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Human papillomavirus and associated factors in patients with unknown cytology treated in northern Peru

Virus papiloma humano y factores asociados en pacientes con citología desconocida atendidas en el norte de Perú

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ABSTRACT

Background: Human papillomavirus is cause of cervical cancer, one of the most common cancers among women. Objective: To determine the prevalence of human papillomavirus and associated factors in patients with unknown cytology. Methods: In gynecology patients with unknown cytology attended at Lambayeque Regional Hospital, at the northern coast of Peru, from April through June 2019, DNA extraction for human papillomavirus identification performed on cervical samples was based on the salting out method. Samples were processed by polymerase chain reaction. All samples were amplified for MY09 and MY11 primers, and PC04 / GH20 primers. Bivariate analysis used the chi-square and t-student tests. Results: 29.9% of the patients studied were infected with human papillomavirus. No statistically significant difference was found between human papillomavirus infection and age, age at first sexual intercourse, promiscuity, number of vaginal deliveries, cervical lesion, history of sexually transmitted infections, use of hormonal contraceptive or condoms, and smoking.

Key words: Human papillomavirus, Polymerase chain reaction, Clinical laboratory techniques.

RESUMEN

Introducción. El virus papiloma humano es el causante del cáncer de cuello uterino, uno de los cánceres más comunes en las mujeres. Objetivo. Determinar la prevalencia del virus papiloma humano y los factores asociados en mujeres con citología desconocida. Métodos. En pacientes de ginecoobstetricia con citología desconocida atendidas en el Hospital Regional Lambayeque, en la costa norte del Perú, entre abril y junio 2019, se realizó la extracción de ADN para identificar el virus del papiloma humano en base al método de *salting out*. Se procesaron las muestras mediante reacción en cadena de la polimerasa y se amplificaron para los primers MY09 y MY11, y PC04/GH20. Se empleó el análisis bivariado mediante la prueba de chi cuadrado y t-student. Resultados. Se encontró que 29,9% de las pacientes atendidas en el área de gineco-obstetricia con citología desconocida tuvieron el virus del papiloma humano. No se encontró diferencia estadística significativa entre la infección por virus del papiloma humano con la edad, edad de primera relación sexual, promiscuidad, número de partos vaginales, lesión de cuello uterino, antecedente de ITS, uso de anticonceptivo hormonal, uso del condón y tabaquismo.

Palabras clave. Papilloma virus humano, Reacción en cadena de la polimerasa, Técnicas de laboratorio clínico.

Introduction

Cervical cancer is one of the most common cancers in women in the world[1]. 585 278 new cases and 327 899 attributable deaths are predicted in 2010, with more than 80% of cases occurring in developing countries. Invasive cervical cancer accounts for 15% of cancers in women and ranks first or second among cancers in women in 13 of the 23 regions of the world[2]. Cervical cancer is caused by human papillomavirus (HPV) and epidemiological knowledge of the distribution of this virus infection in the general population is essential. Human papillomaviruses represent a heterogeneous family of doubly distributed DNA viruses of the taxonomic family *Papillomaviridae*[3]. Some types of human papilloma have a positive tropism for the skin and play a role in cancer[4]. The technique with the most sensitivity and specificity for HPV detection, and

considered the gold standard for many years, is the polymerase chain reaction (PCR) using the MY09 and MY11 consensus primers, followed by genetic sequencing[5]. Molecular assays, specifically PCR, are very effective in the diagnosis of HPV[6].

The objective of this study is to determine the prevalence of human papillomavirus and associated factors in obstetric-gynecological patients with unknown cytology in northern Peru.

Methods

This is a descriptive cross-sectional study. Consecutive non-probabilistic sampling was applied for convenience, which consisted of recruiting all the patients who met the selection criteria during the period April to June 2019. The patients filled out a questionnaire with closed questions with reproductive, demographic and sexual health data. Cervical samples. were collected from the OB / GYN service during the period, which were processed in the molecular biology laboratory of the hospital's research area.

The protocol and the research work were approved by the Ethics Committee of the Lambayeque Regional Hospital, Peru. All participating women signed an individual informed consent, prior explanation and information to carry out the study.

In the molecular characterization of samples, for the previous treatment, the tube containing the cytobrush was vortexed in order to detach all the cells that could remain in it. The cytobrush was carefully removed and the remaining solution was transferred to a 1.5 mL tube. The sample was concentrated by centrifugation at 12 000 rpm for 5 minutes, then all the supernatant was removed and the blank extraction tube was added.

For extraction and purification, 360 µL of lysis buffer and 3 µL of β-mercaptoethanol were added to the sample. It was incubated for 1 hour in a water bath at 65 ° C, vortexing every 10 min. 90 µL of 8M potassium acetate was added and it was centrifuged at 12 000 rpm for 5 minutes; then, the supernatant was recovered in another 1.5 mL tube (this step was repeated twice).

In the precipitation, 450 µL of isopropanol was added and centrifuged at 12 000 rpm for 5 minutes; the supernatant was removed. It was washed with 450 µL of 70% ethanol and centrifuged at 12 000 rpm for 5 minutes. Then it was allowed to dry until the humidity was eliminated. It was resuspended in 25 µL of H2O PCR.

The quality and integrity evaluation of the genomic DNA was carried out by agarose gel electrophoresis, whose concentration was 1% prepared with tris-acetate-EDTA buffer (TAE). For this, the agarose was dissolved in tris-acetate-EDTA buffer and heated in a microwave until boiling. Then it was poured into a holder with the comb, waiting for it to gel. The gel ready was placed in the electrophoresis chamber containing 1X tris-acetate-EDTA buffer. In the wells of the agarose gel, 1 µL of sample previously mixed with 1 µL of loading buffer and 8 µL of H2O PCR was loaded. With the power source, current was applied at 30V for 10 minutes, 70V for 30 minutes and 30V for 10 minutes. After that time, the gel was stained in a solution with ethidium bromide at a concentration of 0.5 mg / mL. The DNA image was then visualized and captured with the help of the Pharos Fx Plus photo-documentator. The quality and quantity of DNA was determined by electrophoresis in 1% agarose gels. Quantification was carried out using a marker with a known concentration.

The extracted DNA was amplified by the polymerase chain reaction technique. All samples were amplified to primers MY09 and MY11, and an internal quality control was done to verify if it was amplifiable DNA (human betaglobin) primers PCo4 / GH20 and to observe its components. All samples were amplified for the 450 bp consensus restriction enzymes of the HPV L1 gene, MY09: (5'-CGTCCMARRGGAWACTGATC-3 ') and MY11: (5'-GCMCAGGGWCATAAYAATGG -3 ') [7–10], and beta subunit hemoglobin (HBB) GH20: (5'-GAAGAGCCAAGGACAGGTAC-3 ') and PC04: (5'-CAACTTCATCCACGTTCACC-3 ') of 268 bp were used[11]. If a sample contained detectable β-globin DNA, then the HPV DNA was intact and amplifiable during the polymerase chain reaction[12].

The preparation of the mix for the β-globin polymerase chain reaction and human papillomavirus was carried out according to tables 1 and 2 at a final volume of 12.5 µL in each reaction.

Table 1. BGH Taq Platinium-Invitrogen concentrations and volumes.

	1 reaction		
	Initial concentration	Final concentration	Initial volume
Buffer 10x	10	1	1.25
Advance primer 10 mM	10	0.2	0.25
Regression primer 10 mM	10	0.2	0.25
dNTPs* 5 mM/C	5	0.2	0.5
MgCl2 50 mM	50	1.5	0.375
Platinum Taq 5 U/µL	5	0.625	0.125
DNA	>100 ng		3
Water			6.75
Final volume			12.5

dNTPs=deoxynucleotide triphosphates

Tabla 2. Concentraciones y volúmenes MY09-11 Taq Platinium-Invitrogen.

	1 reacción		
	Initial concentration	Final concentration	Volumen inicial
Buffer 10x	10	1	1.25
Advance primer 10 mM	10	0.2	0.25
Regression primer 10 mM	10	0.2	0.25
dNTPs* 5 mM/C	5	0.2	0.5
MgCl2 50 mM	50	2.75	0.6875
Platinum Taq 5 U/µL	5	0.625	0.125
ADN	>100 ng		5
Water			4.4375
Final volume			12.5

dNTPs=deoxynucleotide triphosphates

The amplification reactions for the HPV polymerase chain reaction were carried out in an Eppendorf thermocycler and the oligonucleotide sequence MY09 / MY11 with the sequence MY09 (CGTCCMARRGGAWACTGATC), MY11 (GCMCAGGGWCATAAYAATGG) and PC04 / GH20. The optimal thermodynamic conditions consisted of the following steps: pre-naturalization 3 minutes at 94 ° C; annealing 35 cycles for 45 seconds at 94 ° C, 1 minute at 55 ° C and 1 minute at 72 ° C; extension at 72 ° C for 7 minutes.

Regarding electrophoresis and band visualization, the resuspended samples were examined by electrophoresis on a 1.5% agarose gel, using a 100 base pair molecular weight marker. Subsequently, it was taken to electrophoretic run at 70 V for 10 minutes and then at 120 V for 45 minutes. Then the gels were stained for 10 minutes in a 0.5 mg / mL concentration solution of ethidium bromide. For its visualization, a Pharos FX plus brand molecular scanner was used. To identify the association between human papillomavirus infection and demographic characteristics (age, educational level, marital status, number of sexual partners), the t-student and Mann-Whitney tests were performed, using the Stata version 15 program.

Results

186 patient samples were analyzed; of them, 53 had a positive result for human papillomavirus (29.9%) (Table 3) and 124 were negative (70.1%).

No statistically significant difference was found between the factors age, first sexual intercourse, number of vaginal deliveries, cervical injury, history of sexually transmitted infection (STI), use of hormonal contraceptives and condoms, promiscuity, smoker, and a positive result (Table 4).

Discussion

A small group of papillomaviruses are the etiologic agents of several types of human cancers, including carcinomas of the anogenital tract[13]. In the study in Chiclayo, a city in the north coast of Peru, in patients with unknown cytol-

Table 3. General characteristics of patients.

General characteristics		
	Number	Percentage
Age*	45.1	11.9
First sexual intercourse+	18	16 a 21
Number of sexual partners+	1	1 a 2
Sexual partners in the last 6 months+	1	1
Number of vaginal deliveries*	2.2	1.5
Origin		
Chiclayo	79	44.6
José Leonardo Ortiz	26	14.7
La Victoria	16	9.0
Outside Chiclayo	56	31.6
Cervical lesion	23	12.9
History of STI	5	2.8
Hormonal contraceptive	135	76.3
Condom use		
Never	90	50.9
Frequently	82	46.3
Always	5	2.8
Smoker	22	12.4
Positive result	53	29.9

**Mean and standard deviation were used. +Mean and interquartile range were used. STI=sexually transmitted infections.*

Table 4. Differences according to the HPV test result in OB / GYN patients.

	Positive	Negative	p	95% CI
Age*	47.4 (10.7)	44.2 (12.2)	0.091	-7.11 a 0.53
First intercourse+	18 (17 a 22)	18 (16 a 21)	0.489	-1.69 a 0.81
Number of vaginal deliveries*	2.3 (1.6)	2.2 (1.5)	0.574	-0.62 a 0.35
Uterine cervical lesion				
Yes	6 (26.1)	17 (73.9)	0.665	0.34 a 1.97
No	47 (30.5)	107 (69.5)		
History of sexually transmitted infections.				
Yes	1 (20)	4 (80)	0.622	0.66 a 5.11
No	52 (30.2)	120 (69.8)		
Hormonal contraception				
Yes	40 (26.6)	95 (70.4)	0.87	0.82 a 1.18
No	13 (30.9)	29 (62.1)		
Condom use				
Frequently/Always	25 (28.7)	62 (68.9)	0.73	0.67 a 1.31
Never	28 (31.11)	62 (71.3)		
Smoker				
Yes	5 (22.7)	17 (77.3)	0.429	0.26 a 1.76
No	48 (31.0)	107 (69.0)		

**Mean and standard deviation were used. +Mean and interquartile range were used.*

ogy, prevalence of human papilloma virus in the uterine cervix was 29.9%, that is, in almost one third of the participants. A similar study with the same methodological characteristics performed by Serquén-López in the same city of Chiclayo in sex workers, a similar prevalence was found[14]. Studies carried out in other cities of Peru, such as Valderrama et al. (2007) in students from Lima, Peru, the prevalence was 8.4%[15]. Sullcahuaman-Allende et al., in a study at the National Institute of Neoplastic Diseases, Lima, Peru, found that 32.5% tested positive[16]. Manrique-Hinojosa et al. found high prevalence in Lima students (43.4%)[17] and Iwasaki et al., 34.5% in Lima urban population[18]. Santos et al. detected HPV DNA in 95.3% of women with squamous cell carcinoma and in 92.0% of women with adenocarcinoma / adenosquamous carcinoma, compared to 17.7% in control women[19]. These results are similar to those of Mendoza et al.[8], in 2012, with a prevalence of 21% in women with negative cytology in a Paraguayan locality, and by Winer et al., in 603 university students in the state of Washington, USA, at 4-month intervals between 1990 and 2000. At 24 months, the cumulative incidence of first-time infection was 3.3%[20]. The prevalence in the study by Brot et al. was 63% in a Brazilian population[21], and Ingabire et al. found that 8.6% were positive for HPV at the beginning of the study in 2007[22]. These differences are possibly due to the types of study and the region of research. In Peru, the prevalence of the reviewed studies is varied.

Sociodemographic factors such as age and marital status are frequently risk markers for exposure to HPV and other sexually transmitted infections[16]. Results such as age, beginning of active sexual life, number of sexual partners, promiscuity, number of vaginal deliveries, condom use, smokers, cervical injury, history of sexually transmitted infections, use of hormonal contraceptives were not associated with infection of human papillomavirus in our study. Other studies have found an association between HPV and these factors[23–29]. Sathian et al. and Bosch et al. mention that promiscuity and the number of sexual partners are factors of HPV infection[30,31] and the study by Francheschi et al. mentions that there is a relationship between HPV infection and the age of first sexual intercourse[32].

In our study, no statistical significance was found between human papillomavirus infection and the number of vaginal deliveries, similar to that found by Rajkumar et al.[33]. Statistical significance was also not found between having had a cervical lesion and HPV infection, which differs from studies such as Bosch[31], Oliveira[34], Motoyama[35], Naqvi, Wajid and Mitra[36] and Harden and Munger[13]. The history of STIs, use of hormonal contraceptives and / or condoms and smoking did not show statistical significance with the positive result for HPV; This differs from the studies by Hellberg and Vaccarella, who find a relationship between these factors and HPV infection[23,37,38].

The prevalence found in our study coincides with that of other studies. If we refer to associated factors, the results differ from those found in other continents, but are related to studies carried out in Peru. According to our results, the number of sexual partners and HPV infection are unrelated. This may be due to the fact that the study was done in a population with a low number of sexual partners, a conservative society, important factor in the contagion and persistence of the human papilloma virus in the body. A significant percentage of the patients were raped, thus beginning their sexual life. In addition, the patients tended to decrease the number of sexual partners during the interview,

and there was a great lack of knowledge between the types of contraceptive methods.

Only a small part of this population smoked, so this carcinogenic factor would not be reflected in the results and would require the study of a larger population.

In studies similar to this one, there is lack of information in the patients' medical records. A strength of our study was the repetition of the test in case of indeterminate result. The hospital gynecological and oncological services can improve screening programs by performing molecular tests in case of negative and highly suspicious cytology[39]. The present study is one of the first accomplished in the Lambayeque region, Peru.

The World Health Organization has reported that cervical cancer is the second leading cause of malignancy and death in women worldwide[40]. Epidemiological studies have shown that high-risk human papillomavirus (HR-HPV) genotypes are the main cause of this disease[41]. It is necessary to use complementary diagnostic tools that allow detection of the HR-HPV genome, in order to increase the performance of conventional morphological diagnostic methods generally used to detect cervical cancer[42]. One weakness of the study is not having genotyped patients positive for human papillomavirus or having included patients with epithelial carcinoma in any of its stages in another group, which would give a better view of the types circulating in the region. Subsequent studies are recommended to increase the number of samples, better stratifying the patients and extending the test to men, since they are the reservoirs of the disease. Progress has been made with vaccinating girls in Peru, but this should soon cover boys and better focus on the types circulating in the region and in the country. Research on this topic should be strengthened, as it represents an important cause of female mortality.

We conclude that a high prevalence of patients with human papillomavirus was found. No statistically significant difference was found between human papillomavirus infection and age, age of first sexual intercourse, promiscuity, number of vaginal deliveries, cervical lesion, history of STIs, use of hormonal contraceptives, use of condoms, smoking.

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