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Preliminary Study of prevalence of high risk genotypes of human papilloma virus (HPV) in a community located in the northern coast of Ecuador.

Doris Andrea Gartelmann Santamaría

Tesis de grado presentada como requisito para la obtención del título de Medico

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Preliminary Study of prevalence of high risk genotypes of human papilloma virus (HPV) in a community located in the northern coast of Ecuador.

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Quito, 21 Mayo de 2008

Quito, 21 de Mayo 2008-05-21

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Por medio de la presente autorizamos a la estudiante Doris Andrea Gartelmann Santamaría para presentar como tesis de grado para la obtención del titulo de medico el paper: Preliminary Study of prevalence of high risk genotypes of human papilloma virus (HPV) in a community located in the northern coast of Ecuador.

Atentamente,

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DEDICATORIA

A mis padres, Cecilia y Carlos

AGRADECIMIENTO

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Sonia Zapata.

Gabriel Trueba.

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Por el apoyo, dedicación en interés en la realización de mi tesis

Preliminary study of prevalence of high risk genotypes of human papilloma virus (HPV) in a community located in the northern coast of Ecuador.

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Summary: Human Papilloma Virus (HPV) infection is the most common viral sexually transmitted disease worldwide. Although HPV infection has been associated to majority of cervical cancer cases in Ecuador, there is no information about prevalence and infecting genotypes. The aim of this study was to determine the prevalence of HPV high risk genotypes in a community located in the northern coast of Ecuador, and its correlation with age, Pap smear results, and number of sexual partners. Samples (n = 63) were taken from a rural community in the northern coast of Ecuador during April and July 2006. Extraction of DNA was done with Cetyl Trimethyl Ammonium Bromide (CTAB), genotyping was carried out by amplifying a broad region common to all HPV genotypes followed by a second amplification of specific sequences in order to detect only high risk genotypes. Polymerase Chain reaction (PCR) products obtained were run by electrophoresis on an agarose gel in order to recognize genotypes by their specific molecular weights. Of the 63 samples tested, 18 were infected with HPV (28.5%) six of which had 58 genotype, three had 18 and another three 56, one was found to have 31, 45, 52 each. Coinfections were present in three patients, two had genotypes 18 and 52, and one patient had 58 and 59. There was no significant difference in age, Pap smear results, and number of sexual partners between infected and non-infected women.

Key Words: Human Papilloma Virus (HPV), cervical cancer, genotypes, polymerase chain reaction

Introduction

The human papilloma virus is a member of the papovaviridea family. It is a small virus, with only 55nm in diameter. The virus genome is a single molecule of double – stranded, circular DNA. The DNA can be divided into three main regions, a noncoding region which contains promoter, enhancer and silencer sequences that regulate DNA replication. The second region contains protein coding sequences of E2, E4, E5, E6 and E7. The third region encodes L1 and L2, both are structural proteins for the viral capside. (3) Once the virus enters the host cell it begins replication. Cell cycle abnormalities and cancer are the result strongly associated to the transcription of genes E6 and E7 (1, 3)

Genital HPV infection is a sexually transmitted disease caused by the human papilloma virus (HPV). There are more than 200 types of HPV that infect humans, and more than 40 of them are sexually transmitted and infect genitals, cervix, and anus (1, 2).In the majority of cases HPV is transmitted by direct contact, thus the risk of contracting genital HPV infection is influenced by sexual activity, thus the more sexual partners a person has had the greater the probability of acquiring the virus. Another important risk factor is the commencement of sexual activity at an early age. (2)

Most HPV infections are benign and people infected may times ignore their status. However, some genotypes of the virus could produce abnormalities in cervical cells which in the long run may lead to cervical cancer. Clinical range of disease depends on the genotype of the infecting

virus. HPV viruses have been divided into to groups based on their capacity to produce Pap test results compatible with cervical cancer. High risk genotypes are HPV 16, 18, 31, 33, 34, 35, 58, 59, and 66 among other, and low risk genotypes are HPV 6, 11, 42, 43, and 44 (1, 3, 9).

The research on this virus started in the early 80s when the German virologist Harold van Hausen discovered the relationship between cervical cancer and HPV infection (3). Today there is solid evidence of the relationship of cervical cancer and HPV virus. Infections with HPV have been implicated in almost all cervical squamous cell cancer cases (1, 2, 9). Most HPV infections are not curable, but learning about the association between HPV and cervical cancer can help prevent deaths. Unlike most cancers, cervical cancer can be prevented, and also, if detected in an early stage, cured.

In developing countries cervical cancer is the most common cancer in women (1). Worldwide cervical cancer has been recognized as the second most common cause of cancer related deaths in woman (1). HPV is the most common viral Sexually Transmitted Disease (STD) among young, sexually active people and is of increasing public health importance (1, 2). Around 20 million people living in the United States suffer from genital HPV infection. An estimated of 5.5 million people in the United States get infected every year (4). Over 630 million people are infected with HPV worldwide (4).

Although HPV infection has been related to the majority of cervical cancer cases there is no information about prevalence and infecting genotypes in our population. Information about the genotype is important when deciding the best way to treat a patient (12). Cytology and histology

have been so far the most important diagnostic tools for HPV infection (10), however, these procedures are unable to identify the genotype of the infecting virus. It is necessary to identify the virus using molecular techniques.

In this study we have used a PCR assay with the viral E6/E7 oncogenes as the primer target region. This assay targets a broad spectrum of HPV genotypes in the first amplification. In the second amplification type specific primers were used, several of these where combined in cocktails, thereby reducing the number of amplification needed. Genotyping of HPV was done based on PCR product size.

A previous study in a population of Argentina revealed 43% prevalence in healthy women with normal Pap test results (5, 6). Considering that the Argentinean society and health system are similar to the Ecuadorian, we expect approximately the same problem in Ecuadorian communities. In Ecuador, actual incidence and prevalence rates are not known. Epidemiological information of 2002 showed that mortality rate of cervical cancer is around 21%, a high percentage compared with other Latin American countries (5).

A preliminary pilot study with seventeen samples obtained of Afro-Ecuadorian women of a community located in the north coast in the province Esmeraldas, revealed that the main genotypes were HPV 52 and 56, both of them regarded as high-risk genotypes. A more thorough investigation may provide more accurate information of the prevalence of high-risk genotypes of HPV in this Ecuadorian community. The present study had the purpose to not only detect the

prevalence of HPV in the community of Borbon but to correlate the prevalence with age, Pap smear results, and number of sexual partners.

Materials and Methods

Patients

Human subject protocols were approved by the bioethics committee of the Universidad San Francisco de Quito. Verbal explanation of the study and informed written consent was obtained from those who agreed to participate in this study. Patients were kept anonymous by assigning each patient a code. Information obtained was passed on to the community doctor.

A total of 63 samples were taken from a rural community in the northern coast of Ecuador during April and July 2006. Patients filled out a written consent; a questionnaire and Pap smears and cervical scrapes were obtained. Patients whose samples were taken were all woman between the ages of 17 and 68 years of age who agreed to be tested. Exclusion criteria were menses and sexual intercourse during the past 48 hours. Prior to sample collection, patients were subjected to physical examination looking for gross inflammatory changes, bacterial mycotic or viral (other that HPV) infections. If such changes were present the samples were not considered for the study.

Obtained information

The information was gathered through a questionnaire which asked patients about risk factor associated with infection with the HPV. (suggested by IARC-WHO).

Samples

Cytological sample: (It had to be taken before the sample with the citobrush). These samples were taken from the squamocolumnar junction and were fixed on a slide with ethanol so that it could be later analyzed with the usual PAP smear technique.

Cervical cells: this samples were taken from the squamocolumnar junction with a citobrush and then put in 1,5 ml. of sterile phosphate buffered saline (PBS 1X) , the samples were then stored at 4 ^{o}C until further analysis.

Extraction of total genomic DNA from cervical samples was preformed following the Cetyl Trimethyl Ammonium Bromide (CTAB) technique (8).

The sample of cervical cells taken with the citobrush and put in phosphate buffered saline 1X (pH 7, 4) was taken out of refrigeration. The citobrush was stirred in the PBS solution, in order to release cervical cells. The cell suspension was transferred into a Eppendorf tube were it was centrifugated for 5 minutes at 3000 rpm, in order to obtain a cell pellet, supernatant was discarded pellet was allowed to dry on an incubator at 37ºC. The pellet was resuspended in PBS 1X, and centrifuged for 5 minutes at 3000 rpm. The supernatant was eliminated. Washing of the pellet was repeated twice.

The dry pellets were stored at -20 ^{o}C . The concentration of extracted DNA was determined by spectrophotometry, and the final concentration was 20 ug/ul.

The genotyping of HPV was done by the method proposed by Sotlar, et al.. (7)

This technique uses degenerate primers with targets between ORF sequences of E6 and E7b genes which are present in a wide spectrum of HPV viruses, primer sequences used were E6/E7 (GGGWGKKACTGAAATCGGT-- CTGAGCTGTCARNTAATTGCTCA--TCCTCTGAGTYGYCTAATTGCTC).

A first amplification was done (PCR-GP-E6) and when positive a 630 bp product was obtained. This product was used as a template for a second amplification (PCR-NESTED-MULTIPLEX). The PCR-NESTED-MULTIPLEX was carried out with type specific primers for detection of each HPV (8). Primer sequences are described in table 1. In order to reduce the number of PCR reactions and increment the range of detection the primers have been grouped in two cocktails (figure 1), containing the high risk genotypes.

PCR procedures

The first PCRs were preformed using 20 ul as final volume, with 1mM of buffer, 3mM of $MgCl_2$, 200 uM dNTP, 5pM (0,25 uM) of generic primers (E6-E7), 200 ng/ reaction of sample (20 ng/ul), 0,7 U/reaction of Taq polymerase (5U) and H_20 (ultrapure, free of endonucleases) until final volume was obtained.

Amplification of the samples were preformed by a initial denaturalization cycle at 92^0C for 2 minutes, 36 cycles of 50 seconds amplification at 92^0C, hibridation at 45^0C for 50 seconds, one cycle of 72^0C for 50 minutes and a final extension step of two minutes for 72^0C.

The second PCR reactions (PCR-E6-NESTED-MULTIPLEX) were done with 1 mM of buffer, 2.5 Mm of $MgCl_2$, 200 uM of dNTPs, 15 pM of generic primers (coctel 1 and 2), 2ul amplicon from the first amplification, 0.7 U/reaction of Taq polymerase (5U) and enough H_2O (ultrapure, free of endonucleases) to complete final volume of 20 ul.

The cycling conditions were as follows: initial denaturalization at 94^0C for 4 minutes, 35 cycles of amplification at 92^0C for 30 seconds. Hybridation at 56^0C for 30 seconds and then 72^0C for 45 minutes, followed by a final extension step of 72^0C for 2 minutes.

The products were analyzed by electrophoresis on 2, 5 % agarose gel and ethidium bromide staining as shown in figures 3 and 4. Positive and negative controls were also run. Genotypes were recognized by specific molecular weights.

Analysis of data

Questionnaires were filled by hand at the recruitment site. Data was analyzed with the help of Epi Info 6 version 6.04d software.

Results

The mean age of woman who participated in this study was 35.7 years (±11.52).

Of all women who participated 16.4 % had no education, 52.5 % assisted only to school until the age of twelve, and only 31.1 % finished high school. No relationship was found between education levels in woman infected compared with not infected woman (x^2= 2.70, p= 0.259). A 69.8 % of woman tested were housewives and had no other occupation. The great majority of women in this study described themselves as being poor (92.5 %).

Of the 63 samples tested, 18 were infected with HPV (28.5%) six of which had 58 genotype, three had 18 and another three 56, one was found to have 31, 45, 52 each. Co infections were present in three patients, two had genotypes 18 and 52, and one patient had 58 and 59.

The mean age of commencement of sexual activity for the group of woman who tested HPV positive was 17.66 years (± 3.03), while commencement of sexual activity in woman who tested negative was 17.12 years (± 3.28). There was no significant difference (p=0.58) between both groups. There was no significant difference (p=0.11) between both groups concerning number of sexual partners.

In the group of infected woman two had a class I Pap test result (slightly inflammatory), fourteen had a class II Pap test result (moderately inflammatory), and two had a class III Pap test result (severely inflammatory). In the group of not infected woman, 8 had a class I Pap test result, 36 had a class II Pap test result, and one had a class III Pap test result. No relationship was found

between Pap test results of woman in the infected group compared to woman who were not infected (x^2= 2.5, p=0.286).

Discussion

Statistics presented in the WHO webpage (13) report a HPV prevalence of 14.3 % (general population) in South America. In the present study, almost one third of all women tested were infected with HPV of high risk genotype. Genotypes 58 and 18 were found to be the more frequent ones. A study in a rural population in Argentina (Corrientes) revealed the most prevalent genotypes to be 16, 18 and 58 (5). Information gathered in Chile revealed genotypes 33, 16 as the most prevalent (14). A study in the capital city of Colombia, Bogotá revealed genotypes 16, 58 and 56 as the most prevalent genotypes (15). Genotype 16, who is present in most other communities, was not fond in our study.

This study does not inform us about global HPV infection rates, since only high risk genotypes were detected and an unknown number of women could also present infection with low risk genotypes. This is the first description of genotypes of HPV infecting Ecuadorian women.

We found that most women tested had normal Pap test results despite HPV infection. One reason that could explain this is the low sensibility and specificity of a Pap test.

Although literature describes early commencement of sexual activity and an increased number of sexual partners as risk factors (2, 3) we found no statistical difference of these variables in this study. The sample size (n=63) might have been too small to show significant differences. It is also possible that due to social and cultural disapproval of early sexual behaviour, many women may not have disclosed this information.

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Table 1: Sequences of type specific primers. Second amplification was done with type specific primers grouped in two cocktails. (8)

Primer cocktail	Genotype	amplicon Size (bp)	Sequences 5´-3´
I High Risk	16	457	CAC AGT TAT GCA CAG AGC TGC CAT ATA TTC ATG CAA TGT AGG TGT
	18	322	CAC TTC ACT GCA AGA CAT AGA GTT GTG AAA TCG TCG TTT TTC
	31	263	GAA ATT GCA TGA ACT AAG CTC G CAC ATA TAC CTT TGT TTG TCA
	59	215	CAA AGG GGA ACT GCA AGA AAG TAT AAC AGC GTA TCA GCA GC
	45	151	GTG GAA AAG TGC ATT ACA GG ACC TCT GTG CGT TCC AAT GT
II High Risk	33	398	ACT ATA CAC AAC ATT GAA CTA GTT TTT ACA CGT CAC AGT GCA
	6/11	334	TGC AAG AAT GCA CTG ACC AC TGC ATG TTG TCC AGC AGT GT
	58	274	GTA AAG TGT GCT TAC GAT TGC GTT GTT ACA GGT TAC ACT TGT

	52	229	TAA GGC TGC AGT GTG TGC AG CTA ATA GTT ATT TCA CTT AAT GGT
	56	181	GTG TGC AGA GTA TGT TTA TTG TTT CTG TCA CAA TGC AAT TGC

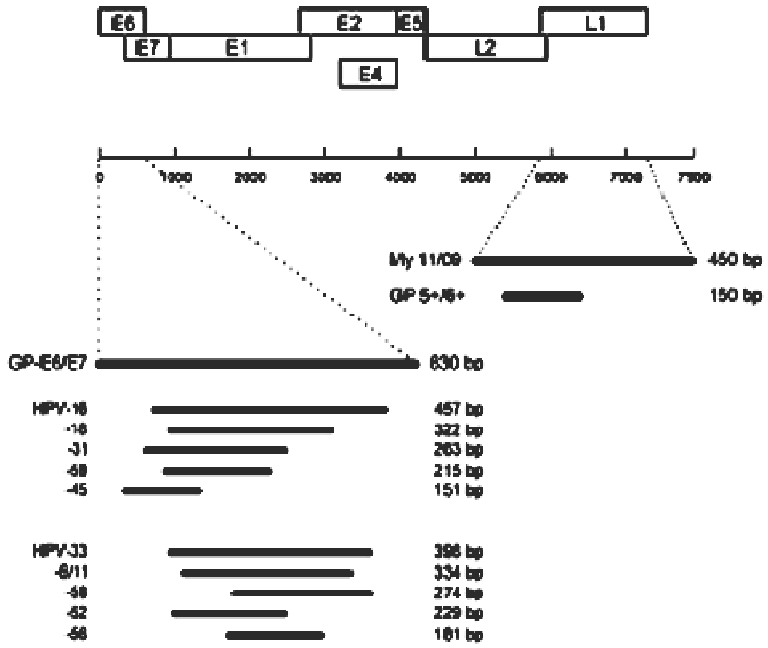

Figure 1. First amplification was done with primers targeting sequences of E6 and E7b genes present in a broad spectrum of HPV genotypes, second amplification used type specific primers grouped in two cocktails (8).

Figure 3. PCR products obtained with cocktail of primers 2, run in 2.5 % agarose gel stained with ethidium bromide. Genotypes were recognized by specific molecular weights. Samples 1, 4, 9, 10, 11, 17 and 18 present infection with HPV genotype 58 (274bp).

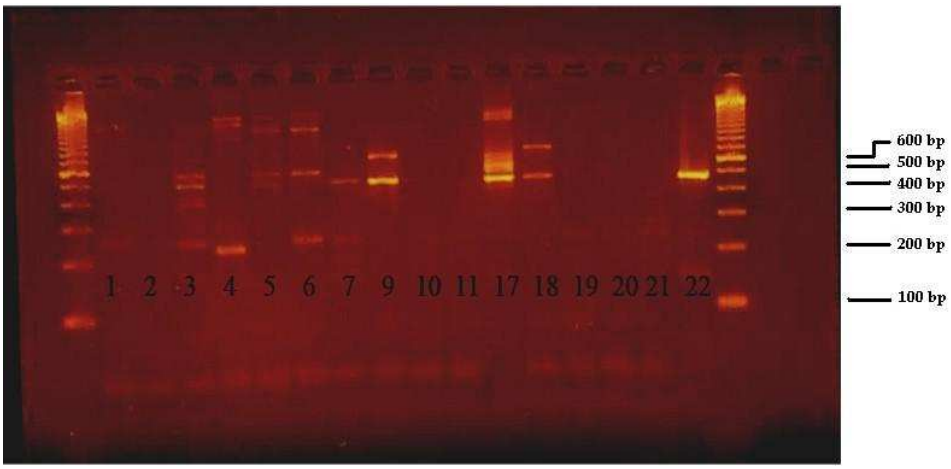

Figure 4. PCR of cocktail 1 products were run in 2.5% agarose gel and stained with ethidium bromide. Genotypes were recognized by specific molecular weights. Samples 9, 17 and 22 presented infection with HPV genotype 16 (457bp).