analysis was done in the statistics program Statistical Package for Social Science (SPSS) using the association kappa test (8). This test measures the level of agreement. Reliability values from 0 to 0.19 are considered "very low," 0.21 to 0.4 "reasonable," 0.41 to 0.6 "moderate," from 0.61 to 0.8 "important," and from 0.81 to 1.0 agreement "perfect."

RESULTS

Of the 40 samples analyzed for HPV by the new technique and compared with the PCRc, it was found that 52.5% (21/40) were positive for the PCRc and 47.5% (19/40) of samples were negative. The results were considered positive when the virus HPV 18 was amplified by using specific primer PCRc for HPV 18 in vaginal secretion samples of women with lesions of low- and high-grade cervical cancer. Women in whom HPV 18 was not found were considered as the control samples (negative) o. In relation to immobilization technique, 55% (22/40) of samples were positive and 45% (18/40) were negative. When we compared the results obtained by both techniques, 100% agreement among samples detected as positive (21 samples) and 95% among sample detected as negative (18 samples) was observed. No disagreement was observed

TABLE 1. Comparison of Concordant and Discordant Results between the PCRc and Immobilization Techniques

	PCRc + (%)	PCRc - (%)	Total	Kappa test
Immobilization +	21 (100)	1 (5)	22	0.949
Immobilization -	0 (0)	18 (95)	18	
Total	21	19	40	

with the samples that showed negative results on immobilization test while the PCRc showed positive results, and in 5% (1 sample) where the immobilization was positive and the PCRc was negative. Thus, we can consider that there is perfect concordance between the techniques studied according to the association test, *kappa* ($\kappa = 0.949$) (Table 1).

A model of the surface charge of APTS-treated glass slides was developed, which provides an indication of the role of ionic interactions in the adsorption of DNA molecules onto aminated glass slides. SEM analysis shows the differences between glass slides with immobilized DNA resulting from hybridization of DNA in HPV-positive and -negative patients (Figure 1).

The use of UV lamps for revelation of DNA bands using a specific dye is well known and used as a complement to PCR. Samples of subgingival and vascular lesions were observed in agarose gel electrophoresis using Blue Green Dye with the results revealed by UV transilluminator (9).

DISCUSSION

A variety of binding techniques have been developed over the last years to immobilize DNA or proteins onto substrates that in general require rather complex fabrication processes and sophisticated surface chemistry (10). In this study, a simple immobilization technique is presented, which was based on the preparation of the glass surface functionalization with APTS allowing the ionic interactions in the adsorption of DNA molecules onto aminated glass slides. This technique is simple and cheap and seems to be promising for a variety of microdiagnostic (11). Hybridization indicators or intercalators are devices used to identify the target probe. Such identification is called indirect detection with the advantage of increased response signal without the use of amplifiers in the biosensor and further reducing costs. Studies have been conducted with methylene blue as an indicator of hybridization due to its affinity for the DNA molecule. These links can occur by electrostatic interaction with the negative charges of phosphate groups present in nucleic acids or by the interaction between guanine bases mainly on single-stranded DNA (12). Usually, chemical compounds such as methylene blue (13), ferrocene, (14) and polyaniline (15) have affinities for some bases present in the DNA with

Fig. 1. Images of scanning electron microscopy of the surface support (glass slides) silanized with APTS: (A) silanized; (B) silanized and immobilized primer HPV18; (C) silanized with HPV18 primer immobilized and tested with ssDNA from patient negative for HPV18; (D) with the immobilized HPV18 primer and hybridized with the ssDNA of HPV18-positive patients. Scanning electron micrograph (increase 3,500 $\times$).

potential to intercalation with the double helix. In many clinical microbiology laboratories, there are several other signal-amplification methods that may be used, such as chemiluminescent rRNA probe hybridization technology that is based on the use probes of target rRNA and the hybridized probe is then detected using a proprietary chemical reaction that results in the generation of light (16) and immunofluorescence techniques, which is based on antibodies chemically conjugated to fluorescent dyes such as fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC). These labeled antibodies bind (directly or indirectly) to the antigen of interest, which allows antigen detection through fluorescence techniques. This technique therefore, is expensive, laborious, and has its limitations for use in diagnosis.

In our study the probe-modified, specific sequence ssDNAHPV/APTS/slide glass was hybridized with ssDNAHPV sequence to form dsDNA, stained with indi-

cator Blue Green visualized with UV. The same protocol was applied to test the hybridization reaction of the probe with noncomplementary oligonucleotide sequence (negative HPV).

CONCLUSION

The HPV 18 detection method based on ultra specific primer immobilized on glass slides proved to be useful and effective for diagnosis of HPV in endocervical specimens. This method has the advantage of being the most inexpensive, fast, and sensitive and presents a new perspective for HPV screening.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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