

Relationship with Factors of Risk and Prevalence to High Risk Human Papillomaviruses among Malagasy Women in Fianarantsoa, Madagascar

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Abstract In this survey, we analyzed by means of polymerase chain reaction (PCR) cervical and blood specimens obtained from consenting women in Fianarantsoa. Among the 1965 agreed women, 537 accept the two withdrawals HPV/HIV. Different PCR techniques were used to detect HPV and HIV infection. Consensus primer sets MY and GP were utilized to obtain HPV typing. For HIV, SK was utilized. The result showed that 89 specimens are positive by MY and GP. For HIV test, all showed negative. From the woman infected by HPV, they all have took tobacco at least 6 times a day, age of the first sexual intercourse 14 years, 8 partners at least and they also have sexually transmitted infections antecedents.

Keywords Human Papillomaviruses (HPV), Madagascar, Sexually Transmitted Infections (STIs), Polymerase Chain Reaction (PCR)

1. Introduction

The cervical cancer is the second most frequent cancer of woman in the world[1]. This illness constitutes a true problem of public health, particularly, in Latin America and in Africa sub-Saharan. On a world scale, cervical cancer kills every year about 200 000 to 250000 women that more of 80% lived in development countries[2].

The human papillomaviruses (HPV) is considered as the main reason but it's not sufficient for the cervical cancer[3]. Majority of women infected by HPV oncogene doesn't develop cervical cancer, that's why other factors, acting at the same time as the HPV, influence the risk to provoke the illness. Some concomitant factors or " cofactors ", as the number of pregnancies, the use of oral contraceptives[4], tobacco, the immunosuppression, in particular when it is linked in HIV, the infections due to other sexually transmitted infections and bad food, have been associated [5][6], in different measures, to the cancer development invasive. However, their specific role in the cervical cancer development remained unclear. The first sexual intercourse age, the number of sexual partners, the sexually transmitted infections historic, and other features of sexual life are linked to the risk to contract the HPV and are not considered like

cofactors of the infection progression in HPV toward the cervical cancer.

More of 50 types of HPV can infect the woman, 15 among them (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82) are considered to very potential oncogene for the cervical cancer and 8 of them (16, 18, 31, 33, 35, 45, 52, and 58) were implied in 95% of the cervical cancer[7]. The most current types of high risk HPV vary according to the countries and the regions[8][9].

HPV infection is the one of the most widespread sexually transmitted infections. However, in most cases, the infection disappears or becomes undetectable in one or two years. The research proved that some negative results become positive after three years. After a follow-up of 60 months, most infections by the HPV had not progressed toward cervical lesions[10].

In Madagascar, according to the statistics held by the oncology service of the CHU/HJRA in Antananarivo that was the unique center of oncology, the cervical cancer occupies the second place after the cancer of the breast at the woman with about 470 new cases per year (yearly report of activity 2011)[11].

Some recent research showed that high risk HPV type 16 and 18 exist to Madagascar, especially in Fianarantsoa[12].

The objective of this survey is to verify the relation between the factors of risk and the prevalence to high risk HPV at the women in rural area of Madagascar, in order to put an efficient and perennial strategy to the preventive struggle of the sexually transmitted infections in developing countries in general and to Madagascar in particular.

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2. Materials and Methods

2.1. Study Population

Women from selected rural health centers in Fianarantsoa (Madagascar) constituted the cohort of this study. The first step in the method consisted of an information session about cervical cancer and HPV being offered in each health center community. Women having suspected something wrong in their cervix were asked to volunteer to participate in the study by filling out a questionnaire requesting her age, education, sexual history, marital status, tobacco consumption habits and sexual transmitted infections. The consent form, the questionnaire and the information pamphlet were written in Malagasy language to facilitate the communication.

Since the HIV/AIDS is one of the factors of risk of the infection of HPV and that this illness especially poses a big problem on the public health in Africa, a tracking of HIV by PCR is done in parallel for the women who accept them.

For the withdrawal, a sterile disposable speculum was used for the gynecological exam to avoid transmission of Sexual Transmitted Infections (STIs). Cervical exfoliated cells were collected from all subjects by sampling the ectocervix and the endocervix using a cytobrush. The head of the cytobrush was kept in 5ml sterile phosphate buffered saline (PBS, pH 7.2) tube and frozen at -20°C until DNA extraction. MY09/MY11[13][14] and GP5+/GP6+ consensus primers set have been used by PCR, amplifying DNA fragments in the conserved L1 region of approximately 450bp and 150bp respectively[15][16][17].

For each woman who has accepted to have both tests of HPV and HIV/AIDS, a blood drawn by lancet from the finger was collected on Guthrie card (Whatman™: 903™ Protein Saver Card) and the HIV proviral DNA was amplified by PCR with the primers SK380/SK390 and SK38/SK39.

The sequences of all primers used in this study are shown in Table 1.

Table 1. Primers sequences for HPV DNA and HIV proviral DNA detection

Primer	Sequence (5'-3')
MY09	CGTCCMARGGAWACTGATC
MY11	GCMCAGGGWCATAAAYAATGG
GP5+	TTTGTTACTGTTGGTAGATAC
GP6+	GAAAAATAAACTGTAATCA
SK380	GAGAACCAAGGGGAAGTGACATAGCAGG
SK390	TAGAACCGGTCTACATAGTCTCTAAAGGG
SK38	ATAACCCTATCCCAGTAGGAGAAAT
SK39	TTTGGTCTTGTCTTATGTCCAGAATGC

All specimens and corresponding questionnaires were carried out to the molecular biology laboratory in Center Hospital University of Fianarantsoa for analysis.

2.2. Analysis of the Investigation Sheet

Every investigation sheet relative to specimen has been analyzed in observing especially the following parameters:

age, socioeconomic statute, marital statute, tobacco addiction, contraception, number of pregnancy, menopause and antecedents of sexually transmitted infections.

2.3. DNA Preparation of HPV

DNA from cervical cell samples was isolated as described previously[18][19]. Briefly, cervical cell suspensions were collected and treated with proteinase K (100 $\mu\text{g}/\text{ml}$) in lysis buffer (10mM Tris-HCl; pH 7.5, 1mM EDTA, pH 7.9; 0.5% SDS) overnight at 37°C . Standard phenol-chloroform extraction and ethanol precipitation were used for DNA purification. Pelleted DNA was resuspended in 50 μL of deionized sterile water and stored at -20°C until further analysis.

2.4. DNA Preparation of HIV-1

The dried blood spot on the circles of the filter (Figure 1) were prepared. Each one of the circles was divided by four and transferred in a tube of 1.5ml. The filters are suspended in 1.25ml lysis solution containing 0.32M sucrose, 10mM Tris-HCl (pH 7.4), 5mM MgCl_2 and 1% Triton X-100, and centrifuged to 3000 rpm for 30min at room temperature. Then the tubes were centrifuged to 12,500 g for 3 min. To eliminate hemoglobin, the supernatant was eliminated. The treated filter is kept to -20°C overnight.

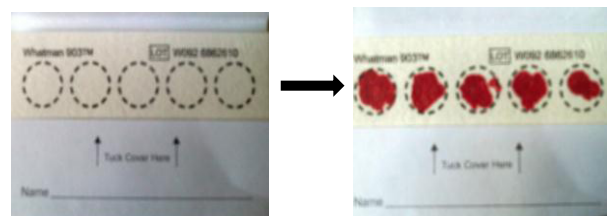


Figure 1. Dried Blood Spot in the circles of Guthrie card (Whatman™: 903™ Protein Saver Card)

Afterwards, the treated filter and sediment has been digested at 37°C in 50 μL of lysis buffer (10 mM Tris hydrochloride[pH 8.3], 50 mM KCl, 2.5 mM MgCl_2 , 0.45% Nonidet-P-40, 0.45% Tween-20, (100 $\mu\text{g}/\text{mL}$) proteinase K). The suspension was digested with proteinase K for 1 h at 56°C and then inactivated by heating at 96°C for 10 min[20][21]. Standard phenol-chloroform extraction and ethanol precipitation were used for DNA purification. Pelleted DNA was resuspended in 50 μL of deionized sterile water and stored at -20°C until further analysis.

In order to determine the quality and the quantity of isolated DNA, each DNA was analyzed by electrophoresis on 1% agarose gels stained with ethidium bromide and spectrophotometrically[22].

2.5. Polymerase Chain Reaction (PCR)

Each amplification reaction was carried out in a total volume of 20 μL overlaid with one drop of mineral oil and contained 10mM Tris-HCl (pH 8.3), 50mM KCl, 0.25U Taq DNA-polymerase, and 100–200ng DNA. The concentration of dNTPs and MgCl_2 varied with each set of primers. Each

PCR was carried out in DNA thermal cycler (Eppendorf).

2.6. PCR with MY09/MY11

The PCR mixture was complemented with 2.5 mM MgCl₂, 0.1 mM of each dNTP, 0.5 mM MY09 and MY11 primers. The DNA amplification was carried out during 30 cycles which included the denaturation at 92°C for 30s, the annealing at 53°C for 30 s, and the primer extension at 72°C for 30 s.

2.7. PCR with the GP5+/GP6+

The PCR mixture was complemented with 2.5 mM MgCl₂, 0.05 mM of each dNTP and 0.5 mM of GP5+ and GP6+ primers. The DNA amplification was carried out during 40 cycles that included the denaturation at 94°C for 30 s, the annealing at 45°C for 30 s and the primer extension at 72°C for 30 s [23],[24].

2.8. PCR with the SK380/SK390 and SK38/SK39

For detection of HIV proviral DNA, two pairs of primers specific to HIV-1 *gag* sequences were used: SK 380 and SK 390 as outer primers and SK 38 and SK 39 as inner primers [25].

The PCR product for HIV-1 was 115 bp in length. The test could detect at least one copy of HIV DNA per sample.

HIV DNA was detected by use of nested PCR. The final volume of 50 µL in each reaction contained the following: template, 100 mM of each dNTP, and 25 pmol/mL of each primer, 0.6 U of Taq polymerase and 200ng DNA. The DNA amplification was carried out during 35 cycles that included the initial denaturation at 95°C for 75 s, denaturation at 95°C for 20s, the annealing at 55°C for 30s and the primer extension at 60°C for 10 mn [25].

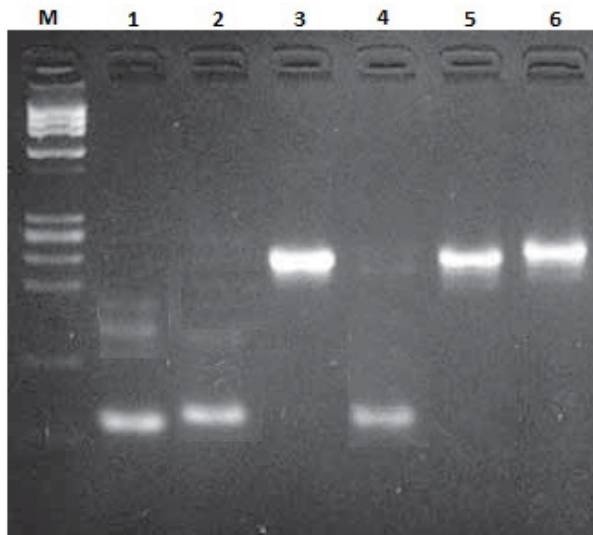


Figure 2. Electrophoresis of PCR products on 2% agarose gel (Image UVP Transilluminator, BIODOC-IT IMAG SYSTEM); M: DNA marker (Φ X174 DNA/HaeIII Marker). (1) (2) (4): PCR with GP5+/GP6+ primers (142 bp), (3) (5) (6): PCR with MY09/MY11 primers (451 bp)

In the PCR assay, positive and negative controls were included in each assay. Great care was taken to avoid

contamination of reactions. Disposable materials and gloves were used. Pre and post amplification stages were physically separated.

Aliquots (10 µL) of each PCR product were separated on 2% agarose gels and visualized with ethidium bromide [26]. The amplified products were identified by UV irradiation of the gels, and photographed by UVP TRANSILLUMINATOR (BIODOC-IT IMAG SYSTEM) (Figure 2 and 3).

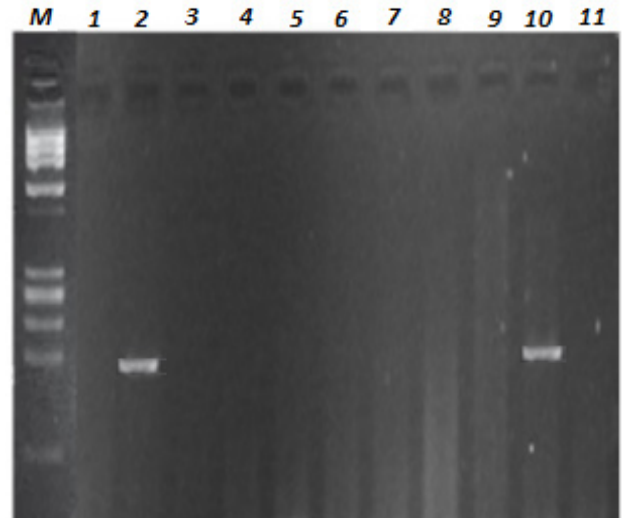


Figure 3. Electrophoresis of PCR products on 2% agarose gel (Image UVP Transilluminator, BIODOC-IT IMAG SYS); M: DNA marker (Φ X174 DNA/HaeIII Marker), HIV primers directed PCR: (1) (11) Controlled Negative, (2) (10) Controlled Positive (115 bp), (3) (4) (5) (6) (7) (8) (9) Specimens

2.9. Statistical Analysis

The statistical analysis of the qualitative and quantitative variables was either of mono-varied type, or of multivariate type using a software Epiinfo version 6. The Student test was used for the comparison of the averages and the variances and the test of Chi-2 for the comparison between the rates and a significant value if $p < 0.05$.

3. Results and Discussions

During the sensitization campaign for the free tracking of the HPV and HIV, 1965 women have been appropriated of which 537 (27.3%) accept the two withdrawals HPV/HIV.

Among these 1965 women, 685 (34.9%) are less of 25 years old, 890 (45.3%) are between of 25 to 44 and 390 (19.8%) are superior than 45. The average age of those women is of 36.5 years old (18 - 60). 1087 (55.3%) are to a weak standard of living, 710 (36.1%) to a standard of living average and 168 (8.6%) to an elevated standard of living.

Concerning the marital statute and the sexual history of the women appropriated, 775 (39.4%) are married, 217 (11%) divorced, 112 (5.7%) widowed and 861 (43.9%) are unmarried. The middle age of the first sexual intercourse is of 16.5 years old (13 - 21). Every woman has at least 4 partners (4 - 26). 1105 (56.3%) have a sexually transmitted infections antecedent.

As for the tobacco addiction, 1718 (87.4%) took at least 3 times (3-8) a day.

After the test of HPV DNA by PCR using consensus primers (MY09/MY11) to the 1965 specimens, 89 of specimens were HPV positive.

These 537 specimens give all negative results by the test HIV DNA of the blood spot by Nested PCR on using the primers SK 380/390 and SK 38/39.

Among the 89 HPV positive, 3 (3.3%) are less than 25 years old, 67 (75.3%) are between 25 to 44 and 19 (21.4%) are superior to 45. 26 (29.2%) of them are to a weak standard of living, 32 (36%) to an average standard of living and 31 (34.8%) to an elevated standard of living[27].

In relation to the marital statute and the sexual history of women infected by HPV, 2 (2.2%) are married, 21 (23.6%) divorced, 20 (22.5%) widowed and 46 (51.7%) unmarried.

The first sexual intercourse average age of these 89 positive HPV is of 14.3 years old (13-15) and they have all at least 8 partners. The results of the investigations also show that they have all one sexually transmitted infection antecedent.

Finally, these 89 positive HPV all took tobacco, minimum 6 times a day.

The set of this results showed that the women in the sites of study don't have will to make HIV and HPV analysis in parallel. This insufficiency number may be on the one hand, the impact positive of national politics Malagasy while creating a National Committee of the Struggle against the HIV/AIDS (CNLS) in 1987 and the state's political engagement from the year 2002. This decision increases the number of the women tracked down in all regions of Madagascar even the most remote zones. On the other hand, in spite of the strong sensitization and the action led by the Malagasy government for the struggle against HIV/AIDS, the majority of Malagasy population especially in rural area only thinks that some traditional medicine is the best solution of preventive and curative struggle of this illness.

Indeed, women who accept blood withdrawal for HIV/AIDS tracking are in majority have an intellectual level, or received an information and education through the different local associations.

Therefore, the women who did HIV/HPV tracking at the same time are already have negative result before by the conventional test and they want to confirm their results by PCR technique.

These results also show that women of 25 to 44 years old are the most infected by the HPV in relation to less than 25 and superior to 45 years old. The difference is meaningful. The HPV infects all women whatever their respective standard of living. The difference is not meaningful. On the other hand, 97.8% of the infected women are divorced, widowed and unmarried that train the tendency to the elevated multi-partner.

Therefore, all infected women have at least 8 partners, age of first sexual intercourse advanced and they have all one sexually transmitted infections antecedent. Besides, they have all took tobacco six times a day[28][29][30][31].

4. Conclusions

The infection rate of high risk HPV in this study is 4.52%. Infection of HPV is not detected on younger women as they are young and still immune-competent.

This survey also shows that HPV infection is independently of the socioeconomic statute. On the other hand, the marital statute, advanced age of the first sexual intercourse, the number of partner as well as the tobacco addiction are in relation with the infection in HPV in Fianarantsoa, Madagascar.

The results of the HIV/AIDS test are all negative but the questionnaires results of woman infected by HPV confirms that in general, the risks of sexually transmitted infection is more elevated.

This study was performed only in Fianarantsoa, and its extension into the remaining regions would be necessary for having a national knowledge of the HPV in Madagascar.

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