RESEARCH COMMUNICATION

Human Papillomavirus Infection among Bolivian Amazonian Women

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Abstract

Cervical cancer is the most common malignancy among women in Latin America. Human papilloma virus infection is known to be an important risk factor. However, HPV infection among Bolivian women has not yet been fully evaluated. The present study aimed to investigate HPV infection among women living in a rural region of the Bolivian Amazon. Cervical swab samples were collected from 151 healthy women in three Amazonian villages. From every woman, two samples were collected by cotton swab; one for cytological examination and the other for ethanol-preservation of cervical epithelial cells for HPV DNA testing. High molecular DNA was extracted from the ethanol-preserved cervical epithelial cells and tested for HPV DNA by a PCR-RFLP protocol.

Ethanol-preserved cervical epithelial cells remained suitable for DNA isolation and PCR amplification of human β-globin and HPV E6/E7 genes, 25 days after sample collection in the field. HPV-31, HPV-58 and HPV-6 were detected in the studied population. The overall prevalence of HPV infection among Bolivian Amazonian women was 8.0%. Neither dual nor multiple HPV infections were found in any of the positive samples.

This is the first report of HPV prevalence and type distribution among Bolivian Amazonian women. Our new method for preservation of cervical epithelial cells in ethanol may be useful for viro-epidemiological studies in rural areas.

Key words: HPV infection-Bolivian Amazonian women-cervical swab samples-ethanol preservation

Asian Pacific J Cancer Prev, **2**, 135-141

Introduction

Cervical cancer is the most common malignancy among women in the third world (Parkin et al., 1992). Eighty percent of the newly diagnosed cases in the world occur in developing countries of Central and South America, the Caribbean, Southern and Eastern Africa and Southern Asia (Pisani, 1993). The annual incidence of cervical cancer in Latin America and the Caribbean is 100 per 100,000 women aged 30-40 years old (OPS, 1990). A high incidence of cervical cancer was also reported in Bolivia (Rios-Dalenz et al., 1995)

Epidemiologic studies have shown a strong association of certain types of human papillomavirus (HPV) with high grade cervical lesions as well as invasive neoplasms of the uterine cervix (zur Hausen, 1994; Gaarenstroom et al., 1994; Lorincz et al., 1992; Koutsky et al., 1992).

Evaluation of HPV infection among the general population is important for surveillance of cervical cancer. A high prevalence has been reported in Latin American countries (Eluf-Neto et al., 1994; De San Jose et al., 1996; Tonnon et al., 1999; Rolon et al., 2000; Ferrera et al., 2000) having high incidences of cervical cancer (IARC, 1997). However, the extent of HPV infection among Bolivian

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Figure 1. Map of the Study Area. Boliviais located in central South-America, bordered in the North and the East by Brazil; in the Southeast by Paraguay; in the South by Argentina; in the Southwest by Chile and in the West by Peru. The country can be divided geographically into three regions: the Andean Highland, the Central Valley and the Amazonian Lowland; which correspond to 28%, 13% and 59% of the total territory, respectively. Shaded Circles (◯) indicate the location of Caranavi, Palos Blancos and Rurrenabaque. Dark squares (■) indicate the location of La Paz, Sucre, Tarija and Tupiza, from where samples used as HPV-positive or negative controls were obtained (Table 2).

women has not yet been fully evaluated.

Two previous studies on cervical pre-cancerous conditions among Amazonian women stated the need to assess the extent of HPV infection among this population (Brito et al., 1996; Taborda et al., 2000).

This study aimed to use a newly-developed method for preservation of cervical swab samples to investigate HPV infection among women living in a rural region of the Bolivian Amazon.

Materials and Methods

Study subjects

Our subjects were healthy women living in three rural villages located in the Bolivian Amazonian lowland: Caranavi, Palos Blancos and Rurrenabaque (Figure 1). The villagers are agriculturalists in a region where medical care and basic living facilities are very limited.

Women were invited to participate in the study by medical personnel and regional authorities through a local radio station, which was the most efficient way to reach even those living far from the village center. Detailed explanation of the study was given and women who agreed to participate were asked to sign an informed consent.

One hundred fifty-one women joined the study: 103 from Caranavi, 23 from Palos Blancos and 25 from Rurrenabaque. General demographic information was requested from the participants.

In addition to the samples from Amazonian women, we collected cervical swab samples from both cervical cancer patients and healthy subjects from other regions of Bolivia (Tupiza, Tarija, Sucre and La Paz) which were used as HPV-positive and negative controls.

Cervical swab samples

Cervical swab samples were collected from the women by two Bolivian gynecologists. Two samples were taken by cotton swab from every subject. One sample was used to prepare a smear for a Papanicolaou test and the other to obtain a cell suspension in 1 ml phosphate-buffered saline (PBS) in a 2 ml Eppendorf tube. Cervical cells in PBS were pelleted at 2000 rpm for 10 min using a portable cyto-centrifuge; the supernatant was discarded and 1 ml of 99% ethanol was added to the cell pellets. Ethanol-preserved samples were kept at ambient temperature until they were transferred to the core laboratories in La Paz (INLASA) and Kagoshima (Department of Virology, Kagoshima University) to be analyzed.

Cervical smears were fixed with propinylglycol spray and brought to the laboratory of Cytopathology at the General Hospital in La Paz, Bolivia.

DNA isolation from ethanol-preserved cervical swab cells

A high molecular DNA was extracted from cervical swab samples by the guanidine-HCl procedure (SMITEST EX-R&D, Sumitomo Metal Industry Japan) with minor modifications. Briefly, cervical cells were pelleted by centrifugation for 15 min at 15,000 rpm and then washed once with PBS. After solubilization by a detergent mixture and digestion by proteolytic enzymes at 55°C for 1 hour, soluble DNA was precipitated with isopropanol, washed with 70% ethanol, air-dried and redissolved in 50 µl of water (Distilled Deionized Sterile Water, WAKO Japan). The DNA concentration was estimated by UV absorbance at a wave length of 260 nm (Gene QuanT II, Pharmacia Biotech - Biochrom Ltd. England).

Polymerase chain reaction (PCR) for human β-globin DNA

All specimens were pre-screened by PCR amplification of Exon 1 human β-globin using gene-specific oligonucleotide primers . The reaction mixture of 50 ul included: 1 µl (0.01-1 µg) of the isolated DNA, 50 mM KCl, 10 mM Tris-HCl pH8.3, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 10 µg/ml gelatin, 2.5 Units thermostable DNA polymerase (AmpliTaq Gold™ polymerase, Perkin Elmer USA) and 25 pmol of each primer: forward primer KM29 (5'-GGTTGGCCAATCTACTCCCAGG-3') and reverse primer KM38 (5'-TGGTCTCCTTAAACCTGTCTTG-3') (Saiki et al., 1988). After a hot-start at 95°C for 9 min the mixture was subjected to 40 cycles of amplification in a DNA thermal cycler (GeneAmp PCR System 9600-R, Perkin-Elmer USA). Each cycle included denaturation at 95°C for 30 sec, annealing at 58°C for 1 min and extension at 72°C for 30 sec, a final extension step was done at 60°C for 10 min. A 264 bp specific PCR product was visualized

by electrophoresis on 1.5% agarose-ME stained with 0.5 μg/ml ethidium bromide (Nacalai Tesque Japan).

Polymerase chain reaction (PCR) for HPV DNA

Detection of HPV DNA was performed using the PCR Human Papillomavirus Typing Kit (TaKaRa Biomedicals Japan) according to the manufacturer's instructions with some modifications of the PCR reaction. In brief, the E6 and E7 genes of the HPV genome were amplified by two pairs of consensus sequence primers. Malignant HPV types (HPV-16, 18 , 31, 33, 52b and 58) were amplified by the pU-1M/pU-2R primer pair; whereas benign HPV types (HPV-6 and 11) were amplified using the pU-31B/pU-2R primer pair. The reaction mixture of 100 μl included 0.2-1 μg of genomic DNA, 50 mM KCl, 10 mM Tris-HCl pH8.3, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 10 μg/ml gelatin, 0.5 Units thermostable DNA polymerase (AmpliTaq Gold™ polymerase, Perkin Elmer USA) and 25 pmol of each primer. After a hot-start at 95°C for 9 min the mixture was subjected to 40 cycles of amplification in a DNA thermal cycler (GeneAmp PCR System 9600-R, Perkin-Elmer USA). Each cycle included denaturation at 94°C for 30 sec, annealing at 57°C for 2 min and extension at 72°C for 2 min. HPV-type specific PCR products were visualized by electrophoresis on 1.5% agarose-ME stained with 0.5 μg/ml ethidium bromide (Nacalai Tesque Japan). Every PCR experiment included HPV positive and negative controls.

HPV Typing by restriction fragment length polymorphism (RFLP)

PCR amplified HPV DNA was precipitated by ethanol and subjected to RFLP analysis using the restriction enzymes: Ava I, Ava II, Afa I (Rsa I), Bgl II and Acc I (Enzyme Set A Kit, TaKaRa Biomedicals Japan) according to the manufacturer's instructions. Size specific fragments of DNA were visualized by electrophoresis on 4% Nusieve agarose 3:1 (FMC Bioproducts USA) stained with 0.5 ug/ml ethidium bromide (Nacalai Tesque Japan).

Statistical analysis

We used one way-ANOVA (analysis of variance) to calculate for statistical differences in age; and Fisher's exact test for differences in the other demographic variables (ethnic group, education, occupation and marital status). Both tests were run on STATA software (Stata Corporation USA). Means and standard deviations were calculated using the StatView program (Abacus Concepts, Inc. USA).

Results

The demographic characteristics of the studied women showed significant difference in ethnicity ($p<0.001$), but not in the other variables (education, occupation and marital status). Women from Caranavi belonged mainly to the Mestizo and Aymara groups. In Palos Blancos the Aymara group predominated. Women from Rurrenabaque were mostly of other ethnic groups of Amazonian origin (Table 1).

The rural setting of our field study required a new method for preservation and transportation of cervical swab samples

Table 1. Demographic Characteristics of the Study Population

Characteristic	Village			P value
	Caranavi	Palos Blancos	Rurrenabaque	
Median Age (range, years)	34.9 (16-65)	38 (23-67)	40.1 (20-70)	0.094**
Ethnic group (%)				
Aymara	42.4	66.7	4.3	
Quechua	13.0	4.8	0.0	<0.001*
Mestizo	42.4	19.0	0.0	
Other	2.2	9.5	95.7	
Education (%)				
None	8.3	13.6	8.3	
Primary	38.5	27.3	33.3	0.812*
Secondary or higher	53.1	59.1	58.3	
Occupation (%)				
Housewife	46.8	45.5	70.8	
Merchant	24.5	13.6	0.0	0.057*
Farmer	9.6	18.2	4.2	
Professional	8.5	9.1	16.7	
Others	10.6	13.6	8.3	
Marital status (%)				
Married or living as married	77.2	69.6	79.2	
Separated/Widowed	17.6	26.1	20.8	0.638*
Single	5.9	0.0	0.0	

**: P value calculated by one-way ANOVA test.
* : P value calculated by Fisher exact test.

Table 2. Non-amazonian Cervical Swab Samples used as HPV-positive and negative Controls

Place	Condition	Time*	DNA Yield (ug)	β-globin PCR	HPV DNA PCR
La Paz	Healthy	1	10.9	+	-
La Paz	Healthy	1	19.1	+	-
Sucre	Cervical cancer	10	21.3	+	+
Tarija	Cervical cancer	13	9.0	+	+
Tarija	Healthy	13	47.6	+	-
Tupiza	Healthy	15	62.5	+	-
La Paz	CIN III	25	9.7	+	+

* Number of days preserved in ethanol prior to DNA isolation.

Table 3. DNA Isolation from Cervical Swab Samples from Amazonian Women

Locality	Yield (ug) Mean	Yield (ug) SD	Purity (260/280) Mean	Purity (260/280) SD	β-globin PCR
Caranavi N=102	11.1	15.8	1.7	0.4	+
Palos Blancos N=23	10.3	9.0	1.6	0.1	+
Rurrenabaque N=25	22.0	25.6	1.7	0.7	+
Total N=150	12.8	17.5	1.7	0.4	+

from the field sites in the Amazon to La Paz or Kagoshima where DNA isolation and analysis were performed. Feasibility of ethanol-preservation of cervical epithelial cells to keep DNA suitable for HPV detection by PCR was evaluated by HPV-positive and negative controls (Table 2). A high molecular DNA was subsequently isolated from all cervical swab samples kept in ethanol for up to 25 days. The mean amount of DNA recovered was 25.5 μg per swab sample, ranging from 3.0 μg to 75.2 μg. Human β-globin gene and HPV E6-E7 genes were successfully amplified from all the control ethanol-preserved cervical epithelial cells (Figure 2A, Figure 2B). HPV DNA was detected in cervical cancer patients and in the CIN III case but not in the healthy controls.

Cervical swab samples obtained from Amazonian women were treated in the same way as the controls. The average amount of extracted DNA was 12.8 μg DNA per swab sample. There was no substantial variation in the amount of the DNA recovered among samples from the three villages (Table 3). All but one of the cervical swab samples were suitable for PCR amplification of human β-globin and HPV DNA even though they were kept in ethanol at ambient temperature for 25 days after their collection in the field until they reached the laboratories (Figure 3).

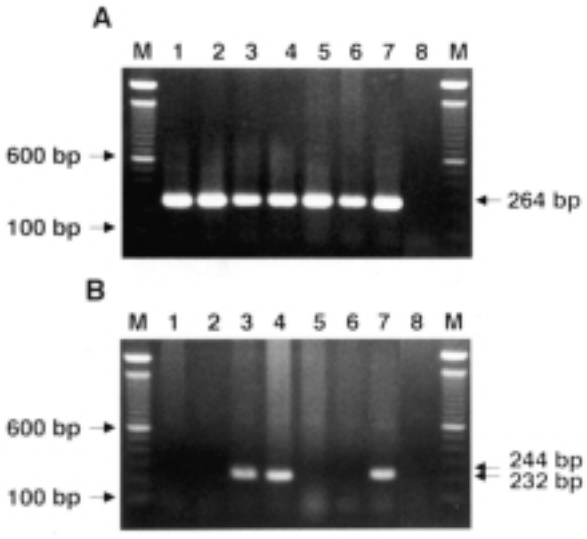

Figure 2. A) PCR amplification of human β-globin DNA (264bp). B) PCR amplification of E6/E7 HPV genes from cervical swab samples of two cervical cancer patients: HPV-31: 232bp (lanes 3 and 4), one case of CIN III: HPV-33: 244bp (lane 7) and four healthy controls (lanes 1, 2, 5 and 6). Samples preserved in ethanol for one day (lanes 1 and 2), 10 days (lane 3), 13 days (lanes 4 and 5), 15 days (lane 6), 25 days (lane 7). Lane 8: Negative control (distilled water). Lanes M: DNA molecular marker (100bp DNA Ladder, Gibco BRL USA).

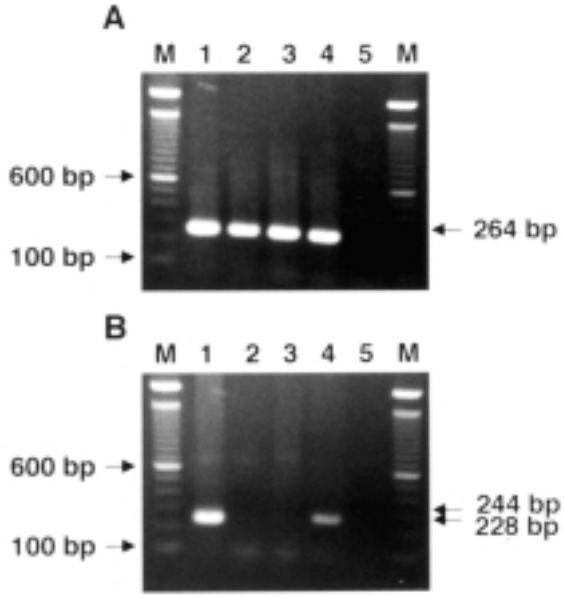

Figure 3. PCR Amplification of A) human β-globin DNA (264bp). B) PCR Amplification of E6/E7 HPV genes from cervical swab samples obtained in Caranavi, Palos Blancos and Rurrenabaque, preserved in ethanol for 25 days: HPV-6: 228bp (lane 1) and HPV-58: 244bp (lane 4). Lanes 2 and 3: HPV-negative samples. Lane 5: Negative control (distilled water). Lanes M: DNA Molecular Marker (100bp DNA Ladder, Gibco BRL USA).

Table 4. Prevalence of HPV Infection Among the Study Population

Village	Total No. of samples	HPV DNA Positive samples	HPV Type	HPV Prevalence(%)
		1	HPV-6	
Caranavi	102	8 4	HPV-31	7.8
		3	HPV-58	
Palos Blancos	23	0 0	-	0.0
		1	HPV-6	
Rurrenabaque	25	4 1	HPV-58	16.0
		2	HPV-X	
		2	HPV-6	
Total	150	12 4	HPV-31	8.0
		4	HPV-58	
		2	HPV-X	

HPV-X: Unassigned HPV Type (based on positive PCR amplification with the pU-31B / pU-2R primer pair, but negative RFLP)

Overall prevalence of HPV infection among the Amazonian women was 8.0%, although the prevalence in each village varied considerably; 7.8% in Caranavi, 0% in Palos Blancos and 16% in Rurrenabaque (Table 4). Restriction fragment length polymorphism (RFLP) analysis revealed that HPV type 31 and type 58 predominated among the Amazonian women studied (Figure 4). HPV-X refers to samples where HPV type could not be assigned by the kit used. Neither dual nor multiple HPV infections were found in our study subjects.

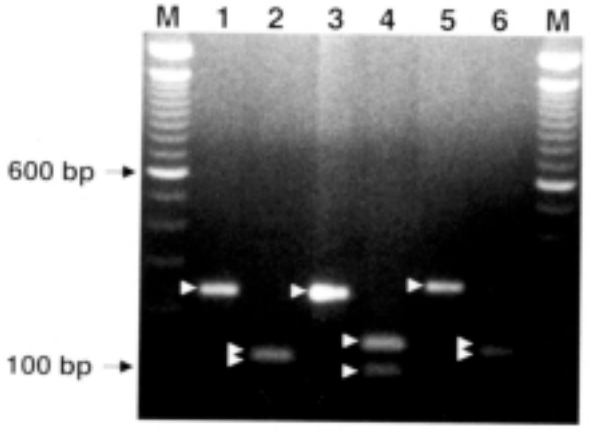

Figure 4. Restriction fragment length polymorphism (RFLP) of HPV types found among the Amazonian population: Lane 1: Undigested HPV-31 (232bp). Lane 2: Afa I-digested HPV-31 (117bp and 115bp). Lane 3: Undigested HPV-6 (228bp); Lane 4: Afa I-digested HPV-6: 132bp and 96bp. Lane 5: Undigested HPV-58: 244bp; Lane 6: Acc I-digested HPV-58: 126bp and 118bp. Lanes M: DNA Molecular Marker (100bp DNA Ladder, Gibco BRL USA).

Among 151 women studied, one case of CIN I was found, the remaining smears were Papanicolaou grade II, which corresponds to reactive changes associated with inflammation (data not shown).

Discussion

We investigated the natural occurrence of HPV infection among women living in a rural region of the Bolivian Amazon. Evidence of cervical cancer and pre-cancerous conditions among Amazonian women have been previously reported (Brito et al., 1996; Taborda et al., 2000).

Our studied subjects represent a general population of Bolivian Amazonian women. Since they voluntarily joined the study, they were not subjected to selection bias through being hospitalized or seeking a Papanicolaou test (Table 1).

Limited laboratory facilities in the field required a new method to preserve cervical specimens for molecular diagnosis of HPV infection. Preservation of cervical epithelial cells in 99% ethanol yielded a good recovery of DNA (Table 2 and Table 3). Isolated DNA allowed a satisfactory PCR-amplification of both human β-globin and HPV E6/E7 DNA (Figure 2 and Figure 3) even though cervical swab samples were kept for up to 25 days at ambient temperature before DNA isolation.

The overall prevalence of HPV infection among the Bolivian Amazonian women was 8% (Table 4). This value was unexpectedly low taking into account the high incidence of cervical cancer among urban populations in Bolivia: 53.1 per 100,000 women (Rios-Dalenz et al., 1995). It also contrasted with high prevalence of HPV infection among healthy women in urban cities such as Sao Paulo-Brazil (17%) (Eluf-Neto et al., 1994); Cali-Colombia (13.3%) (De San Jose et al., 1996); Hat-Yai-Thailand(16%) (Chichareon et al., 1998); Rabat-Morocco (20.5%) (Chaouki et al., 1998); Posadas-Argentina (43%) (Tonnon et al., 1999); Asuncion-Paraguay (19.8%) (Rolon et al., 2000) and Tegucigalpa-Paraguay (29.3%) (Ferrera et al., 2000).

Based on the results of the present study in the Bolivian Amazon, it is reasonable to speculate that prevalence of HPV infection among women living in rural areas of Bolivia is lower than in urban areas. Ethnicity distribution varied between the three villages (Table 1). An HPV infection prevalence difference was also observed, although not statistically significant, probably due to sample size (Table 4).

HPV-6, HPV-31 and HPV-58 types were found among the Amazonian women (Table 4). HPV-31 was previously reported in 26.5% of cervical cancer cases from Bolivia (Bosch et al., 1995). This value is close to the HPV-31 prevalence we found among healthy Amazonian women. (Table 4).

HPV-31 and HPV-58 appear to be more relevant to the Bolivian population than HPV-16 or HPV-18, since none of our samples were positive for these classic high risk types for cervical cancer (Bosch et al., 1995; Lorincz et al., 1992; Burger et al., 1996). Higher prevalence of HPV-31 as

compared to HPV-16 and 18 was reported in the Eskimo population in Alaska (Sebbelov et al., 2000). HPV-58 appears to be common in non-Caucasian populated countries, like China (Chan PK et al., 1999; Chan R et al., 2001; Huang et al., 1997; Wong et al., 2000), Thailand (Chichareon et al., 1998), Japan (Sasagawa et al., 2001; Sugase, 1992; Matsumoto et al., 1997) and Korea (Hwang, 1999). Thus, we suggest that Bolivian Amazonian population may be ethnically related to Asian Mongoloids.

We did not find either dual or multiple HPV infection among our samples, probably due to the relative geographic isolation of the villages. In fact, the highest HPV infection prevalence as well as both HPV-X samples belonged to Rurrenabaque, the village with the highest influx of tourists and strongest urban influence. Conversely, HPV DNA was not detected in any sample from Palos Blancos, which was the smallest and most isolated of the three villages.

Reactive changes associated with inflammation (Papanicolaou grade II) were found in all but one of the cervical smears. A high proportion of women with a cervical inflammatory condition was also reported among other Amazonian women (Brito et al., 1996; Taborda et al., 2000) and it seems to occur commonly in rural areas.

In conclusion, 8% of the Bolivian Amazonian women were infected with human papilloma virus with HPV-31 and HPV-58 predominating. Ethanol-preservation of cervical swab samples was found to be a feasible method for viro-epidemiological studies in rural areas.

Acknowledgements

We are grateful to all participating women from Caranavi, Palos Blancos and Rurrenabaque as well as the local authorities: Mr. Luis Ramirez S., Mr.Felipe Kittelson H., Agr.Rolly Sanchez T., Ing.Willy Cahuana T. and Mr.Fidel Estrada P. for their kind collaboration. We also thank Dr.Chihaya Koriyama for her collaboration in the data analysis and Mr.Glenn Forbes for his help in English editing.

This study was supported in part by Grants in Aid for Scientific Research on Priority Areas for Cancer from the Ministry of Education, Science, Sports and Culture of Japan (No.12218232, No.12218104) and the Kodama Memorial Fund for Medical Research.

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